EXPERIMENTAL STUDIES ON THE ANTI-ATHEROSCLEROTIC PROPERTIES OF LACIDIPINE

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A thesis submitted to the University of London in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

December 2005

This thesis describes research conducted at GlaxoSmithKline (GSK), Verona, between 1999 and 2004 under the supervision of Dr. John Turton (School of Pharmacy, University of London). I certify that the research described is original and any parts of the work that have been conducted as part of collaboration with other workers are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature

Date 12 December 2005
ABSTRACT

Atherosclerosis is the major form of heart disease in Western countries. Atherosclerosis is a slowly progressing complex disease in which deposits build up in the inner lining of arteries causing the formation of plaques. Plaque formation is a multi-stage process which results in decreased arterial blood flow. The long-lasting calcium antagonist lacidipine is believed to affect many of the steps related to plaque formation and plaque development.

Various animal models have been used to date to study experimental atherosclerosis and reproduce lesions comparable to those occurring in man. In the present studies the hamster and the apoE-decient mouse were the animal models of choice to investigate the basis of the anti-atherosclerotic properties of lacidipine.

Experiments demonstrated that in the hamster and the apoE-deficient mouse fed a standard rodent diet, lacidipine treatment reduced the extent of atherosclerotic activity, without lowering hypercholesterolemia. The drug also significantly reduced endothelial cell changes associated with atherosclerosis.

Moreover, in the apoE-deficient mouse fed a high-fat diet, lacidipine decreased plasma low density lipoprotein (LDL) oxidation and confirmed that the susceptibility of LDL to oxidation is related to increased atherosclerotic risk. In addition, it was demonstrated that lacidipine can maintain endothelial nitric oxide levels at nano molar concentrations, which protects against vascular injury. Finally, lacidipine was shown to have a significant inhibitory
effect on cholesterol esterification and matrix metalloproteinases activity, properties which may also contribute to increased plaque stability.

In conclusion, it is proposed that lacidipine influences the atherogenic process by specific mechanisms, which are the result of a combined potent and long-lasting calcium antagonism, in conjunction with powerful antioxidant properties. These activities are further enhanced by the high lipophilicity of the drug.
ACKNOWLEDGEMENTS

The studies reported in this thesis were performed in the Department of Pathology and Toxicology, Safety Assessment Division, Glaxo Smith Kline R&D, Verona. I am grateful to the Directors and all the staff for allowing the work to take place; in particular I would like to thank Dr Piemica Marchiori for encouragement at the outset of the project and Dr Mike Jackson for support and availability. Particular thanks to Dr Gaetano Bertolini who gave me the best possible start anyone could hope for in this profession.

I would not have been able to undertake these investigations without technical assistance. In this respect I am in debt to Daniela Spagnolo, Sara Munaro and Valentina Zantedeschi for their excellent technical assistance in histology and to Monica Bonato for her help with clinical chemistry. I am also grateful to colleagues from the Universities of Verona and Milan who have always found time to give helpful advice and provide insights into the comparative pathology of atherosclerosis. Special thanks are due to Federica Crivellente who kindly assisted in gathering information and in reviewing with endurance drafts of the thesis.

Throughout this protracted project, and particularly at times when progress seemed difficult, my internal supervisor Dr John Turton was a steady source of help and guidance, providing a special inspiration and source of encouragement at times when progress was difficult.
Finally, I would like to acknowledge the sacrifices made by my husband and my daughter, who have been my main source of support and who have had to bear the brunt of my commitment to this project.
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<tr>
<td>ACAT</td>
<td>acyl-CoA-cholesterol acyltransferase</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
</tr>
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<td>AcLDL</td>
<td>acetyl LDL</td>
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<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AgNO</td>
<td>silver nitrate</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immuno-deficiency syndrome</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>analysis of covariance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activated protein-1</td>
</tr>
<tr>
<td>APAAP</td>
<td>streptavidin alkaline phosphate conjugate</td>
</tr>
<tr>
<td>ApoE-deficient mouse</td>
<td>apolipoprotein E knockout mouse</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BM-8</td>
<td>rat anti-mouse monocyte/macrophage monoclonal antibody</td>
</tr>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
</tr>
<tr>
<td>CA</td>
<td>calcium antagonist</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium ion</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>CBM</td>
<td>far walls of common carotids and bifurcations</td>
</tr>
<tr>
<td>CCB</td>
<td>calcium channel blocker</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>wild type mouse strain</td>
</tr>
<tr>
<td>CCR2</td>
<td>chemokine receptor 2</td>
</tr>
<tr>
<td>CCR3</td>
<td>chemokine receptor 3</td>
</tr>
<tr>
<td>CFM</td>
<td>cell free medium</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanidine monophosphate</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
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<tr>
<td>cm²</td>
<td>centimeter square</td>
</tr>
<tr>
<td>Cmax</td>
<td>maximum concentration</td>
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HMG-CoA  hydroxyl methyl glutaryl Coenzyme A
HPLC  high performance liquid chromatography
HSPs  heat-shock proteins
HUVEC  human umbilical vein endothelial cell
IHC  immunohistochemistry
ICAM-1  intra-cellular adhesion molecule-1
i.e.  that is
IMT  intima-media thickness
IP  isolated platelets
i.p.  intraperitoneal
IP-10  interferon-gamma-inducible protein of 10 kDa
ITT  intent-to-treat
KCl  potassium chloride
kDa  kilodalton
kg  kilogram
l  liter
Lac  lacidipine
LDL  low density lipoprotein
LDLR  low density lipoprotein receptor
L-NNA  N-omega-nitro-L-arginine
Lp(a)  lipoprotein (a)
LPL  lipoprotein lipase
M  molar
MCP-1 or MCP1  monocyte chemoattractant protein-1
mCFE  coated carbon fibre micro-electrode
mCi  millicurie
μCi  microcurie
M-CSF  macrophage colony stimulating factor
MDA  malonyldialdheyde
mg  milligram
μg  microgram
min  minute
μM  micromolar
<table>
<thead>
<tr>
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<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>μm²</td>
<td>micrometer square</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>mmHg</td>
<td>millimetres of mercury</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MMP-9</td>
<td>matrix metalloproteinase-9</td>
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<td>mol</td>
<td>mole</td>
</tr>
<tr>
<td>mN</td>
<td>millinewton</td>
</tr>
<tr>
<td>MPM</td>
<td>mouse peritoneal macrophage</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>mV</td>
<td>millivolt</td>
</tr>
<tr>
<td>nA</td>
<td>nanoAmpers</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric (or nitrogen) oxide (or monoxide)</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric (or nitrogen) oxide synthetase</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>OM</td>
<td>original magnification</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>oPD</td>
<td>ortho-phenylenediamine</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil red O</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>oxidized low density lipoprotein</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet-rich plasma</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>r</td>
<td>Pearson's correlation coefficient</td>
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<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>rpm</td>
<td>revolution per minute</td>
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<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SD</td>
<td>standard diet</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SM</td>
<td>smooth muscle</td>
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<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
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<td>SNAP</td>
<td>S-nitroso-N-acetylpenicillamine</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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<tr>
<td>SPSHR</td>
<td>stroke-prone spontaneously hypertensive rat</td>
</tr>
<tr>
<td>TBARS</td>
<td>thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper lymphocytes type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper lymphocytes type 2</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>t.l.c.</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>U.I.</td>
<td>international unit</td>
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<tr>
<td>uPA</td>
<td>urokinase plasminogen activator</td>
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<td>v</td>
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<td>V</td>
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<tr>
<td>VCAM-1</td>
<td>vascular cellular adhesion molecule-1</td>
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<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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<tr>
<td>WHHL</td>
<td>Watanabe heritable hyperlipidemic rabbit</td>
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<tr>
<td>WTD</td>
<td>Western-type diet</td>
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<tr>
<td>Zn^{2+}</td>
<td>zinc ion</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>zinc chloride</td>
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Chapter 1

INTRODUCTION

1.1 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) includes dysfunctional conditions of the heart, arteries, and veins that supply oxygen to vital life-sustaining areas of the body such as the brain, the heart itself, and other major organs. If oxygen is not supplied, the tissue or organ will die (Bing 1968).

Coronary artery disease is a condition in which fatty deposits (atheroma) accumulate in the cells lining the wall of the coronary arteries. These fatty deposits build up gradually and irregularly in the large branches of the two main coronary arteries which encircle the heart and are the main source of its blood supply. The excess build up of fat or plaque are termed arteriosclerosis and atherosclerosis, respectively. Of equivalent significance is inadequate oxygen supply to the brain, which causes stroke (Keaney 2000).

High blood pressure (hypertension) often results from excess fat or plaque build up in blood vessels due to the extra effort required to circulate the blood. Even though the heart works harder, the blockages in the vessels still reduce the required blood supply to all areas of the body. The survival systems of the body may mask for a time the subtle damage that is occurring due to this extra “wear and tear”. High blood pressure has been called "The Silent Killer" because the first warning sign may be an angina attack, or a fatal heart attack, or a stroke (Seedat 1981).
Kidney disorders (which may cause the retention of extra fluid, sodium ions, and toxic metabolites in the body), obesity, diabetes, contraceptive drugs, pregnancy, smoking, excess alcohol, stress, and thyroid and adrenal gland problems can also cause and exacerbate high blood pressure conditions (Rao & White 1993).

Damage to heart tissue from CVD, or from heart surgery, may disrupt the normal electrical impulses of the heart and result in cardiac arrhythmias (an abnormally high or abnormally low heart rate). For the patient, sudden fluctuations in heart rate may cause noticeable palpitations, with associated faintness, or dizziness, and if these are severely abnormal may interfere with blood flow and initiate a heart attack (Rossi 2003).

The maintenance of normal levels of cholesterol is also important in the prevention of heart attack or stroke. Total blood cholesterol above 200 mg/dl, low density lipoprotein (LDL) cholesterol above 130 mg/dl, high density lipoprotein (HDL) cholesterol below 35 mg/dl, and lipoprotein(a) levels greater than 30 mg/dl, are all indicators of cholesterol abnormalities. Changes in cholesterol levels may not actually cause injury in themselves, but are more an indicator of compromised liver function, and an increased risk of heart attack (Marques-Vidal et al. 1995).

Inflammation of the heart, myocarditis and endocarditis, are additional complications that may occur as a result of a compromised immune system, liver disease, heart surgery, or autoimmune disease such as rheumatic fever. Endocarditis is quite common in persons with compromised immune systems resulting from human immunodeficiency virus (HIV) or acquired immuno-
deficiency syndrome (AIDS). If not appropriately handled, permanent heart muscle damage can occur from such infections (Wasi & Shuter 2003).

1.1.1 Atherosclerosis

Atherosclerosis is a form of arteriosclerosis. The word atherosclerosis comes from the Greek “athero” (meaning gruel or paste) and “sclerosis” (hardness). Atherosclerosis involves the deposition of fatty substances, cholesterol, cellular waste products, calcium and fibrin, in the inner lining of an artery (Campbell & Campbell 1994). Atherosclerosis can affect, in particular, the arteries of the brain, heart, kidneys, other major organs, and the arms and legs (Weller 1979). When atherosclerosis develops in the arteries that supply the brain (Kwon et al. 1998), a stroke may occur (d'Uscio et al. 2001); when atherosclerosis develops in the arteries that supply the heart, a heart attack may result (Hess et al. 1993).

In Western countries and in the United States, atherosclerosis is the leading cause of morbidity and death. Despite significant medical advances, coronary artery disease (resulting from atherosclerosis), and atherosclerotic stroke, together are responsible for more deaths than all other causes combined (Komatsu & Sakurai 1996).

Atherosclerosis is a disease of the arterial intima (Fig. 1.1a and 1.1b) (Werns et al. 1989) leading to the formation of fibrous (atheromatous) plaques and to stenosis/occlusion of the arterial lumen. The condition involves the proliferation of smooth muscle cells and the accumulation of lipids. Atherosclerosis affects both large and medium-sized arteries. The type of artery and the site at which the plaque develops, varies from person to person.
The disease is progressive, involving many factors and a variety of mechanisms (Chilton 2004; Chahoud et al. 2004; Choudhury et al. 2004; Kolodgie et al. 2004).

Fig. 1.1a: Normal coronary artery: a normal coronary artery with a widely patent lumen and able to carry the normal requirement of blood to the myocardium. From Werns et al. (1989).

Fig. 1.1b: Severe coronary atheroclerosis: the lumen is narrowed by half. A small area of calcification is seen. From Werns et al. (1989).

1.1.2 Atherosclerotic lesions

The American Heart Association classification divides atherosclerotic lesions into six types, beginning with isolated foam cells ("fatty dots"), through stages of fatty streaks, atheromas, and fibroatheromas, to the more complicated lesions (Table 1.1) (Robbins 1999).
Table 1.1 Classification of atherosclerotic lesions.
From Robbins (1999)

Nomenclature and main histology

**Type I** (initial) lesion
Isolated macrophage foam cells

**Type II** (fatty streak) lesion
Mainly intracellular lipid accumulation

**Type III** (intermediate) lesion
Type II changes and small extracellular lipid pools

**Type IV** (atheroma) lesion
Type II changes and core of extracellular lipid

**Type V** (fibroatheroma) lesion
Lipid core and fibrotic layer, or multiple lipid cores and fibrotic layers, or mainly calcific, or mainly fibrotic

**Type VI** (complicated) lesion
Surface defect, hematoma-hemorrhage, thrombus

1.1.3 Arterial changes in the ageing process

Atherosclerosis causes harm by occluding the arteries slowly over time, or by occluding arteries suddenly by the rupture of plaques or weakening of the walls of the arteries. Arterial changes occurring with age may be due to three different causes (Oxenham & Sharpe 2003), such as intimal thickening, reduction in the elasticity of elastic tissues, or changes in lipoprotein composition; these changes are illustrated in Fig. 1.2 (Franklin 2002).
BIRTH

Endothelium
Fibroelastic intima
Internal elastic lamina
Media

AGEING

The intima thickens, and muscle cells penetrate the intima

Circular elastic fibers develop

Connective tissue develops internal to the intimal elastic fibers

Fig. 1.2: Arterial changes during the ageing process. From Franklin (2002).

1.2 ATEROMA

Atherosclerosis begins when monocytes migrate from the intimal blood into the intimal wall of the artery and begin to accumulate fatty material (Ludewig & Laman 2004). In time, increasing number of fat-laden monocytes accumulate, leading to a patchy thickening in the inner lining of the artery. Each area of thickening (an atherosclerotic plaque or atheroma) is filled with a
soft “cheeselike” substance consisting of various fatty materials, principally cholesterol, and smooth muscle cells and connective tissue cells (Stein & Stein 1995; Moreno & Mitjavila 2003). Atheromas may be scattered throughout the medium and large arteries, but usually form where arteries branch off; this is thought to be because the constant turbulence at these sites causes injury to the arterial wall, making it more susceptible to atheroma formation (Badak et al. 2003). The stages of atheroma development are shown in Fig. 1.3 (Riccioni et al. 2003).

**Fig. 1.3a:** Fibrous plaque: structure of a raised fibrous plaque. From Riccioni et al. (2003).

**Fig. 1.3b:** Calcification: hardening of the plaque by deposition of calcium salts, leading to possible rupture. From Riccioni et al. (2003).
Fig. 1.3c: Haemorrhage: blood may enter the ruptured atheroma, increasing lesion size. 
From Riccioni et al. (2003).

Fig. 1.3d: Ulceration: the contents of the ruptured atheroma may be discharged, leading to ulceration of the vessel wall. 
From Riccioni et al. (2003).

Fig. 1.3e: Thrombosis: the discharge of the ruptured atheroma and further injury of the vessel wall may lead to the formation of a thrombus (thrombosis) with possible occlusion of the artery. 
From Riccioni et al. (2003).
Arteries affected with atherosclerosis lose their elasticity, and as the atheromas increase in size, the arteries narrow. With time, the atheromas develop calcium deposits, and consequently may become brittle, and may rupture (Bini et al. 1999). Blood may then enter a ruptured atheroma, increasing the size of the lesion, so that the artery is narrowed even more. The ruptured atheroma may then discharge the contained fatty contents and trigger the formation of a thrombus (Falk & Fernandez-Ortiz 1995). The thrombus may further narrow, or occlude the artery, or possibly detach and cause vessel occlusion at another site (Hess et al. 1993; Casscells et al. 1996). A scheme illustrating the various possible stages of atheroma formation are shown in Fig. 1.4.

Fig. 1.4: Scheme summarizing the process of atheroma formation. From Libbi (2002).
1.3 LOW DENSITY LIPOPROTEIN

LDL is a plasma lipoprotein particle whose lipid component includes cholesterol and triglycerides. LDL has been referred to as “bad cholesterol” due to the role of LDL in promoting atherogenic heart disease. Plasma LDL originates from very-low density lipoprotein (VLDL) which is produced by the liver in association with the apoprotein, B-100. VLDL is converted to LDL by the action of lipoprotein lipase, an enzyme that hydrolyzes the triglycerides in VLDL, thus removing triglycerides from the VLDL particle and releasing free fatty acids. The removal of triglycerides from the VLDL particle by lipoprotein lipase leaves a greater proportion of cholesterol, increasing the density of the particle and changing it to LDL.

Atherogenic heart disease, as stated earlier, involves the formation of plaques in the arterial walls that narrow the arterial passage, restricting blood flow and increasing the risk of occlusion of the blood flow resulting in a myocardial infarction. One of the first steps in the development of atherogenic heart disease is the passage of LDL from blood in the arterial lumen and into the arterial wall. Once in the arterial wall, the lipids in LDL are chemically modified and oxidized. Oxidized LDL stimulates inflammatory signaling by neighboring endothelial cells, releasing chemokines and cytokines such as macrophage colony stimulating factor (M-CSF) and monocyte chemoattractant protein-1 (MCP-1) with the result that monocytes are recruited into the arterial wall (Babiy et al. 1992; Chang et al. 1993; Hayek et al. 1994; Cominacini et al. 1994a). Once in the arterial wall, monocytes differentiate into macrophages and internalize oxidised LDL. Macrophages continue to internalize LDL, becoming enlarged and full of lipid. The macrophages accumulate in the
arterial wall and are transformed into foam cells, which eventually die and form part of the atherogenic plaque. The involvement of LDL particle in atherosclerotic process is illustrated in Fig. 1.5.

**Fig. 1.5:** The involvement of LDL in atherosclerosis.
From Libbi (2002).

ACAT: acyl-CoA-cholesterol acyltransferase; IL-6: interleukin-6; LDL: low density lipoprotein; LDLR: LDL receptor; LPL: lipoprotein lipase; MCP1: monocyte chemoattractant protein-1; M-CSF: macrophage colony stimulating factor; VLDL: very low density lipoprotein.
1.4 ATHEROSCLEROSIS AND INFLAMMATION

It is only recently that there is a better appreciation of the mechanisms that link inflammation processes and dyslipidemia to atheroma formation. Leucocyte recruitment and the expression of pro-inflammatory cytokines characterize early atherogenesis, and the malfunctioning of inflammatory mediators is known to be associated with atheroma formation in mice. Moreover, inflammatory pathways promote thrombosis, a late and feared complication of atherosclerosis which is responsible for myocardial infarctions and the majority of strokes (Watanabe & Fan 1998).

At the beginning of the atherosclerotic process, light microscopy reveals the attachment of blood leukocytes to the endothelial cells of the intima lining. Under normal circumstances, the endothelial monolayer of cells in contact with the blood flow resists the adhesion of leukocytes. One endothelial-leukocyte adhesion molecule has recently emerged as a particularly attractive candidate for the early adhesion of mononuclear leukocytes to the arterial endothelium at sites of atheroma initiation (Libbi 2002). It is now known that vascular cell adhesion molecule-1 (Nakashima et al. 1998) binds in particular those leukocytes found in nascent atheroma: the monocyte and the T lymphocyte. Once adherent to the activated endothelial layer, the monocyte diapedeses between intact endothelial cells to penetrate into the tunica intima. However, this directed migration requires a chemoattractant gradient. Various chemokines appear to participate in this process, particularly the interaction of monocyte chemoattractant protein-1 (MCP-1) with its receptor chemokine receptor 2 (CCR2) (Libbi 2002). Once resident in the intima, the monocyte acquires characteristics of the tissue macrophage. In the atheroma, in
particular, the macrophage expresses scavenger receptors that bind internalized lipoprotein particles, modified, for example, by oxidation or glycation. These processes give rise to the arterial foam cell, a hallmark of the arterial lesion, so named because of the foamy appearance of the cell under the microscope, which is the result of the accumulation of lipid droplets within the cytoplasm. Within the arterial intima, the macrophage serves many functions related to atherosclerosis and the complications of atherosclerosis (Libbi 2002). Notably, the foam cell secretes pro-inflammatory cytokines that amplify the local inflammatory response in the atheromatous lesion, as well as producing reactive oxygen species (ROS). The activated mononuclear phagocyte has a key role in the thrombotic complications of atherosclerosis by producing matrix metalloproteinases (MMPs) that can degrade extracellular matrix which lends strength to the fibrous cap of the plaque (Newby 2005). When the plaque ruptures as a consequence, the blood comes into contact with another macrophage product, tissue factor, which is a potent pro-coagulant protein. Eventually the macrophages congregate in a central core in the typical atherosclerotic plaque. Macrophages may die in this location, some by apoptosis, hence producing the so-called “necrotic core” of the atherosclerotic lesion (Libbi 2002).
Blood monocyte Monocyte adhered to epithelium Monocyte migrating into intima Arterial lumen Monocyte becoming intimal macrophage From Libbi (2002).

Fig. 1.6: Mononuclear phagocytes in atherogenesis. Steps in the recruitment of mononuclear phagocytes into the nascent atherosclerotic plaque and some of the functions of the macrophage in the mature atheroma.

As in the case of the mononuclear phagocyte, lymphocytes also enter the intima, facilitated by binding to adhesion molecules including VCAM-1 (Nakashima et al. 1998) and also in response to chemoattractants selective for lymphocytes. Known chemoattractants include interferon and inducible chemokines of the alpha chemokines (CXC) family, and interferon-gamma-inducible protein of 10 kDa (IP-10) (Libbi 2002). These chemokines bind to chemokine receptor type 3 (CXCR3) expressed by T cells in the atherosclerotic lesion. Once resident in the arterial intima, the T cell may encounter antigens
such as oxidized low-density lipoprotein (Ox-LDL) and heat-shock proteins (HSPs) of endogenous or microbial origin, among others (Libbi 2002). Upon activation by the engagement of the receptor and antigen, the T cell can produce cytokines that can influence the behaviour of other cells present in the atheroma. Notably, CD154 binding to CD40 ligand, particularly on macrophages, may induce the expression of tissue factor, MMPs and pro-inflammatory cytokines. The production of these mediators provides an amplification loop resulting from crosstalk between the prototypical cell of acquired immunity (the T lymphocyte) and the cell of innate immunity, the mononuclear phagocyte. Within the atheroma, as in other tissues, the helper T cells (CD4) can polarize into those cells secreting generally pro-inflammatory cytokines, known as T helper lymphocytes type 1 (T\(_{H1}\) cells) and/or those cells secreting predominantly anti-inflammatory cytokines (denoted as T helper lymphocytes type 2 - T\(_{H2}\) cells). In general, T\(_{H1}\) cells predominate in the atheroma, but experimental data in mice suggest that with extreme levels of hypercholesterolaemia the balance may shift towards T\(_{H2}\) predominance. Recent evidence indicates that in abdominal aortic aneurysms, T\(_{H2}\) cytokines predominate, in contrast with the situation in occlusive atherosclerotic disease (Libbi 2002).

The leukocytic infiltrate within the atheromatous plaques includes a small but potentially important population of mast cells. Eotaxin, a chemoattractant that interacts with the chemokine receptor 3 (CCR3), may mediate the trans-endothelial migration of this specialized leukocyte. Once resident in the intima, the mast cell can undergo degranulation, releasing preformed tumour necrosis factor (TNF), heparin (with its anti-coagulant and
potentially growth inhibitory effects on smooth muscle cells), and the serine proteinases tryptase and chymase. These proteinases may activate the inactive zymogen forms of MMPs to their proteolytic forms. Chymase may also generate active forms of angiotensin from their precursor, angiotensin I (Libbi 2002).

1.4.1 Inflammation and plaque disruption

Two processes related to inflammation may participate in endothelial desquamation. The first, endothelial cell death (perhaps by apoptosis) may result from the local production of inflammatory mediators, or by cytolytic attack by activated killer T cells. Additionally, inflammatory mediators and oxidized lipoproteins can stimulate the expression and activation of MMPs specialized in degrading components of the sub-endothelial basement membrane (Newby 2005).

1.4.2 Metalloproteinases

MMPs are a family of Zn$^{2+}$-endopeptidases capable of cleaving components of extracellular matrix. This ability to modify the structural integrity of tissues is essential for certain aspects of normal physiology, including embryonic development, cell migration, wound healing, and tissue resorption. MMP modification of integrins, and the activation of certain cell-signaling cascades, also contribute to the regulation of platelet function (Newby 2005). However, the disregulation or activation of MMP expression is a feature of numerous pathological conditions, such as tumour metastasis, vascular and cardiac remodeling, and certain rheumatic conditions. Recently,
several lines of evidence have implicated MMPs in the rupture of atherosclerotic plaques and subsequent acute coronary syndromes. An enhanced expression of MMPs in the atherosclerotic lesion and their contribution to the weakening of the vascular wall by degrading the extracellular matrix has been reported (Watanabe & Ikeda 2004). The transcription, enzyme processing, and specific inhibition of MMPs by tissue inhibitors of matrix metalloproteinase (TIMPs) regulate these effects (Fig. 1.7) (Loftus et al. 2000). These processes are also modified by inflammatory cytokines and cell-cell contact signaling. Both animal experiments and the analysis of clinical samples have shown that a balance in expression and activation of MMPs, and inhibition by TIMPs, is critical for the development of stenotic and aneurysmal changes. Polymorphism in the MMP gene promoter contributes to inter-individual differences in susceptibility to coronary heart disease (Watanabe & Ikeda 2004). The development of therapeutic drugs specifically targeting MMPs may thus be useful for the prevention of atherosclerotic lesion progression, plaque rupture, and restenosis.
Fig. 1.7: Possible mechanisms involving metalloproteinases in plaque disruption processes. Proposed interaction of various matrix metalloproteinases (MMPs) that results in a cascade of activation of matrix degradation, tightly controlled by tissue inhibition. From Loftus et al. (2000).

MT: membrane; TIMP: tissue inhibitor of MMPs; uPA: urokinase plasminogen activator.

1.5 SYMPTOMS OF ATHEROSCLEROSIS

Usually, atherosclerosis does not produce symptoms until the condition severely narrows the artery, or until a sudden obstruction is caused. Symptoms depend upon where the atherosclerosis develops; thus, symptoms may reflect a problem in the heart, the brain, the legs, or other sites in the body (Chilton 2004).

As the developing atherosclerosis begins to severely narrow an artery, the areas of the body served by the artery may not receive sufficient blood, and
therefore sufficient oxygen. The first symptom of a narrowing artery may be pain, or cramps, at times when the blood flow can not keep up with the demand for oxygen in the tissues. For instance, during exercise the patient may feel chest pain (angina) due to a lack of oxygen to the heart, or alternatively while walking, the individual may feel leg cramps (intermittent claudication) because of a lack of oxygen to the legs (Chilton 2004). Typically, these symptoms develop gradually as the atheroma slowly narrows the artery. However, if an obstruction occurs suddenly, for example when a blood clot lodges in an artery, the symptoms develop rapidly.

1.6 RISK FACTORS

The risk of developing atherosclerosis increases with high blood pressure, high blood cholesterol levels, cigarette smoking, diabetes, obesity, a lack of exercise, and advancing age. Having a close relative who has developed atherosclerosis at an early age also puts a person at risk. Men have a higher risk than women, though after the menopause, the risk increases in women and eventually equals that in men (Chilton 2004).

People with the inherited disease homocystinuria develop extensive atheroma formation, particularly at a young age (Samson et al. 2004). This disease affects many arteries but does not primarily affect the coronary arteries. In contrast, in the inherited disease familial hypercholesterolemia, extremely high levels of blood cholesterol cause atheromas to form in the coronary arteries rather than in other arteries.
1.7 PREVENTION OF ATHEROSCLEROSIS

To help prevent atherosclerosis, the individual needs to eliminate the controllable risk factors, high blood cholesterol levels, high blood pressure, cigarette smoking, obesity, and lack of exercise (Stoy 1997). So, depending on the risk factors of a particular person, prevention may consist of lowering blood cholesterol levels, lowering blood pressure, quitting smoking, losing weight, and beginning an exercise programme. Fortunately, taking steps to achieve some of these goals also helps to achieve others. For instance, starting an exercise program assists a person to lose weight, which in turn helps lower cholesterol levels and reduce blood pressure. Quitting smoking helps lower cholesterol levels and reduce blood pressure.

In people who already have a high risk of heart disease, smoking is particularly dangerous (Lu & Creager 2004). Cigarette smoking decreases the level of “good” cholesterol (i.e. HDL cholesterol) and increases the level of “bad” cholesterol (LDL cholesterol). Smoking also raises the level of carbon monoxide in the blood, which may increase the risk of injury to the lining of the arterial wall, and smoking also constricts arteries already narrowed by atherosclerosis, further decreasing the amount of blood reaching the tissues. Furthermore, smoking increases the tendency of the blood to clot, and so smoking also increases the risk of peripheral arterial disease, coronary artery disease, stroke, and the obstruction of an arterial graft after surgery (Lu & Creager 2004).

The risk of coronary artery disease in a smoker is directly related to the number of cigarettes smoked daily. Individuals who quit smoking have only half the risk of those who continue to smoke, regardless of how long they
smoked before quitting. Stopping smoking also decreases the risk of death after coronary artery bypass surgery, or a heart attack. Additionally, quitting decreases morbidity, and the risk of death, in those who have atherosclerosis in arteries other than those that supply the heart and brain.

In short, the best treatment for atherosclerosis is prevention. When atherosclerosis becomes severe enough to cause complications, the medical practice is currently to treat the complications themselves, angina, heart attack, abnormal heart rhythms, heart failure, kidney failure, stroke, or obstructed peripheral arteries.

1.8 TREATMENT OF ATHEROSCLEROSIS

The treatment of atherosclerosis and the diseases related to atherosclerosis is at present undergoing a period of great change. Specific treatments have evolved largely through a better understanding of the pathophysiology of the disease. Much of this ground breaking research is the result of recent discoveries made by pathologists.

1.8.1 Cholesterol reducing drugs

Numerous studies (Sirtori & Colli 1993; Jacobson 2001; Raza et al. 2004) confirm the beneficial effects of controlling blood cholesterol levels by the use of drugs. Maintaining cholesterol levels within the normal range not only assists in preventing heart attacks and strokes, but may also prevent the progression of atherosclerosis. The drugs currently in use (Bellosta et al. 1998) include hydroxyl methyl glutaryl coenzyme A inhibitors (statins e.g. atorvastatin, fluvastatin, lovastatin, pravastatin, etc.), resin drugs (e.g.
colesevelam, colestipol, etc.), and fibrate drugs (e.g. bezafibrate, gemfibrazole, clofibrate, finofibrate, etc.). A combination of dietary modification and medication may be necessary to maintain cholesterol levels within the desirable range.

1.8.2 Anti-hypertensive agents

While anti-hypertensive drugs do not control the process of atherosclerosis, these agents are successful in controlling blood pressure levels. These drugs include diuretics, beta-blockers, alpha blockers, alpha-beta blockers, sympathetic nerve inhibitors, angiotensin-converting enzyme (ACE) inhibitors, calcium channel blockers, calcium antagonists, and angiotensin receptor blockers (sartans) (Kohlman-Trigoboff 2004).

1.8.3 Calcium antagonists

It has been shown that various calcium antagonists (CA) may delay plaque formation in animal models (Ginsburg et al. 1983b; Lichtor et al. 1989b; Skepper & Kappagoda 1992; Cristofori et al. 2000b). Although the mechanism of this beneficial effect is not well clarified, it appears unrelated to the antihypertensive properties of this class of drugs. CA have been shown to modulate low density lipoprotein metabolism and directly interfere with the major process of atherogenesis occurring in the arterial wall. These drugs inhibit smooth muscle cell (SMC) migration and proliferation, and cholesterol accumulation is prevented in macrophages by inhibiting acyl-CoA-cholesterol acyltransferase (ACAT) activity, both in vitro and in vivo (Grossman & Messerli 2004).
First generation CA (e.g. verapamil, diltiazem, nifedipine, etc.) were characterised by a short duration of action, in the order of a few hours, and this was paralleled by an equally short plasma half-life. Therefore, the activity of a CA with such properties was strongly coupled with the length of time the drug remained in the plasma compartment. These first generation CA were characterised by a rapid onset of action, since they reached an optimal plasma concentration in less than 2 hours. They were also shown to be highly amphiphilic, because they could rapidly transport into, and out of, and across cellular membranes. However, the problem with drugs in this category included an elevated degree of side effects, including headaches, nausea, and ankle oedema and flushing (Grossman & Messerli 2004).

1.8.4 Lipophilic calcium antagonists

The new lipophilic, and intrinsically long-acting CA, namely lacidipine, amlodipine, and lercanidipine, are now available for clinical use (Hernandez et al. 2003). The long duration of action of these drugs is derived from distinct molecular mechanisms. Amlodipine has a rate-limiting transport across membranes and has a long plasma half-life, and this is equal to its duration of action. Lacidipine and lercanidipine are more lipophilic than amlodipine (Gaviraghi & Trist 1998).

The clinical efficacy of CA results from their physiological effect on vasodilating the arterial network. This activity depends on the high solubility of the drug in the vascular smooth muscle cell (SMC) membrane. The drugs bind to calcium-channel receptors and block calcium ion movement into the cell, resulting in the relaxation of smooth muscle cells with a concomitant
dilatation of blood vessels, thus lowering peripheral resistance and blood pressure. Studies have shown that most CA of the 1,4-dihydropyridine type (e.g. lacidipine, amlodipine, felodipine, lercanidipine, nifedipine, etc.) interact with calcium channels in a location near the surface of the cell membrane (Zanchetti et al. 2002). Diseases such as atherosclerosis affect the amount of cholesterol in the cell membrane where the calcium channels are located. The presence of increasing amounts of non-esterified cholesterol in membranes affects the binding of CA to the membranes and increases the concentration of CA within the membrane bilayer compartment. Thus, the cholesterol content in a target membrane can be a determinant of biological activity, and the clinical half-life of the drug. This may be important in the light of the finding that hypertension is often associated with atherosclerosis and elevated levels of cholesterol in membranes. It has been shown that the antihypertensive action of CA cannot be considered as being simply due to vasodilatation, and that prevention of the secondary complications of hypertension is not necessarily related only to a reduction in blood pressure (Paoletti et al. 1996; Gaviraghi & Trist 1998; Sobal et al. 2001; Hernandez et al. 2003; Grossman & Messerli 2004).

1.8.5 Lacidipine

Lacidipine (Fig. 1.8), like other dihydropyridine CA drugs, impairs vascular smooth muscle contraction by decreasing transmembrane calcium-influx as a result of blocking voltage-dependent L-type calcium channels through binding to the dihydropyridine receptor (Zanchetti et al. 2002).
By virtue of its lipophilic properties, lacidipine has a slow onset and a long duration of action. Lacidipine readily accumulates in the lipid bilayers of the plasma membrane, and possibly other tissue compartments, forming a reservoir in close proximity to calcium channel dihydropyridine receptors. The continuous and slow release of the drug from these reservoirs is considered to account for the slow onset of activity, and the sustained vasodilatory action, which is characteristic of lipophilic dihydropyridine calcium antagonists.

Dihydropyridine CA are vascular selective in that they have greater effects on vascular smooth muscle than on myocardial or nodal cardiac tissues. Their vascular selectivity is greater than those of the phenylalkylamine or benzothiazepine classes of CA. Therefore, dihydropyridine CA cause less cardiodepression. Lacidipine has greater vascular selectivity than most other dihydropyridine CA (Bernini et al. 1996).

As with dihydropyridine CA as a class, the major effect of lacidipine is vasodilation, predominantly on arteriolar vessels, rather than on venous blood vessels. Accordingly, lacidipine reduces total peripheral vascular resistance,
the elevation of which is the most consistent haemodynamic alteration in essential hypertension.

There is evidence to suggest that CA, especially the dihydropyridines, may retard the development of atherosclerotic lesions in the coronary circulation. It has been hypothesized that both the antioxidant and the CA properties of lacidipine might interfere with the early stages in the cascade of events that ultimately leads to the development of atherosclerotic plaques. In vitro and animal studies have shown that lacidipine reduces the levels of proinflammatory oxidized LDL, prevents adhesion molecule upregulation, inhibits macrophage cholesterol esterification, reduces the proliferation and migration of smooth muscle cells into the vascular intima, and reduces the incidence and size of atheromatous lesions (Napoli et al. 1999).

1.8.5.1 European Lacidipine Study on Atherosclerosis

The European Lacidipine Study on Atherosclerosis (also called ELSA), the largest study of its kind to date, was designed to compare the effects of lacidipine at 4-6 mg/day, with those of atenolol at 50-100 mg/day, for 4 years, on the progression of carotid artery intima-media thickness (IMT) as an index of atherosclerosis in patients with hypertension. After 4 years of therapy, the mean change in IMT was lower in lacidipine- than atenolol-treated patients, but the difference did not reach statistical significance in the intent-to-treat (ITT) population. However, the mean change in IMT was significantly (P<0.01) lower in lacidipine- than atenolol-treated patients for the population of patients completing the full 4 years of the study (the “completers”). Analysis of estimated treatment effect (lacidipine minus atenolol) showed significant
(P<0.001) differences favouring lacidipine for both ITT and completer populations. Plaque formation was also significantly (P<0.05) less frequent in the lacidipine-treated patients.

In the long-term treatment of elderly patients with isolated systolic hypertension, the incidence of cardiovascular events (stroke, sudden death, myocardial infarction, congestive heart failure or transient ischaemic attacks) with lacidipine was similar to that with chlorthalidone over 5 years. Similarly, in the ELSA study, there was no significant difference between the lacidipine and atenolol treatment groups in the incidence of cardiovascular events and death during 4 years of treatment (Zanchetti et al. 2002; Guidelines Committee 2003).

1.9 ANIMAL MODELS OF ATHEROSCLEROSIS

Given the complexity of atherosclerotic lesion development in man, there is a challenge to develop animal models that closely mimic the human disease (Armstrong & Heidstad 1990). One must accept, however, that there is no one perfect animal model that completely replicates the stages of human atherosclerosis, but several models are useful in studying specific pathologic processes associated with the disease. Irrespective of species, there are various common features shared by most models of atherosclerosis. Firstly, the induction of the vascular lesion in most animal models is dependent upon development of a plasma hypercholesterolemia (Fekete 1993). Plasma cholesterol elevation can either be induced by dietary supplementation with cholesterol, hepatic overproduction of lipoproteins or genetic mutation of receptors and/or receptor ligands responsible for lipoprotein clearance.
Secondly, to accelerate the development of atherosclerotic lesions in hypercholesterolemic animals, various forms of acute or chronic endothelial damage have been employed. The animal models differ with respect to the degree of dietary cholesterol supplementation, length of hypercholesterolemia, dietary regimen and type, and the duration and the degree of mechanical endothelial injury (Armstrong & Heidstad 1990).

1.9.1 Rabbit

The cholesterol-fed rabbit has been extensively used as a model of atherosclerosis since the identification by Anitschkow in 1913 that short-term cholesterol feeding results in the formation of foamy lesions within the aorta. Historically, supplementation of commercial rabbit chow with 1 to 3% cholesterol and 4 to 8% fat for 6 to 8 weeks has resulted in marked elevations in plasma cholesterol, i.e., from 1000 to 3000 mg/dl, cholesteryl ester accumulation in hepatic and peripheral tissues and the development of aortic macrophage foam cell enriched lesions (Jokinen et al. 1985). Development of atherosclerosis in the coronaries is limited to the small intra-myocardial vessels and not the larger epicardial vessels as has been found in man. The rabbit atherosclerotic lesions were reminiscent in cellular composition to Type I to III human lesions (see Table 1.1). Kritchevsky (Kritchevsky 1975) has performed numerous cholesterol feeding experiments in which the cholesterol supplementation was maintained as a constant, i.e. 2%, and the type of dietary fat was altered to further refine the role of cholesterol metabolism in atherosclerosis progression. A notable finding was that with the addition of 6% peanut oil, or 6% coconut oil, to a 2% cholesterol diet, two histologically
distinct atherosclerotic lesions developed. Peanut oil supplementation produced aortic lesions that contained relatively little lipid but abundant smooth muscle cell proliferation and collagen deposition. In contrast, addition of 6% coconut oil to the diet resulted in lesions with demonstrable intracellular lipid and intima proliferation; however, less collagen and elastin were evident. Although elevated plasma cholesterol levels induce atherosclerotic lesions, and dietary fat composition may affect the cellular composition of the lesion in rabbits, prolonged hypercholesterolemia results in exponential cholesterol enrichment of many peripheral organs (Vesselinovitch 1988).

Rabbit models of atherosclerosis have been developed which limit the amount of dietary cholesterol supplementation; however, such models are time consuming and for that reason may have limited utility for screening anti-atherosclerotic agents. Wilson and colleagues (Wilson et al. 1992) fed rabbits an agar-gel diet containing 19% butter and 1% corn oil for up to 5 years. Plasma total cholesterol levels were approximately 300 mg/dl, and over the course of 5 years atherosclerotic lesions representing Type I to V lesions (see Table 1.1) were noted. Advanced atherosclerosis can also be induced in a shorter time by the intermittent feeding of a 1% cholesterol, 5% cottonseed oil diet for 2 months, followed by 6 months of a chow diet and 2 additional months of the cholesterol diet (Kratky et al. 1993). While plasma cholesterol levels fluctuated with dietary cholesterol supplementation, the five stages of atherosclerosis were present in both aorta and coronary arteries. Protracted feeding of a low cholesterol diet or intermittent feeding of high and low cholesterol diets produced histologically similar atherosclerotic lesions. Given the disparate plasma cholesterol levels, these data suggest that the lipoprotein
profile may play an important role in the rate at which atherosclerotic lesions develop (Kratky et al. 1993). Feeding studies have indicated that beta-VLDL was the primary lipoprotein in rabbits fed a cholesterol diet while LDL-like particles predominated in animals fed a semi-synthetic casein-enriched diet. Morphologic and morphometric analysis of rabbits fed either a 0.125% to 0.5% cholesterol or casein-enriched diet for 6 months revealed that atherosclerotic lesions developed in both models; however, the nature and extent of lesions varied (Daley et al. 1994). At comparable plasma cholesterol levels, the cholesterol-fed rabbits had approximately twice the extent of aortic atherosclerosis relative to the casein-fed animals and Type IV-V lesions (Table 1.1) predominated. In the casein-fed rabbits, 30% of the aorta contained atherosclerotic lesions that ranged in appearance from fatty dots to fibrous plaques with a necrotic lipid-rich core (Daley et al. 1994). These data indicate that under a similar time frame and plasma cholesterol level the type of dietary supplementation can affect the quantity and type of atherosclerotic lesion that develops primarily by altering the major cholesterol carrying lipoprotein, i.e., beta-VLDL or LDL.

Genetic rabbit models of atherosclerosis, namely, the homozygote Watanabe heritable hyperlipidemic rabbit (WHHL) (Buja et al. 1983), which lacks functional LDL receptors, have also been compared to cholesterol-fed rabbit models. Like the casein-fed rabbit, plasma cholesterol was primarily distributed in LDL (Buja et al. 1983). In WHHL rabbits, leukocyte margination, subendothelial accumulation of isolated lipid-filled macrophages, accumulation of SMC and formation of fatty streaks occurred over the first 4 weeks of life (Buja et al. 1983). A similar sequence of lesion formation was
noted in New Zealand White rabbits fed a 0.1% to 0.2% cholesterol diet (Chiba et al. 1997). Expansion of the lipid-filled monocyte-macrophage rich lesions, i.e., Type I-III fatty streaks, occurred during the first 6 months in both types of rabbits while complex Type V fibrous plaque lesions were noted in the WHHL and cholesterol-fed rabbits by 13 months of age. An enrichment of cholesteryl ester, primarily cholesteryl oleate, was noted in the aorta of both animals over the course of 13 months and such a finding was consistent with the morphologic data previously seen (Stewart-Lee & Burnstock 1991).

Hypercholesterolemia in conjunction with mechanical denudation of the endothelium in various vascular regions of the rabbit have been utilized to develop shorter-term models of atherosclerosis with a high degree of predictability as to the location and type of atherosclerotic lesion (LeVeen et al. 1982). Acute mechanical injury of the arterial vessel wall can be achieved using a variety of methods (Dufourcq et al. 1997).

In summary, hypercholesterolemic rabbits are valuable models and the most widely used model for the evaluation of pharmacologic agents. Different types of human-like atherosclerotic lesions can be induced in the rabbit; however, the model is limited in that evidence of complicated ruptured fibrous plaques cannot be found. Rabbits are also valuable for atherosclerosis research because unlike other models, atherosclerotic lesions progress even after removal of dietary cholesterol supplementation. Evaluation of the direct anti-atherosclerotic properties of hypocholesterolemic agents requires normalization of plasma cholesterol levels by diet prior to drug administration. Since rabbit anti-atherosclerotic lesions will develop further and become more complex following cholesterol removal, agents such as ACAT inhibitors,
which act by directly altering cellular processes that limit macrophage accumulation, can be evaluated and their effect on lesion progression/regression can be monitored (Fekete 1993).

1.9.2 Hamster

Another model of atherosclerosis that has received attention recently is the hypercholesterolemic hamster (Bocan 1998; Cristofori et al. 2000a). Male hamsters fed a 3% cholesterol, 15% butterfat diet for up to a month had elevated plasma cholesterol levels and the presence of Type I fatty dots and fatty streaks within the aortic arch. Within 3 to 4 months of the administration of the very high cholesterol/fat diet, expansion of the fatty streaks into the thoracic aorta around sites of the intercostal ostia was noted. By 10 months of cholesterol supplementation, when plasma cholesterol levels were 17-times normal, advance Type V lesions were observed in the aortic arch of the hamster but their extent was quite limited, i.e., 30% of the cross-sectional vessel surface. Feeding hamsters a 0.2% cholesterol, 10% coconut oil diet for 10 weeks, or a 0.05% cholesterol, 10% coconut oil diet for 8 weeks, resulted in the accumulation of monocyte-macrophages within the aortic arch (Mangiapanne et al. 1999). Thus, short-term feeding of a cholesterol and either a coconut oil or butter-fat diet to hamsters is a model of subendothelial monocyte-macrophage foam cell formation. Atherosclerotic lesions can be found predictably in this animal model within the inner curvature of the aortic arch and can be identified by staining with the lipid dye, Oil Red O. A model such as the hamster is useful due to its size for the acute evaluation of agents
that may interfere with the early stages of lesion formation, e.g., monocyte adherence, transmigration and foam cell formation.

The hypercholesterolemic hamster has also been used for the evaluation of numerous pharmacologic agents with varying mechanisms of action. This model has proven to be useful for the assessment of compounds; however, the changes in plasma cholesterol and blood pressure tend to confound the interpretation of the anti-atherosclerotic data, and limit the ability of the experimenter to ascribe any activity to a direct effect of the compound (Bocan 1998).

1.9.3 Swine

Swine are a useful non-rodent model of atherosclerosis in which atherosclerotic lesions have been found to develop spontaneously. The pathogenesis of the lesion development in pigs has been shown to closely parallel the stages of lesion formation in man. Unlike the rabbit and hamster, where lesions predominate in the aorta, atherosclerotic lesions have been observed in both the cerebral and coronary vessels of the pig. Thus, swine are a useful model for the evaluation of atherosclerosis from the perspective that lesions develop spontaneously, the circulatory system and the localisation of lesions in the pig are similar to man, and the lesions are also responsive to dietary intervention by exhibiting regression after prolonged periods (Carey 1997).

However, despite their similarity to man, swine are not widely used for the evaluation of anti-atherosclerotic agents, and considering that miniature swine weigh approximately 12 kg at 4 months of age, such a model may be
limited to the evaluation of the anti-atherosclerotic potential of compounds during their development stages rather than discovery phases (Hughes 1986).

1.9.4 Monkeys

Non-human primates have often been portrayed as ideal models for human atherosclerosis due to their close phylogenetic relationship to man. The morphological characterisation of atherosclerotic lesion progression and regression has been performed in both cynomolgous and rhesus monkeys. Spontaneous development of atherosclerotic lesions is rare in non-human primates; however, cholesterol feeding has been shown to promote the development of atherosclerosis in the monkey (Armstrong & Heidstad 1990). Few studies have been performed using either dietary or pharmacologic intervention to promote lesion regression.

Despite an elaborate characterization of atherosclerosis in primates, their usage has been relatively limited. New World monkeys are rarely used, with the exception of the squirrel monkey. Old World monkeys however have been of primary interest and at least seven species of macaques have been used in atherosclerosis research. Common macaque models in atherosclerosis research are the stumptail, rhesus, cynomolgous, and the pigtail (Vesselinovitch 1988). Primates share similar advantages and disadvantages, with few specific individual features. Significant disadvantages include large sizes, difficulty in handling, high cost, maintenance difficulties, decreasing availability, and government regulations toward species protection. However, the phylogenetic resemblance of primates to humans is a large singular advantage. Lesions are extremely similar to the human type and also
demonstrate plaque mineralization and other complications (Taylor & Cox 1962). Myocardial infarction, a common sequela of atherosclerotic thrombosis in the coronary artery in humans, is also a complication found in the cynomolgous macaque (Pick et al. 1974).

The rhesus monkey has been the most extensively used primate as an animal model (Taylor & Cox 1962). Most macaque species are endangered and are, therefore, banned for research purposes, unless domestically bred in captivity (Pick et al. 1974). In the presence of hypertension, the stumptail macaque demonstrates an increase in the extent and severity of lesions and, consequently, this species has been used as a model to study the effects of hypertension on atherosclerosis (Pick et al. 1974). Sexual differences to the susceptibility of the disease are evidenced in both the pigtail and the cynomolgous (Bond et al. 1976; Hamm et al. 1983). Pigtail macaques have been used to study the neurophysiologic control of cardiovascular function (Bond et al. 1976).

Apart from the squirrel monkey, which is the most extensively studied New World primate, two other species that have also been used as models for atherosclerosis research are the African green monkey and the baboon. Limitations to the use of the squirrel monkey include its small size and limited availability because importation of this animal is banned. Both natural and induced forms of the disease occur in the squirrel monkey and mature plaques and lipoprotein levels, similar to those of humans, can be induced (Middleton et al. 2005). Individual variability to disease development is high and is influenced by age, sex, genetic predisposition, and co-morbidities (Middleton et al. 2005). Dietary induction is successful in the African green monkey and
this primate has been used to study the effects of oral contraceptives on plasma cholesterol (Clarkson et al. 1976; Kritchevsky et al. 1977). The baboon, on the other hand, is resistant to dietary induction. It does, however, develop spontaneous disease and has been used for studies on cholesterol metabolism (Strong & McGill 1967).

1.9.5 Transgenic mice

Due to advances in molecular biology and the realization that mice, in general, are normally resistant to the development of atherosclerosis, genetically engineered mice have been developed which are predisposed to hypercholesterolemia-induced disease. Two well-characterized transgenic mouse models of atherosclerosis are the apolipoprotein E (apoE)-deficient mouse and the low density lipoprotein (LDL) receptor-negative mouse. ApoE is a major component of plasma lipoproteins that has a high affinity for LDL receptors and chylomicron remnant receptors and may be important in facilitating reverse-cholesterol transport from peripheral tissues (Nakashima et al. 1994; Paigen et al. 1994). The apoE-deficient mouse has been shown to be hypercholesterolemic, i.e., 400 to 700 mg/dl at 5 to 55 weeks of age (Yoshida et al. 2001), while maintained on a chow diet. Atherosclerotic lesions develop naturally over the time frame of 11 to 64 weeks within the aortic sinus and exhibit a similar histologic appearance as Type I to V lesions. Monocyte-macrophage foam cells predominate either as individual cells or clusters in the early stage of lesion development, i.e., less than 28 weeks, while fibrosis, intimal necrosis, acellular and necrotic lipid-rich cores with evidence of cholesterol clefts can be found after 32 weeks of age (d’Uscio et al. 2001).
similar histologic pattern can be seen in apoE-deficient mice fed a Western-type diet, i.e., 0.15% cholesterol; however, the timecourse of lesion development is shorter and the extent of atherosclerosis is increased. Cholesterol-fed apoE-deficient mice have plasma cholesterol levels of 1000 to 4400 mg/dl over 6 to 40 weeks of age. Evidence of Type IV-V complex fibrous plaques (Table 1.1) can be seen as early as 15 weeks and the lesions are not only present in the aortic sinus but are also associated with the bifurcations of such major branch vessels as the common carotids, celiac, mesenteric, renal and iliac arteries (Nakashima et al. 1994; Paigen et al. 1994).

The LDL receptor-negative transgenic mouse has also been developed. Unlike the apoE-deficient mouse, atherosclerotic lesions do not occur naturally during the timeframes currently studied, i.e., 6 months. Dietary supplementation with 0.15% cholesterol results in plasma cholesterol levels of 900 to 1000 mg/dl over 6 months in conjunction with the development of atherosclerotic lesions within the aortic sinus. The morphologic appearance and extent of atherosclerosis in the LDL receptor-negative mouse is similar to the comparably fed apoE-deficient mouse; however, plasma cholesterol levels are half that noted for the apoE-deficient mouse. Thus, both the apoE and LDL receptor-deficient mouse are viable small animal models for the evaluation of atherosclerotic lesion progression (Nakashima et al. 1994; Paigen et al. 1994).

Numerous other relevant transgenic models have also been developed. Some of these transgenic mouse models of potential relevance to atherosclerosis, from the perspective of lipoprotein metabolism, are the human apolipoprotein B model, the apolipoprotein (a) model, and the Lp(a) and the cholesteryl ester transfer protein (CETP) transgenic models. In addition, one
might predict that site specific deletions or overexpression of pro-
atherosclerotic molecules such as adhesion molecules, growth factors,
cytokines or integrins, for example, would be useful models for the assessment
of direct acting anti-atherosclerotic agents. A caveat to such an approach is
exemplified by the comparison of the apoE- and LDL receptor-deficient mice.
Both genetic defects have resulted in similar atherosclerotic lesion pathology
and have required some degree of hypercholesterolemia (Nakashima et al.
1994; Paigen et al. 1994). Therefore, temporal evaluations of lesion
development in the presence and absence of pharmacologic agents may be
more informative in assessing whether the specific gene product/defect
exacerbates disease progression and whether pathologic redundancies limit the
efficacy of the specific pharmacological entity.

1.10 THESIS RATIONALE AND STRATEGY

The primary objective of the present investigations was to collect in
vivo experimental evidence on the anti-atherosclerotic properties of lacidipine.

In addition to the anti-hypertensive activity of lacidipine, the ability of
lacidipine to provide vascular protection was previously demonstrated in
spontaneously hypertensive rats (SHRs) and salt-sensitive Dahl-S rats
(Cristofori et al. 1991).

Atherosclerosis is a progressive disease affecting the arterial wall. The
formation of atherosclerotic lesions is a multifactorial process that involves
several mechanisms including: endothelial damage, lipid accumulation in the
intimal tissue of the arterial vessel, smooth muscle cell proliferation and
migration from the medial layer to the intimal layer of the artery, and the
synthesis and accumulation of components of the extracellular matrix. Several pieces of evidence from in vitro studies indicated that lacidipine could have a role in mediating these mechanisms. In particular, the antioxidant properties of lacidipine could play an important role by reducing intracellular reactive oxygen species, a key component in pathogenetic pathways of the atherosclerotic process (Gaviraghi & Trist 1998).

Initially it was planned to investigate the effect of lacidipine treatment on the progression of atherosclerotic lesions in the hamster model. In the hamster, as in man, cholesterol is carried in the LDL fraction. In addition, the hamster is susceptible to diet-induced atherosclerosis.

The next stage of investigation was to use transgenic animals as these represent a genetically reproducible, and therefore a more controlled, experimental model. The apoE-deficient transgenic mouse was considered to be a promising model, according to the recent literature, since these mice develop spontaneous hyperlipidaemia and hypercholesterolemia, as well as lesions of atherosclerosis closely resembling, in both appearance and distribution, those observed in man (Zhang et al. 1992; Nakashima et al. 1994; Paigen et al. 1994; Qiao et al. 1994; Tangirala et al. 1995b). Initial work was to be aimed at characterizing the extent of the atherosclerotic lesions in the aorta by morphometry, both with en face entire aortic preparations and on histological sections of the aortic root. The relationship with plasma endothelin levels, a biomarker of atherosclerotic progression, would also be evaluated in control and lacidipine-treated mice.

Therefore the effect of a high fat diet would be assessed, as this is a well-known risk factor for human atherosclerosis, in control and lacidipine-treated
apoE-deficient mice. In addition, the susceptibility of plasma LDL to oxidation would be evaluated in biochemical investigations (malonyldialdehyde-MDA measurement) and in an in vitro model (human umbilical vein cell cultures-HUVECs). Furthermore, the involvement of the vascular nitrogen monoxide system, an important mediator of vascular functionality, would be characterized using voltammetric techniques in aortic samples taken from both control and lacidipine-treated apoE-deficient mice.

Finally, the immunohistochemical characterization of atherosclerotic lesions would be performed to compare lesions in control and lacidipine-treated apoE-deficient mice. In particular, the role of lacidipine in matrix metalloproteinase (Bellosta et al. 1998) expression in atheroma would be assessed. MMP activity is considered to be responsible for acute plaque disruption and the subsequent onset of clinical ischemic events.
Chapter 2

MATERIALS AND METHODS

2.1 ANIMALS

Experimental work has been conducted using different animal models: the golden Syrian hamster, the apoE-deficient mouse and the C57BL/6J mouse. Animals were housed three per cage on wood shavings; a temperature of 22°C ±1°C was maintained, with a relative humidity of 45-70% and a 12:12 light:dark cycle (lights on at 06.00 h). Animals were acclimatised for at least 5 days before the start of each experiment.

The research complied with national legislation and the work was performed under a Project Licence obtained according to Italian law (art.7, Legislative Decree n. 116, 27 January 1992) with acknowledgement of the European Directive 86/609/EEC and with the GlaxoSmithKline policy on the Care and Use of Animals, and with related codes of practice.

2.1.1 Hamster

Male golden Syrian hamsters 10 weeks old (mean body weight 110 g) were obtained from Charles River Ltd., Italy. Animals were observed daily, and body weights and food intake were determined weekly; animals were randomly assigned to dose groups using computer-generated tables.
2.1.2 ApoE-deficient and C57BL/6J mice

Homozygous, female, apoE-deficient mice were obtained from GlaxoSmithKline Research and Development, Ware, UK. This colony was established from animals purchased from the Jackson Laboratory, which originated from apoE-deficient mice first engineered at the University of North Carolina in the laboratory of Dr. Nobuyo Maeda (Chapel Hill, North Carolina, Bar Harbor, ME, USA) (Piedrahita et al. 1992; Zhang et al. 1992). Dr. Maeda inactivated the mouse apoE locus by homologous recombination. Animals homozygous for the modified apoE gene are devoid of apoE protein in their sera. These mice appear to develop normally, however they exhibit five times the normal serum plasma cholesterol level and develop spontaneous atherosclerotic lesions. The mice are on a C57BL/6J genetic background and females of this inbred strain (obtained from GlaxoSmithKline Research and Development) were used as one of the control groups.

In the first study, apoE-deficient and C57BL/6J mice were fed a standard rodent diet containing 4.4% fat (weight/weight) and 0.2% cholesterol (weight/weight) (Altromin R; Altromin Rieper S.p.A, Vandoies, Bolzano, Italy). In the subsequent studies, mice received a Western-type rodent diet (Adjusted Calories Diet, Harlan Tekland TD88137, Madison WI, USA), containing 42% fat from milk fat and 0.15% cholesterol. In all studies, diet and drinking water were available ad libitum. Animals were observed daily, and body weights and food intake were determined weekly.
2.2 DOSING

Lacidipine (GlaxoSmith Kline) was administered daily by gavage at dose levels of 0 (control), 0.3, 1.0, 3.0, 10.0 mg/kg body weight in 0.5% methyl cellulose (Sigma, St. Louis, USA), at a standard dose volume of 10 ml/kg body weight. Control animals (apoE and wild type) were dosed with vehicle alone.

2.3 BIOCHEMICAL MEASUREMENTS: IN VIVO STUDIES

2.3.1 Blood samples for lipidic profile

*In the hamster study:* at the end of the treatment period, animals were sacrificed under ether anesthesia and blood was collected from the abdominal aorta for determination of serum lipids and lipoprotein cholesterol. Blood was anticoagulated with 1mM tripotassium ethylenediaminetetraacetic acid (EDTA), and centrifuged at 13,000 rpm for 3 min at 4°C. Total cholesterol and triglycerides were quantified by enzymatic methods with commercial kits (Boehringer Mannheim GmBH), using a Cobas Bio analyzer. Calibration for the different species was not necessary. High density lipoprotein (HDL) cholesterol was assayed after apo-b precipitation by a phosphotungstate method using a commercial kit (Boehringer Mannheim GmBH). Low density lipoprotein (LDL) cholesterol was calculated by subtracting the HDL value from the total cholesterol value.

*In apoE-deficient mouse studies:* at the end of treatment period, mice were fasted overnight and killed under pentobarbital anaesthesia (60 mg/kg, i.p.) by withdrawal of blood from the vena cava. Blood was anticoagulated with 1mM tripotassium ethylenediaminetetraacetic acid (EDTA), and centrifuged at 13,000 rpm for 3 min at 4°C. Total plasma cholesterol and
triglyceride levels were determined with an automated enzymatic technique (Boehringer Mannheim GmBH). Separation of total lipoprotein was performed by sequential density ultracentrifugation with a TLA-100.2 rotor (Optima TL Ultracentrifuge, Beckman Instruments, Palo Alto, CA, USA) in NaBr solution at a final density of 1.019, 1.063 and 1.210 for very low density lipoprotein (VLDL), LDL and HDL, respectively, with a total centrifugation time of 5 h at 16°C (Cristofori et al. 2000b). Physical separation of LDL was required in the apoE-deficient mouse studies in order to evaluate some LDL functions (susceptibility to oxidation, hydroperoxide content, etc.).

2.3.2 Plasma endothelin

Endothelin-1 is a 21 amino acid peptide and was measured by an enzyme immunoassay (EIA) (Cayman Chemical Co. MI, USA) on plasma, from apoE-deficient and C57BL/6J mice, which was stored at -70°C. The antibody provided by the kit is specific for human endothelin; however it has been reported that human and mouse endothelin-1 are almost identical in their sequence (Biondo et al. 2003). This immunometric assay is based on a double-antibody “sandwich” technique. Each well of the microtiter plate supplied with the kit has been coated with a monoclonal antibody which is specific for endothelin (endothelin capture antibody). This antibody will bind any endothelin introduced into the well. An acetylcholinesterase:Fab’ conjugate (AChE:Fab’), which binds selectively to a different epitope on the endothelin molecule, is also added to the well. This allows the two antibodies to form a “sandwich” by binding on opposite sides of the endothelin molecule. The “sandwiches” are immobilized on the plate so the excess reagents may be
washed away. The concentration of the analyte is then determined by measuring the enzymatic activity of the AChE. Addition of Ellman's reagent produces a yellow-colored product which can be measured spectrophotometrically. The intensity of the colour is directly proportional to the amount of bound conjugate, which in turn is proportional to the concentration of the endothelin. No specific calibration for mouse endothelin was required since this had been performed during the validation of the assay and it was known that human ranges were also acceptable for the mouse. A schematic description of the assay is given in Fig. 2.1.

![Diagram of the assay](image)

**Fig. 2.1:** Enzyme Immunometric Assay (EIA) method for endothelin.
2.4. BIOCHEMICAL MEASUREMENTS: IN VITRO ASSAYS

2.4.1 LDL preparation

LDL-like particles (d=1.006-1.063) were isolated from pooled plasma (2.5 ml) from each group by sequential density ultracentrifugation, as previously described in Section 2.3.1. To minimise LDL oxidation during the isolation process, bubbling with argon to deoxygenated all solutions was used in the isolation. LDL was stored in the dark under nitrogen at 4°C, in sterile conditions, and used within 3 days. Immediately before the oxidation, LDL was separated from EDTA and from diffusible low molecular mass compounds by gel filtration on PD-10 Sephadex G-25M gel (Pharmacia, Upsala, Sweden) in 0.01 mol/l phosphate-buffered saline, pH 7.4.

2.4.2 Human umbilical vein cell culture

Human umbilical vein cell culture (HUVECs) were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 12, and subsequently used at passages 14-16 in order to re-equilibrate the cells after thawing, as suggested by the supplier. A limited number of incubations were also performed with endothelial cells, isolated from human umbilical veins according to the method of Jaffe (Jaffe et al. 1973) and used at passage 2-4. The cells were plated at a concentration of 1.5x10^6 cells and grown in 75 cm^2 culture flasks (Falcon, Becton Dickinson, Lincon Park, USA), filled with 10 ml of Ham’s F-12 medium (Sigma, St. Louis, USA); the flasks contained 10% fetal calf serum (Seromed, Berlin, Germany), 2 mM glutamine (Seromed), 30 μg/ml endothelial cell growth supplement (Sigma), 100 μg/ml heparin (Sigma), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) and
2.5 μg/ml amphotericin (Sigma). The flasks were incubated at 37°C under an atmosphere of 95% air and 5% CO₂ for 2-4 weeks. The medium was replaced every 2 days. At the beginning of each experiment the cells were harvested 5 min by trypsinisation, using 0.05% trypsin (Sigma) and 0.537 mM EDTA in phosphate-buffered saline (pH 7.4) without calcium and magnesium (Seromed). The trypsin was inactivated by dilution, and the cells were washed and counted. Cells were plated at a concentration of 20,000 cell/cm² on a multiwell plate (9.6 cm²/well) (Falcon), grown for 3 days and then used for the incubations. At that time the cells were nonconfluent and the occupied surface area was between 60 and 80%. HUVECs at different passages were harvested and characterized in terms of acetylated LDL binding and factor VIII expression by previously described techniques (Smith et al. 1989).

2.4.3 Incubation of low density lipoproteins

HUVEC-modified LDL was prepared by adding 1.5 ml of serum-free F-12 medium (Seromed) containing 200 μg/ml LDL protein to each 35 mm well of HUVECs, and incubating for 24 h at 37°C. Parallel control incubations of LDL in cell free medium (CFM) were performed in every experiment. LDL (200 μg protein/ml) was also oxidized in the absence of cells, by exposure to different concentrations of CuSO₄ in F-12 medium at 37°C (Ren et al. 1997). The final concentration of Cu²⁺ (0.5 μM) was chosen from preliminary experiments, to modify LDL to the same extent as with the cells evaluated by gel electrophoresis and fluorescence. Oxidation was arrested by adding 200 μM EDTA and refrigeration. LDLs corresponding to the different times of
incubation were isolated by ultracentrifugation at d=1.15, before further analysis.

Protein was measured by the Pierce BCA protein assay reagent (Smith et al. 1985).

2.4.4 Low density lipoprotein lipid peroxidation

The extent of LDL oxidation after incubation with HUVECs was determined in the medium as the thiobarbituric acid adduct, by a published high performance liquid chromatography method (Carbonneau et al. 1991). Analytical separations were performed with a Hewlett-Packard 1050 HPLC connected to a reverse phase C18, 15x0.46 cm column (Bio-Rad, California, USA); the system included a guard column (Microguard System, Bio-Rad) with a Hewlett-Packard spectrophotometric UV-VIS detector at 532 nm. Malonyldialdehyde (MDA) standard was prepared by dissolving 220.3 mg of 1,1,3,3-tetraethoxypropane (Aldrich-Chemie, Steinheim, Germany) in 100 ml of deionized water, to give a 10 mmol/l stock solution. Vitamin E was measured by HPLC with fluorescence detection as described by Cominacini (Cominacini et al. 1991c).

2.5 CHOLESTEROL ESTERIFICATION ACTIVITY MEASUREMENT

Cholesterol esterification activity was measured on mouse peritoneal macrophages (MPMs) obtained from mice three days after intraperitoneal injection of 4% thioglycollate as described by Bernini et al. (2001). Cells (3 x10^6 in 35 mm Multidish, Nunclon, Roskilde, Denmark) were plated and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Seromed) with 10% foetal
calf serum (FCS, Mascia Brunelli, Milano, Italy). Unattached cells were eliminated by washing with phosphate-buffered saline (PBS) at 3 h after plating.

Cell viability was evaluated as described by a previous method (Marks et al. 1956). The dimethylthiazol-diphenyltetrazolium bromide (MTT) assay relies on the ability of viable cells to actively metabolise a tetrazolium dye. Briefly, cells were washed with PBS and MTT (Sigma) was added at a concentration of 10 μg/ml in the culture medium. Following 90 min of incubation, the supernatant was decanted; the formazan precipitates were solubilised by the addition of 100% dimethyl sulfoxide (DMSO) (Merck) and placed on a plate shaker for 10 min. Absorbance was evaluated at 620 nm.

Macrophages were incubated for 8 h at 37°C with 50 μg/ml of acetyl LDL (AcLDL), followed by a second incubation for 16 h with lipoprotein-free medium. Cholesterol esterification was evaluated as the incorporation of radioactivity into cellular cholesterol esters after addition of [1-14C]-oleic acid (Amersham, Buckinghamshire, UK; 0.68 μCi sample⁻¹, 54 mCi mmol⁻¹) albumin complex (Brown & Goldstein 1980). At the end of incubation, cells were washed with PBS and lipids were extracted with hexane/isopropanol (3:2). The extracted lipids were separated by thin layer chromatography (t.l.c.) (isooctane/diethyl ether/acetic acid, 75:25:2, v/v/v). Cholesterol radioactivity in the spots was determined by liquid scintillation counting (Insta-Fluor, Packard, Groningen, The Netherlands).
2.6 MATRIX DEGRADATING METALLOPROTEINASES: MMP-9 ACTIVITY

Matrix degradating metalloproteinases (MMPs) activity was measured by gelatin zimography. MPMs were collected by peritoneal lavage with PBS from mice given a 3 ml intraperitoneal injection of 4% thioglycollate in water. The MPMs were pelleted, washed twice with serum-free DMEM, plated at a density of 3 x 10^6 cells/35 mm dish, and allowed to adhere to the dishes for 2 hours in DMEM containing 10% fetal bovine serum (FBS, Seromed). Then, plates were washed 3 times with DMEM to remove nonadherent cells and incubated in DMEM containing 10% FBS until the day of the experiment (Bellosta et al. 1998). Samples (40 μl for MPMs of conditioned medium per lane) underwent electrophoresis at 4°C on 7.5% polyacrilamide gels containing 10% sodium dodecyl sulphate (SDS) and gelatin (1 mg/ml) under non-reducing conditions and without boiling. After electrophoresis, SDS was removed from the gels in two washes with 2.5% Triton X-100 (Sigma, St. Louis, USA) at room temperature. After washes, the gels were incubated overnight at 37°C with gentle shaking in TRIS 50 mmol/l pH 7.5 containing NaCl 150 mmol/l, CaCl₂ 10 mmol/l, ZnCl₂ 1 μmol/l, to activate the MMPs ability to digest the substrate. For inhibition studies, and to confirm the identity of MMPs, identical gels were incubated in the above buffer containing either EDTA 20 mmol/l (an inhibitor of MMPs), or phenylmethylsulphonylfluoride (PMSF) 1 mmol/l (an inhibitor of serine proteinases). The addition of PMSF did not alter the MMP-9 activity, while the treatment with EDTA completely abolished it (data not shown). At the end of the incubation, the gels were stained with a solution of 0.1% Comassie brilliant blue R-250 (Sigma, St.
Louis, USA) in 25% methanol and 7% acetic acid. Clear zones against the blue background indicated the presence of proteinolytic activity. It is important to note that in this SDS-containing gel, both the latent form of MMP-9, the pro-MMP-9, and the activated gelatinase develop gelatinolytic activity (Kleiner & Stetler-Stevenson 1994). For this reason, the word “activity” was used to indicate the total gelatinolytic capacity measured in the conditioned media. In our experimental conditions, this activity was due entirely to the 92-kDa pro-MMP-9. Results were normalized by cellular protein content and expressed as optical density units (OD/cellular protein).

2.7 Lacidipine Plasma Levels

2.7.1 Sample Assay

Plasma concentrations of lacidipine were determined with the HPLC-RIA method. The limit of quantification of this method is 0.125 ng/ml starting with a 0.5 ml plasma sample. Quality control samples at the concentration of 0.5 ng/ml were assayed to guarantee the reliability of the results.

2.8 Tissue Collection and Preparation for Macroscopic and Microscopic Examinations

2.8.1 Hamster study

At term, animals were sacrificed under ether anesthesia. To evaluate the fatty streak, the aorta was fixed by perfusing with 3% glutaraldheyde in 0.1 M cacodylate buffer, pH 7.4, for 20 min, at 110 mmHg, followed by staining with 0.06% AgNO₃ in water and Harris hematoxylin for 30 min. The right atrium was punctured for an outflow. The segment of aortic arch that was
dissected free started at 1mm above the aortic valves, and extended 3-4 mm to the top of the arch near the branch of the right common carotid artery. The tissue was immersed in the same fixative for at least 3 days and was then thoroughly cleaned of adventitial fat. The aortic arch was then dissected, starting from the aortic valve, up to the left subclavian artery and the thoracic aorta was taken in toto up to the diaphragm; finally, the samples were stained with 0.3% Oil red O (ORO) (Sigma), cut open, mounted on a glass slide under a coverslip and examined en face by light microscopy (Rogers & Karnovsky 1988). Oil red O stain is a routinely used histochemical method for the localisation of fats, followed by a nuclear stain, in formalin-fixed samples. Fats (simple lipids) are neutral esters of glycerols with saturated or unsaturated fatty acids. After Oil red O staining, the appearance, with partially crossed polars, allows lipid identification in conjunction with the distribution of unstained, birefringent, cholesterol (Cheville 1994).

2.8.1.1 Quantitation of the atherosclerotic lesion

Morphometric evaluation was performed on colour photographs, obtained in standardized conditions, by using the digitizing board of an image analyzer (Image Measure 3100, Microscience Phoenix Technology Inc., Washington, USA) installed on a HP computer (Vectra 386/20N). Macrophage-foam cells attached to the endothelial surface and in the subendothelial space (red dots with ORO) were counted (i.e. the total area of foam cells) to give the area of the fatty streak in square micrometers, and divided by the total area of the specimen to define the fatty streak area as percentage of the whole segment (Legg et al. 1980).
2.8.2 ApoE-deficient mouse studies

At the end of lacidipine treatment period, mice were fasted overnight and killed under pentobarbital anaesthesia. After the removal of blood, animals were perfused with 10% buffered formalin for 10 min. The heart and the aorta (ascending aorta, aortic arch with main arterial branches, and the thoracic descending aorta up to the diaphragm) with surrounding adventitial tissues, were excised under a stereo-microscope. The aorta was separated from the heart at the base of the aortic arch, and stained with Oil Red O by flotation. The aorta was opened longitudinally with microscissors along the ventral side, and along the inner curvature of the aortic arch, and pinned out on a wax block with fine pins. The extent of the atherosclerotic lesions in this \textit{(en face)} preparation was quantified.

In addition, the heart was sectioned according to a specific protocol as described by Tangirala et al. (1995); it was sectioned transversely just below the level of the atria, postfixed in 10% buffered formalin overnight and embedded in paraffin. Sequential 7 \textmu{}m sections were cut from the apex towards the base of the heart until the aortic valve leaflets appeared. From this point, 17 sections of the aortic origin, representing every second serial section, over a distance of approximately 240 \textmu{}m, were taken and stained with haematoxylin and eosin (H&E). The area of the atherosclerotic lesions in the aortic sinus was measured in the 17 sections using a computerised image analysis system made of the image analysis software and board, and a black and white video camera (Jay; 711.00-CV) mounted on a Leitz Diaplan microscope.
2.8.2.1 Quantitation of the atherosclerotic lesion

The extent of the atherosclerotic lesions in the aortic en face preparations was quantified by morphometry on colour photographs taken with a camera (Olympus; OM 101) mounted on a stereo-microscope (Olympus; SZ-PT). A Kodak Gold 100 film was used. For each aorta, two sets of photographs were taken. Firstly, the entire aortic tree (ascending aorta, arch with branches, and thoracic segment) was photographed at low magnification (x6.7, original magnification, OM). Then the arch (aortic root to left subclavian artery) and the thoracic tract (from the left subclavian artery to the position of diaphragm) were separately photographed at higher magnification (x40, OM).

Image analysis was performed under standardized conditions using an image analysis computerized system composed of image analysis software and board (Image Measure 3100, Microscience Phoenix Technology Inc, Washington, USA) installed on a HP computer (Vectra 486/33) and a digitizing board. Measurements consisted of the manual tracing of the perimeter of ORO positive areas and of the whole sample on the photographs, to obtain the extent of the lesion area, or the total surface area, respectively. The extent of both areas was determined by the software. All measurements were performed by the same operator and verified by a second investigator who was "blind" to the identity of the image being observed.

The extent of the atherosclerotic lesion in aortic sinus sections was quantified according to Tangirala et al. (1995b). The areas of the lesions were measured in 17 H&E sections using a computerised image analysis system composed by the image analysis software and board described above, and a black and white video camera (Jai; 711.00-CV) mounted on a Leitz Diaplan...
microscope. For each section, three images were captured, each comprising the area under one value leaflet (x4, OM). The outlines of the lesions were traced manually and the lesion size determined by the program. Lesion areas from the three images were summated to obtain the total lesion area for each section. Results are expressed as the average ($\mu m^2$) of the data from the 17 sections analysed. All determinations were carried out by the same operator, in a "blind" fashion.

2.9 TRANSMISSION ELECTRON MICROSCOPY AND SCANNING ELECTRON MICROSCOPY

For transmission electron microscopy (TEM), tissues were perfused-fixed at a constant, near physiological pressure (80 mmHg), via a cannula inserted in the left ventricle, with 10% buffered formalin, pH 7.4, for 10 min. Some aortic tree samples were post-fixed in 1% osmium tetroxide in phosphate buffer, dehydrated in graded ethanols, embedded in epoxy resin (Epon-Araldite) and sectioned in an Ultracut E ultramicrotome (Reichert-Jung). Ultrathin sections were stained with uranyl acetate/lead citrate and observed with an EM 10 electron microscope (Zeiss).

For scanning electron microscopy, SEM, tissues were postfixed as above, dehydrated in graded ethanols, critical point dried (CPD 030 Balzers), fixed to stubs with colloidal silver, sputtered with gold (MED 010, Balzers), and examined with a SEM (DSM 690, Zeiss).
2.10 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is a technique that allows the recognition of individual antigens in tissues and cells. In principle, sections are treated with an antibody, and the sites of antibody binding are identified by a marker, e.g. fluorescent dye or enzyme. IHC is a morphological tool. In this work, monoclonal and polyclonal antibodies were used to characterize atheromatous lesion in the aorta from control apoE-deficient mice (vehicle-treated) and lacidipine-treated.

Sections of aortic sinus, fixed in 10% formalin for 10 min, were stained with a polyclonal anti-mouse MMP-9 (Bellosta et al. 1998) antibody (Santa Cruz Biotechnology; dilution 1:20); anti-α-smooth muscle (SM) actin was identified using a polyclonal antibody sheep anti-mouse (Cymbus Bioscience Ltd.; dilution 1:100; DakoK675). As negative controls, non-immune IgGs (Dako) were used. Antibody binding was visualized with the Avidin Biotin peroxidase method with the diaminobenzidine (DAB).

Samples of heart tissue were removed and washed in saline solution, frozen by immersion in liquid nitrogen and than stored at -20°C until sectioning. Cryosections, 10 μm thick, were fixed in 1:1 acetone/chloroform mixture and stained with the respective antibodies. Rat anti-mouse monocyte/macrophage monoclonal antibodies (BM-8, BMA Biomedicals AG; dilution 1:50) were used as marker for activated macrophages. To assess antibody specificity a section of lymphonodes (external positive control) was used. Expression of vascular cell adhesion molecules (VCAM-1, Pharmingen; dilution 1:200) and intracellular adhesion molecules (ICAM-1, Pharmingen; dilution 1:200) was performed using a monoclonal rat anti-mouse and hamster anti-mouse primary antibody,
respectively. After incubation, antibody binding was visualized with streptavidin alkaline phosphates conjugate (APAAP). Controls included omission of primary antibody, use of non-immune sera and use of frozen sections of heart from wild-type control group from C57BL/6J mice. The slides were counterstained with Carazzi haematoxylin.

2.11 VASCULAR FUNCTIONAL STUDIES

The evaluation of vascular reactivity was performed on aortic samples obtained from apoE-deficient mice (vehicle control and lacidipine-treated). The aorta was placed in physiological solution containing glucose 11.10 mmol/l, on a silicone plateau on ice. Adventitial tissue was removed carefully avoiding damage to the endothelium. After the dissecting procedure, the aorta was cut into 2.5 mm long aortic rings identifying four different locations in the aorta: the first corresponded to ascending aorta, the second was taken below the aortic arch, and the third and fourth rings were taken in the middle, and at the end of thoracic aorta, respectively.

The aortic rings were mounted on small Ni-Co/Fe metal triangles connected to an isometric force transducer (Multi Myograph System, Model 610 M, J.P.Trading I/S, Denmark), in the glass chamber containing 20 ml physiologic buffered solution heated to 37°C and aerated with the mixture of 94%O₂/6%CO₂, at pH 7.4. The resting tension of the preparation was equal to 10 millinewton (Dieber-Rotheneder et al. 1991). During a 1 h resting period, the solution was changed every 20 min. The artery contraction was induced by the addition of norepinephrine at concentration varying from 10⁻⁹ to 3x10⁻⁶ M. When the maximum response reached a steady-state, a dose-dependent
response to acetylcholine (Ach, endothelial-dependent muscarinic agonist) was elicited within a concentration gradient from $10^{-9}$ to $3 \times 10^{-6}$ M (10 nM to 1 mM). The preparation was then washed and stabilised for 1 h with changing of the bathing solution containing $3 \times 10^{-4}$ M N-omega-nitro-L-arginine (L-NNA), NO-synthase blocker every 20 min. The second dose-response curve was then carried out by the addition of norepinephrine. After reaching a stable contraction a dose-dependent response to S-nitroso-N-acetylpenicillamine (SNAP, a NO-donor) was elicited within the concentration range as used above for Ach. Sometimes, after washing, the preparation was again stabilised in the manner described above and was exposed for the last 30 min to superoxide dismutase (Stewart-Lee & Burnostock 1991), 30 U.I./ml. The contraction and relaxation study performed by means of lacidipine followed the same procedure described for SNAP.

2.12 VOLTAMMETRIC STUDIES

Voltammetry associated with carbon fibre micro-electrodes (mCFEs, 10-30 μm diameter, 0.5-3 mm length) "working electrode" is an electrochemical methodology allowing continuous, and in real time, and in situ detection of oxidizable chemicals. This technique can be used both in vitro, with the active tip of the mCFE immersed in buffered solution, and also ex vivo, with the active tip of the mCFE in contact with one or more tissues (e.g. the endothelium of rat aortic rings). This methodology has been also used in vivo, for example in brain extracellular fluid, where the mCFE is stereotactically implanted in the brain area of anaesthetised and freely moving animals. The technique can also be used with blood and platelet-rich plasma...
(PRP) and/or with isolated platelets (IP). The active tip of the mCFE allows the measurement of the oxidation of chemicals (Crespi et al. 2000).

In a conventional set-up, a voltage waveform (lasting μseconds, or few seconds, depending on the type of voltammetry used) is applied by means of a polarograph to a three way electrode system (Fig. 2.2) (Crespi et al. 2001b).
Fig. 2.2: Voltammetry associated with carbon fibre micro-electrodes. A working electrode and voltage waveform are applied by means of a polarograph to a three way electrode system.
For the present purposes, amperometry, involving the use of NO sensitive mCFEs (3 mm length, 1.5 mm diameter), has been used to selectively monitor NO on the surface of the endothelial layer in rat aortic rings. This technique was used according to methodology described by Crespi et al. (2001b), and is shown in Fig. 2.3 and Fig. 2.4.

Fig. 2.3: A working electrode (a carbon fibre micro-electrode) applied on the surface of the endothelium of an aortic ring.
Fig. 2.4: Amperometry of the endothelium of an aortic ring using the carbon fibre microelectrode (mCFE).

Diagramatic representation of (a): mCFE (working electrode); b) the positioning of the active tip of the Nafion-oPD mCFE parallel and in contact with the endothelium of the rat aortic ring immersed in 200 µl PBS. The amperometric analysis is performed at +850 mV and allows the detection of the electrons generated by the oxidation of endogenous NO, resulting in the basal current shown in the two voltammograms. Note that the level of such a current is higher in the aortic ring incubated with lacidipine (d) when compared to the ring incubated in vehicle (c). At approximately 80 sec of the amperometric analysis, the single addition of PBS or substance P are followed by the appearance of a transient peak upon the basal amperometric current, which is related to stimulated NO release. From Crespi et al. (2001b).
The current monitored by the working electrode, and due to electrons generated (or captured) by oxidation and/or the reduction of chemicals at the electrode surface, gives the concentration of a chemical species that is, in addition, identified by its oxidation (and/or reduction) specific potential (Crespi et al. 2001a; Crespi et al. 2002). Chemically and/or electrically (Nafion and/or ortho-phenylenediamine, oPD) treated mCFEs, have been developed to increase the sensitivity, selectivity and reliability of the sensor. In particular, mCFEs treated in this way have been applied mainly involving two types of voltammetry: Differential Pulse Voltammetry (DPV) and Direct Current Amperometry (DCA). DPV is characterized by the application of a dynamic potential to a treated mCFE, based upon an initial potential (Ei) and a final potential (Ef) that results in the oxidation of chemicals. This scanning method (scan speed 10-100 mV/sec) is routinely used between an Ei of -0.2 V and an Ef of +1 V scan range. This allows the simultaneous, selective detection of various oxidation signals related to the oxidation of endogenous electroactive chemicals such as monoamine neurotransmitters and peptides. DCA involves the immediate increase of the input voltage to a value that is sufficient to oxidise the chemical, and this is then held for the time of measurement. This DCA method is accurate in evaluating the amount of oxidisable species present and is in "real time", as the corresponding current level can be measured in time intervals of 0.1 sec. In Fig. 2.4, the potentiostat used, and the principle of the technique, is shown as well as a particular application. The amperometric analysis was performed at +850 mV and allows the detection of the electrons generated by the oxidation of endogenous NO, resulting in the basal current shown in the two voltammograms. The single addition of PBS or substance P
are followed by the appearance of a transient peak on the basal amperometric current, which is related to stimulated NO release.

2.13 STATISTICAL ANALYSIS

Results are generally expressed as the mean ±SEM. To test for differences in mean/SEM between control and treated groups a one sided Dunnett's test was used.
ANTI-ATHEROSCLEROTIC ACTIVITY OF
LACIDIPINE IN CHOLESTEROL FED HAMSTERS

3.1 INTRODUCTION

Most of the existing information on which the understanding of the atherosclerotic process is based has been acquired through animal studies. Since the early work carried out in the rabbit (Giordano et al. 1970), other animal species have been utilised and numerous induction methods have been used in attempts to reproduce human-like lesions. Various animal models have been used to study experimental diet-induced atherosclerosis (Jokinen et al. 1985; Vesselinovitch 1988; Armstrong & Heidstad 1990; Fekete 1993; Bocan 1998; Narayanaswamy et al. 2000). The early lesion, the fatty streak, can be induced in the aorta of rabbits, hamsters, monkeys and transgenic mice and by dietary supplementation with cholesterol, which causes hypercholesterolemia. Investigations of the intimai changes which precede the formation of the fatty streak, have focused on the increased number of monocytes adhering to the endothelium, and their migration into the subendothelial space (Watanabe & Fan 1998). Ultrastructural changes in the intima, at the early stage of lesion formation, were also reported by some researchers using cholesterol fed-animals (Takasu et al. 1990b). However, little is known about the morphological changes in the cellular and extracellular components of the intima at the prelesion stage. This is due to the difficulty associated with both the prediction, and the collection, of the vascular regions
where the lesions may occur (Takasu et al. 1990b). In the past, some authors
(Nistor et al. 1987b) investigated the processes involved in the progression of
atherosclerotic lesions in the aorta of the hamster fed a high cholesterol diet for 12
months, and in this way clearly demonstrated the usefulness of the hamster as a
model for atherosclerosis research. Also, the hamster has been considered by some
to be the animal of choice (Nistor et al. 1987b; Takasu et al. 1990b) because, as in
humans, the major plasma cholesterol carrier is LDL. As determined by
electrophoresis in agarose gel, in the hamster about 50% of cholesterol is carried in
the LDL fraction and during the consumption of a hyperlipidemic diet, this
lipoprotein fraction increases. Another advantage of the hamster is the high
frequency with which the atherosclerotic lesions develop. In addition, the
relatively small size of the hamster cardiovascular system facilitates the
morphometric analysis of the developing lesions. Also, the hamster is physically
small, easy to handle, and is susceptible to diet-induced atherosclerosis. Another
relevant point is that the hamster is not a vegetarian, like the rabbit. The
cholesterol metabolism of the rabbit is different from that of humans and this
species develops a "cholesterol storage disease" where the lesion topography is
significantly different to that seen in man (Vesselinovitch 1988).

Previous studies in rabbit models of atherosclerosis have demonstrated that
lacidipine treatment, at dosages that do not affect blood pressure, decreased the
development of atheromatous lesions without lowering plasma lipids (Boyd et al.
1989; Mao et al. 1991; Wiklund et al. 1991). This pharmacological activity of
lacidipine prompted the present study to investigate further the antiatherogenic
properties of the drug in the hamster. The small size of the hamster is also an
advantage in that it reduces the amount of compound required to carry out a
study. In the hamster, the fatty streaks develop consistently along the “lesion-prone area” which follows the inner curvature of the aortic arch; the development of the fibrous-fatty plaques in this highly predictable site during hypercholesterolemia is a considerable advantage in such experimental investigations.

The present study set out to examine the morphological changes occurring in hamsters receiving an atherogenic diet, and the effect of lacidipine on this process when administered at three dose levels (0.3, 1.0 and 3.0 mg/kg). The development of atherosclerosis in animals receiving the test compound was followed, in conjunction with monitoring the plasma lipid profile, and comparing such changes with those of non-drug-treated (control) animals at two time points (14 and 24 weeks). To characterise the lesion, morphometric assessment was used at the light microscope level to quantify the effect of lacidipine treatment, and electron microscopy examination was also carried out to investigate morphological changes at the cellular level. Therefore, the overall objective of the investigation was to evaluate whether, in the hamster, the once-daily treatment with a long-lasting calcium antagonist influenced the atherogenic process, and to identify the ultrastructural targets of lacidipine action using an animal model other than the rabbit.

3.1.1 Acknowledgements

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3.2 MATERIALS AND METHODS

Male golden Syrian hamsters (n=200), 10 weeks old with a mean body weight of 110 ±6 g were randomised into five equal groups.

A control group (Group 1) of 40 animals was fed a standard diet (SD; Standard Diet). Four groups of hamsters (n=40 per group) were fed an atherogenic, high cholesterol diet (Boyd et al. 1989; Cominacini et al. 1997a) consisting of the SD supplemented with 2% cholesterol and 5% butter. The animals fed the atherogenic diet were distributed into the different treatment groups as shown in Table 3.1 and treated daily by gavage with lacidipine at 0 (Group 2), 0.3 (Group 3), 1.0 (Group 4) and 3.0 (Group 5) mg/kg (Chapter 2, Materials and Methods).

Animals from each group were sacrificed under ether anaesthesia at 14 and 24 weeks after the start of the study. Analysis of plasma lipids and lipoprotein cholesterol was carried out on approximately ten animals from each group at each time-point and were quantified by enzymatic methods using a Cobas bio-analyser. Tissue collection and preparation for the examination and the quantification of atherosclerosis was performed on colour photographs on en face preparations (Chapter 2, Material and Methods), using approximately ten animals in each group at each timepoint. Ultrastructural examination of the aorta (aortic and thoracic segments) were performed using both scanning electron microscopy and transmission electron microscopy (Chapter 2, Material and Methods), on approximately three animals per group per time-point.

Statistical analysis was performed by comparison of treated and control groups using Duncan’s test.
3.3 RESULTS

3.3.1 Clinical observations

The body weight gain of lacidipine-treated hamsters was generally comparable with the Group 1 controls (initial mean body weight: 110 ±16 g; mean body weights at week 24: Group 1, 121 ±8 g, Group 2, 128 ±11 g, Group 3, 123 ±10 g, Group 4, 119 ±16 g, Group 5, 127 ±16 g). No significant differences were seen between the lacidipine-treated animals and the controls (Groups 1 and 2), in food consumption (approximately 10 g per animal per day). No adverse clinical events were observed during the lacidipine dosing period.

3.3.2 Plasma lipid profile

Marked increases in total cholesterol, triglycerides and lipoproteins were detected at week 14 in animals receiving the HC atherogenic diet (Group 2 to 5) in comparison with hamsters receiving the SD (Group 1) (Table 3.2). This increase was statistically significant (P<0.05) for Group 2 (fed the HC diet and treated with vehicle), and also for Groups 3 to 5 fed the HC diet and given lacidipine at 0.3, 1.0 and 3.0 mg/kg, in comparison with Group 1 controls. Furthermore, at week 14, the administration of lacidipine to Groups 3 to 5 induced a slight but not significant decrease in total cholesterol, triglycerides and lipoproteins, in comparison with Group 2 treated with vehicle. The pattern of findings for total cholesterol, triglycerides and lipoproteins at week 24 was directly comparable to the results at week 14 (Table 3.2).
3.3.3 Atherosclerotic lesions: examination and quantification

In en face preparations, a fatty streak area was observed at week 14 in the aortic arch of the majority of hamsters receiving the HC atherogenic diet (Groups 2 to 5). At the light microscope level, in the aortic arch of Group 2 (control) hamsters at week 14, many subendothelial macrophage-foam cells were seen to have accumulated along the inner curvature of the arch (Fig. 3.1a). The foam cells were engorged with neutral lipid and were often located near clusters of small extracellular lipid droplets. Sections stained with Oil red O were viewed with partially crossed polars to show both red-stained glycerol esters, mainly triglycerides (neutral lipids), and the unstained birefringent crystalline cholesterol. The en face view of the aortic arch at the light microscopy level demonstrated that fewer and smaller macrophage-foam cells were present at week 14 in all groups of hamsters fed the HC diet and treated with lacidipine (Groups 3, 4 and 5) (Fig. 3.1b). Quantitative analyses of the area of the fatty streak was planned to be performed on the aortic arch and the thoracic aorta at both week 14 and 24. However, measurements performed at week 14 showed only a sporadic apparent reduction of the fatty streak area in the groups receiving lacidipine, without any dose-relationship, and without any significant difference in relation to the control animals. For this reason, data on week 14 findings are not reported. At 24 weeks, quantitative analysis of the fatty streak area in the aortic arch indicated that in hyperlipidemic hamsters fed the HC diet (Group 2), the mean area covered by the lesion was $375 \pm 145 \mu m^2 \times 100$; this accounted for 2.7% of the total aortic arch surface area (Fig. 3.2a). In contrast, the area of fatty streak involvement in hyperlipidemic hamsters fed the HC diet and treated with lacidipine at 0.3, 1.0 and 3.0 mg/kg was
significantly reduced by 67%, 41% and 71%, respectively, in comparison with the hyperlipidemic controls of Group 2 (Fig. 3.2a); these reductions were statistically significant (P<0.05). No fatty streaks were evident in the aortic arch of Group 1 hamsters fed the SD at 14 and 24 weeks.

In the thoracic aorta, at week 24, hyperlipidemic animals showed early lipid deposits with foamy macrophages and/or small clusters of extracellular lipids. Relevant differences in the incidence and the degree of lesion severity were noted between hyperlipidemic controls (Group 2) and the lacidipine-treated animals (Groups 3, 4 and 5) (Fig. 3.3). Quantitative analysis at week 24 demonstrated that the area affected by lipid deposition was reduced by 85%, 78% and 97% in Groups 3, 4 and 5, treated with lacidipine at 0.3, 1.0 and 3.0 mg/kg respectively, when compared with the control hyperlipidemic group (Group 2) (Fig. 3.2b).

3.3.4 Ultrastructure of the aortic arch (lesion prone area)

Group 1: Standard diet (control). The ultrastructural features of the lesion prone area on the inner curvature of the aortic arch remained the same throughout the experiment (week 14 and 24). On SEM examination, the endothelial cells showed a polygonal shape with the nucleus protruding into the aortic lumen. Short microvilli were visible close to the cell borders. On TEM examination (Fig. 3.4a), the endothelial cells showed infrequent organelles and numerous micro-pinocytotic vesicles. The elastic lamina was thin, but was thicker than in other areas of the aorta. The subendothelial layer was usually devoid of cells showing only rare collagen fibres.
Group 2: High cholesterol diet (control) without lacidipine. At SEM examination (Fig. 3.5a) the surface of the aortic arch showed irregular endothelial cells with an increased number of microvilli. Crater-like lesions in the endothelial cell layer were often present. Lympho-monocytic cells and platelets (bearing in mind differences in the size and morphology of the cells; monocytes demonstrated to be in diapedesis) were often attached to the endothelial cell surface. On TEM examination (Fig. 3.4b), the endothelial cells appeared rich in organelles with hypertrophic Golgi complex and endoplasmic reticulum. Lysosomal inclusions were numerous. The sub-endothelial layer was thickened and showed a large number of liposomal particles. Foam and smooth muscle cells in the sub-endothelial space were visible. These smooth muscle cells (myointimal cells) appeared rich in mitochondria with a well-developed endoplasmic reticulum but with a few often fragmented microfilaments. Liposomes, small moderately to markedly electron-dense lipid droplets, were present in a large area of the subendothelial space; liposomes represent very small triglyceride globules in the cisternae of the endoplasmic reticulum, becoming ultimately large globules of triglycerides in the cytosol (Cheville 1994; Ghadially 1997). These lesions were visible but less frequent at 14 weeks of treatment. On SEM examination in the animals sacrificed at week 24 of treatment, the endothelial surface was much more irregular than at 14 weeks, with the presence of numerous crater-like lesions, and there was an increase in the thickness and cellularity of the subendothelial space. Also, at 24 weeks, the smooth muscle cells showed degenerative features and there were interruptions of the internal elastic lamina.
Groups 3 and 4: High cholesterol diet with lacidipine at 0.3 and 1.0 mg/kg. In these animals the lesions showed an intermediate development, in between those evident in Group 2 and those in Group 5 animals.

Group 5: High cholesterol diet with lacidipine at 3.0 mg/kg. On SEM examination, the surface of the endothelial cells appeared rather regular and the cells did not show signs of cytoplasmic changes nor prominent microvilli (Fig. 3.5b). Occasionally an elevated region of the endothelium was seen, with a large basal area and a rounded surface, and it was considered that these may have been above areas rich in sub-endothelial cells. Occasionally isolated crater-like lesions were also visible. On TEM examination (Fig. 3.4c), the endothelial cells did not show signs of the proliferation of cell organelles. The basal lamina tended to be thickened and the cellularity of the subendothelial space, and the density of the liposomal particles, were reduced in comparison with animals in Group 2, and only rarely were myointimal cells visible.

3.3.5 Ultrastructure of the thoracic aorta

Group 1: Standard diet (control). The morphological appearance of the thoracic segment was normal throughout the study (week 14 and 24), both at the SEM and TEM level. On SEM examination, the endothelial cells showed their typical polygonal shape with a protruding nucleus towards the aortic lumen. On TEM examination the intima consisted of an intact endothelium, subendothelial matrix and a thin elastic lamina; no smooth muscle cells or macrophages were found in the intima of the specimens examined.

Group 2: High cholesterol diet (control) without lacidipine. On SEM, the intimal surface was generally well preserved, but elevations of endothelial
cells were sometimes seen and crater-like lesions were evident (Fig. 3.6a). At the TEM level, the subendothelial space was enlarged and often contained liposomal particles, foam cells and muscle cells.

Groups 3 and 4: High cholesterol diet with lacidipine at 0.3 and 1.0 mg/kg. On SEM, the intimal surface appeared irregular and occasionally crater-like lesions were present. The same findings were evident in specimens from animals of both lacidipine groups (0.3 and 1.0 mg/kg/day) without any significative differences between the two groups. On TEM, one animal from Group 3 (0.3 mg/kg/day) and one from Group 4 (1.0 mg/kg/day) showed an intima which consisted of an intact endothelium, a thin subendothelial matrix, and an intact elastic lamina; no smooth muscle cells or macrophages were found in the intima of the specimens examined. However, the other aortic samples from both groups showed an enlarged subendothelial space with liposomal particles, foam cells and muscle cells. No major relevant differences between the specimens from Group 3 and Group 4 were noted.

Group 5: High cholesterol diet with lacidipine at 3.0 mg/kg. Using SEM, the intimal surface appeared generally regular with only isolated elevations of the endothelium (Fig. 3.6b). On TEM, in comparison with Group 2, the cellularity of the subendothelial space was reduced, as was the density of the liposomal particles; muscle cells were rarely evident in the intima.
3.4 DISCUSSION

Previous studies in a variety of animal models have clearly demonstrated that atherogenesis can be reduced by the administration of calcium antagonists (Ginsburg et al. 1983a; Lichtor et al. 1989a; Skepper & Kappagoda 1992). The present study characterized the antiatherogenic properties of the long-lasting calcium antagonist, lacidipine, when administered at 0.3, 1.0 and 3.0 mg/kg using a once-daily treatment regimen.

In the present hyperlipidemic hamster model, we have identified vascular lesions which compare closely with those reported by previous investigators (Nistor et al. 1987b; Kowala et al. 1991). Using light microscopy, it has been demonstrated that lacidipine treatment significantly reduces the area of fatty streaks and there is also a reduction in the presence of foam cell deposits. Similarly, ultrastructural examination has confirmed that treatment with lacidipine greatly reduced the severity of the intimal lesion. In particular, TEM examination demonstrated that lacidipine administration markedly reduced the presence of liposomal particles in the region of the basal lamina and in the sub-endothelial space. The accumulation of liposomes appeared to be a relatively early event, occurring at the 14 week sampling point and this accumulation occurred in the absence of foam cells. Lacidipine treatment reduced the accumulation of liposomes in hyperlipidemic hamsters without lowering serum lipids, and this suggests that a mechanism is involved which is different from the mode of action of drugs which are active in reducing atherosclerosis by lowering plasma lipids. Similar observations have been reported in comparable studies on the suppression of atherogenesis by other calcium antagonists (Ginsburg et al. 1983a; Lichtor et al. 1989a). However, it is
considered possible that lacidipine influences the atherogenic process by an unusual mechanism and it has been suggested by some authors (Micheli et al. 1991) that this may be related to a combination of the long-lasting calcium antagonism of the compound, in conjunction with the strong antioxidant properties of the drug; both of these activities would be further enhanced by the very high lipophilicity of the compound (Herbette et al. 1993). The protective effect of lacidipine in the present study was clearly evident on the endothelial cell phenotype, both at the TEM and the SEM level. It was seen that lacidipine decreased irregularities of the endothelial cells in the hypercholesterolemic animals, lowered the incidence of crater-like lesions, and reduced the hypertrophy of the cytoplasmic organelles. Therefore, in this hamster model of atherogenesis, the present results demonstrate clearly that lacidipine reduces the extent of atherosclerotic activity, without lowering hypercholesterolemia, and also significantly reduces, or prevents, the endothelial cell changes typically associated with atherosclerosis (Tashiro et al. 1994).
Fig. 3.1: *En face* views of the aortic arch at the light microscope level. All x16 (OM); (Oil red O stain).

(a) Lesion-prone area of the aortic arch from control hamster fed the high cholesterol atherogenic diet for 24 weeks. At this low power, many lipid inclusions forming a well organised fatty streak area are located along the inner curvature of the arch, i.e. the lesion-prone area.

(b) Aortic arch from a hamster fed the high cholesterol atherogenic diet and treated with lacidipine at 3.0 mg/kg of for 24 weeks. There are less lipid deposits in the lesion-prone area compared to the control.
Fig. 3.2: Measurements of fatty streak area in the aortic arch and the thoracic aorta.

Effect of feeding a standard diet (SD) and a high cholesterol diet, and the administration of lacidipine at 0, 0.3, 1.0 and 3.0 mg/kg, on the mean total surface area occupied by the fatty streak in the aortic arch (a) and in the thoracic aorta (b), at 24 weeks of treatment.

Values represent the mean total surface area occupied by the fatty streak expressed as a percentage of the total surface area of the aortic arch and the thoracic aorta, (n=9-11); *, significantly different from Group 2, P<0.05 (Duncan’s test).
Fig. 3.3: *En face* views of the thoracic aorta at the light microscope level. All x64 (OM); (Oil red O stain).

(a) Thoracic aorta specimen from a control hamster fed the high cholesterol atherogenic diet for 24 weeks. At low magnification purple dots are visible (arrowheads), these correspond to lipid inclusions positive to the Oil red O stain; the dots represent intimal macrophage-foam cells engorged with lipid.

(b) Thoracic aorta specimen from a hyperlipidemic hamster fed the atherogenic diet for 24 weeks and treated with lacidipine at 3.0 mg/kg. The macrophage-foam cells (arrowhead) are fewer in number and smaller than those observed in control animals fed the high cholesterol atherogenic diet but not treated with lacidipine.
Fig. 3.4: Transmission electron microscopy of the aortic arch. All x16,000 (OM).

(a) Electron microphotograph of a control hamster (Group 1) fed the standard diet for 24 weeks. The intima of this normolipidemic animal shows an intact endothelium with endothelial cells (E), and a very thin
subendothelial layer (basal lamina) that has laid down the lamina elastica interna (*). No foamy macrophages or smooth muscle cells were identified in the intima.

(b) A control hypercholesterolemic hamster (Group 2) fed the high cholesterol diet for 24 weeks. The intima appears thickened, the lamina elastica interna (*) is fragmented and elastic fragments are visible in subendothelial space (S). Large foamy macrophages (F), accumulation of liposomes (arrowheads) and smooth muscle (SM) cells are visible in the intima below the endothelial cells (E). The endothelial cells have an irregular, villous surface and hypertrophy of organelles.

(c) A hyperlipidemic hamster (Group 5) fed the atherogenic diet for 24 weeks and treated with lacidipine at 3.0 mg/kg. The intima appears less thick than in the untreated control animal of Group 2, the subendothelial space (S) is reduced, as is the number of liposomes; the lamina elastica interna (*) is intact with SM located below (media) and the endothelial cell (E) does not show evidence of organelle hypertrophy.

* lamina elastica interna; E: endothelial cell; F: foam cell; SM: smooth muscle cell; S: subendothelial space.
Fig. 3.5: Scanning electron microscopy of the aortic arch. All x2,000 (OM).

(a) Control hypercholesterolemic hamster (Group 2) fed the high cholesterol diet for 14 weeks. The intima surface is irregular, showing swelling into the vessel lumen as a result of foam cell accumulation beneath the endothelium, which contains crater-like lesions. Lymphomonocytic cells and platelets are attached to the endothelial surface.

(b) Hyperlipidemic hamster fed the atherogenic high cholesterol diet (Group 5) for 14 weeks and treated with lacidipine at 3.0 mg/kg. The intimal surface shows endothelial cells in a regular pattern. No crater-like lesions are present.
Fig. 3.6: Scanning electron microscopy of the endothelial surface of the thoracic aorta. All x4,000 (OM).

(a) SEM micrograph from a control hamster (Group 2) fed the high cholesterol atherogenic diet for 24 weeks. The endothelial surface appears well preserved; however some crater-like lesions are present.

(b) Hyperlipidemic hamster (Group 5) fed the atherogenic diet for 24 weeks and treated with lacidipine at 3.0 mg/kg. The intimal surface shows endothelial cells in a regular pattern. No crater-like lesions are present.
Table 3.1: Experimental design showing groups of animals, diet and lacidipine treatment *.

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Treatment</th>
<th>Dose of lacidipine (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group 1</td>
<td>Standard diet</td>
<td>Vehicle</td>
<td>-</td>
</tr>
<tr>
<td>Control Group 2</td>
<td>High cholesterol diet</td>
<td>Vehicle</td>
<td>-</td>
</tr>
<tr>
<td>Group 3 Treated</td>
<td>High cholesterol diet</td>
<td>Lacidipine</td>
<td>0.3</td>
</tr>
<tr>
<td>Group 4 Treated</td>
<td>High cholesterol diet</td>
<td>Lacidipine</td>
<td>1.0</td>
</tr>
<tr>
<td>Group 5 Treated</td>
<td>High cholesterol diet</td>
<td>Lacidipine</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* There were 40 hamsters in each dose level group.
Table 3.2: Plasma levels of total cholesterol, triglycerides and lipoproteins in male Syrian hamsters fed a standard diet (SD) or a high cholesterol diet (HC), and treated with lacidipine at 0.3, 1.0 and 3.0 mg/kg for 14 and 24 weeks.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cholesterol (mmol/l)</th>
<th>Triglycerides (mmol/l)</th>
<th>HDL cholesterol (mmol/l)</th>
<th>LDL cholesterol (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 weeks</td>
<td>24 weeks</td>
<td>14 weeks</td>
<td>24 weeks</td>
</tr>
<tr>
<td>Group 1</td>
<td>Control SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>Control HC</td>
<td>24.1±4.9*</td>
<td>21.4±2.4*</td>
<td>16.4±6.2*</td>
</tr>
<tr>
<td>Group 3</td>
<td>HC+lacidipine 0.3 mg/kg</td>
<td>19.0±3.1</td>
<td>28.2±3.8</td>
<td>14.4±6.3</td>
</tr>
<tr>
<td>Group 4</td>
<td>HC+lacidipine 1.0 mg/kg</td>
<td>15.4±2.9</td>
<td>26.3±9.1</td>
<td>13.3±0.8</td>
</tr>
<tr>
<td>Group 5</td>
<td>HC+lacidipine 3.0 mg/kg</td>
<td>16.4±2.4</td>
<td>20.7±2.9</td>
<td>12.9±4.3</td>
</tr>
</tbody>
</table>

Values are expressed as means; n=9-11. Results of Group 2 were compared with Group 1 (control) and Groups 3, 4, 5 with Group 2. Group 1: hamsters receiving the SD; Group 2: hamsters fed the HC diet and treated with vehicle; Groups 3 to 5: hamsters fed the HC diet and given lacidipine at 0.3, 1.0 and 3.0 mg/Kg. *P<0.05.
Chapter 4

REDUCTION IN THE DEVELOPMENT OF
ATHEROSCLEROTIC LESIONS IN THE APOE-
DEFICIENT MOUSE BY LACIDIPINE

4.1 INTRODUCTION

In the previous study carried out in the hypercholesterolemic hamster model of atherosclerosis, it was demonstrated that lacidipine administration reduced the development of atheromatous lesions without affecting the levels of plasma lipids (Cristofori et al. 2000a). In addition, Donetti and colleagues (Donetti et al. 1997) had demonstrated that lacidipine effectively reduced the extent of neointimal hyperplasia induced in rabbits, and this was independent of blood pressure variations. Therefore, it was considered likely that lacidipine influenced the atherogenic process by an unusual mechanism which could be related to a combination of both the long-lasting calcium antagonism of the drug, and the significant antioxidant activity of the compound.

The objective of the present study was therefore to investigate the anti-atherogenic potential of lacidipine in a transgenic animal model. Transgenic mice provide a convenient source of experimental animals, and such mice represent a genetically reproducible model that could help to overcome the many problems and deficiencies of larger laboratory animals; also, in particular, the use of transgenic mice would permit studies on possible therapies that may require relatively large number of animals.
The apoE-deficient transgenic mouse (also referred to as the apolipoprotein E knockout mouse) had been used in several earlier studies. It had been found that the mouse represented a very useful model for the study of atherosclerosis, since apoE-deficient mice develop spontaneous hyperlipidaemia and hypercholesterolemia, as well as lesions of atherosclerosis closely resembling, in appearance and distribution, those observed in man (Zhang et al. 1992; Nakashima et al. 1994; Paigen et al. 1994; Qiao et al. 1994; Tangirala et al. 1995b). The apoE-deficient mouse appears to develop normally, however the animal exhibits serum plasma cholesterol levels which are five times normal and also shows the development of spontaneous atherosclerotic lesions. As seen in the hypercholesterolemic hamster, another important feature of the apoE-deficient model is that the small size of the animals makes them easy to handle and this feature of small size also reduces the amount of compound required to carry out a study involving drug administration.

Furthermore, in the present study, the role of endothelin was examined, particularly with regard to the usefulness of the peptide as a plasma marker of atherosclerotic progression (Mathew et al. 1996a). Indeed, Mathew and colleagues (Mathew et al. 1996a) had previously demonstrated that endothelin can be considered as an atherogenic peptide because of its mitogenic and proliferative properties, as well as the inter-actions of the peptide with known atherogenic factors. In addition, it was considered that endothelin could have a specific role in the evolution of atherosclerosis. This possibility was also relevant as lacidipine had previously been demonstrated to affect endothelin-1 gene expression in the stroke prone hypertensive rat (Godfraind & Salomone 1996),
and therefore it was of considerable interest to investigate the effect of lacidipine administration on plasma endothelin levels.

The present study therefore set out to examine the development of atherosclerosis in the apoE-deficient mouse fed a standard rodent diet and receiving lacidipine at the dose level of 0.3, 1.0 and 3.0 mg/kg for 10 weeks, by monitoring the plasma lipid profile and levels of endothelin, and by comparing these changes with those of vehicle-treated (control) animals. We also wished to follow the development of atherosclerosis in animals receiving the test compound by evaluating the extent of the atherosclerotic lesions by means of morphometry.

4.1.1 Acknowledgements

The author would like to acknowledge and thank Dr. Antonio Pastorino and Dr Luciano Cominacini of the Department of Biomedical and Surgical Sciences, Section of Internal Medicine, University of Verona for their assistance in the analysis of plasma endothelin and Dr Carlo Zancanaro, Institute of Human Anatomy and Histology, the University of Verona, for his scientific advice on quantitative analyses of lesions.
4.2 MATERIALS AND METHODS

Female apoE-deficient (n=24) and C57BL/6J mice (n=6) were used in the present study. Homozygous, apoE-deficient mice were approximately 13 weeks old at the beginning of the experiment and were randomly allocated to four groups of 6 animals each (one vehicle-treated control and three lacidipine-treated groups). The apoE-deficient mice were on a C57BL/6J genetic background and a further control group of females of this inbred strain (n=6) were also used as a vehicle-treated group. Animals were housed three per cage on wood shavings, fed a standard rodent diet containing 4.4% fat (weight/weight) and 0.2% cholesterol (weight/weight). Lacidipine in 0.5% methylcellulose was administered daily for 10 weeks by gavage at dose levels of 0 (control), 0.3, 1.0 and 3.0 mg/kg body weight (as described in Chapter 2, Materials and Methods); C57BL/6J control mice received 0.5% methylcellulose vehicle without the drug.

After 10-weeks of lacidipine treatment, animals from each group were fasted overnight and sacrificed under pentobarbital anaesthesia by withdrawal of blood from vena cava. Blood was anti-coagulated with EDTA, centrifuged and total plasma cholesterol and triglyceride levels were determined with an automated enzymatic technique (as reported in Chapter 2, Materials and Methods). Separation of lipoproteins was performed by sequential density ultracentrifugation (see Chapter 2, Materials and Methods), for very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL), with a total centrifugation time of 5 h at 16°C.

Plasma endothelin levels were measured by enzyme-linked immunosorbent assay in plasma stored at -70°C (see Chapter 2, Materials and Methods).
In addition, the heart and aorta were examined to assess the extent of atherosclerosis. The aorta (ascending aorta, aortic arch, and descending aorta to the diaphragm) was formalin-fixed, stained with Oil red O (ORO) and the extent of the atherosclerotic lesions quantified in en face preparations. The heart after fixation was embedded in paraffin and sectioned according to Tangirala et al. (Tangirala et al. 1995b) and the quantification of the area of the atherosclerotic lesions measured in the aortic sinus, in 17 sections, using a computerised image analysis system (see Chapter 2, Materials and Methods).

Statistical analysis was performed by comparison of treated and control groups using a one-sided Dunnett’s test. Analysis of covariance (ANCOVA) was used to test for differences between treatment groups in the lesion areas in en face aortic samples. Mixed model analysis of variance (ANOVA) was used to test for statistically significant differences between lacidipine-treated groups and the apoE-deficient vehicle control group, with regard to the lesion size measured in the aortic sinus preparations. Statistical tests were performed on atherosclerotic lesions in the aortic sinus versus plasma endothelin values; the correlation coefficient (Pearson’s r) was calculated (P < 0.05).
4.3 RESULTS

4.3.1 Clinical observations

During the 10-week period of treatment with lacidipine, the drug was well tolerated at all the dose levels used (0.3, 1.0 and 3.0 mg/kg) and there were no clinical signs attributable to the administration of the compound. The body weight gain in apoE-deficient vehicle-treated control mice, and in the C57BL/6J control mice, was the same as in the lacidipine-treated groups. At the end of the study the mean body weights in the control apoE-deficient and C57BL/6J mice were 27 g ±2 and 28 g ±2, respectively; the mean body weights of lacidipine-treated mice were: 29 g ±1, 28 g ±3 and 28 g ±2 at 0.3, 1.0 and 3.0 mg/kg, respectively. No significant differences in diet consumption were seen between lacidipine-treated animals and the control groups.

4.3.2 Plasma lipid profile

Results for plasma lipid profiles are presented in Table 4.1. The apoE-deficient control group showed an increase of about 7.1 times the plasma cholesterol level of the wild-type control C57BL/6J animals (P<0.01). Mean levels of total cholesterol ranged from 11.75 mmol/l (vehicle-control group) to 13.49 mmol/l (1.0 mg/kg lacidipine group) in the apoE-deficient groups, in comparison with 1.65 mmol/l in C57BL/6J vehicle-control mice. Mean plasma total cholesterol levels measured in apoE-deficient mice treated with lacidipine (at 0.3, 1.0, 3.0 mg/kg/day) were not significantly different to the apoE-deficient control animals. Mean plasma triglycerides levels were comparable in C57BL/6J control animals and the four groups of apoE-deficient mice (controls and lacidipine-treated) (Table 4.1). Comparison of lipoprotein cholesterol profiles
showed that in apoE-deficient mice the major cholesterol-carrying plasma lipoproteins were the VLDL and LDL fractions (about 98% of total); however, in the C57BL/6J animals, the main cholesterol-carrying plasma lipoprotein was the HDL fraction (making up about 83% of the total). The administration of lacidipine did not alter the lipoprotein profile in apoE-deficient mice, nor the cholesterol distribution in the different lipoprotein fractions (Table 4.1).

4.3.3 Plasma endothelin

The effect of lacidipine on plasma endothelin concentrations was evaluated (Fig. 4.1). A value of 0.48 ±0.03 pg/ml was detected in the C57BL/6J control mice; however, in the apoE-deficient vehicle control mice, values about fourfold higher were observed (2.07 ±0.12 pg/ml; P<0.01). Administration of lacidipine to apoE-deficient mice induced a significant dose-dependent decrease in plasma endothelin levels; the values detected were 1.50 ±0.05 pg/ml (NS), 1.15 ±0.02 pg/ml (P<0.01) and 1.15 ±0.03 pg/ml (P<0.01) in the lacidipine-treated groups (0.3, 1.0 and 3.0 mg/kg), respectively.

4.3.4 Atherosclerotic lesions

All apoE-deficient mice developed spontaneous atheromatous lesions in the aortic tract. No lesions were detected in the wild-type control C57BL/6J mice. Under the stereo-microscope, atheromatous lesions appeared as raised, Oil red O positive deposits (Fig. 4.2a). Sites of predilection for lesion development were, in order of severity: the inner curvature of the aortic arch, the right common carotid artery, the left common carotid artery, and the left subclavian artery. In the thoracic aortic tract, focal spotty lesions were seen at
the sites of the branching of the arteries. The distribution of lesions was not significantly affected by the treatment with lacidipine at 0.3, 1.0 and 3.0 mg/kg (Fig. 4.2b).

### 4.3.5 Quantitation of atherosclerotic lesions

Morphometry of en face aortic preparations (Fig. 4.3) in control apoE-deficient mice showed that the extent of lesions in the entire aorta (aortic arch and thoracic aorta) was equal to 2.18 ±0.33% of the mean total surface area examined (corresponding to a mean lesion area of $779 \times 10^3$ μm$^2$ in comparison with a mean total aorta surface area of $35,789 \times 10^3$ μm$^2$). In lacidipine-treated apoE-deficient mice, a reduction was observed in the total extent of lesions over the entire aortic surface examined (Fig. 4.4), from 2.18 ±0.33% (vehicle control) to 1.40 ±0.33% (0.3 mg/kg lacidipine), 1.24 ±0.30% (1.0 mg/kg lacidipine), and 1.23 ±0.33% (3.0 mg/kg lacidipine). The extent of the atherosclerotic lesions was significantly reduced at all three dosages of lacidipine (P<0.05) (Fig. 4.4).

The extent of atherosclerosis was also quantified in transverse sections of the aortic root at the level of the aortic sinus (Fig. 4.5). In the apoE-deficient control group the average lesion area was $144 \times 10^3$ μm$^2$. The daily oral administration of lacidipine at 0.3, 1.0, 3.0 mg/kg, reduced the mean lesion area in a dose-related manner by 10%, 17% and 53%, respectively (P<0.05, at 3.0 mg/kg lacidipine) (Fig. 4.6).

The relationship between plasma endothelin levels and the extent of the atheromatous lesions in control C57BL/6J and apoE-deficient mice was also
examined. A statistically significant correlation was observed (P<0.05; r = 0.81) in plasma endothelin values and the extent of the atherosclerotic lesions in the aortic sinus (Fig. 4.7).
4.4 DISCUSSION

Transgenic apo-E deficient mice are a genetically defined murine model in which the pathogenesis of atherosclerosis can be investigated. The strain is considered to be of particular value because the mouse develops spontaneous atheromatous lesions that are morphologically similar to those found in man (Bocan 1998). The atheromatous lesions form in a reproducible manner, and the transition from the lipid-laden lesion to the fibrous plaque can be easily evaluated microscopically. In addition, the progression of lesion development can be accelerated by feeding the animals with dietary fat and cholesterol (Plump et al. 1992). In fact, when challenged with a high fat Western-type diet, these animals reach plasma cholesterol levels ten times higher than controls. This marked hypercholesterolemia is primarily due to elevated levels of very low and intermediate density lipoproteins. At 10 weeks of age, apoE-deficient mice have already developed atherosclerotic lesions in the aorta, and in the coronary and pulmonary arteries (Nakashima et al. 1994).

In the present experiment the advantages of the apoE-deficient mouse model of atherosclerosis have been re-assessed, and the usefulness confirmed. The doses of lacidipine used in the present study (0.3, 1.0 and 3.0 mg/kg) were chosen on the basis of preliminary measurements of drug levels in humans, and in apo-E deficient mouse plasma. In general terms, on average, in man, 4 mg/day of lacidipine gives rise to a maximal plasma concentration (Cmax) ranging from 2.76 to 4.29 ng/ml (Da Ros et al. 2003). In the apo-E deficient mouse, a similar Cmax (ranging from 1.2 to 4.0 ng/ml) was obtained in earlier studies by administering lacidipine at a daily dose of 3 mg/kg body weight. Earlier preliminary studies have also shown similar ranges of blood pressure in
CD-1 strain control mice (89.8 ±5.0 mmHg), the C57BL/6J strain (84.5 ±3.1 mmHg), and the Apo-E deficient mouse (87.2 ±8.6 mmHg). Furthermore, in these studies, the daily administration of lacidipine at 3.0 mg/kg to apo-E deficient mice did not cause any affect on blood pressure, the level remaining 86.8 ±3.8 mmHg.

In the present study, it was observed for the first time (Fig. 4.1) that the atherosclerotic apoE-deficient mouse has plasma endothelin levels about 4-fold higher than the control wild-type C57BL/6J mouse. These results are in agreement with the data published by Barton et al. (1998) who demonstrated the elevated aortic endothelin-1 protein content in apo-E deficient mice, compared with C57BL/6J mice. Increased vascular endothelin production inhibits endothelial nitrogen oxide release thereby impairing endothelium-dependent relaxation and promoting atheroma formation (Barton et al. 1998). In addition, it was possible to demonstrate in the present experiment that the endothelin values obtained in the apoE-deficient mouse are closely correlated to the extent of the atherosclerotic lesions (Fig. 4.7). Several studies have demonstrated that, in a clinical situation, plasma endothelin values are elevated in hypercholesterolemic patients (Bath & Martin 1991; Haak et al. 1994), in ischemic heart disease (Yasuda et al. 1990), and in symptomatic atherosclerotic patients. Furthermore, a significant correlation between plasma endothelin concentrations and the particular vascular sites with atherosclerotic lesions has also been described (Lerman et al. 1991; Arendt et al. 1993). The expression of endothelin in the process of atherosclerosis, observed both in human patients and in apo-E deficient mice, lends additional support to the relevance and usefulness of this murine model in the study of human atherosclerosis, and this also suggests a role.
for endothelin as a monitorable marker in both the evolution and in the treatment of cardiovascular disease.

The present work clearly demonstrates that the calcium antagonist, lacidipine, significantly reduced the development of atherosclerotic lesions in the apoE-deficient mouse. These results therefore provide further useful support to the previous evidence of lacidipine-mediated anti-atherosclerotic activity, which has been reported in a range of other animal models (Paoletti et al. 1996; Soma et al. 1996; Cristofori et al. 2000a), and in \textit{in vitro} studies with the drug (Bernini et al. 1996; Bernini et al. 2001). From a mechanistic viewpoint, lacidipine is known to interfere with major processes of atherogenesis occurring within the arterial wall (Bernini et al. 1996). In particular, the drug is known to inhibit smooth muscle cell migration and proliferation, and through the inhibition of acyl-Co A transferase (ACAT) activity, prevents cholesterol accumulation in macrophages \textit{in vitro}, as well as in the aorta of hypercholesterolemic rabbits (Bernini et al. 2001). Many components of plaque formation rely on oxidative stress for their expression, and lacidipine is one of the most potent antioxidant calcium channel blockers, having activity similar to vitamin E (Van Amsterdam et al. 1992).

The anti-atherosclerotic activity of lacidipine in the hypercholesterolemic apoE-deficient mouse, in the present study, was not associated with any reduction in the very high levels of plasma cholesterol; this result suggests a different mechanism of action for lacidipine in comparison to that described for other lipid lowering drugs (Tawara et al. 1986; Reckless et al. 1997). A reduction of atheromatous lesions, without any accompanying changes in plasma lipids, has been reported in previous studies which investigated the suppression of atherogenesis by other calcium antagonists (Paoletti et al. 1996). However, it is
considered possible that lacidipine may influence the atherogenic process by a more specific mechanism, which is the result of a combined potent and long-lasting calcium antagonism in conjunction with the known powerful antioxidant properties of the drug. These activities of lacidipine are considered to be further enhanced by the high lipophilicity of the drug (Herbette et al. 1993; Gaviraghi & Trist 1998). In addition, it is unlikely that the anti-atherosclerotic properties of lacidipine could be related to the well-reported antihypertensive effects of the drug, since the dose levels of 3.0mg/kg/day has been shown not to affect blood pressure values in the apoE-deficient mouse (Gaviraghi et al. 1998). However, on the basis of the present data, we cannot exclude the possibility that vasodilatation may have occurred, with a compensatory increase in cardiac output, and therefore that the effects seen might also be accounted for by vascular dilatation.

In the present work, lacidipine-treatment significantly reduced both plasma endothelin levels and the development of atherosclerosis in the apoE-deficient mouse. Other authors have demonstrated the effects of lacidipine on endothelin expression in the salt-loaded stroke prone hypertensive rat, where a cardioprotective effect was observed which was not correlated with a detectable reduction in blood pressure (Ferron et al. 1996). Expression of the endothelin gene in endothelial cells is subject to a complex pattern of regulatory pathways involving numerous factors (Rubanyi & PoloKoff 1994). In particular, endothelin gene expression is linked to the activation of the transcription factor which has been named activated protein-1 (Lee et al. 1991; Meyer et al. 1993).

Transcription factors are proteins typically located in the cytosol. These readily-available elements are therefore activated after transcription, and they are considered to rapidly regulate the expression of selective genes (Lee et al. 1991).
It has also been shown that AP-1 is activated by many oxidative stress-inducing stimuli. For example, previous studies have demonstrated that oxidized LDL increases the release of endothelin by endothelial cells through an oxidative stress-inducing stimulus (Meyer et al. 1993). Since lacidipine has also been shown to possess a strong antioxidant activity (Van Amsterdam et al. 1992), and to reduce the production of intracellular reactive oxygen species induced by oxidized LDL in endothelial cells (Cominacini et al. 1998), it may be concluded that the decrease in plasma endothelin, identified in the present experiments in apoE-deficient mice treated with lacidipine, may be related to a specific effect on cellular oxidative stress. Furthermore, there is evidence that endothelin plays an important role in the initiation and progression of cellular pathways leading to atherogenesis (Berghese et al. 1996). Also, the potent coronary vasoconstrictive properties of endothelin may potentiate the atherosclerotic process by reducing blood flow, and subsequently initiate an enhancement of platelet aggregation and thrombus formation (Berghese et al. 1996; Cominacini et al. 1998). We propose therefore that the anti-atherogenic activity of lacidipine may be related to a specific effect on endothelin over-production which occurs in atherosclerosis, and the drug may thus interfere with the proatherogenic activity of endothelin.

In conclusion, it is considered that the present results show that lacidipine, administered at the dose levels of 0.3, 1.0 and 3.0 mg/kg reduces the extent of atherosclerosis in the apoE-deficient mouse, and that this useful effect may be due, at least in part, to a direct protection of endothelial cell function.
Fig. 4.1: Effect of lacidipine treatment on plasma endothelin levels in apoE-deficient mice.

Values of plasma endothelin in the apoE-deficient mouse treated with vehicle (control), or lacidipine at 0.3, 1.0 and 3.0 mg/kg daily, and in vehicle-treated (control) C57BL/6J mice. n=4 in each group; values are expressed as mean ±SEM; **P<0.01 in lacidipine-treated mice in comparison with apoE-deficient controls, and in C57BL/6J mice in comparison with apoE-deficient controls.
Fig. 4.2: Stereo-microscope photograph of the aortic arch after Oil red O staining in apoE-deficient mice. All x0.67 (OM).

(a) Gross appearance of the atherosclerotic lesions in the aortic arch from a control apoE-deficient mouse; red areas, stained by Oil red O, represent lesions, and are evident at different sites in the aortic arch, particularly on the inner curvature and at the right common carotid artery bifurcation.

(b) Aortic arch of a hypercholesterolemic apoE-deficient mouse treated for 10 weeks with 3 mg/kg of lacidipine. Only slight Oil red O positivity is evident along the inner curvature, the most prone area of the aortic arch.
Fig. 4.3: *En face* preparations: gross appearance of the atherosclerotic lesions in the aorta (arch and thoracic segments) opened longitudinally, of apoE-deficient mice; Oil red O positive spots correspond to lesions which appear as raised red areas. All x0.67 (OM); (Oil red O stain).

(a) Control apoE-deficient mouse treated with vehicle; large lesions are evident in the aortic arch and smaller red areas are present at the sites of arterial branching in the thoracic aorta.

(b) Lacidipine-treated apoE-deficient mouse (3.0 mg/kg); the extent of the lesions is clearly reduced in comparison with the control.
Fig. 4.4: Extent of atherosclerosis measured in en face preparations of the aorta (arch and thoracic segments) in control apoE-deficient mice or in animals treated with lacidipine at 0.3, 1.0 and 3.0 mg/kg for 10 weeks.

Figure shows the lesion area, expressed as percentage of total aortic surface area, covered by atherosclerotic lesions in control apoE-deficient mice and in mice treated with lacidipine at 0.3, 1.0 and 3.0 mg/kg lacidipine. Values are expressed as means, ±SEM; n=6 in each group. The reduction in atherosclerosis in lacidipine-treated mice versus control animals was statistically significant (*P<0.05).
Fig. 4.5: Cross-section through the aortic origin at the level of the aortic sinus. x4 (OM); (H&E stain)

(a) Aortic sinus from an apoE-deficient control mouse fed a standard diet. Extensive lesions are visible beneath all three valve leaflets. Lesions appear advanced showing a well-defined fibrous cap, a necrotic core with cholesterol clefts, and thinning of the vessel wall.

(b) Photomicrograph shows the histological effects of lacidipine treatment on the lesions in the aortic sinus. The extension of the lesions in this apoE-deficient mouse treated with lacidipine at 3.0 mg/kg for 10-weeks appears to be markedly reduced, and the lesion is less complex in composition suggesting a less advanced pathological condition.
Fig. 4.6: Atherosclerotic lesion area measured in cross-sections of the aortic root at the level of the aortic sinus.

Figure shows the average lesion area ($10^3 \mu m^2$) measured for each mouse in 17 cross-sections through the aortic origin, over a distance of 240 $\mu m$. Values are expressed as means, ±SEM; $n=6$ in each group; *$P<0.05$, for lacidipine-treated mice at 3.0 mg/kg compared with the vehicle-treated controls.
Fig. 4.7: Correlation between the extent of the atherosclerotic lesion areas and plasma endothelin values.

Aortic lesion areas were measured in cross-sections of the aortic origin at the level of the aortic sinus, and plasma endothelin values, in individual (n=4) apoE-deficient mice, and in wild-type C57BL/6J control mice. Individual values are C57BL/6J control mice (●), apoE-deficient control mice (■), apoE-deficient mice treated with lacidipine at 0.3 (○), 1.0 (●) and 3.0 mg/kg (▲). Dashed lines indicate the 95% confidence intervals of the regression line. The coefficient of correlation was r=0.81; P<0.05.
Table 4.1: Plasma lipid profile of control vehicle-treated apoE-deficient and wild-type C57BL/6J mice, and apoE-deficient animals treated with lacidipine at 0.3, 1.0 and 3.0 mg/kg for 10 weeks.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cholesterol (mmol/l)</th>
<th>Triglycerides (mmol/l)</th>
<th>VLDL&lt;sup&gt;b&lt;/sup&gt; cholesterol (mmol/l)</th>
<th>LDL&lt;sup&gt;b&lt;/sup&gt; cholesterol (mmol/l)</th>
<th>HDL&lt;sup&gt;b&lt;/sup&gt; cholesterol (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE-deficient (control)</td>
<td>11.75±0.28</td>
<td>0.87±0.03</td>
<td>10.02</td>
<td>1.52</td>
<td>0.22</td>
</tr>
<tr>
<td>ApoE-deficient (0.3 mg/kg)</td>
<td>13.27±0.31</td>
<td>1.23±0.07</td>
<td>11.30</td>
<td>1.72</td>
<td>0.25</td>
</tr>
<tr>
<td>ApoE-deficient (1.0 mg/kg)</td>
<td>13.49±0.22</td>
<td>1.18±0.04</td>
<td>12.01</td>
<td>1.43</td>
<td>0.33</td>
</tr>
<tr>
<td>ApoE-deficient (3.0 mg/kg)</td>
<td>12.24±0.33</td>
<td>1.19±0.03</td>
<td>10.40</td>
<td>1.59</td>
<td>0.23</td>
</tr>
<tr>
<td>C57BL/6J (control)</td>
<td>1.65±0.02**</td>
<td>0.90±0.03</td>
<td>0.07</td>
<td>0.17</td>
<td>1.17</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are expressed as means; n=6. Data from the apoE-deficient vehicle-treated control mice were compared with mice in the three lacidipine-treated groups, and also with the C57BL/6J control mice; **P<0.01.

<sup>b</sup> VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein. The level of total cholesterol in the three main lipoprotein classes (VLDL, LDL, and HDL) was quantified from pooled plasma from the mice in each group; for this reason the results were not analysed statistically.
Chapter 5

ANTI-ATHEROSCLEROTIC AND ANTI-OXIDANT EFFECTS OF LACIDIPINE IN APOE-DEFICIENT MICE FED A HIGH FAT DIET

5.1 INTRODUCTION

Several lines of evidence from both in vitro and in vivo studies suggest that oxidation of low density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis, although the reasons for this are not fully understood (Avogaro et al. 1988; Boyd et al. 1989; Steinberg et al. 1989; Wiklund et al. 1991; Rosenfeld 1991).

The ability of oxidized LDL to induce cholesterol accumulation in macrophages was the first pro-atherogenic property of oxidized LDL to be described (Henriksen et al. 1981). Over the following years a number of additional properties of oxidized LDL were described that could, in principle, contribute to its atherogenicity. Oxidized LDL is itself a chemotactic agent for monocytes (Quinn et al. 1987), and for T cells (McMurray et al. 1993), but not for B cells. This is consistent with the fact that atherosclerotic lesions contain primarily monocytes and T cells. Also, oxidatively modified LDL is taken up by macrophages at an increased rate in comparison to native LDL, and thus promotes cellular cholesterol accumulation and foam cell formation which are both the hallmarks of early atherosclerotic lesions (Steinberg 1997). Moreover, the oxidation process can induce the expression of adhesion molecules and
facilitate transcription factor expression, which both play an important role in the further development of atherosclerosis (Cominacini et al. 1997a).

LDL oxidation, occurring inside the artery wall, can be inhibited by many defence systems in plasma such as antioxidants, if such systems are preserved and are able to perform their activity (Steinberg et al. 1989). Since oxidized-LDL can not be obtained from whole blood, considerable research has focussed on the measurement of the susceptibility of isolated LDL particles to \textit{in vitro} oxidation (Esterbauer et al. 1987; Cominacini et al. 1991b).

Antioxidant supplementation has been shown to reduce the progression of atherosclerotic lesions in Watanabe hyperlipidemic rabbits (Mao et al. 1991), and to increase the resistance of LDL to oxidation in both healthy and diabetic subjects (Dieber-Rotheneder et al. 1991; Cominacini et al. 1991a; Babié et al. 1992).

1,4-dihydropyridine (1,4-DHP) calcium channel blockers (CCBs), although markedly different in their chemical structures and antihypertensive effects, contain aromatic rings capable of stabilizing oxygen radicals, and a hydrogen-donating reaction may also account for their anti-oxidant activity (Napoli et al. 1999). Of the several DHP CCBs, lacidipine has been demonstrated to possess clear anti-oxidant properties in that the drug has activity comparable to the reference antioxidant compound, vitamin E, in biological membrane models (Van Amsterdam et al. 1992). In addition, lacidipine has been shown to be able to reduce the extent of atherosclerotic lesions in the cholesterol-fed hamster model (Cristofori et al. 2000a), as well as in the apoE deficient mouse model fed a conventional rodent diet (Cristofori et al. 2000b), and also in man (Zanchetti et al. 2002).
The present study investigated the anti-atherosclerotic activity of lacidipine in the apoE-deficient mouse. It has been previously demonstrated that LDLs in this strain of mouse are highly susceptible to oxidation (Hayek et al. 1994), and therefore this animal model could prove to be invaluable in assessing the atherogenic relevance of factors involved in the oxidative modifications of lipoprotein (Breslow 1993). In particular, the effect of lacidipine on the development of atherosclerotic lesions in the apoE-deficient mouse was to be assessed in mice which also would be challenged with a high fat diet, in order to evaluate the associated susceptibility of LDL to oxidation under conditions of oxidative stress. Considering that it was known that in apoE-deficient mice the 3 mg/kg lacidipine dose level induced plasma drug levels comparable to that achieved with the human therapeutic dose of 4 mg/kg (Zanchetti et al. 2002) it was decided to choose the 3 mg/kg dose, and the 10 mg/kg dose was also chosen to maximise any effect of the lacidipine-treatment.

5.1.1 Acknowledgements

The author would like to acknowledge and thank Dr Luciano Cominacini and his colleagues from the Departement of Biomedical and Surgical Sciences, Section of Internal Medicine, University of Verona for their assistance with the experiments to assess the oxidation profile of low density lipoprotein.
5.2 MATERIALS AND METHODS

Homozygous female apoE-deficient mice (n=60) were used for the present study. Mice were approximately 6 weeks old at the beginning of the experiment. ApoE-deficient mice were randomly allocated to three groups of 20 animals each (one vehicle-treated control and two lacidipine-treated groups), and treated daily by gavage. Animals were housed three per cage on wood shavings and fed a Western-type diet to accelerate the development of atherosclerosis (see Chapter 2, Materials and Methods). During the 8-week period of treatment, lacidipine was administered daily by gavage at 0 (control), 3.0 and 10.0 mg/kg body weight in 0.5% methylcellulose at a standard dose volume of 10 ml/kg body weight, or animals were given vehicle (0.5% methylcellulose, as described in Chapter 2).

After the 8-week period of lacidipine-treatment, animals from each group were fasted overnight and sacrificed under pentobarbital anaesthesia by withdrawal of blood from vena cava. Blood was anti-coagulated with EDTA, centrifuged and total plasma cholesterol and triglyceride levels were determined with an automated enzymatic technique (as reported in Chapter 2). Separation of lipoproteins was performed by sequential density ultracentrifugation (see Chapter 2, Materials and Methods), for very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL), with a total centrifugation time of 5 h at 16°C. LDLs derived from apo-E deficient mice were oxidized either with copper ions or with human umbilical vein endothelial cells (HUVECs). The method for LDL oxidation with Cu^{2+} and for the evaluation of LDL susceptibility to oxidation (i.e. evaluation of the length of time of the lag phase), was based on the
development of fluorescence during copper-catalysed LDL oxidative modification. The hydroperoxide content of native LDL was determined by evaluating the level of malonyldialdehyde (MDA) and the extent of LDL oxidation was determined as the thiobarbituric acid adduct (full details in Chapter 2, Materials and Methods). LDL vitamin E consumption during HUVEC oxidation was also measured. In addition, the extent of the atherosclerotic lesions was measured in paraffin sections of the heart, in the aortic sinus, and quantified using a computerized image analysis system (Chapter 2, Materials and Methods).

Statistical analysis was performed by comparison of treated and control groups using a one-sided Dunnett's test. Mixed model analysis of variance (ANOVA) was used to test for statistically significant differences between ladinpine-treated groups and the apoE-deficient vehicle control group, with regard to the lesion size measured in the aortic sinus preparations.
5.3 RESULTS

5.3.1 Clinical observations

During the 8-week period of treatment with lacidipine, the drug was well tolerated at the dose levels used (3.0 and 10.0 mg/kg) and there were no clinical signs attributable to the administration of the compound. No significant differences were seen between the lacidipine-treated animals and the controls in the consumption of diet. The increases in body weight observed during the 8 week period of the study in vehicle-treated control apoE-deficient mice were in the same range as in the lacidipine-treated groups (mean increases of 15.6% and 16.3%, respectively).

5.3.2 Plasma lipid profile

The administration of lacidipine at 3.0 and 10.0 mg/kg did not affect either plasma total cholesterol levels or the cholesterol distribution in the different lipoprotein fractions, as the values observed were not significantly different to the apoE-deficient control (vehicle-treated) animals (Table 5.1). Mean levels of total cholesterol were 36.88 ±0.28 mmol/l in the apoE-deficient control group, and 35.20 ±0.31 mmol/l and 35.20 ±0.22 mmol/l in the apoE-deficient mice treated with lacidipine at dosages of 3.0 and 10.0 mg/kg/day, respectively.

5.3.3 Quantitation of atherosclerotic lesions

In control apoE-deficient mice, severe atherosclerosis developed during the 8-week period of the study and lesions covered almost the entire aortic surface at the level of the aortic sinus. When the extent of the atherosclerosis
was quantified in the aortic root of the heart, a significant dose-related reduction in aortic lesion development was found in mice given lacidipine at 3.0 and 10.0 mg/kg (P<0.01 at both dose levels). The average lesion area was $305.5 \pm 8.8 \times 10^3 \mu m^2$ in the apoE-deficient control group (Fig. 5.1), $173.7 \pm 6.7 \times 10^3 \mu m^2$ in animals treated at 3.0 mg/kg lacidipine, and $152.6 \pm 7.6 \times 10^3 \mu m^2$ in the 10.0 mg/kg lacidipine group (reductions of 43.1%, and 50.1% of the control lesion area, respectively). The histological assessment of the aortic arch demonstrated advanced atherosclerotic disease with plaques showing foamy macrophages, calcification, and fibrous caps, a morphology which closely resembles human atherosclerosis. However, no significant differences in the histological appearance of the lesions in vehicle-treated control mice, and in mice treated with lacidipine at 3.0 and 10.0 mg/kg, were noted.

Statistical analysis was performed by ANOVA one-way analysis following Dunnett’s test to compare lacidipine-treated groups and the vehicle-treated (control) group, where P<0.05 was considered significant.

5.3.4 Susceptibility of mouse low density lipoproteins to undergo lipid peroxidation

After treatment with lacidipine at 3.0 and 10.0 mg/kg daily for 8 weeks, the susceptibility of mouse LDL to undergo lipid peroxidation following incubation with CuSO$_4$ was significantly reduced (P<0.01) in comparison with the control vehicle-treated group (Fig. 5.2). In the vehicle-treated control group the mean lag time required for the initiation of CuSO$_4$-induced LDL oxidation was $100.3 \pm 12.7$ min. In contrast, in the two lacidipine-treated groups, the mean time required for LDL oxidation was increased, being $125.8 \pm 15.2$ min
with lacidipine at 3.0 mg/kg, and 146.8 ±10.0 min in the 10.0 mg/kg lacidipine group.

This result paralleled the significantly (P<0.01) lower concentrations of hydroperoxide content (MDA), detected in the LDL-like particles, derived from both lacidipine-treated groups, compared with the vehicle-treated controls (Fig. 5.3). The mean MDA values, measured in vehicle controls and lacidipine-treated groups at 3.0 and 10.0 mg/kg, were 1.93 ±0.31, 1.21 ±0.30 and 0.93 ±0.07 nmol/mg LDL, respectively. By plotting the lag phase values detected in individual samples from apoE-deficient mice treated with lacidipine or vehicle, with the corresponding MDA values (Fig. 5.4), a significant relationship was identified (r=0.89; P<0.01).

5.3.5 Umbilical vein cell culture-mediated oxidation of low density lipoprotein-like particles

Changes in MDA production of LDL-like particles derived from apoE-deficient mice were also monitored and quantified during a 24h incubation period with HUVECs. The amount of MDA formation in the cell free medium (CFM) over the 24h period was less than 1 nmol/ml/h. However, a significant increase in MDA production was detected (P<0.01) with HUVECs incubated for 3, 5, 8, 11 and 24h at 37°C with LDL-like particles from apoE-deficient mice treated with vehicle. The formation of MDA was significantly (P<0.01) reduced by HUVECs incubation with lacidipine, since lower quantities of MDA were detected from the fourth to the twenty-fourth hour of the incubation period. Expressing these results as area under the curve (Kwon et al. 1998), calculated by using the mean values of MDA production at several
time intervals during the 24h incubation, a significant (P<0.01) dose-related reduction in MDA production of LDL-like particles from lacidipine-treated mice was detected (Fig. 5.5).

5.3.6 Vitamin E content of low density lipoprotein-like particles

No difference was detected in the basal LDL-like particle concentrations of vitamin E between apoE-deficient mice treated with vehicle or lacidipine (mean and SEM: 5.43 ±0.09 µg/mg LDL in vehicle-treated control mice versus 5.85 ±0.29 and 5.86 ±0.16 µg/mg LDL in the 3.0 and 10.0 mg/kg lacidipine treated groups, respectively). However, after 24h exposure to HUVECs, a significant decrease in the LDL-like particle concentration of vitamin E in the vehicle (control) group was observed as, at the end of the incubation period, a value below 15 nmol/ml/h was measured. Nevertheless, in apoE-deficient animals treated with lacidipine at 3.0 and 10.0 mg/kg, levels of vitamin E were maintained with concentrations significantly (P<0.01) higher in both lacidipine-treated groups in comparison with the vehicle-treated control mice. Expressing the data as AUC, calculated as the mean values of vitamin E content, at specific time points during the 24 h incubation period, a significant (P<0.01) and dose-related preservation of vitamin E levels in the lacidipine-treated mice was demonstrated (Fig. 5.6).
5.4 DISCUSSION

The present study demonstrates that lacidipine treatment of atherosclerotic apoE-deficient mice significantly reduced the progression of accelerated aortic atherosclerosis induced by a high fat diet. The extent of atherosclerosis in the aortic origin of the heart was significantly reduced after the 8-week period of treatment with lacidipine at 3.0 and 10.0 mg/kg. In apoE-deficient mice it has been shown that the 3.0 mg/kg lacidipine dose level induces plasma drug levels comparable to that achieved with the human therapeutic dose of 4 mg/kg (Zanchetti et al. 2002); the 10.0 mg/kg dose was chosen in this study to maximise any effect of lacidipine-treatment. In the present experiments, the reduction of atherosclerotic lesions caused by lacidipine was paralleled by a marked decrease in the propensity for oxidation of plasma LDL, induced by different modes of oxidative stress. However, the anti-atherosclerotic activity of lacidipine in the apoE-deficient mice was not caused by a reduction in plasma cholesterol levels, as the plasma lipid profiles in vehicle-treated and lacidipine-treated groups were similar (Table 5.1). This last finding is in line with previously published work (Cristofori et al. 2000b), although in that study apoE-deficient mice were fed a standard rodent diet. Moreover, in the earlier study (Cristofori et al. 2000b), it was reported that lacidipine did not affect blood pressure, which would suggest that it is unlikely that the anti-atherosclerotic properties of lacidipine, at least at a daily dose of 3.0 mg/kg, could be related to the anti-hypertensive effect of the drug. These findings are also in agreement with another study in the stroke-prone spontaneously hypertensive rat (SPSHR), in which lacidipine exerted a powerful antioxidant effect in vivo without affecting blood pressure (Napoli et al. 1999). Therefore, the sum total of these findings tends to reinforce the concept that the
activity of lacidipine on the atherosclerotic processes may be related to the known antioxidant properties of the compound (Van Amsterdam et al. 1992).

Several previous studies have demonstrated the important role of the inhibition of LDL oxidation in reducing the atherosclerotic process. Angiotensin-converting enzyme (ACE) inhibiting drugs such as captopril (Hayek et al. 1998), and fosinopril (Hayek et al. 1999), have also been reported to inhibit LDL oxidation and the atherosclerotic process in apoE-deficient mice. Other potent antioxidative agents, such as vitamin E, also attenuated atherosclerosis in this animal model (Maor et al. 1997). Similarly, Tangirala et al. (Tangirala et al. 1995a) also demonstrated anti-atherogenic effects in apoE-deficient mice using the antioxidant N,N'-diphenyl-1,4-phenylenediamine.

Lacidipine belongs to the class of 1,4-dihydropyridine calcium channel blocking drugs; analysis of the structural-functional relationships of the effects of 1,4-dihydropyridines on the oxidative modification of LDL suggests an important role for the 2-substitution of the phenyl ring (Napoli et al. 1999). In addition, calcium antagonists of the dihydropyridine type are highly lipophilic and therefore their antioxidative effects could be explained by hypothesising a direct interaction with plasma lipoproteins (Rojstaczer & Triggle 1996). The insertion of the hydrophobic drug directly into plasma lipoproteins may cause a change in the physical state of the lipid phase, and such a process could lead to a decrease in the susceptibility of the LDL lipids to oxidation. In the present work, the effect of lacidipine on the progression of aortic atherosclerosis was effectively associated with a reduced susceptibility of LDL to oxidation. Our data are also consistent with other studies in which an increase of LDL antioxidants, by incubation or oral supplementation, always resulted in a strictly proportional increase in the length of
the lag phase (Dieber-Rotheneder et al. 1991; Cominacini et al. 1991a; Regnstrom et al. 1992; Sasahara et al. 1994; Cominacini et al. 1997a; Sobal et al. 2001). There is now growing evidence of a relationship between susceptibility of LDL to oxidation and atherosclerotic risk (Cominacini et al. 1991a; Regnstrom et al. 1992; Sasahara et al. 1994; Cominacini et al. 1994a; Cominacini et al. 1994b; Cominacini et al. 1997b; Sobal et al. 2001). Oxidatively modified LDL is taken up by macrophages at an increased rate, in comparison to native LDL, and thus can promote cellular cholesterol accumulation and foam cell formation, which are the hallmarks of early atherosclerotic lesions (Steinberg 1997).

Vitamin E is the major lipid-soluble antioxidant present in blood. The vitamin acts synergistically with other circulating and cellular antioxidants to protect cells from damage and lysis induced by oxidative stress. Most of the vitamin E in blood plasma is present in the LDL fraction, and hence vitamin E is optimally placed to prevent free-radical-mediated modification of this lipoprotein. In the present study the amount of vitamin E was measured in apoE-deficient mice treated with lacidipine at 3.0 and 10.0 mg/kg and in a vehicle-treated control group. Levels of vitamin E were maintained in animals treated with lacidipine at both dose levels, since the vitamin E concentrations were significantly (P<0.01) higher in both lacidipine-treated groups in comparison with the vehicle-treated control mice. However, significant differences in the basal LDL-like particle concentrations of vitamin E were found between the apoE-deficient mice treated with lacidipine and the vehicle-treated control group. This could mean that lacidipine may have reduced the progression of aortic atherosclerosis by lengthening the LDL lag phase, working as a radical scavenger. This finding is consistent with the classic
kinetic model proposed by Niki for lipid peroxidation (Niki 1987), where the length of the lag phase is directly related to the amount of antioxidant contained in the lipoprotein. Another possibility in explaining the effect of lacidipine on LDL lag phases in apoE-deficient mice derives from the scheme proposed by (Esterbauer et al. 1989). According to these authors, the role of the copper in the oxidative process of LDL, is to catalyse the conversion of trace amounts of pre-formed lipid hydroperoxides to alkoxy and peroxy radicals, which in turn then start another lipid peroxidation reaction. More recently, some authors (Yoshida & Niki 1992) reported that the rate of copper-catalysed oxidation of methyl linoleate was proportional to the half power of the initial preformed peroxide concentration. In our study, hydroperoxides were reduced in the LDL-like particles of apoE-deficient mice treated with lacidipine in comparison with the vehicle-treated control group, and there was a direct correlation between the hydroperoxide concentrations in the LDL-like particles and the length of the lag phase. Therefore, lacidipine may have lengthened the LDL lag phase also by reducing the LDL content of the lipid hydroperoxides.

In the present study the anti-oxidative properties of lacidipine were also assessed in the in vitro model of oxidized LDL obtained by incubation with HUVECs, and interesting results were obtained. It is well known that the process of LDL modification induced by HUVEC endothelial cells in vitro closely resembles the corresponding process in vivo. The present experimental results may suggest that lacidipine plays a crucial role in the sequence of events leading to the formation of foam cells and the development atherosclerotic plaques. Although cell-mediated oxidative modification of LDL has been the subject of several
studies in recent years, the mechanism by which cells initiate LDL oxidation still remains unclear. Both extracellular superoxide radicals (Cathcart et al. 1989) and lipoxygenase activity (Cathcart et al. 1991; Chamulirat et al. 1991) have been proposed. Attention has also focused on the reaction of superoxide with nitric oxide (Bruckdorfer 1993). Nitric oxide can react with superoxide to form the peroxynitrite anion. Decomposition of the peroxynitrite anion generates a strong oxidant with reactivity similar to the hydroxyl radical, and which has been shown to initiate lipid peroxidation (Bruckdorfer 1993). Whichever mechanism of oxidation may be involved, this led to the initial hypothesis that the action of modifying cells is to accelerate the formation of lipid peroxides within the LDL particle. Recently, it has also been proposed that the modifying cells could directly provide peroxidative products to the LDL (Ezaki et al. 1995; Cominacini et al. 1996). Lacidipine could increase the potential of scavenging lipid peroxyl radicals, but the drug may also modify the potential of the modifying cells to produce peroxidative products and consequently delay the start of the lipid peroxidation chain. In the present study, the reduction in the content of hydroperoxides found in LDL-like particles of apoE-deficient mice treated with lacidipine, may therefore also be the result of the action of the drug on endothelial cells. This conclusion is consistent with the demonstrated inhibitory effect of lacidipine on the generation of reactive oxygen species induced by pro-oxidant stimuli in endothelial cells (Cominacini et al. 1998). The results of the present study also show that LDL vitamin E consumption during HUVEC oxidation was reduced in apoE-deficient mice treated with lacidipine, in comparison with the vehicle-treated control group. Although the mechanism underlying this effect of lacidipine on vitamin E is not clear, one hypothesis was proposed by some authors
(Thomas et al. 1995) which was that lacidipine acts as co-antioxidant. In the model proposed by Thomas and his colleagues, vitamin E does not act as a chain-breaking antioxidant, but facilitates the transfer of radical reactions from the aqueous phase into LDL, and mediates radical chain reactions within the lipoprotein particle. The vitamin thus exhibits pro-oxidant activity. Since it is the isolation of vitamin E within a lipoprotein undergoing oxidation that forces the vitamin to react with the polyunsaturated fatty acids of LDL, prevention of this oxidation depends on the rapid destruction of vitamin E. To interfere with such a process a compound must have a significant capacity to reduce and interact with LDL-associated vitamin E, and we have demonstrated that lacidipine does have the capacity to do this (see Section 5.3.6). The conversion of the lipophilic vitamin E into a harmless aqueous radical is the process that actually prevents lipid hydroperoxide formation. Results obtained recently with lacidipine containing the pyridinic group instead of the dihydropiridinic group (Cominacini et al. 1998) favour the hypothesis that the drug also works as a reductive compound.

In conclusion, in the present study it has been demonstrated that lacidipine reduces the extent of the atherosclerotic lesion in the hypercholesterolemic apoE-deficient mouse. Furthermore, this reduction in the area of the atherosclerotic lesion is associated with a decrease in the susceptibility of LDL to oxidation. These effects of lacidipine may be related to a radical scavenging activity of the molecule and/or to a decrease in the potential of the modifying cells to produce peroxidative products and/or to a reducing activity of the compound.
Fig. 5.1: Effect of lacidipine treatment at a dose of 3.0 or 10.0 mg/kg/day on the extent of atherosclerotic lesions measured in the aortic sinus of apoE-deficient mice after 8 weeks feeding a Western-type diet.

Values are expressed as mean ±SEM of the lesion area (x10^3 μm²) measured for each mouse in 17 cross-sections through the aortic origin, over a distance of 240 μm. n=20 in each group; **P<0.01 in lacidipine-treated animals compared to the vehicle-treated controls.
Fig. 5.2: The susceptibility of apoE-deficient mouse LDL to undergo lipid peroxidation following incubation with CuSO₄, measured as lag phase in minutes. ApoE-deficient mice treated with 3.0 or 10.0 mg/kg of lacidipine for 8 weeks showed a prolongation of the time required for oxidation of LDL.

The results are expressed as means ±SEM of the lag phase (min); n=6 pooled samples per dose level group; *P<0.05 in comparison with the 3.0 mg/kg lacidipine group; **P<0.01 in comparison with the vehicle-treated control group.
Fig. 5.3: Effect of lacidipine on the hydroperoxide content of native LDL.

The thiobarbituric acid reactive substances (TBARS) were analysed by the measurement of malondialdehyde (MDA) content, in LDL-like particles derived from apoE-deficient mice after 8 weeks treatment with vehicle or lacidipine at the dose of 3.0 or 10.0 mg/kg. The results are expressed as means ±SEM; n=6 pooled samples; **P<0.01 in comparison with the vehicle-treated control group.
Fig. 5.4: Relationship between the hydroperoxide content of native LDL, measured as malonyldialdehyde (MDA), and the resistance to oxidation of LDL-like particles, expressed as lag phase in minutes.

The results are expressed as means of lag phase (min) and MDA content, in LDL-like particles derived from the plasma of apoE-deficient mice treated with vehicle (control, solid squares) or lacidipine at a dose of 3.0 mg/kg (open circles) or 10.0 mg/kg (solid triangles). n=6 pooled plasma samples per dose group. A significant (r=0.89; P<0.01) relationship was identified.
Fig. 5.5: HUVEC-mediated oxidation of LDL-like particles.

The oxidation of LDL-like particles is expressed as the area under the curve (AUC in nmol/ml/h; mean ±SEM) of MDA production during a 24 h-incubation period with HUVEC. LDL-like particles were derived from the plasma of apoE-deficient mice after an 8 week period of treatment with vehicle (control), and lacidipine at 3.0 and 10.0 mg/kg. A dose-related reduction in AUC values was observed at both doses of lacidipine. **P<0.01 in comparison with the cell free medim (CFM), and the vehicle control; n=6 for vehicle control; n=11 for lacidipine at 3.0 mg/kg; n=6 for lacidipine at 10.0 mg/kg, and n=8 for CFM.
Fig. 5.6: Vitamin E content of LDL-like particles derived from the plasma of apoE-deficient mice after 8 weeks treatment with vehicle, or lacidipine at 3.0 or 10.0 mg/kg.

Vitamin E content, expressed as the area under the curve (AUC in nmol/ml/h; mean ±SEM) during a 24 h-incubation period with HUVECs. Dose-related increases in the AUC values were observed at both dose levels of lacidipine in comparison with the vehicle-treated control group; *P<0.01; n=6 for vehicle control; n=11 for lacidipine at 3.0 mg/kg; n=6 for lacidipine at 10.0 mg/kg.
Table 5.1: Plasma lipid profile of apoE-deficient mice and mice treated with lacidipine at 3.0 and 10.0 mg/kg for 8 weeks.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cholesterol (mmol/l)</th>
<th>Triglycerides (mmol/l)</th>
<th>VLDL&lt;sup&gt;b&lt;/sup&gt; cholesterol (mmol/l)</th>
<th>HDL&lt;sup&gt;b&lt;/sup&gt; cholesterol (mmol/l)</th>
<th>LDL&lt;sup&gt;b&lt;/sup&gt; cholesterol (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE-deficient (control)</td>
<td>36.88±0.28</td>
<td>0.87±0.03</td>
<td>10.00±0.07</td>
<td>1.52±0.22</td>
<td>0.22±0.02</td>
</tr>
<tr>
<td>ApoE-deficient (3.0 mg/kg)</td>
<td>35.20±0.31</td>
<td>1.23±0.07</td>
<td>11.30±0.05</td>
<td>1.72±0.01</td>
<td>0.25±0.02</td>
</tr>
<tr>
<td>ApoE-deficient (10.0 mg/kg)</td>
<td>35.20±0.22</td>
<td>1.18±0.04</td>
<td>12.00±0.03</td>
<td>1.43±0.03</td>
<td>0.33±0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are expressed as means; n=20 mice in each dose level group. For each dose level group, 6 pooled plasma samples of 2.5 ml each were assayed. Each pooled sample was the result of mixing the plasma from 3 or 4 mice.

<sup>b</sup> VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.
Chapter 6

EVIDENCE THAT LACIDIPINE ACTIVATES THE NITROGEN MONOXIDE SYSTEM IN APOE-DEFICIENT MICE

6.1 INTRODUCTION

Nitrogen monoxide (NO) is a highly reactive molecule with a wide range of distribution throughout the body; it is involved in signal transduction, in blood pressure homeostasis (Griffith & Kilbourn 1997) and in oxidative processes throughout the body. NO was first characterized in 1987 as endothelial derived relaxing factor (Palmer et al. 1987; Akopov 1996). Successively, there has been an explosion of research activity that indicates NO as an important bioregulatory molecule (Hibbs et al. 1988; Palmer et al. 1988; McCall et al. 1989; Bredt et al. 1990) that acts in many tissues to regulate a diverse range of physiological processes (Moncada et al. 1991). NO has a fleeting existence as it reacts extremely swiftly with superoxide, other active free radicals and with molecular oxygen (Stamler 1994).

Recent findings indicate that the vasorelaxant action of calcium antagonists is facilitated by NO (Salomone et al. 1996). In fact, NO is a potent vasodilator that activates guanylate cyclase, resulting in the generation of cGMP which is presumed to be the principal effector of NO-induced vasorelaxation in various tissues. It has been reported that the production of the natural vasoconstrictor endothelin is increased by a high-salt diet in
spontaneous hypertensive stroke-prone rats, an effect which is reduced in the presence of lacidipine (Godfraind & Salomone 1996). In addition, Salomone et al. (1996) demonstrated that the NO/cyclic guanosine monophosphate pathway facilitates the inhibitory effect of Ca^{2+} antagonists on KCl-evoked contraction in the rat aorta.

Microdialysis experiments have shown that NO metabolites (NOx; i.e. nitrites+nitrates) can be monitored in vivo in the rat treated with N-methyl-D-aspartate (NMDA) (Luo et al. 1993). By means of the electrochemical method of voltammetry applied with Nafion and ortho-phenylenediamine (oPD) coated carbon fiber micro-electrodes (mCFE, 30 μm diameter, 3 mm length) used as a biosensor, it has been recently demonstrated that the NMDA stimulated release of NO can be monitored in vivo in real time in the rat striatum (Crespi et al. 2001a). Furthermore, the same method has been used for the measurement of Substance P (endothelial NO synthase, eNOS, activator) stimulated release of NO in rat aortic rings (Crespi et al. 2000; Crespi et al. 2002). Substance P is an amidated peptide known to have a vascular dilating function and has been associated with the calcium-dependent activation of the enzyme nitrogen oxide synthetase (NOS), and thus is limited to an increased production of NO (Nava et al. 1996; Oldham et al. 1997).

The present study was therefore carried out to investigate the antiatherosclerotic activity of lacidipine in apoE-deficient mice treated for 8 weeks with either vehicle or lacidipine at 3.0 or 10.0 mg/kg/day. In parallel with histological studies of atherosclerotic lesions in the aorta, functional ex vivo studies were performed in order to examine the vascular properties of aortic ring preparations and analysis of voltammetric levels of NO in the aortic rings.
These studies allowed the substantiation of the efficacy of lacidipine in protecting and maintaining vascular properties, and provide further supporting evidence on the interaction between lacidipine and the vascular NO system.

6.1.1 Acknowledgements

The author would like to acknowledge and thank Dr Francesco Crespi, from Biology Department at GSK (Verona), for help in the interpretation of voltametric and functional studies and his colleagues for the technical assistance in performing analyses. Also Dr Carlo Zancanaro, Institute of Human Anatomy and Histology at the University of Verona, for his scientific advice in the interpretation of the scanning electron microscopy.
6.2 MATERIALS AND METHODS

Female apoE-deficient mice (n=18) were approximately 6 weeks old at the beginning of the experiment. They were randomly allocated to three groups of 6 animals each (one vehicle-treated control and two lacidipine-treated groups) and fed a Western-type diet (WTD) (containing 42% fat from milk fat and 0.15% cholesterol). Mice were treated daily for 8 weeks with lacidipine at dose levels of 0 (vehicle control), 3.0 and 10.0 mg/kg/day, as described in Chapter 2, Materials and Methods.

Additional “satellite” groups of apoE-deficient and wild-type C57BL/6J mice were fed a standard diet (containing 4.4% fat and 0.2% cholesterol) for 8 weeks. Some of these satellite animals (both naive apoE-deficient and wild-type C57BL/6J) (n=5) were sacrificed at the beginning of the study to assess the vascular reactivity of the aortic ring before starting lacidipine treatment; other animals (n=3), were killed at the end of the study to measure endogenous NO production.

After 8-weeks of lacidipine treatment, animals from each group were fasted overnight and sacrificed by carbon dioxide asphyxiation and withdrawal of blood from vena cava. Blood was anti-coagulated with EDTA, centrifuged and total plasma cholesterol and triglyceride levels were determined with an automated enzymatic technique, as reported in Chapter 2.

The extent of the atherosclerotic lesions was measured in paraffin sections of the heart, in the aortic sinus, and quantified using a computerized image analysis system (Chapter 2, Materials and Methods).
To further characterise the morphology of the intimal surface of aortic arch at the level of the inner curvature, i.e. the lesion prone area, scanning electron microscoscopy (Kritchevsky et al. 1977) examination was performed.

In parallel with histological studies of atherosclerotic lesions in the aorta, functional studies on the vascular reactivity of the aorta were carried out by measurement of the relaxant effect of various concentration of acetylcholine (Ach), and the analysis of voltametry levels of nitric oxide (NO) (Chapter 2, Materials and Methods).

Statistical analysis was performed using a one-sided Dunnett's test to determine differences between means; the correlation coefficient (Pearson's) was calculated with P values <0.05.
6.3 RESULTS

6.3.1 Clinical observations

During the 8-week period of treatment with lacidipine, the drug was well tolerated at the dose levels used (3.0 and 10.0 mg/kg) and there were no clinical signs attributable to the administration of the compound. No significant differences were seen between treated animals and controls in food consumptions. Starting mean body weights in apoE-deficient and C57BL/6J mice were 18 g ±2 and 17 g ±2, respectively; body weights in lacidipine-treated mice were: 20 g ±2 and 19 g ±3 for lacidipine at 3.0 and 10.0 mg/kg, respectively.

6.3.2 Plasma cholesterol profile

The administration of lacidipine at doses of 3.0 and 10.0 mg/kg did not affect the plasma total cholesterol levels. Mean levels of total cholesterol were 36.88 ±0.28 mmol/l in the apoE-deficient control (vehicle-treated) group, and 35.20 ±0.31 mmol/l and 35.20 ±0.22 mmol/l in apoE-deficient mice treated with lacidipine at 3.0 and 10.0 mg/kg/day, respectively.

6.3.3 Morphological examination and quantitation of atherosclerotic lesions

The histological assessment of the atherosclerotic lesions at the level of the aortic origin in all 3 groups demonstrated advanced atherosclerotic disease with plaques containing foamy macrophages, calcification, and fibrous caps, a morphology which closely resembles human atherosclerosis. Furthermore, the intimal surface appearance of the aortic arch was evaluated by scanning electron microscopy at the level of the inner curvature of the aortic arch. Although
treatment with lacidipine did not change the histological appearance of the above lesions in comparison with the vehicle treated control animals, the SEM examination of the inner curvature of the aortic arch clearly demonstrated less extensive endothelial damage in animals treated with lacidipine in comparison with vehicle-treated controls (Fig. 6.1).

ApoE-deficient control mice fed the WTD developed severe atherosclerosis during the 8-week period of the study. The extent of the mean lesion area (Fig. 6.2) in the aortic origin was $313.8 \pm 31.7 \times 10^3 \mu m^2$. In contrast, a significant dose-related reduction in aortic lesion development was found in mice given lacidipine at the dose levels of 3.0 and 10.0 mg/kg (*$P<0.05$, **$P<0.01$, respectively). The average extent of the lesion areas was $199.7 \pm 23.4 \times 10^3 \mu m^2$ and $178.0 \pm 27.1 \times 10^3 \mu m^2$ in animals treated at 3.0 and 10.0 mg/kg lacidipine, respectively. The area affected by lesions was therefore reduced by 36.4% and 43.3% in groups treated with lacidipine at doses of 3.0 and 10.0 mg/kg, respectively (Fig. 6.2).

6.3.4 Functional studies: vascular reactivity

The aortic rings from C57BL/6J wild-type mice showed a high relaxation response to increasing levels of Ach (0.1 nM to 1000 nM). A similar response was shown by the aortic rings of the naive apoE-deficient mice sacrificed at the beginning of the study to assess basal vascular reactivity.

The aortic rings from control (vehicle treated) apoE-deficient mice fed the WTD for 8 weeks resulted in lower relaxation in response to Ach at 0.1, 1.0 and 10.0 nM concentrations; no relaxation was recorded in the presence of higher concentrations of Ach (100 and 1000 nM).
In contrast, the aortic rings from apoE-deficient mice receiving the WTD and lacidipine at 3.0 and 10.0 mg/kg for 8 weeks, displayed a response to increasing levels of Ach similar to that of the naive apoE-deficient mice sacrificed at the beginning of the study. In particular the aortic rings obtained from the apoE-deficient mice treated with lacidipine 3.0 mg/kg displayed a response to Ach at 1000 nM very close to that of aortic rings from the control wild type C57BL/6J mice.

It is also of interest to note the low variability (i.e. a small standard error) of the C57BL/6J wild type mice values. A similar degree of variability was also observed in the apoE-deficient mice treated with lacidipine at 3.0 and 10.0 mg/kg, and in the naive apoE-deficient group sacrificed at the beginning of the study. In contrast, high variability (i.e. a large standard error) was seen in the apoE-deficient vehicle-treated mice, when the aortic rings were challenged with high concentrations of Ach (100 and 1000 nM). This finding further underlines the abnormal condition of the aorta in this group of mice and tends to support the appropriateness of the range of concentrations of Ach selected for these studies. The results from the functional studies are presented in Fig. 6.3.

6.3.5 Voltammetric analysis of nitrogen monoxide

To measure the basal production and release of endothelial NO, amperometry involving the use of NO sensitive mCFE was used on the endothelial surface of the aortic rings from six-week old wild type C57BL/6J mice and naive apoE-deficient mice. The aortic rings of these animals gave
similar amperometric basal current levels (in nanoAmperes; nA) of endothelial NO (0.58 ±0.02 nA and 0.53 ±0.02 nA, respectively) (Fig. 6.4a).

A similar response to substance P-stimulated release of vascular endogenous NO was observed in wild type C57BL/6J mice, and in the naive apoE-deficient mice groups (2.68 ±0.34 nA and 2.79 ±0.39 nA, respectively) (Fig. 6.4b).

In contrast, in the aortic rings of the apoE-deficient vehicle-treated mice fed the WTD for 8 weeks, a significant increase in the basal endogenous NO levels was seen (Fig. 6.4a), while the substance P-stimulated endogenous NO (Fig. 6.4b) was significantly decreased. Conversely, in the aortic rings of the apoE-deficient mice fed the WTD and treated with lacidipine (3.0 and 10.0 mg/kg), both the basal NO related signals (Fig. 6.4a) and the substance P-stimulated endogenous NO signals (Fig. 6.4b) were significantly increased. To assess a possible effect of the WTD on NO production, these data were compared with voltammetric results from the aortic rings of wild type C57BL/6J and naive apoE-deficient mice fed the standard diet (for 8 weeks) and treated with vehicle or lacidipine at 10.0 mg/kg (n=3 each treatment) and a similar trend of increase in substance P-stimulated NO levels was observed after treatment with lacidipine.
6.4 DISCUSSION

The present study demonstrated that lacidipine treatment significantly reduced the progression of atherosclerotic lesions in apoE-deficient mice fed a high fat diet, and also that the anti-atherosclerotic activity of lacidipine was not associated with a reduction of plasma cholesterol levels. This would suggest a different mechanism of action for lacidipine in comparison with that described for other lipid lowering drugs such as probucol and tamoxifen (Tawara et al. 1986; Reckless et al. 1997). It has been demonstrated that probucol lowers serum cholesterol mainly by increasing catabolic excretion of cholesterol into bile (Tawara et al. 1986). While tamoxifen is able to abolish the development of lipid-filled vascular lesions in apoE knockout mice by changing the lipoprotein profile (there is a decrease in the LDL fraction and increase in HDLs) and by elevating levels of transforming growth factor beta (TGF-β) (Reckless et al. 1997), both of which are thought to be protective against atherosclerosis in both human and animal models.

The data obtained in the present study provides further support for the previously accumulated evidence of a lacidipine-mediated anti-atherosclerotic activity, which has been reported in several other animal models (Soma et al. 1996; Bernini et al. 1996; Crespi et al. 2001b), and also in in vitro studies (Piedrahita et al. 1992; Oldham et al. 1997).

Studies have shown that lacidipine is capable of vascular protection when administered both prophylactically and therapeutically at nonsustained anti-hypertensive dose levels to salt sensitive Dahl-S rats (Cristofori et al. 1991). Furthermore, lacidipine has been shown to reduce the extent of atherosclerotic
lesions in cholesterol-fed hamsters (Cristofori et al. 2000a), in apoE-deficient mice (Cristofori et al. 2000b), and in humans (Zanchetti et al. 2002).

In particular, the findings of the present work appear to indicate that lacidipine “enhances” the activity of the endogenous NO system, both at basal levels and in response to substance P-stimulated NO release. This capacity of the drug would result in a facilitation of vasodilatation in the presence of atherosclerosis. This observation is supported by the present functional studies that demonstrate that the aortic rings from control apoE-deficient mice fed the WTD, with extensive atherosclerotic lesions, showed a severely impaired relaxation in response to Ach. In contrast, the aortic rings from apoE-deficient mice fed the WTD and treated with lacidipine at 3.0 and 10.0 mg/kg, maintained vascular functional activities close to supra-normal levels.

In the voltammetric studies carried out in parallel with the investigations on vascular reactivity, other supporting evidence was forthcoming. The present results clearly demonstrate that voltammetry is able to measure basal, as well as substance P-stimulated NO release in vascular tissue of mice. In particular, in both wild type C57BL/6J and naive apoE-deficient control groups, the values of basal and substance P-stimulated release of NO were similar, suggesting no significant differences in the activity of their respective NO systems. These results correlate well with the lack of appreciable atherosclerotic lesions in the aorta of both, and the evidence of similar functional reactivity. In contrast, in apoE-deficient, vehicle-treated animals fed a WTD, it appeared that basal NO levels were increased when compared to naive (6-week old) apoE-deficient mice. This could indicate increased activity of the NO system with the development of atherosclerosis.
This increase in the activity of the NO system could be either passive, i.e. simply a consequence of the presence of the lesion, or possibly an "active" response, i.e. as a "protective" vasodilatory action of NO in presence of a damaged and malfunctioning vascular tissue.

Moreover, the substance P-stimulated NO signal in apoE-deficient vehicle-treated mice fed the WTD was reduced when compared to control mice (naïve and wild-type). Substance P is known to have vascular dilating properties (Oldham et al. 1997) via the stimulation of the NO system (Crespi et al. 2000), and thus one can suggest that the NO system is adversely affected in the presence of abnormal, pathological vascular lesions (i.e. rapidly developing atherosclerotic plaques induced by the WTD). This effect appears to be related in particular to the severity of the lesion. The presence of substance P receptors within the endothelium has been described (Tawara et al. 1986; Reckless et al. 1997), and Oldham et al. (1997) reported also that substance P acts as a vasodilator via NOS activation in endothelial cells. This indicates a reduced availability of endogenous NO in the WTD-fed and vehicle-treated mice following the application of substance P. Therefore, the positive levels of substance P-stimulated NO release in lacidipine-treated mice fed the WTD, would indicate that lacidipine is able to maintain, and possibly even increase, the efficacy of the NO system, even in the presence of very large atherosclerotic lesions. Thus, lacidipine may be able to prevent the negative effects of atherosclerosis on the activity of the NO system.

Akopov (1996) has reported that the vasodilatory effect of lacidipine was significantly attenuated by the presence of the NO-synthase inhibitor, N-nitro-L-arginine. Bearing Akopov's (Akopov 1996) work in mind, the present
investigations show that lacidipine enhances the activity and the levels of endothelial NO. Therefore this may indicate that the factor responsible for the arterial endothelium modulating vascular relaxation, in presence of DHPs, is probably a DHP-induced increase in the release of NO, rather than an enhancement of a calcium antagonist activity due to the basal release of NO at the level of vascular smooth muscle.

In conclusion, the reduced levels of atherosclerosis in apoE-deficient mice fed the WTD and treated with lacidipine at doses that do not control hypertension (Crespi et al. 2002), would indicate that the beneficial effects of this calcium antagonist in hypertensive patients are not simply restricted to the reduction of blood pressure. On the basis of the present findings it can be suggested that an additional property of lacidipine exists which may be related to the capacity of the drug to maintain endothelial NO levels (and consequently ONOO\(^-\) levels) at “useful” (i.e. nano molar) concentrations; this therefore results in protection against vascular damage. And finally, we consider that our studies further support the use of voltammetry with treated micro-biosensors, as an appropriate technique for the analysis of rapid, and short lasting, biochemical events.
Fig. 6.1: Scanning electron microscopy photomicrographs of atherosclerotic lesions on the inner curvature of the aortic arch of apoE-deficient mice. All x150; insert x4,000 (OM).

(a) Control vehicle-treated apoE-deficient mouse fed a Western-type diet (WTD) for 8 weeks. The intimal surface appears irregular and contains crater-like regions. At higher magnification, extensive damage to the endothelial cells is evident and shows an increased number of microvilli. Monocytic cells and platelets are frequently attached to the intimal surface.

(b) ApoE-deficient mouse fed the WTD for 8 weeks and receiving 3.0 mg/kg lacidipine. The extent of the lesion is reduced and is associated with an evident preservation of the integrity of the endothelial cells.
Fig. 6.2: Effect of lacidipine at 3.0 and 10.0 mg/kg on the atherosclerotic lesions measured in the aortic origin in apoE-deficient mice fed a Western-type diet and receiving vehicle control, or lacidipine at 3.0 and 10.0 mg/kg/day for 8 weeks.

Results are expressed as means ±SEM of the lesion area (x10^3 μm²) measured for each mouse in 17 cross-sections through the aortic origin, over a distance of 240 μm. n=6 in each group; *P<0.05 and **P<0.01 in lacidipine treated groups at 3.0, and 10.0 mg/kg, respectively, compared with vehicle-treated controls.
Fig. 6.3: Relaxation response of aortic rings.

Vascular reactivity was measured in noradrenaline (0.1 μM) contracted aortic rings, following exposure to increasing concentrations of Ach. The state of contraction (%) is shown in control apoE-deficient mice (n=6) fed a Western-type diet and treated with vehicle (♦), or lacidipine at 3.0 (▲) and 10.0 (●) mg/kg/day. In addition, at the beginning of the study, the vascular reactivity was measured in C57BL/6J control mice (□) and in naive apoE-deficient mice (■) (n=5), to assess the basal relaxation response. Results are expressed as mean ±SEM.
Fig. 6.4: Aperometric current levels of endothelial NO (nA) in aortic rings.

(a) Wild-type C57BL/6J mice and naive apoE-deficient mice (n=3) are compared to apoE-deficient mice fed a Western-type diet and receiving vehicle (control), or lacidipine at 3.0 and 10.0 mg/kg/day (n=6). Results are expressed as mean ±SEM; *P<0.05 versus vehicle control (C57BL/6J and Naive apoE receiving the Standard diet); **P<0.05 versus vehicle treated control receiving the Western-type diet.

(b) Substance P-stimulated amperometric current levels of NO (nA): wild-type C57BL/6J mice and naive apoE-deficient mice are compared to apoE-deficient mice fed the Western-type diet and receiving vehicle (control) or lacidipine at 3.0 and 10.0 mg/kg/day.
Results are expressed as mean ±SEM; \(^*\)P<0.05 versus vehicle control (C57BL/6J and naive apoE receiving the Standard diet); \(^*\)P<0.05 versus vehicle treated control receiving the Western-type diet.
Chapter 7

ANTI-ATHEROSCLEROTIC AND PLAQUE STABILIZING EFFECTS OF LACIDIPINE IN THE APOE-DEFICIENT MOUSE

7.1 INTRODUCTION

Acute disruption of an atherosclerotic plaque may be the prelude to the onset of clinical ischemic events, including stroke and myocardial infarction (Kolodgie et al. 2004).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that act as the physiological regulators of the extracellular matrix (Watanabe & Ikeda 2004). Activated macrophages and other cells present in the lipid-rich atherosclerotic plaque in the vessel wall secrete matrix degrading MMPs that weaken the atherosclerotic plaque and contribute to the formation of a fissure (Dollery et al. 1995). Plaque instability (manifesting as the ulceration of the fibrous cap), plaque rupture, and intra-plaque haemorrhage, are characteristic of plaques with a high content of lipid, and an excess of macrophages in the cap (Fernandez-Ortiz et al. 1994). The size and consistency of the atheromatous core, which is rich in extracellular lipid (i.e., cholesterol and cholesterol esters), are critical for the stability of individual lesions. The central lipid-rich core of a typical lesion contains many lipid-laden macrophage foam cells derived from blood monocytes (Libby 1995). Such foam cells are able to produce large amounts of tissue factor, a powerful pro-
coagulant that stimulates thrombus formation when in contact with blood (Wilcox et al. 1989). Thus the integrity of the fibrous cup overlying the lipid-rich core determines the stability of an atherosclerotic plaque and protects the blood compartment in the arterial lumen from a potentially disastrous contact with the underlying thrombogenic lipid core (Libby 1995).

The 92-kDa gelatinase B or metalloproteinase-9 (Bellosta et al. 1998) is expressed by virtually all activated macrophages and, through the degradation of the basement membrane, facilitates macrophages extravasation.

Stabilising a plaque may bring about clinical benefits, even in the absence of plaque regression (Brown et al. 1993). Several lipid-lowering trials with angiographic follow-up have shown that the stability of coronary plaques, over the short term, is associated with a good long-term prognosis (Jukema et al. 1998). Experimental observations indicate that the lowering of serum lipid, brought about by dietary intervention, may stabilise vulnerable plaques by reducing the expression and activity of enzymes that degrade the arterial extracellular matrix (Aikawa et al. 1998). Besides lipid-lowering therapies, plaque stabilisation could also be achieved by a direct inhibition of MMPs in the arterial wall. Along with transforming growth factor-beta, corticostetroids, and heparin, several synthetic inhibitors have been investigated (Brown et al. 1993), namely, tetracycline, anthracyclines, and synthetic peptides. A number of MMP inhibitors have now entered development.

The anti-atherosclerotic activity of lacidipine has been demonstrated in different animal models (rabbit, hamster and apo-E deficient mouse) at doses that did not affect blood pressure and which do not lower plasma lipids (Paoletti et al. 1995; Soma et al. 1996; Bernini et al. 1996; Donetti et al. 1997;
Furthermore, it has been demonstrated that lacidipine inhibits cholesterol esterification \textit{in vitro} and \textit{ex vivo} through the inhibition of acyl-CoA:cholesterol acyltransferase (ACAT) activity, thus preventing \textit{in vitro} cholesterol accumulation in macrophages (Donetti et al. 1997), and 92-kD gelatinase B secretion \textit{in vitro} (Bellosta et al. 1998; Bellosta et al. 2001).

In the present study, the effect of lacidipine on atherosclerotic progression in apoE-deficient mice has been evaluated, focusing on the assessment of the ability of peritoneal macrophages to accumulate cholesterol, and on 92-kDa gelatinase B expression (Bellosta et al. 1998). The results provide further experimental observations on how lacidipine interferes with the major processes of atherogenesis occurring in the arterial wall. In this study a further characterisation of the atherosclerotic lesion was performed using immunohistochemistry.

\subsection*{7.1.1 Acknowledgements}

The author would like to acknowledge and thank Dr Stefano Bellosta from the Department of Pharmacological Sciences, University of Milan, for his scientific advice in the interpretation of metalloproteinase activity in the context of the present studies.
7.2 MATERIALS AND METHODS

Female apoE-deficient and C57BL/6J mice were approximately 6 weeks old at the beginning of the experiment. Both apoE-deficient (n=27) and C57BL/6J (n=27) mice were each allocated into three groups of 9 animals each (one vehicle-treated control and two drug-treated groups). All mice were fed the Western-type diet. During the 2-week period of treatment, lacidipine was administered daily at dose levels of 0 (control), 1.0 and 3.0 mg/kg body weight in 0.5% methyl cellulose (details in Chapter 2, Materials and Methods). Three days before the end of the study, n=6 mice from each group were intraperitoneally injected with 4% thioglycollate in order to produce a localised inflammation and increased white blood cell count for peritoneal macrophage collection (Hau & Simmons 1980). At the end of the two-week period of lacidipine, or vehicle-treatment, mice were sacrificed under deep isofluorane anaesthesia (5% isofluorane, Abbott).

Peritoneal macrophages were collected by peritoneal lavage with phosphate-buffered saline (PBS) (Cohn & Benson 1965). The action of lacidipine on intracellular cholesterol metabolism in peritoneal macrophages was evaluated by assessing the incorporation of radioactivity into cellular cholesteryl esters after the addition of [1-14C]-oleic acid albumin complex to the culture medium. In addition, the gelatinolytic capacity of secreted metalloproteases (Bellosta et al. 1998) was analysed by zymography on the collected conditioned media using 7.5% polyacrylamide gels (Chapter 2, Materials and Methods).

Blood was collected from all mice (n=9 per group), anti-coagulated with EDTA, centrifuged, and total plasma cholesterol levels were determined.
with an automated enzymatic technique (Chapter 2, Materials and Methods). The extent of the atherosclerotic lesions was measured in paraffin sections of the heart, and the aortic sinus (on 13 sections), and quantified using a computerized image analysis system (Chapter 2, Materials and Methods).

In addition, in this study, immunohistochemical methods (IHC) were applied to further characterise the atherosclerotic lesions; for this purpose three sections of the aortic sinus (sections number 1, 10 and 17), were stained with fluorescent anti-mouse MMP-9 monoclonal antibodies to investigate the expression of matrix MMPs, and one section (number 9) was stained with anti-α-smooth muscle (SM) actin monoclonal antibody (sheep monoclonal anti-mouse) (Abcam, UK) to assess the integrity of the media of the aortic sinus (as described in Chapter 2, Materials and Methods). Verhoeff elastic stain was used for the evaluation of the architecture of the elastic fibres in the media of the vessel wall. Antibody binding was visualised with the Avidin Biotin Peroxidase method with diaminobenzidine. Furthermore, from three randomly selected mice from each lacidipine-treated and vehicle-treated group, samples of heart were frozen and cryosections stained with monoclonal antibodies specific for the identification of mature macrophages (rat anti-mouse monocyte/macrophage monoclonal antibody (BM-8)), and for the analysis of the expression of vascular cellular adhesion molecules (Nakashima et al. 1998), and inter-cellular adhesion molecules (ICAM-1) in the endothelium. These investigations were carried out as the focal recruitment of monocytes and lymphocytes is one of the earliest detectable cellular responses in the formation of the lesions of atherosclerosis (Nakashima et al. 1998).
Results are expressed as mean ±SD and analysed by one sided Dunnett's test to determine the differences between means with P values <0.05 and <0.01.
7.3 RESULTS

7.3.1 Clinical observations

During the 2-week period of treatment with lacidipine, the drug was well tolerated at the two doses used (1.0 and 3.0 mg/kg), and there were no clinical signs of toxicity attributable to the compound. The body weight gain in apoE-deficient vehicle-treated control mice, and in the C57BL/6J (wild-type) control mice, was the same as in the lacidipine-treated groups (mean body weight increases of approximately 2% at the end of the 2-week study).

7.3.2 Plasma cholesterol profile

Lacidipine-treatment did not significantly affect plasma cholesterol level in both apoE-deficient and C57BL/6J mice in comparison with their counterpart controls. Mean levels of total cholesterol were equal to 24.38 ±0.86 mmol/l in the apoE-deficient control group, and 27.15 ±0.82 mmol/l and 25.20 ±0.62 mmol/l in the apoE-deficient mice treated with lacidipine at doses of 1.0 and 3.0 mg/kg, respectively. Mean levels of total cholesterol were equal to 1.80 ±0.05 mmol/l in the C57BL/6J control group, and 1.82 ±0.02 mmol/l and 1.80 ±0.02 mmol/l in C57BL/6J mice treated with lacidipine at doses of 1.0 and 3.0 mg/kg, respectively.

7.3.3 Quantitation of atherosclerotic lesions

ApoE-deficient mice developed atheromatous lesions in the aortic sinus. No lesions were detected in the wild-type C57BL/6J mice (control and lacidipine-treated groups). The extent of atherosclerosis was quantified in the aortic sinus (Fig. 7.1). In the apoE-deficient control group the average lesion
area was $222.0 \pm 1.2 \times 10^3 \mu m^2$, $199.2 \pm 9.8 \times 10^3 \mu m^2$ in animals treated at 1.0 mg/kg lacidipine, and $120.7 \pm 2.0 \times 10^3 \mu m^2$ in the 3.0 mg/kg lacidipine group (reductions of 10.0% and 45.0% of the control lesion area, respectively).

### 7.3.4 Immunohistochemistry of atherosclerotic lesions

Microscopic examination of the aortic sinus of apoE-deficient mice showed atherosclerotic lesions rich in foam macrophages with the adhesion of mononuclear cells to the endothelial surface, and the subendothelial accumulation of foam cells and smooth muscle cells (Fig. 7.2a).

Immunohistochemical analysis demonstrated that areas of macrophage accumulation appeared to stain positively with the monoclonal antibody BM-8, suggesting that the cells were of the macrophage lineage. The BM-8 positive staining was consistently present in lesions, both in the areas rich with foam cells (shoulders of the plaque) and in the necrotic core, while no staining was present in the fibrous cap region (Fig. 7.2b). When lesion areas of apoE-deficient mice receiving vehicle (control) were compared with those of apoE-deficient mice receiving lacidipine, BM-8 staining was positive where foam cells and necrotic core were identified. No staining was observed in the aortic sinus in the control and lacidipine-treated wild-type C57BL/6J mice.

The immunohistochemical patterns of the lesions were also investigated after staining with monoclonal antibodies to MMP-9. In control apo-E deficient mice an intense immunoreactivity was seen in the areas of macrophage infiltration, mainly in the shoulders of the plaques, and the positivity was predominantly in the cytoplasm of the macrophages (Fig. 7.3a).
No MMP-9 positivity was identified in smooth muscle cells. In the lesion areas of apoE-deficient mice treated with lacidipine, a similar pattern of distribution was present, but a significantly reduced immunoreactivity related to MMP-9 expression was evident, in comparison with control (vehicle-treated) apoE-deficient mouse lesions (Fig. 7.3c). This reduction in immunoreactivity of the atherosclerotic lesions was seen in aortic sinus samples of apoE-deficient mice dosed with lacidipine at both 1.0 and 3.0 mg/kg; there was no appreciable difference between the two dose levels of lacidipine.

In apoE-deficient mice, characteristically below the atherosclerotic lesions, a complete loss of positivity of anti-α-actin was observed, both in the vehicle-treated control group and in the lacidipine-treated groups. This feature corresponded to the degradation and/or fragmentation of the elastic laminae, and was highlighted by the loss of positivity in the media of the aortic sinus to Verhoeff’s stain (Fig. 7.4a). This pattern is considered to indicate a loss of the contractile function of the smooth muscle cells, and this view is supported by morphological changes, namely abundant basophilic cytoplasm, a large nucleus, and the orientation of smooth muscle cells upwards toward the vascular lumen. In addition to the fragmentation of the elastic fibres in the media, plaque development was associated with a thinning of the aortic wall and loss of α-smooth muscle actin immunostaining (Fig. 7.4b).

The aortic sinus of C57BL/6J mice, both vehicle or lacidipine-treated, did not show any staining after incubation with both BM-8 and MMP-9 antibodies; in contrast, the normal distribution of positivity of anti-α-actin of smooth muscle cells was present in the tunica media of the aorta of the C57BL/6J mice.
In the aorta of apoE-deficient mice, VCAM-1 staining appeared diffusely expressed in the endothelium over lipid-laden lesions, as well as in more fibrotic lesion areas, and in general occurred in areas where macrophages were accumulated (Fig. 7.5a). ICAM-1 is thought to be involved in the cell adhesion step of leukocyte infiltration. Antibodies to ICAM-1 stained both individual endothelial cells, and focal areas, in the aortic sinus (Fig. 7.5b). The intensity of the staining appeared clearly localised over a macrophage-rich plaque, due to the marked positivity of the stain to foamy macrophages. Moreover, only a weak signal was detectable where a fibrotic plaque was present, and here the red staining was limited to the necrotic core; no staining was present in the fibrous cap region (Fig. 7.5b). When lesion areas of apoE-deficient mice receiving vehicle (control) were compared with those of apoE-deficient mice receiving lacidipine, no significant difference in the degree of immunoreactivity was evident.

7.3.5 Effect of lacidipine on cholesterol accumulation

Results showed that the rate of cholesterol esterification in peritoneal macrophages from apoE-deficient control mice, when compared to the C57BL/6J wild type controls, was significantly higher (3038 ±132 and 404 ±59 pmol/mg of cell protein x h, respectively; P<0.05) (Fig. 7.6). Lacidipine influenced cholesterol esterification in apoE-deficient mice receiving 1.0 and 3.0 mg/kg of lacidipine, by decreasing the esterification efficiency by 28.0 and 34.0%, respectively. The effect of lacidipine on cholesterol esterification was even more pronounced in the C57BL/6J mice, where the inhibitory effect was
approximately 50% in both C57BL/6J groups of mice receiving lacidipine at 1.0 and 3.0 mg/kg, respectively (Fig. 7.6).

7.3.6 Effect of lacidipine on MMP-9 activity

In the present study, attention was also focused on MMP-9 activity, the most prevalent form of gelatinase secreted by macrophages. The aim was to evaluate if lacidipine was also effective in reducing MMP production. As shown in Fig. 7.7, both apoE-deficient and C57BL/6J derived macrophages displayed a similar basal MMP-9 activity. Lacidipine at both 1.0 and 3.0 mg/kg significantly reduced the gelatinolytic capacity of macrophages from apoE-deficient and from C57BL/6J mice. Treatment with lacidipine at 1.0 mg/kg induced a reduction of MMP-9 activity of 22.3% (P<0.01) and 30.4% (P<0.05) in apoE-deficient and C57BL/6J mice, respectively. Similar reductions in MMP-9 activity of 27.1% (P<0.01) and 34.1% (P<0.05) were seen in apoE-deficient and C57BL/6J mice, respectively, treated with lacidipine at 3.0 mg/kg.
7.4 DISCUSSION

In this study, two key features in the development of atherosclerosis which are crucial for plaque instability have been evaluated: lipid accumulation and the secretion of MMPs. The effect of lacidipine on the metabolism of cholesterol in an experimental model of atherogenesis has been studied, both in vivo in the aortic arch of apoE-deficient mice fed a hypercholesterolemic diet (Chapter 5), and in vitro (this Chapter) in cholesterol rich mouse peritoneal macrophages, which is a widely accepted model for studying foam cell formation (Hussain et al. 1992).

The results obtained in Chapter 5 demonstrated that lacidipine treatment reduced the extension of atherosclerotic lesions in the aortic sinus of apoE-deficient mice, and the the ex vivo data in the present Chapter showed that the drug reduced esterified cholesterol accumulation, by inhibiting the cholesterol esterification rate in peritoneal macrophages. In addition, in the present study, it was observed for the first time that atherosclerotic apo-E deficient mice have a significantly higher basal esterification rate compared with C57BL/6J mice (Fig. 7.6). This is a novel finding that could potentially contribute further to the already high susceptibility of the apoE-deficient mouse to atherosclerosis. The ex vivo data also showed that lacidipine was effective in inhibiting MMP-9 gelatinolytic activity in macrophages obtained from lacidipine-treated mice.

In this thesis, transgenic apoE-deficient mice have been shown to be a valuable model for the study of the pathogenesis of atherosclerosis; for example, the lesions develop quickly, in a reproducible manner, and the transition from lipid-laden lesion to fibrous plaque formation can be evaluated, and the
progression of lesion development may be accelerated by feeding animals with
dietary fat and cholesterol (Mathew et al. 1996b).

The doses of lacidipine used in the present study (Chapter 7) were chosen
on the basis of preliminary measurements of drug levels in humans and in apo-E
deficient mouse plasma. On average, in man, 4 mg/day of lacidipine brings about
a maximal plasma concentration (Cmax) ranging from 2.7 to 4.3 ng/ml. In the
apoE-deficient mouse, a similar Cmax (ranging from 2.7 to 7.3 ng/ml) was
obtained by administering a daily dose of 3 mg/kg body weight of lacidipine.
Earlier studies on blood pressure have shown similar ranges, in control mice of
the CD-1 strain (89.8 ±5.0 mm Hg), the C57BL/6J strain (84.5 ±3.1 mm Hg), and
the apo-E deficient mouse (87.2 ±8.6 mm Hg); the daily administration of
lacidipine at 3.0 mg/kg to apoE-deficient mice did not affect blood pressure, the
level being in this case 86.8 ±3.8 mm Hg.

The present work (Chapter 7) also demonstrated that the calcium
antagonist lacidipine significantly reduced the development of atherosclerotic
lesions in the apoE-deficient mouse after a short treatment period (i.e. 2 weeks).
These results therefore provide support for previous evidence of lacidipine-
mediated anti-atherosclerotic activity, which has been reported in other animal
models (Lerman et al. 1991; Paoletti et al. 1996; Soma et al. 1996), and in in vitro
studies (Bernini et al. 1996; Bernini et al. 2001). Lacidipine is known to interfere
with major processes of atherogenesis occurring in the arterial wall (Bernini et al.
1996). In particular, the drug inhibits smooth muscle cell migration and
proliferation, and through the inhibition of acyl-Co A transferase (ACAT)
activity, prevents cholesterol accumulation in macrophages in vitro as well as in
the aorta of hypercholesterolemic rabbits (Bernini et al. 2001). Many components
of plaque formation rely on oxidative stress for their expression. Interestingly, lacidipine was demonstrated to be effective in reducing the overall gelatinolytic capacity of macrophages (Fig. 7.7). Galis and colleagues (1998) showed that MMPs are sensitive to antioxidant treatment. The possibility that macrophage gelatinolytic activity is redox-dependent was suggested by previous studies showing activation of gelatinase zymogens by reactive oxygen species (ROS) known to be produced by macrophage foam cells (Rajagopalan et al. 1996), as well as by the detection of active MMP-9 in an isolated cell system along with inhibition of macrophage-derived gelatinolytic activity by treatment with N-acetyl-cysteine, a ROS scavenger. Activated macrophages, especially those of atherosclerotic lesions, are a major source of ROS; thus, such an activation mechanism would result in the activation of MMP zymogens secreted by the macrophages themselves as well as by neighbouring cells (Galis et al. 1998). Lacidipine is a potent antioxidant, having activity similar to vitamin E (Van Amsterdam et al. 1992) and this chemical property could complete the inhibitory action of the drug on MMP-9 secretion, regardless of the calcium antagonist activity of the compound. The reduced level of MMP-9 secreted into the media by peritoneal macrophages could also be the result of a lacidipine-mediated decrease of MMP-9 gene expression. However, although no evidence to propose such a mechanism has been provided in this present study, previous data (Bemini et al. 2001) indicated that this is not the case, in that mRNA levels did not appreciable change in human monocyte-derived macrophages treated with or without lacidipine. This would suggest a mechanism of action for the drug in affecting post transcriptional processes of MMP-9.
Immunohistochemistry demonstrated that the expression of MMP-9, was localized to the area of atherosclerosis, and that lacidipine treatment resulted in an evident reduction of MMP-9 expression in the lesion area of apoE-deficient mice fed a high fat diet. These findings further support a possible role for lacidipine in enhancing atherosclerotic plaque stability. By contrast, an immunohistochemistry investigation of adhesion molecules by Nakashima et al. (1998) failed to detect any differences between apoE mice treated with lacidipine and their counterpart controls. ApoE mice fed the Western-type diet quickly develop a complex lesion, and this could make the model suitable for studying differences in the expression of adhesion molecules. In fact, adhesion molecules represent one of the earliest detectable cellular responses in the formation of atherosclerotic lesions.

The anti-atherosclerotic activity of lacidipine in the hypercholesterolemic apo-E deficient mouse was not associated with any reduction in the high levels of plasma cholesterol; this suggests a different mechanism of action to that described for other lipid lowering drugs (Tawara et al. 1986; Arendt et al. 1993). The reduction of atheromatous lesions, without accompanying changes in plasma lipids, has been reported in studies on the suppression of atherogenesis by other calcium antagonists (Paoletti et al. 1996). However, it is considered possible that lacidipine influences the atherogenic process by a specific mechanism, which is the result of a combined potent and long-lasting calcium antagonism in conjunction with the powerful antioxidant properties of the drug. These activities are furtherly enhanced by the high lipophilicity of the drug (Herbette et al. 1993; Reckless et al. 1997). In addition, it is unlikely that the anti-atherosclerotic properties of lacidipine could be related to the well known antihypertensive effect of the drug, since the administration of 3.0 mg/kg/day has been shown not to
affect blood pressure values in other strains of mice (CD-1, C57BL/6J), as well as in the apo-E deficient mouse (Gaviraghi & Trist 1998). However, on the basis of the present data (Chapter 7), it is not possible to exclude the possibility that vasodilatation may have occurred, with a compensatory increase in cardiac output, and therefore that the effects seen may also be accounted for by vascular dilatation.

In conclusion, the present study demonstrates that lacidipine administered at different dose levels reduces the extent of atherosclerosis in apoE-deficient mice and inhibits cholesterol esterification ex vivo in cultured macrophages. These results support a unique effect of lacidipine on the cellular metabolism of cholesterol which may also contribute to the potential anti-atherosclerotic effect of this drug. Moreover, lacidipine has a significant inhibitory effect on MMP-9 activity, a property which may contribute to increased plaque stability. These results therefore support the concept that lacidipine, in vivo, at plasma levels of clinical relevance, may accumulate in the vessel wall and achieve a concentration which is effective in modifying the cellular functions involved in atherogenesis.
Fig. 7.1: Effect of lacidipine at 1.0 and 3.0 mg/kg on atherosclerotic lesions measured in the aortic sinus in apoE-deficient mice.

Results are expressed as mean ±SEM of the lesion area ($10^3 \mu m^2$) measured for each mouse in 13 cross-sections through the aortic origin, over a distance of 240 μm. n=9 in each group; **P<0.01 compared with the methocel-treated control animals.
Fig. 7.2: Histology sections of atherosclerotic lesions in the aortic sinus of an apoE-deficient mouse fed a Western-type diet.

(a) Photomicrograph of an early plaque showing a small necrotic core (*), a fibrous cap (arrowhead) and several lipid-loaden, monocyte-derived macrophages that typically accumulate in the shoulders of the plaque (arrow); beneath the lesion smooth muscle cells (SM) of the tunica media show changed morphology and orientation in comparison to the outer layers. Azan stained, x100 (OM).

(b) Photomicrograph shows a section immunostained with the macrophage antibody BM-8. Intense red staining is present in the shoulders of the plaque (arrows) where macrophages are starting to accumulate; * indicates a necrotic core. x40 (OM).
Fig. 7.3: Immunostaining of lesions in the aortic sinus of apoE-deficient mice. Serial sections.

(a) Immunofluorescence staining for MMP-9 in the aorta of a control (vehicle-treated) apoE-deficient mouse. Fluorescence in the lesion is related to the contained macrophages, in the shoulders of the plaque and in the necrotic core. Fluorescence appears clearly localised in the cytoplasm of macrophages. x100 (OM).

(b) H&E stained section corresponding to the lesion presented in (a). Areas of macrophage accumulations are evident in the shoulders of the plaque (arrows) and in the necrotic core (*). x40 (OM).

(c) Immunofluorescence staining for MMP-9 in the aorta of an apoE-deficient mouse treated with lacidipine at 3.0 mg/kg. Immunostaining of the lesion is less diffuse in the plaque and appears to be localised in single cells; positive staining is also evident in the adventia where a mononuclear cell infiltration is present. x100 (OM).

(d) H&E stained section corresponding to the lesion presented in (c). A localised plaque, rich in macrophages in a segment of intact media (SM) is seen, with a focal inflammatory cell infiltration in the adventitia (mci). x40 (OM).
Fig. 7.4: Histochemistry of sections of atherosclerotic lesions in the aortic sinus of an apoE-deficient mouse fed a Western-type diet.

(a) Cross-section of the aortic sinus stained with Verhoeff-VanGieson’s stain for elastin, demonstrating the architecture of the elastic fibres of the media (arrows), and which is thinned (arrowhead) below the most developed plaque (*). x4 (OM).

(b) Higher-power micrograph of a section of the aortic sinus immunostained with anti-α-actin antibody for the contractile apparatus of smooth muscle cells. Intense brown staining is present in the normal smooth muscle cells (arrowhead), while loss of positive staining (arrow) is evident in the segment of media beneath the plaque (*); in addition, the spindle-shaped cells that made up the fibrous cap of the plaque are shown to be smooth muscle cells by immunohistochemical staining for α-actin at the top of the plaque. x40 (OM).
Fig. 7.5: Cryosections of the aortic sinus of an apoE-deficient mouse fed a Western-type diet.

(a) Cross-section of the aortic sinus stained with VCAM-1 antibody. Diffuse staining (red) on the aortic surface is present and corresponds to lesion area (*), but staining is also seen in the region where no morphological abnormalities were evident in the underlying subendothelial space (arrows). x40 (OM).

(b) Cross-section of aortic sinus stained with ICAM-1 antibody. Localised cytoplasmic positivity (red staining) of some cells located over the lesion; areas of weak extracellular positivity are present at the periphery of the lesion (rectangular box). x40 (OM).
Fig. 7.6: Cholesterol esterification rate in peritoneal macrophages.

Results are expressed as the mean ±SEM. Basal cholesterol esterification rate in peritoneal macrophages isolated from apoE-deficient mice is higher in comparison with C57BL/6J mice. Lacidipine-treatment at 1.0 and 3.0 mg/kg significantly lowered the esterification rate in both strains of mice; *P<0.05 in lacidipine-treated animals (1.0, and 3.0 mg/kg groups), respectively, compared with the methocel-treated control animals.
Fig. 7.7: MMP-9 activity in peritoneal macrophages.

Results are expressed as mean ±SEM. The basal MMP-9 activity in macrophages isolated from the two mouse strains, apoE-deficient and C57BL/6J mice, is similar. Treatment with 1.0 and 3.0 mg/kg of lacidipine significantly reduced MMP-9 activity in macrophages from both apoE-deficient mice and C57BL/6J mice; O.D., optical density; *P<0.05, **P<0.01.
Chapter 8

GENERAL DISCUSSION

The approach taken in the present experimental investigations was to examine the morphological changes occurring in two animal models of atherosclerosis (hamsters, apoE-deficient mice) and investigate the effect of lacidipine on this process. The aim was to follow the development of atherosclerosis in the animals receiving the test compound (lacidipine), in conjunction with monitoring the plasma lipid profile, and compare the changes taking place with those of non-drug treated (control) animals. A characterisation of the lesion was also performed using the morphometric assessment at the light microscope level to quantify the effect of lacidipine treatment; electron microscopy examination to investigate morphological changes at the cellular level. In parallel with the histological characterizations of atherosclerotic lesions in the aorta, functional ex vivo analyses were conducted to examine the vascular properties of aortic ring preparations in studies of the voltammetric levels of nitric oxide. Finally, the ability of peritoneal macrophages to accumulate cholesterol was examined in association with 92-kDa gelatinase B in apoE-deficient mice and their wild-type counterpart C57BL/6J (Bellosta et al. 1998).

The hamster model of atherosclerosis was chosen in the first experiments (Chapter 3) because it has been considered by many investigators to be the animal of choice (Nistor et al. 1987a; Takasu et al. 1990a) since, as in humans,
the major plasma cholesterol carrier in the hamster is LDL. Other advantages of
the hamster model are the high frequency with which the atherosclerotic lesions
develop, the susceptibility to diet-induced atherosclerosis, and the relatively
small size of the animal, which enables the morphometric analysis of the
atherosclerotic lesions to be carried out.

In the hyperlipidemic hamster model, in the present studies (Chapter 3),
vascular lesions which compare closely with those reported by previous
investigators were identified (Nistor et al. 1987a; Kowala et al. 1991). Using
light microscopy, it was demonstrated that lacidipine treatment significantly
reduced the area of developing fatty streaks and there was also a reduction in
the amount of foam cell deposits. Similarly, ultrastructural examination
confirmed that treatment with lacidipine greatly reduced the severity of the
intimal lesion. In particular, TEM examination demonstrated that lacidipine
administration markedly reduced the presence of liposomal particles in the
region of the basal lamina and in the sub-endothelial space. The accumulation
of liposomes appeared to be a relatively early event, occurring at the 14 week
sampling point (Chapter 3) and this accumulation occurred in the absence of
foam cells. Furthermore, lacidipine treatment reduced the accumulation of
liposomes in the hyperlipidemic hamster without lowering serum lipids, and
this suggests that a mechanism is involved which is different from the mode of
action of drugs (e.g. pravastatin, atorvastatin, fenofibrate, etc.) which are active
in reducing atherosclerosis by lowering plasma lipids. Similar observations
have been reported in comparable studies investigating the suppression of
atherogenesis by other calcium antagonists (Ginsburg et al. 1983a; Lichtor et
al. 1989a). However, it is considered possible that lacidipine influences the
atherogenic process by an unusual mechanism and it has been suggested by Micheli et al. (1991) that this may be related to a combination of the long-lasting calcium antagonism of the compound, in conjunction with the strong antioxidant properties of the drug; both of these activities would be further enhanced by the very high lipophilicity of the compound (Herbette et al. 1993).

The protective effect of lacidipine was clearly evident on the endothelial cell phenotype, both at TEM and SEM level (Chapter 3). It was seen that lacidipine decreased the abnormalities of the endothelial cells in the hypercholesterolemic animals, lowered the incidence of crater-like lesions, and reduced the hypertrophy of cytoplasmic organelles. Therefore, in this hamster model of atherogenesis, the results clearly demonstrated that lacidipine reduces the extent of atherosclerotic activity, without lowering hypercholesterolemia, and also significantly reduces, or prevents, the endothelial cell changes typically associated with atherosclerosis.

Following the studies in the hamster, all the other experiments were carried out in transgenic apo-E deficient mice because these animals develop spontaneous atheromatous lesions that are morphologically similar to those found in man (Bocan 1998). The model also presents other interesting features. For example, the atheromatous lesions form in a reproducible manner, and the transition from the lipid-laden lesion to the fibrous plaque can be easily evaluated microscopically. In addition, the progression of lesion development can be accelerated by feeding the animals increased dietary fat and cholesterol (Plump et al. 1992). At 10 weeks of age, apoE-deficient mice have already developed atherosclerotic lesions in the aorta, and in the coronary and pulmonary arteries.
In the first experiments carried out using apoE-deficient mice fed a standard rodent diet (Chapter 4), the advantages of this model of atherosclerosis were re-assessed, and the usefulness of the animal confirmed. The doses of lacidipine used (0.3, 1.0 and 3.0 mg/kg for 10 weeks) were chosen on the basis of preliminary measurements of drug levels in humans, and in apo-E deficient mice plasma, and it was also demonstrated that the daily administration of lacidipine at up to 3.0 mg/kg did not cause any effects on blood pressure. Moreover, it was observed for the first time that the atherosclerotic apoE-deficient mouse had plasma endothelin levels about 4-fold higher than those found in the control wild-type C57BL/6J mouse. These results are in agreement with data recently published by Barton et al. (1998), who reported the elevated aortic endothelin-1 protein content in apo-E deficient mice, and compared the increased levels with those found in C57BL/6J mice. Increased vascular endothelin production inhibits endothelial NO release, thereby impairing endothelium-dependent relaxation and promoting atheroma formation (Barton et al. 1998). In addition, it was possible to demonstrate that the endothelin values obtained in the apoE-deficient mouse were closely correlated to the extent of the atherosclerotic lesions. Several studies have demonstrated that, in a clinical situation, plasma endothelin values are elevated in hypercholesterolemic patients (Bath & Martin 1991; Haak et al. 1994), in ischemic heart disease (Yasuda et al. 1990), and in symptomatic atherosclerotic patients. Furthermore, a significant correlation between plasma endothelin concentrations and the particular vascular sites with atherosclerotic lesions has also been described (Lerman et al. 1991; Arendt et al. 1993). An interesting finding in the present studies (Chapter 4) was that the
administration of lacidipine in apoE-deficient mice induced a significant dose-dependent decrease in plasma endothelin levels. The expression of endothelin in the process of atherosclerosis, observed both in human patients and in apo-E-deficient mice, lends additional support to the relevance and usefulness of this murine model in the study of human atherosclerosis, and this also suggests a role for endothelin as a monitorable marker in both the evolution and in the treatment of cardiovascular disease.

In the second group of experiments (Chapter 5) carried out using apoE-deficient mice, lacidipine was administered to mice at the same dosages used in the previous experiment (0.3, 1.0 and 3.0 mg/kg/day) and the animals were fed a Western-type rodent diet in order to accelerate the development of atherosclerosis. It was further demonstrated here that lacidipine was able to significantly reduce the development of atherosclerotic lesions in the apoE-deficient mouse fed a Western-type diet (WTD). These results therefore provided further useful support to the previous evidence of lacidipine-mediated anti-atherosclerotic activity, which has been reported in a range of other animal models (Paoletti et al. 1996; Soma et al. 1996; Cristofori et al. 2000a), and in in vitro studies with the drug (Bernini et al. 1996; Bernini et al. 2001). From a mechanistic point of view, lacidipine is known to interfere with major processes of atherogenesis occurring within the arterial wall (Bernini et al. 1996). In particular, the drug is known to inhibit smooth muscle cell migration and proliferation, and through the inhibition of acyl-Co A transferase (ACAT) activity, prevents cholesterol accumulation in macrophages in vitro, as well as in the aorta of hypercholesterolemic rabbits (Bernini et al. 2001). Many components of plaque formation rely on oxidative stress for their expression, and lacidipine is
one of the most potent antioxidant calcium channel blockers, having activity similar to vitamin E (Van Amsterdam et al. 1992). In this study, where apoE-deficient mice were fed a WTD (Chapter 5), the anti-atherosclerotic activity of lacidipine was not associated with any reduction in the very high levels of plasma cholesterol; this result suggests a different mechanism of action for lacidipine in comparison to that described for other lipid lowering drugs (Tawara et al. 1986; Reckless et al. 1997). A reduction in the extent of atheromatous lesions, without any accompanying changes in plasma lipids, has been reported in previous studies which investigated the suppression of atherogenesis by other calcium antagonists (Paoletti et al. 1996). However, it is considered possible that lacidipine may influence the atherogenic process by a more specific mechanism, which is, it is suggested, the result of a combined potent and long-lasting calcium antagonism, in conjunction with the known powerful antioxidant properties of the drug. These activities of lacidipine are considered to be further enhanced by the high lipophilicity of the drug (Herbette et al. 1993; Gaviraghi & Trist 1998). In addition, it is considered unlikely that the anti-atherosclerotic properties of lacidipine could be related to the well-reported antihypertensive effects of the drug, since the dose level of 3.0 mg/kg/day has been shown not to affect blood pressure values in the apoE-deficient mouse (Gaviraghi et al. 1998). However, on the basis of the present data, it is not possible to exclude the possibility that vasodilatation may have occurred in the animals, with a compensatory increase in cardiac output, and therefore that the effects seen might also be accounted for by vascular dilatation.

It was found (Chapter 5) that lacidipine treatment significantly reduced both plasma endothelin levels and the development of atherosclerosis in the
apoE-deficient mouse fed a WTD, as was observed in the earlier experiments (Chapter 4). Other authors have demonstrated the effects of lacidipine on endothelin expression in the salt-loaded stroke prone hypertensive rat, where a cardioprotective effect was observed which was not correlated with a detectable reduction in blood pressure (Ferron et al. 1996). Expression of the endothelin gene in endothelial cells is subject to a complex pattern of regulatory pathways involving numerous factors (Rubanyi & PoloKoff 1994). In particular, endothelin gene expression is linked to the activation of the transcription factor which has been named activated protein-1 (Lee et al. 1991; Meyer et al. 1993). Transcription factors are proteins typically located in the cytosol. These readily-available elements are therefore activated after transcription, and are considered to rapidly regulate the expression of selective genes (Lee et al. 1991). It has also been shown that AP-1 is activated by many oxidative stress-inducing stimuli. For example, previous studies have demonstrated that oxidized LDL increases the release of endothelin by endothelial cells through an oxidative stress-inducing stimulus (Meyer et al. 1993). Since lacidipine has also been shown to possess strong antioxidant activity (Van Amsterdam et al. 1992), and to reduce the production in endothelial cells of intracellular reactive oxygen species induced by oxidized LDL (Cominacini et al. 1998), it may be concluded that the decrease in plasma endothelin, identified in the present experiments in apoE-deficient mice treated with lacidipine, may be related to a specific effect on cellular oxidative stress. Furthermore, the potent coronary vasoconstrictive properties of endothelin may potentiate the atherosclerotic process by reducing blood flow, and subsequently initiate an enhancement of platelet aggregation and thrombus formation (Berghese et al. 1996; Cominacini et al. 1998). In conclusion therefore,
it is considered likely that the anti-atherogenic activity of lacidipine is related to a specific effect on endothelin over-production which occurs in atherosclerosis, and the drug may thus interfere with the proatherogenic activity of endothelin.

In a third study (Chapter 6), the extent of atherosclerosis in the aortic origin of the heart of apoE-deficient mice was significantly reduced after an 8-week period of treatment with lacidipine at 3.0 and 10.0 mg/kg. In these mice fed a WTD, the 3.0 mg/kg lacidipine dose level has been shown to induce plasma drug levels comparable to those achieved with the human therapeutic dose of 4.0 mg/kg (Zanchetti et al. 2002); the 10.0 mg/kg dose was chosen in this study to maximise any effect of treatment. The reduction of atherosclerotic lesions caused by lacidipine in this experiment was paralleled by a marked decrease in the propensity for oxidation of plasma LDL, induced by different modes of oxidative stress. However, the anti-atherosclerotic activity of lacidipine in the apoE-deficient mouse was not caused by a reduction in plasma cholesterol levels as the plasma lipid profiles in all groups were similar. This last finding is in line with the results obtained in all the previous experiments, although in the first study (Chapter 4) apoE-deficient mice were fed a standard rodent diet. Moreover, in that earlier study (Chapter 4), lacidipine did not affect blood pressure, which would suggest that it is unlikely that the anti-atherosclerotic properties of lacidipine, at least at a daily dose of 3.0 mg/kg, could be related to the antihypertensive effect of the drug. These findings are also in agreement with another study in the stroke-prone spontaneously hypertensive rat (SPSHR), in which lacidipine exerted a powerful antioxidant effect in vivo without affecting blood pressure (Napoli et al. 1999). Therefore, the sum total of these present findings tend to reinforce the concept that lacidipine activity on the atherosclerotic processes may be related to the known
antioxidant properties of the drug (Van Amsterdam et al. 1992). In the present work, the effect of lacidipine on the progression of aortic atherosclerosis was effectively associated with a reduced susceptibility of LDL to oxidation (Chapter 5). These data are also consistent with other studies in which an increase of LDL antioxidants, by incubation or oral supplementation, always resulted in a strictly proportional increase in the length of the lag phase in studies investigating the susceptibility of LDL to oxidation (Dieber-Rotheneder et al. 1991; Cominacini et al. 1991a; Regnstrom et al. 1992; Sasahara et al. 1994; Cominacini et al. 1997a; Sobal et al. 2001). There is now growing evidence of a relationship between susceptibility of LDL to oxidation and atherosclerotic risk (Cominacini et al. 1991a; Regnstrom et al. 1992; Sasahara et al. 1994; Cominacini et al. 1994a; Cominacini et al. 1994b; Cominacini et al. 1997b; Sobal et al. 2001). Oxidatively modified LDL is taken up by macrophages at an increased rate, in comparison to native LDL, and thus can promote cellular cholesterol accumulation and foam cell formation, which are the hallmarks of early atherosclerotic lesions (Steinberg 1997).

The amount of vitamin E in apoE-deficient mice treated with lacidipine and in vehicle-treated control groups was also measured (Chapter 5), since most of the vitamin E in blood plasma is present in the LDL fraction, and hence vitamin E is optimally placed to prevent free-radical-mediated modification of this lipoprotein. Levels of vitamin E were maintained in animals treated with lacidipine at 3.0 and 10.0 mg/kg, and the vitamin E concentrations were significantly (P<0.01) higher in both lacidipine-treated groups, in comparison with the control mice. However significant differences in the basal LDL-like particle concentrations of vitamin E were found between
the apoE-deficient mice treated with lacidipine and the vehicle-treated control group. This finding could suggest that lacidipine may have reduced the progression of aortic atherosclerosis by lengthening the LDL lag phase, with the drug working as a radical scavenger. This finding is consistent with the classic kinetic model proposed by Niki for lipid peroxidation (Niki 1987), where the length of the lag phase is directly related to the amount of antioxidant contained in the lipoprotein. Another possibility in explaining the effect of lacidipine on LDL lag phases in apoE-deficient mice derives from the scheme proposed by (Esterbauer et al. 1989). According to these authors, the role of the copper in the oxidative process of LDL is to catalyse the conversion of trace amounts of pre-formed lipid hydroperoxides to alkoxy and peroxy radicals, which in turn start another lipid peroxidation reaction. In our present studies (Chapter 5), hydroperoxides were reduced in the LDL-like particles of apoE-deficient mice treated with lacidipine, in comparison with the vehicle-treated control group, and there was a direct correlation between the hydroperoxide concentrations in LDL and the length of lag phase. Therefore, lacidipine may have lengthened the LDL lag phase also by reducing the LDL content of the lipid hydroperoxides.

The anti-oxidative properties of lacidipine were also assessed (Chapter 5) in the in vitro model of oxidized LDL obtained by incubation with HUVECs; interesting results were obtained. It is well known that the process of LDL modification induced by endothelial cells in vitro closely resembles the corresponding process in vivo. The present experimental results may suggest that lacidipine plays a crucial role in the sequence of events leading to the formation of foam cells and atherosclerotic plaques. Although cell-mediated oxidative
modification of LDL has been the subject of several studies in recent years, the mechanism by which cells initiate LDL oxidation still remains unclear. Both extracellular superoxide radicals (Cathcart et al. 1989) and lipoxygenase activity (Cathcart et al. 1991; Chamulitrat et al. 1991) have been proposed. Attention has also focused on the reaction of superoxide with NO (Bruckdorfer 1993). NO can react with superoxide to form the peroxynitrite anion. Decomposition of the peroxynitrite anion generates a strong oxidant with reactivity similar to the hydroxyl radical, and which has been shown to initiate lipid peroxidation (Bruckdorfer 1993). Whichever mechanism of oxidation may be involved, this led to the initial hypothesis that the action of modifying cells is to accelerate the formation of lipid peroxides within the LDL particle. Recently, it has also been proposed that the modifying cells could directly provide peroxidative products to the LDL (Ezaki et al. 1995; Cominacini et al. 1996). Lacidipine could increase the potential of scavenging lipid peroxyl radicals, but the drug may also modify the potential of the modifying cells to produce peroxidative products and consequently delay the start of the lipid peroxidation chain. In the present studies (Chapter 5), the reduction in the content of hydroperoxides found in LDL-like particles of apoE-deficient mice treated with lacidipine, may therefore also be the result of the action of the drug on endothelial cells. This conclusion is consistent with the demonstrated inhibitory effect of lacidipine on the generation of reactive oxygen species induced by pro-oxidant stimuli in endothelial cells (Cominacini et al. 1998). The results of the present studies (Chapter 5) also show that LDL vitamin E consumption during HUVEC oxidation was reduced in apoE-deficient mice treated with lacidipine, in comparison with the vehicle-treated control group. Although the mechanism underlying this effect of lacidipine on vitamin E is not
clear, one hypothesis was proposed by Thomas et al. (1995) which was that lacidipine acts as co-antioxidant. In the model proposed by these authors, vitamin E does not act as a chain-breaking antioxidant, but facilitates the transfer of radical reactions from the aqueous phase into LDL, and mediates radical chain reactions within the lipoprotein particle. The vitamin thus exhibits pro-oxidant activity. Since it is the isolation of vitamin E within a lipoprotein undergoing oxidation that forces the vitamin to react with the polyunsaturated fatty acids of LDL, prevention of this oxidation depends on the rapid destruction of vitamin E. To interfere with such a process a compound must have a significant capacity to reduce and interact with LDL-associated vitamin E, and we have demonstrated that lacidipine does have the capacity to do this. The conversion of the lipophilic vitamin E into a harmless aqueous radical is the process that actually prevents lipid hydroperoxide formation. Results obtained recently with lacidipine containing the pyridinic group instead of the dihydropiridinic group (Cominacini et al. 1998) favour the hypothesis that the drug also works as a reductive compound.

In conclusion, in the present investigations (Chapter 5) it has been demonstrated that lacidipine reduces the extent of the atherosclerotic lesion in the hypercholesterolemic apoE-deficient mouse. Furthermore, this reduction in the area of the lesion is associated with a decrease in the susceptibility of LDL to oxidation and/or the reducing property of the lacidipine molecule, as well as the capacity of the drug to interfere in the cellular production of peroxidative products. These effects of lacidipine may be related to a radical scavenging activity of the molecule and/or to a decrease in the potential of the modifying cells to produce peroxidative products and/or to a reducing activity of the compound.
In a fourth study (Chapter 6), apoE-mice were fed a WTD and treated with lacidipine at 3.0 and 10.0 mg/kg/day for 8 weeks. The results obtained using these mice were compared with apoE-deficient and C57BL/6J mice fed a standard rodent diet. The results obtained provide further support for the previously accumulated evidence of a lacidipine-mediated anti-atherosclerotic activity, which has been reported in several other animal models (Soma et al. 1996; Bernini et al. 1996; Crespi et al. 2001b), and also in in vitro studies (Piedrahita et al. 1992; Oldham et al. 1997). In particular, the findings of the present work appeared to indicate that lacidipine “enhances” the activity of the endogenous NO system, both at basal levels and in response to substance P-stimulated NO release. This capacity of the drug would result in a facilitation of vasodilatation in the presence of atherosclerosis. This observation is supported by the functional studies (Chapter 6) which demonstrate that the aortic rings from control apoE-deficient mice fed the WTD, which have therefore developed extensive atherosclerotic lesions, showed a severely impaired relaxation in response to Ach. In contrast, the aortic rings from apoE-deficient mice fed the WTD and treated with lacidipine at 3.0 and 10.0 mg/kg, maintained vascular functional activities close to normal levels.

In the voltammetric studies carried out in parallel with the investigations on vascular reactivity (Chapter 6), other supporting evidence was forthcoming. The results obtained clearly demonstrated that voltammetry can measure basal, as well as substance P-stimulated NO release in vascular tissue of mice. In particular, in both wild type C57BL/6J and naive apoE-deficient control groups, the values of basal and substance P-stimulated release of NO were similar, suggesting no significant differences in the activity of their respective
NO systems. These results correlate well with the lack of appreciable atherosclerotic lesions in the aorta of both the wild type C57BL/6J and the naïve apoE-deficient control groups, and the evidence of similar functional reactivity. In contrast, in apoE-deficient vehicle-treated animals fed the WTD, it appeared that basal NO levels were increased when compared to naive (6-week old) apoE-deficient mice. This could indicate increased activity of the NO system with the development of atherosclerosis. This increase in the activity of the NO system could be either passive, i.e. simply a consequence of the presence of the lesion, or possibly an “active” response, i.e. as a “protective” vasodilatatory action of NO in presence of a damaged and malfunctioning vascular tissue. Moreover the substance P-stimulated NO signal in apoE-deficient vehicle-treated mice fed the WTD was reduced when compared to control mice (naïve and wild-type). Substance P is known to have vascular dilating properties (Oldham et al. 1997) via the stimulation of the NO system (Crespi et al. 2000), and thus one can suggest that the NO system is adversely affected in the presence of abnormal, pathological vascular lesions (i.e. rapidly developing atherosclerotic plaques induced by the WTD). This effect appears to be related in particular to the severity of the lesion. The presence of substance P receptors within the endothelium has been described (Tawara et al. 1986; Reckless et al. 1997), and Oldham et al. (1997) also reported that substance P acts as a vasodilator via NOS activation in endothelial cells. This indicates a reduced availability of endogenous NO in the WTD-fed-vehicle-treated mice following the application of substance P. Therefore, the positive levels of substance P-stimulated NO release in lacidipine-treated mice fed the WTD, would indicate that lacidipine is able to
maintain and possibly even increase the efficacy of the NO system, even in the presence of very large atherosclerotic lesions. Thus, lacidipine may be able to prevent the negative effects of atherosclerosis on the activity of the NO system.

Akopov (Akopov 1996) has reported that the vasodilatory effect of lacidipine was significantly attenuated by the presence of the NO-synthase inhibitor, N-nitro-L-arginine. Bearing the work of Akopov (1996) in mind, the present investigations (Chapter 6) show that lacidipine enhances the activity, and the levels, of endothelial NO (Cristofori et al. 2004b). Therefore this may indicate that the factor responsible for the arterial endothelium modulating vascular relaxation, in presence of dihydropyridines (DHPs), is probably a DHP-induced increase in the release of NO, rather than an enhancement of a calcium antagonist activity due to the basal release of NO at the level of vascular smooth muscle.

The reduced levels of atherosclerosis in apoE-deficient mice fed the WTD and treated with lacidipine at doses that do not control hypertension (Crespi et al. 2002) would indicate that the beneficial effects of this calcium antagonist in hypertensive patients are not simply restricted to the reduction of blood pressure. On the basis of the present findings it can be suggested that an additional property of lacidipine exists which may be related to the capacity of the drug to maintain endothelial NO levels (and consequently ONOO\(^{−}\) levels) at “useful” (i.e. nano molar) concentrations; this therefore results in protection against vascular damage. It is also considered important to state that the present studies (Chapter 6) further support the use of voltammetry with treated micro-biosensors, as an appropriate technique for the analysis of rapid, and short lasting biochemical events.
In the final study carried out in the apoE-deficient mouse model of atherosclerosis (Chapter 7), it was further demonstrated that lacidipine treatment reduces the extension of atherosclerotic lesions in apo E-deficient mice. In particular, the *ex vivo* data show that lacidipine is able to affect two key features of the unstable plaque: lipid accumulation and matrix metalloproteinase-9 (Bellosta et al. 1998) production. In fact, the results show that the drug reduced esterified cholesterol accumulation by inhibiting the cholesterol esterification rate and MMP-9 gelatinolytic potential in macrophages obtained from lacidipine-treated mice. In this study, it was observed for the first time that atherosclerotic apo-E deficient mice have a significantly higher basal esterification rate compared with C57BL/6J mice.

It is of interest to note that lacidipine was demonstrated to be effective in reducing the overall gelatinolytic capacity of macrophages. Galis and colleagues (Galis et al. 1998) showed that MMPs are sensitive to antioxidant treatment. The possibility that macrophage gelatinolytic activity is redox-dependent is suggested by previous studies showing activation of gelatinase zymogens by reactive oxygen species (ROS) which are known to be produced by macrophage foam cells (Galis et al. 1998). The suggestion that gelatinolytic activity may be redox-dependent is also indicated by the detection of active MMP-9 in an isolated cell system along with the inhibition of macrophage-derived gelatinolytic activity by treatment with N-acetyl-cysteine, a ROS scavenger. Activated macrophages, especially those contained in atherosclerotic lesions, are a major source of ROS; thus, such an activation mechanism would result in the activation of MMP zymogens secreted by the macrophages themselves as well as by the neighboring cells.
The results obtained in this last study (Chapter 7) confirm that lacidipine administered at different dose levels reduces the extent of atherosclerosis in apoE-deficient mice. Moreover, lacidipine has a significant inhibitory effect on cholesterol esterification and MMP-9 activity, properties which may contribute to increased plaque stability.

In conclusion, the investigations described in this thesis clearly demonstrate that lacidipine reduces the extent of atherosclerotic activity in different animal models of atherosclerosis without lowering hypercholesterolemia. This activity is strictly related to the different properties of this compound since it has been demonstrated that lacidipine can: a) reduce plasma endothelin levels; b) lengthen the LDL lag phase by reducing the LDL content of the lipid hydroperoxides; c) enhance the activity of the endogenous NO system; d) inhibit the effect on cholesterol esterification and MMP-9 activity, properties which may contribute to increase plaque stability.

The studies carried out in the hamster model of atherosclerosis (Chapter 3) and in apoE-deficient mice (Chapters 4, 5, 6 and 7) find a parallel in the European Lacidipine Study on Atherosclerosis (Zanchetti et al. 2002), published in 2002 (Zanchetti et al. 2002). This was a randomized, double-blind trial in 2334 patients with hypertension that compared the effects of a 4-year treatment based on either lacidipine or atenolol using an index of carotid artery atherosclerosis. The index used was the mean of the maximum intima-media thickness (IMT) in the walls of the common carotids and their bifurcations \((CBM_{\text{max}})\). The ELSA study has provided conclusive evidence of the anti-atherosclerotic activity of lacidipine. In addition, it was demonstrated in the ELSA study that lacidipine was more effective than atenolol in slowing the
progression of carotid wall IMT. Finally, the ELSA study further supports the evidence that the anti-atherosclerotic action of lacidipine is independent of antihypertensive effects.

Future studies on the activity of lacidipine in atherosclerosis progression and/or regression in apoE-deficient mice, or in other animal models of atherosclerosis, might involve non-invasive and safe techniques such as high-resolution magnetic resonance imaging (MRI). This technique could provide several benefits since it would allow the carrying out of repeated analyses in the same animal, rather than the study of multiple experimental groups with large number of animals killed at different time points. In addition, MRI techniques could accurately quantify atherosclerosis in different sites of the cardiovascular system such as the abdominal aorta, aortic arch, aortic root and brachiocephalic artery in vivo. Finally, this technique would allow much longer term studies to be planned to investigate the basis of the mode of action of lacidipine and its long-term activity and safety.
PUBLISHED WORK RELEVANT TO THIS THESIS


REFERENCES


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Vascular Protection of Lacidipine in Salt-Loaded Dahl-S Rats at Nonsustained Antihypertensive Doses

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Summary: The aim of this study was to characterize the antihypertensive and vasoprotective properties of lacidipine in salt-loaded Dahl-S rats, a suitable animal model of malignant hypertension. After 9 weeks of a high (8%) sodium chloride (NaCl) diet, 80% of the untreated Dahl-S rats died (20% survival rate) whereas a 100% survival rate was observed with chronic treatment with lacidipine at doses of 0.1 (equivalent to the recommended dose in humans), 0.3, 1, and 10 mg/kg once daily by gastric gavage. The most interesting results included the following: (a) Only the highest dose tested (10 mg/kg once daily) was able to control the increase in blood pressure, which was measured 24 h after the preceding administration of drug, yet a 100% survival rate was maintained. (b) There appeared to be prevention of brain lesions, which is very likely the cause of the survival of all of the lacidipine-treated rats in this study. (c) A clear dose-related vascular protection was observed in other tissues. In conclusion, lacidipine protects against the vascular damage and concomitant increase in mortality of salt-loaded Dahl-S rats even at doses that do not adequately control the development of hypertension. Key Words: Dahl-S rat—Hypertension—Vascular protection—Calcium antagonist.

Arterial hypertension is considered one of the most suitable models for showing the functional and morphologic consequences of intracellular accumulation of calcium into the vascular smooth muscle. In this context, spontaneously hypertensive rats (SHRs) and sodium chloride (NaCl)-fed, salt-sensitive Dahl-S rats represent well-described and reproducible animal models that can be used to follow the pathogenetic consequences of excessive calcium uptake by vascular smooth muscle together with the protective effects of various calcium antagonists.

The most striking vasoprotective effects of calcium antagonists, namely nifedipine, nitrendipine, nisoldipine, and anipamil, have been recently reported in salt-loaded Dahl-S rats; thus, we have investigated the antihypertensive and vasoprotective properties of lacidipine, a new vascular-selective and long-acting 1,4-dihydropyridine, in this accelerated model of vascular damage (1-3).

METHODS

Male Dahl-S rats (Mollegaard Breeding Center, Skensved, Denmark), 7 to 8 weeks old, were randomly allocated into one of six groups and housed two to a cage. Altromin-MT 0.3% NaCl (standard) diet (A. Rieper SpA, Vandoies Bolzano, Italy) or Altromin-MT 8% NaCl (salt-rich) diet and water from the normal domestic supply were provided ad libitum from the first day of the study. Rats were treated daily by gastric gavage with lacidipine at doses of 0.1, 0.3, 1, and 10 mg/kg or vehicle (0.5% methylcellulose). The rats were fed and treated from week 1 of the study according to the scheme reported in Table I.

Systolic blood pressure (SBP) was measured indirectly by the tail-cuff method in conscious animals before starting the study and at weekly intervals during the treatment period. All evaluations were performed before the daily oral treatment. Ophthalmoscopic examination was carried out before starting the study and once a week during the study, before daily treatment and 4 h after treatment. Ophthalmoscopy, performed with a mydriatic (Visumidriatic 1%, Merck Sharp & Dohme Italia, Rome, Italy) and an indirect ophthalmoscope, was followed by photographic documentation of the ocular fundus. Individual body weights were recorded once during the predosing period, twice weekly during the treatment period, and shortly before necropsy. Final necropsy was performed when 80% of the untreated hypertensive rats (group 2) had died. After death, a full macroscopic examination was performed.
TABLE 1. Study design

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet (% NaCl)</th>
<th>Treatment</th>
<th>Dosage* (mg/kg o.d.)</th>
<th>No. of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
<td>Vehicle</td>
<td>—</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>Vehicle</td>
<td>—</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>Lacidipine</td>
<td>0.1</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>Lacidipine</td>
<td>0.3</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>Lacidipine</td>
<td>1</td>
<td>12</td>
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<td>6</td>
<td>8</td>
<td>Lacidipine</td>
<td>10</td>
<td>12</td>
</tr>
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</table>

* Administered by gastric gavage.

carried out of all animals, including those that had died or were killed during the study. All tissues to be examined were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained in hematoxylin and eosin. The hearts of all animals were weighed when fresh.

Statistical analysis
To allow for the variability of the baseline values, differences between treatments with respect to time were evaluated using analysis of covariance. The development of hypertension was assessed in terms of slope of the time/SBP regression line. Comparisons between each value before and after drug administration were made by paired t test when acute antihypertensive effects were studied; p < 0.05 was considered significant. For the heart weights, Dunnett’s two-tailed test for multiple comparison was carried out to compare the means of the treated group with the untreated hypertensive group.

RESULTS

Mortality
From week 4 of the study, the vehicle-treated hypertensive rats (group 2) began to die or were killed in extremis and, by week 9 of the study, an 80% mortality rate had been achieved, at which time the study was stopped. In contrast, all rats treated with any dosage of lacidipine survived during the same period (Fig. 1).

Blood pressure
Vehicle-treated Dahl-S rats fed with an 8% NaCl diet (group 2) developed sustained hypertension: at week 3, SBP exceeded 200 mm Hg, and then progressively increased to a maximum value of 241 mm Hg at week 8 in the surviving 9 of 24 rats (slope of 11.6 for the whole treatment period; Fig. 2). The animals in group 3, treated with 0.1 mg/kg of lacidipine once daily in addition to the high-salt diet, also showed high values of SBP (245 mm Hg at week 9 with a slope of 13.6 for the period 0–9 weeks); the time course in this group in the first 5 weeks was not significantly different from that in the vehicle-treated group (group 2; slope of 19.2 vs. 16.8; Fig. 2). Similarly, the animals in group 4 (0.3 mg/kg of lacidipine once daily) developed fulminant hypertension during the first 5 weeks (slope of 15.9), although the SBP was significantly decreased during the subsequent 4 weeks. Indeed, the slope for the entire 9-week period was significantly different from both group 2 and group 3 (7.1 vs. 11.6 and 13.6, respectively; Fig. 2).

The SBP in the animals in group 5 (1 mg/kg of lacidipine once daily) rose from a mean baseline value of 159 to 204 mm Hg at week 3, and then decreased to slightly lower values at weeks 4 and 5 to eventually fluctuate around 205 to 215 mm Hg during the last 3 weeks of the study period. The slope for the whole treatment period was significantly lower (6.6) in comparison to either group 2 or 3, but was not significantly different from that of group 5. The slope (7.3) for the first 5 weeks, however, was significantly different from those of groups 2, 3, and 4 (Fig. 3).
VASCULAR PROTECTION WITH LACIDIPINE

In group 6 animals (10 mg/kg of lacidipine once daily), no significant increase in SBP was observed during the 9-week study treatment period, as indicated by the slope value of 0.7. In fact, a significant decrease in SBP was measured during the first 5 weeks of treatment (slope of -3.4, Fig. 3).

The animals in group 1 (vehicle-treated 0.3% NaCl-fed Dahl-S rats) showed progressive increases in SBP from a mean baseline value of 162 to 195 mm Hg at week 8, with a slope of 3.2, which was significantly higher than that of group 6 (Fig. 4).

With regard to the acute antihypertensive effect, investigated at weeks 8 or 9 in animals that had already developed sustained hypertension, lacidipine induced a dose-related decrease in SBP (Fig. 5): with 0.1 mg/kg once daily, a 27% reduction was observed by the first hour after treatment and the last significant effect (l.s.e.) at 7 h; with 0.3 mg/kg once daily, there was a 39% reduction with peak effect within the first hour and the effect was only slightly significant at 12 h (-5.7%); with 1 mg/kg once daily, SBP was reduced by 53% within the first hour and the l.s.e. was observed at 12 h (-20%).

Ophthalmoscopic examination

The increase in systemic arteriolar tone can be directly visualized by examination of the ocular fundus. The caliber of the retinal arterioles diminishes during the progressive blood pressure elevation with the appearance of marked arteriolar vasoconstrictions and aneurysm-like lumen irregularities (1,4,5). Lacidipine completely prevented any arteriolar alteration at the dose of 10 mg/kg once daily and dose-dependently retained the reversibility of the altered arteriolar tone, as assessed in daily examinations performed after treatment in the other groups (Figs. 6 and 7). The aneurysm-like formations were completely prevented by lacidipine at doses of 0.3, 1, and 10 mg/kg once daily whereas a delayed and lower incidence was observed with the 0.1 mg/kg dose
0.3% NaCl Vehicle-treated (n=12)

8% NaCl+Lacidipine 10 mg/Kg p.o.(n=12)

FIG. 4. Time course of systolic blood pressure in Dahl-S standard diet-fed controls and hypertensive rats treated with the highest study dose of lacidipine. Means ± SEM.

dose (Fig. 8). This suggests that arteriolar vasoconstrictions, reversible after lacidipine administration, are of a spastic nature whereas aneurysm-like proliferations are probably a more pronounced structural alteration of arteriolar walls.

Heart weight

In hypertensive untreated rats (group 2), an increased heart weight, indicating cardiac hypertrophy, was evident (6–8) as both absolute and relative weights. At all doses of lacidipine, relative heart weights were significantly lower than that of the group 2 rats (Fig. 9); this was also true for rats treated with 1 and 10 mg/kg of lacidipine once daily in terms of absolute heart weights. The relative heart weights of lacidipine-treated rats, however, were not significantly different from those of rats fed a standard diet (group 1).

Pathology

Salt-loaded vehicle-treated rats (group 2). All rats showed diffuse and marked vascular and tissue damage characteristic of malignant essential hypertension (1,2,9). The most common pathologic macroscopic findings were as follows: in the brain, generalized edema or patchy discoloration, and focal or large hemorrhage mainly in the occipital cortex (Fig. 10); in the mesenteric arteries, marked thickening of the distal branches with increases in transverse diameter and evident tortuosity, sometimes with diffuse intestinal hemorrhage (Fig. 11); in the eye, occasionally protruding eye, very often with chromodacryorrhea.

On microscopic examination of the tissues, the most prominent feature was the vascular damage that appeared with various degrees of organization (acute and chronic damage) and with an apparent involvement of the adjacent tissues. Arteritis with peripheral fibrotic reaction, accumulation of fibrinoid material in the intima, and frank necrosis of the arterial wall (fibrinoid necrosis), sometimes leading to complete disorganization of the normal vessel architecture, were common microscopic features in the gastrointestinal tract (Fig. 12), kidney, liver, testis, lungs, heart, eye, and lymphoid tissues.
In the brain, earlier lesions within the gray matter were characterized by slight deposits of acidophilic exudates with a few extravasated erythrocytes in close proximity to the leaking blood vessels (penetrating arterioles). The surrounding tissue was spongy due to small vacuoles and, in the spongy parenchyma, most neurons had well-preserved nuclei surrounded by a thin rim of cytoplasm; condensed neurons were most often found in the peripheral parts with early activated astrocytes. The sponginess of the gray matter became further increased, resulting in more confluent loss of neuronal tissue and formation of sparsely trabeculated cystic cavities (Fig. 13). Findings indicative of hemorrhage (Fig. 13) or an edematous state, such as widely dispersed myelinated fibers with activated astrocytes, were seen mainly in the corpus callosum and generally in the white matter.
Most of these rats showed neuropathologic signs such as depression, spatial disorientation, jerky movements, and leg paralysis.

Salt-loaded rats treated with 10 mg/kg of lacidipine once daily (group 6). No macroscopic abnormalities were seen at necropsy in this group of rats, and microscopic examination of the tissues revealed no vascular damage.

Salt-loaded rats treated with 1 mg/kg of lacidipine once daily (group 5). No macroscopic abnormalities were seen at necropsy (Fig. 11) and, on microscopic examination of the tissues, minimal pathologic changes (arterial wall hypertrophy) in the coronary artery branches were seen and only in three rats. No microscopic abnormalities were seen in the mesenteric artery (Fig. 12).

Salt-loaded rats treated with 0.3 mg/kg of lacidipine once daily (group 4). No macroscopic abnormalities were seen at necropsy and, on microscopic examination, only 4 of 12 rats showed early signs of vascular damage in the more sensitive tissues. The brain of one animal, which had not displayed abnormal neurologic signs in vivo, was found to have two small foci of necrosis in the hippocampus.

Salt-loaded rats treated with 0.1 mg/kg of lacidipine once daily (group 3). At necropsy, the mesenteric vessels in nine rats appeared to be thickened, but only in their most distal portions adjacent to the intestinal wall. The brain in two animals was found to be irregularly enlarged by edema. Generally, histologic examination of this group revealed vascular damage in a wide range of tissues, although with a clearly lower incidence in comparison to vehicle-treated hypertensive rats.

Dahl-S rats fed a standard diet (group 1). No macroscopic or microscopic abnormalities were seen in these animals.

DISCUSSION AND CONCLUSION

In addition to their vital physiologic function, calcium ions have pathogenetic potential if they accumulate excessively in cytoplasm. This is partic-
VASCULAR PROTECTION WITH LACIDIPINE

In this context, since 1970, Fleckenstein has focused interest on the pathogenetic consequences of excessive calcium uptake by arterial smooth muscle cells and the vascular protection by various calcium antagonists (2). Most of his experimental observations were made in SHR and NaCl-fed, salt-sensitive Dahl-S rats, whose common anomaly is a marked inability to keep intracellular calcium concentrations of vascular smooth muscle at a normal level. Fleckenstein has hypothesized that calcium overload is the common denominator of arterial lesions in SHR and salt-loaded Dahl-S rats, although these hypertensive animals develop vascular damage over different time courses, within 4-6 weeks in salt-loaded Dahl-S rats compared with 18-20 months in SHRs. In addition, Dahl-S rats have a preferential calcium overload of the small arterial branches and arterioles that induces functional and structural alterations. The functional consequence is an increased peripheral vascular resistance that leads to hypertension if vasoconstriction is generalized, whereas the structural consequence is that permanent calcium overload of resistance vessels produces vascular damage similar to certain forms of arteriosclerosis in humans (10).

Calcium antagonists have proved their therapeutic value as antihypertensive agents by preventing arterial calcium overload and thereby protecting these hypertensive animals from all functional and structural alterations. Some calcium antagonists will prolong the life expectancy of SHRs considerably: less than 20% of untreated animals reach the age of 21 months whereas a 65% survival rate has been observed at this age when verapamil was added to the standard diet (1).

Ever more remarkable are the results reported from studies involving salt-sensitive Dahl rats. Only 20% of untreated 8% NaCl-loaded animals survived a 6-week period whereas life expectancy markedly increased in nitrendipine-treated rats: 100% of the rats were still alive after 10 weeks of salt loading. However, it is noteworthy that a very high dosage of nitrendipine was used (150 mg/kg p.o. twice daily) to inhibit the development of fulminant hypertension (1).

Lacidipine is a new vascular-selective 1,4-dihydropyridine calcium antagonist that has been reported to induce a potent and long-lasting antihypertensive effect in animal studies (11). The present article describes studies performed in salt-loaded Dahl-S rats to investigate the prevention of hypertension as well as vascular protection exerted by lacidipine when administered prophylactically as once-daily oral treatment. To this end, lacidipine has been studied in a wide range of doses, starting from 0.1 mg/kg (roughly equivalent to the human therapeutic dose of 4 to 6 mg once daily) to 10 mg/kg (presumably equipotent to the dose of nitrendipine (150 mg/kg p.o. twice daily) used by Fleckenstein in a similar experiment (1)).

In accordance with previous observations, salt-sensitive Dahl-S rats developed fulminant hypertension and showed pathologic alterations, together with accelerated mortality, when fed an NaCl-rich (8%) diet (9). Vehicle-treated salt-loaded animals very quickly attained high levels of SBP, exceeding 200 mm Hg by the third week and progressing to approximately 240 mm Hg by the eighth week. However, when salt-loaded Dahl-S rats were given lacidipine at 10 mg/kg once daily, this marked increase in SBP was completely prevented; in fact,
their SBP was even lower than that of Dahl-S rats treated with a standard (0.3% salt) diet. In contrast, lacidipine at the lowest dose tested (0.1 mg/kg once daily) had no effect on the development of hypertension.

At the intermediate doses of 0.3 and 1 mg/kg once daily, lacidipine did not prevent high values of blood pressure, especially during the first 3 to 5 weeks, although significantly lower slopes of the regression line, relating SBP with time, were obtained, suggesting a damped progression of hypertension. Nevertheless, an acute antihypertensive effect of lacidipine was apparent in hypertensive animals at week 8 or 9, although lasting always less than 24 h. This most probably explains why the two intermediate doses, each with a duration of action of approximately 12 h, only slightly modified the development of hypertension whereas the highest dose was completely effective.

As for prevention of early death, our study is in agreement with previous observations: less than 20% of vehicle-treated, salt-loaded Dahl-S rats survived a 9-week period. In contrast, the daily administration of lacidipine in addition to the salt-rich diet produced a striking increase in survival rate: 100% of the treated animals were still alive after the same period of observation. It is important to emphasize that all animals survived even when the dosage level was as low as 0.1 mg/kg once daily. No other calcium antagonist tested in this animal model has exhibited prevention of mortality at such a low dose.

In the vehicle-treated hypertensive rats, fulminating hypertension was characterized by a diffuse
and severe necrotizing vasculopathy; one of the most dramatic macroscopic alterations was apparent in the distal branches of the mesenteric arteries and comprised extreme wall thickening, and partial or total luminal obliteration. Furthermore, an unexpectedly common finding in our strain of Dahl-S rats was lesions in the brain, varying from a diffuse edematous enlargement to pinpoint or focal hemorrhages in the cortex. Cerebrovascular lesions are known to occur in stroke-prone SHR as a consequence of multifocal breakdown of the blood-brain barrier, resulting in an excessive influx of macromolecules and other blood constituents into the arterial wall and perivascular parenchyma (12).

A noteworthy observation in our studies was a selective prevention of the brain lesions, which is very likely why all of the lacidipine-treated rats survived. This selectivity is supported by the very low incidence of brain lesions in rats treated with 0.1 mg/kg of lacidipine once daily, a dose that, in this animal model, is completely ineffective in controlling blood pressure. This finding suggests that different mechanisms may be involved in the control of hypertension and in vascular protection. Further evidence of prevention of vascular damage has been provided by direct examination of the ocular fundus, which revealed that lacidipine at doses of 0.3, 1, and 10 mg/kg once daily completely prevented retinal arteriolar aneurysm-like irregularities. A delayed and lower incidence of this structural alteration was observed with the 0.1 mg/kg once-daily dose (1-4). Vascular protection was even more evident on detailed histologic examination, which showed a clearly dose-related vascular protection in the majority of tissues. Microscopically, the protective effect was particularly evident in the brain.

**FIG. 12.** Histological cross-sections (low-power views) through a distal branch of the mesenteric artery of hypertensive Dahl-S rats fed an 8% NaCl diet show severe periarteritis and vascular necroses (A) compared with the normal appearances (B) with 1 mg/kg of lacidipine once-daily treatment.
FIG. 13. Histology (low-power view) of a hypertensive NaCl-loaded Dahl-S rat brain showing (A) severe loss of parenchyma leading to cyst formation, following acute edema, and (B) a large hemorrhage in the cortex near the corpus callosum. Hematoxylin and eosin stains.

as only three rats treated with lacidipine at 0.1 mg/kg once daily showed lesions in those tissues.

In vehicle-treated hypertensive rats, the indication of cardiac hypertrophy resulted from the high heart weight whereas, in the lacidipine-treated rats, the relative heart weight values were significantly decreased independent of blood pressure values (6–8). This interesting evidence may be explained by the fact that all doses of lacidipine acutely reduced blood pressure for at least 7 h after daily oral treatment.

As a whole, these findings support Kazda’s and Fleckenstein’s assumption that hypertension per se is not the crucial factor for early death in SHR and salt-loaded Dahl-S rats. Their suggestion was that intracellular calcium overload is the main cause of calcinotic and sclerotic arterial lesions, and hypertension is simply a result of calcium-induced spastic hyperactivity of the peripheral resistance vessels. Moreover, it is well established that calcium ions play an important role in a number of processes involved in arterial calcinosis (2,10,13,14). Thus, calcium antagonists should inhibit calcium overload-dependent pathogenetic factors through the maintenance of calcium homeostasis in the arterial tissue, thereby prolonging the life expectancy of hypertensive rats.

That lacidipine proved to be beneficial at ex-
tremely low doses is most probably due to its potent and long-lasting calcium entry-blocking activity, but raises the possibility that this dihydropyridine derivative is endowed with additional properties that require further investigation. In conclusion, the results of our trial give further support to the possibility that the beneficial effects of calcium antagonists in hypertensive patients are not restricted to simply the lowering of elevated blood pressure.

REFERENCES


DISCUSSION

Professor Zanchetti: This is a very good experimental model, particularly because of the speed of the hypertensive disease. Were the lesions mostly microaneurysms? Did these animals die mostly by cerebral hemorrhage?

Dr. Bertolini: That is correct. The cause of death was probably the brain lesions.

Professor Zanchetti: Was there any evidence of kidney involvement, as in traditional malignant hypertension in humans?

Dr. Bertolini: Yes. On microscopic examination, most of the organs, including the liver, kidney, and testes, showed diffuse vasculopathy. The dramatic findings were mainly in the gastrointestinal tract and in the brain. We were very surprised by the protection provided by doses of lacidipine that did not prevent hypertension because, until now, all of the previous studies with other drugs used doses that prevent hypertension completely. So, we are trying to understand why, and other studies are now ongoing to explain this.

Professor Zanchetti: It is of further interest because most of these microaneurysms are thought to be strongly blood pressure-dependent; this has been given as the reason for an advantage seen with strokes, but not with cardiac events, in humans. Will you speculate on how calcium or calcium antagonists, but not blood pressure, may be involved in these types of lesions, which are different from those discussed by Dr. Frey?

Dr. Bertolini: We are undertaking research to explain these mechanisms. I believe that the specific pharmacological characteristics of lacidipine that endow it with vascular selectivity may explain the differences, but there may be other hypotheses. Perhaps Dr. Gaviraghi would like to comment further.

Dr. Gaviraghi: I think it is difficult to explain the mechanism at this time. What Dr. Bertolini has shown is very interesting for mainly two reasons. I think this is the first time that a calcium antagonist has proved to be able to prevent a vascular disease at a low dose in contrast to the experiment published with nitrendipine, in which they used huge doses — 150 mg twice daily. This was a very controlled experiment and, although the very low dose does not provide complete protection, it is still very good protection.

The second mechanism, in my view, is the mechanism by which lacidipine is so long lasting. I believe that the molecule is very lipophilic; the molecule sticks close to the receptor for a long time, which we have demonstrated in vitro with washout experiments. The compound is distributed, and then bound to the receptor and, once there, stays for a long time, giving a sort of local protection against the factors that destroy the arteries; for example, the compound could block calcium overload in situ continuously.

The other calcium antagonists, such as amlodipine, achieve their long duration of action through a long kinetic profile and not by the mechanism proposed for lacidipine—sticking to the receptor. Lacidipine possesses comparatively very high antioxidant properties. It is at least 10 times more potent than any of the other dihydropyridines, having a similar antioxidant activity to vitamin E. It may well
be that this blocks the formation of free radicals, which is one of the mechanisms by which the endothelium is destroyed.

Professor Mancia: Was pulse pressure also unchanged? An additional possibility is that the drug did not have an effect on mean pressure, but reduced pulse pressure, which could be a factor involved in tissue damage and in stroke.

Dr. Bertolini: Yes, we measured the systolic blood pressure and you are right—the difference between diastolic and systolic pressures was well correlated.
Anti-atherosclerotic activity of the calcium antagonist lacidipine in cholesterol-fed hamsters

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Summary — We have investigated the activity of the calcium antagonist lacidipine in male hamsters fed an atherogenic diet containing 2% cholesterol and 5% butter. Animals were examined at 14, 20 and 24 weeks of treatment. At 14 weeks, in hamsters fed the atherogenic diet and without lacidipine treatment, there were significant increases in serum levels of total cholesterol, triglycerides and lipoproteins; these values were approximately similar at week 24.

Lacidipine treatment at 0.3, 1.0 and 3.0 mg/kg/d did not affect levels of serum cholesterol, triglycerides and lipoproteins. At 24 weeks, in hyperlipidemic hamsters fed the atherogenic diet, the area of the fatty streak in the aortic arch covered a mean area of $375 \pm 145$ micron$^2$, which accounted for 2.7% of the total surface area of the aortic arch. In hamsters fed the atherogenic diet and treated with lacidipine at 0.3, 1.0 and 3.0 mg/kg, at 24 weeks, the surface area of the aortic arch lesion was significantly reduced by 41 to 71%. In the thoracic aorta at 24 weeks, in lacidipine-treated animals, both the incidence and degree of severity of the lesions was reduced, the area of the fatty streak being lowered by 78 to 97% in comparison with non-lacidipine-treated control animals. Ultrastuctural examination demonstrated that the early changes in the aorta in hamsters fed the atherogenic diet involved the intima and smooth muscle cells; lacidipine treatment reduced the severity of the intimal lesions significantly. With SEM, lacidipine administration was seen to reduce endothelial irregularity and the presence of crater-like lesions. At TEM, treatment with lacidipine reduced the number of foam cells and the presence of liposomes in the subendothelium. This investigation demonstrates that in the hyperlipidemic hamster, lacidipine treatment decreases atheromatous lesions without lowering serum lipids. It is suggested that lacidipine influences the atherogenic process by an unusual mechanism which may be related to a combination of both the long-lasting calcium antagonism of the drug and significant antioxidant activity. © 2000 Éditions scientifiques et médicales Elsevier SAS

Lacidipine is a calcium antagonist (calcium channel blocker) belonging to the 1,4-dihydro pyridine class. The drug has been shown, in both in vivo and in vitro preclinical studies, to possess potent and long-lasting antihypertensive properties [5]. We have previously described the protective activity of lacidipine, with increased survival rate, in the salt-loaded Dahl-S rat [1]. Similarly, we have reported that lacidipine inhibits the development of alterations in the arterial wall, and induces significant regression of cardiac hypertrophy, in a dose-dependent fashion [3]. Furthermore, using the spontaneously hypertensive stroke-prone rat, we have demonstrated the protective activity of lacidipine in preventing cardiac hypertrophy [2].

The pharmacologic profile of lacidipine prompted us to investigate further the anti-atherogenic properties of the drug which have not been previously examined in detail. The hyperlipidemic hamster is a suitable experimental model of atherosclerosis, being susceptible to diet-induced atherosclerosis, and for showing cholesterol metabolism and atheromatous lesions comparable to man’s [7-10]. Other advantages of the hamster model are the frequency with which the atherosclerotic lesions develop, and their relatively small size, which facilitates morphometric analysis. The fatty streaks develop consistently along the ‘lesion-prone area,’ following the inner curvature of the aortic arch; this evolution of the fibro-fatty plaques in a highly predictable site during hypercholesterolemia is a considerable advantage in experimental investigations.

In the present study we have examined in the hamster model the pathophysiological and morphological
changes occurring in animals receiving an atherogenic diet, and the effect of lacidipine administered at three dose levels, at different time points, using light and electron microscopy. The objective of the investigation was to evaluate whether the once-daily treatment with a long-lasting calcium antagonist influences the atherogenic process, and to identify the ultrastructural targets of drug action.

MATERIALS AND METHODS

Animals and drug administration

Two hundred male golden Syrian hamsters (10 weeks old, mean body weight 120g; Charles River Ltd., Italy), were randomised into five equal groups. Group 1 was fed a standard diet (Standard Diet n49; Piccioni, Milan, Italy [SD]). Groups 2 to 5 were fed an atherogenic diet (high cholesterol diet [HCD]) consisting of the SD supplemented with 2% cholesterol (supplied by Piccioni, Milan, Italy) and 5% butter (weight/weight [w/w]). Animals in groups 1 and 2 were treated once daily by oral gavage with vehicle (Methocel; Sigma Chemical Co Ltd) at 0.5% weight/volume (w/v), or with lacidipine at 0.3 (group 3), 1.0 (group 4) and 3.0 (group 5) mg/kg.

The research complied with national legislation and with the company policy on the Care and Use of Animals and with related codes of practise.

Serum Analysis

Animals (n = 9–11) from groups 1 to 5 were sacrificed under ether anaesthesia at 14 and 24 weeks after the start of diet and drug treatment. Blood was collected from the abdominal aorta and serum prepared by centrifugation for the determination of serum lipids and lipoprotein cholesterol. Serum total cholesterol and triglycerides were quantified using enzymatic methods with commercial kits (Boehringer Mannheim GmbH) with a Cobas Bio analyser. High-density lipoprotein cholesterol (HDL cholesterol) was assayed using a commercial kit after apo-b precipitation by a phosphotungstate method (Boehringer Mannheim). Low-density lipoprotein cholesterol (LDL cholesterol) was calculated by subtraction of HDL cholesterol from total cholesterol.

Histological examination

To evaluate the fatty streak, the aorta was fixed by perfusion with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), for 20 min at 110 mm Hg, followed by staining with 0.06% AgNO₃ and Harris’s haematoxylin. The aortic arch was dissected, starting at the aortic valve, to the left subclavian artery, and the thoracic aorta was taken in toto up to the diaphragm; aortic samples were stained with 0.3% Oil Red O, and examined as en face preparations by light microscopy.

Morphometric evaluations were performed on colour photographs, obtained under standardised conditions, using the digitising board of an image analyser (Image Measure 3100, Microscience Phoenix Technology Inc., Washington, USA) installed on an Hp computer (386/20N; Vectra).

For transmission electron microscopy (TEM), glutaraldehyde-fixed tissues were postfixed in 1% osmium tetroxide in phosphate buffer, dehydrated in graded ethanols, embedded in epoxy resin (Epon-Araldite) and sectioned using an ultra microtome (Ultracut E; Riechert-Jung). Ultrathin sections were stained with uranyl acetate/lead citrate and observed in an electron microscope (EMIO; Zeiss). For scanning electron microscopy (SEM), fixed and postfixed tissues (as above) were dehydrated in graded ethanols, critical point-dried (CPD 030; Balzers), fixed to stubs with colloidal silver, sputter-coated with gold (MED 010; Balzers) and examined with an SEM (DSM 690; Zeiss).

Statistical Analysis

Treated and control groups were compared using Duncan’s test.

RESULTS

Serum lipids and lipoprotein cholesterol

At 14 weeks, there were significant increases in mean values for serum total cholesterol, triglycerides and lipoproteins in group 2 hamsters fed the atherogenic HCD diet, in comparison with group 1 animals fed the SD normal diet (table I). Levels of total cholesterol, triglycerides and lipoproteins at 14 weeks were also increased in hamsters treated with lacidipine at 0.3 mg/kg (group 3), 1.0 mg/kg (group 4) and 3.0 mg/kg (group 5), in comparison with the control animals in group 1. However, in lacidipine-treated hamsters (groups 3 to 5), values for cholesterol, triglycerides and lipoproteins were directly comparable with group 2 animals fed the HCD diet and without lacidipine treatment. In all groups at 24 weeks there was some evidence of a general trend of increasing mean values for cholesterol, triglycerides and lipoprotein, in comparison with week 14; however, this did not achieve statistical significance. At 24 weeks there was a direct parallel with the pattern of changes seen at week 14 for cholesterol, triglycerides and lipoprotein, with group 2 animals
Table I. Mean values (±SEM) for serum lipids in hamsters fed a standard diet (SD) or an atherogenic diet (HCD), treated with lacidipine at 0.3, 1.0, and 3.0 mg/kg, and sampled at 14 and 24 weeks.

<table>
<thead>
<tr>
<th>Group (diet; lacidipine dose level, mg/kg)</th>
<th>Cholesterol (mmol/L) 14 weeks</th>
<th>Triglycerides (mmol/L) 14 weeks</th>
<th>HDL cholesterol (mmol/L) 14 weeks</th>
<th>LDL cholesterol (mmol/L) 14 weeks</th>
<th>24 weeks 14 weeks</th>
<th>24 weeks 14 weeks</th>
<th>24 weeks 14 weeks</th>
<th>24 weeks 14 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (SD)</td>
<td>2.0 ± 0.1*</td>
<td>2.5 ± 0.1*</td>
<td>1.5 ± 0.2*</td>
<td>2.1 ± 0.1*</td>
<td>1.2 ± 0.1*</td>
<td>1.3 ± 0.1*</td>
<td>0.7 ± 0.1*</td>
<td>0.7 ± 0.1*</td>
</tr>
<tr>
<td>2. (HCD; 2% Chol)</td>
<td>24.1 ± 4.9</td>
<td>21.4 ± 2.4</td>
<td>16.4 ± 6.2</td>
<td>21.0 ± 4.5</td>
<td>4.8 ± 0.6</td>
<td>6.4 ± 1.2</td>
<td>14.4 ± 4.9</td>
<td>16.5 ± 4.2</td>
</tr>
<tr>
<td>3. (HCD; 0.3%)</td>
<td>19.0 ± 3.1</td>
<td>28.2 ± 3.8</td>
<td>14.4 ± 6.3</td>
<td>31.0 ± 8.4</td>
<td>5.2 ± 0.3</td>
<td>8.4 ± 1.6</td>
<td>10.3 ± 3.3</td>
<td>18.2 ± 5.0</td>
</tr>
<tr>
<td>4. (HCD; 1.1)</td>
<td>15.4 ± 2.9</td>
<td>26.3 ± 9.1</td>
<td>13.3 ± 0.8</td>
<td>18.3 ± 10.0</td>
<td>4.9 ± 0.4</td>
<td>8.4 ± 1.8</td>
<td>7.8 ± 2.2</td>
<td>18.2 ± 4.3</td>
</tr>
<tr>
<td>5. (HCD; 3.0)</td>
<td>16.4 ± 2.4</td>
<td>20.7 ± 2.9</td>
<td>12.9 ± 4.3</td>
<td>13.4 ± 6.5</td>
<td>6.1 ± 1.0</td>
<td>7.5 ± 1.6</td>
<td>7.9 ± 1.6</td>
<td>15.1 ± 3.9</td>
</tr>
</tbody>
</table>

1 n = 9 to 11; *, significantly different to group 1 animals at the same time period, P < 0.05 and no significant differences between group 2 animals receiving high-cholesterol diet (HCD) and lacidipine-treated groups (statistical analysis by Duncan’s test).

Table II. The effect of lacidipine, administered at 0.3, 1.0 and 3.0 mg/kg, on the mean total surface area occupied by fatty streak in the aortic arch and thoracic aorta of hamsters fed a standard diet (SD), or an atherogenic diet (HCD), at 24 weeks of treatment.

<table>
<thead>
<tr>
<th>Group (diet; lacidipine dose level, mg/kg)</th>
<th>Aortic arch</th>
<th>Thoracic aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fatty streak area¹</td>
<td>Reduction in fatty streak area²</td>
</tr>
<tr>
<td>1. (SD; –)</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>2. (HCD; –)</td>
<td>2.7 ± 0.96*</td>
<td>–</td>
</tr>
<tr>
<td>3. (HCD; 0.3)</td>
<td>0.9 ± 0.54*</td>
<td>67%</td>
</tr>
<tr>
<td>4. (HCD; 1.0)</td>
<td>1.6 ± 0.21*</td>
<td>41%</td>
</tr>
<tr>
<td>5. (HCD; 3.0)</td>
<td>0.8 ± 0.31*</td>
<td>71%</td>
</tr>
</tbody>
</table>

¹ Values represent the mean total surface area (±SEM) occupied by the fatty streak as a percentage of the total surface area of the aortic arch or the thoracic aorta; n = 9 to 11; *, significantly different to group 2, P < 0.05; ² Values represent the reduction in fatty streak area in comparison with group 2.

En face preparations of aortic arch and thoracic aorta

At 14 weeks, in en face preparations of the aortic arch stained with Oil Red 0 to evaluate the development of the fatty streak, the lesion was observed in the majority of group 2 hamsters fed the atherogenic diet. The lesion then increased in size and severity, and in group 2 animals at 24 weeks the mean area covered was 375 ± 145 (micron² x 100); this accounted for 2.7% of the total surface area of the aortic arch (table II). In contrast, at week 24, in hamsters treated with lacidipine at 0.3, 1.0 and 3.0 mg/kg (groups 3 to 5, respectively), the area occupied by the fatty streak, expressed as a percentage of the total aortic arch surface area, was significantly reduced in comparison with group 2, by 67% (group 3), 41% (group 4) and 71% (group 5). No fatty streaks were evident in the aortic arch of group 1 hamsters fed the SD diet, at any point in time.

In the thoracic aorta, at 14 weeks, the hyperlipidemic hamsters of group 2 showed the formation of early lipid deposits. These consisted of foamy macrophages and/or small clusters of extracellular lipid deposits. There were significant differences in the incidences of the lesions, and the degree of severity of the lesions, between group 2 hamsters fed the HCD and the animals of groups 3 to 5 fed HCD and treated with lacidipine. Morphometric assessment at week 24 (table II) demonstrated that, in comparison with group 2, the fatty streak area was reduced by 85, 78 and 97% in groups 3 to 5 treated with lacidipine, respectively.

Ultrastructure of the aortic arch (lesion-prone area)

Group 1 (SD; without lacidipine)

The ultrastructural features of the aortic arch remained unchanged throughout the experiment. At SEM, the endothelial cells were polygonal in shape with the nucleus protruding into the vessel lumen; short microvilli were evident close to the cell borders. At TEM, the endothelial cells showed few organelles, but...
numerous micropinocytotic vesicles were present. The basal lamina was thicker than in other regions of the aorta. The subendothelial area was generally devoid of cells and showed infrequent collagen fibres (figure 1).

**Group 2 (HCD; without lacidipine)**

At 14 weeks, using SEM, the surface of the aortic arch showed irregular endothelial cells with an increased number of microvilli (figure 2A). Crater-like lesions were often evident on the endothelial surface. Lymphomonocytic cells and platelets were frequently attached to the intimal surface. At TEM, at 14 weeks, the endothelial cells were seen to be rich in organelles, with hypertrophy of the Golgi complex and endoplasmic reticulum; lysosomal inclusions were numerous and the basal lamina was thickened. The subendothelial space was enlarged and showed many liposomal particles (figure 3A). Foam cells and muscle cells were present in the subendothelial space. The muscle cells were rich in mitochondria, with a well-developed endoplasmic reticulum, but microfilaments were few.

In hamsters at 24 weeks, the intimal surface was more irregular than at 14 weeks and numerous crater-like lesions were evident. The basal lamina was increased in thickness, and the cellularity of the subendothelial space was also greater. Muscle cells at this time were showing evidence of degenerative changes, and an irregularity of the internal elastic lamina was also evident.

**Group 3 (HCD, 0.3 mg/kg lacidipine) and group 4 (HCD; 1.0 mg/kg lacidipine)**

The lesions in these animals were intermediate between those of group 2 and group 5.

**Group 5 (HCD, 3.0 mg/kg lacidipine)**

At SEM level the intimal surface appeared generally regular (figure 2B); the endothelial cells did not show
anti-atherosclerotic activity of lacidipine in hamsters

Figure 3A, B. A) Aortic arch from a hyperlipidemic hamster: accumulation of liposomes in a clearly evident and enlarged subendothelial space. B) Aortic arch from a hyperlipidemic hamster treated with 3.0 mg/kg lacidipine: there is a reduced number of liposomes in the subendothelial space.

A) TEM, x 16,000; B) TEM, x 16,000.
E: endothelial cell; F: foam cell; M: muscle cell; S: subendothelial space; SM: smooth muscle cell; *: elastic lamina.

signs of cytoplasmic activation, and the microvilli were normal. Where the intimal surface lay above a presumed area rich in subendothelial cells, the endothelial cells were elevated, with a large basal area and a rounded surface membrane; some isolated crater-like lesions were visible. At TEM, the endothelial cells did not show evidence of organelle hypertrophy; however, the basal lamina was increased in thickness. In comparison with the appearance of the tissues in group 2 hamsters, the cellularity of the subendothelial space was reduced, as was the density of the liposomal particles (figure 3B). Smooth muscle cells were rarely visible.

Ultrastructure of the thoracic aorta

Group 1 (SD; without lacidipine)
The morphological appearance was normal throughout the study, both at SEM and TEM level.

Group 2 (HCD; without lacidipine)
At SEM, the intimal surface was generally well preserved, but elevations of endothelial cells were sometimes seen and crater-like lesions were evident (figure 4A). At TEM level, the basal lamina was increased in thickness: the subendothelial space was enlarged and often contained liposomal particles, foam cells and muscle cells.

Group 3 (HCD; 0.3 mg/kg lacidipine) and group 4 (HCD; 1.0 mg/kg lacidipine)
In these animals the lesions appeared intermediate in structure between those of group 2 and group 5.

Group 5 (HCD; 3.0 mg/kg lacidipine)
Using SEM, the intimal surface appeared generally regular with only isolated elevations of the endothelium (figure 4B). At TEM, in comparison with group 2, the cellularity of the subendothelial space was reduced, as was the density of the liposomal particles; muscle cells were rarely evident.

DISCUSSION AND CONCLUSION

Previous studies in a variety of species have demonstrated that atherogenesis can be reduced by the administration of calcium antagonists [4-11]. The present investigation characterizes the anti-atherogenic properties of the long-lasting calcium antagonist, lacidipine, when administered using a once-daily treatment regimen. In the hyperlipidemic hamster model we have identified vascular lesions which compare with those reported by previous investigators [7-10]. Using light microscopy we have demonstrated that lacidipine treatment significantly reduces the area of fatty streaks, and there is also a reduced presence of foam cell deposits.

Similarly, ultrastructural examination confirmed that treatment with lacidipine greatly reduced the severity of the intimal lesion. TEM examination, in particular, showed than an effect of drug treatment was to markedly lower the number of liposomal particles in the region of the basal lamina and the subendothelial space. The accumulation of liposomes appeared to be a relatively early event, and one which occurred in the absence of foam cells.
Another important finding was that lacidipine treatment was able to reduce the accumulation of liposomes in the hyperlipidemic hamster without lowering serum lipids. A reduction in atheromatous lesions, without the reduction of serum lipids, has been reported in other studies on the suppression of atherogenesis by calcium antagonists [4-8]. However, it is considered possible that lacidipine influences the atherogenic process by an unusual mechanism, and it has been suggested by Micheli et al. [9] that this may be related to a combination of the long-lasting calcium antagonism of the compound, in conjunction with the antioxidant properties of the drug, both of these activities being further enhanced by the high lipophilicity of the drug [6].

An alternative hypothesis on a possible mechanism of drug action emerges from previously reported ultrastructural observations [12], and this is supported by evidence from the present model of atherosclerosis and the examination of the early endothelial cell lesion. The protective action of lacidipine was seen to be manifested on the endothelial cell phenotype, both at the TEM and SEM level. In particular, lacidipine decreased endothelial cell irregularities and the presence of crater-like lesions, and reduced the hypertrophy of cytoplasmic organelles. Therefore in this hamster model of atherogenesis, our results demonstrate that the action of lacidipine, at dose levels that do not lower serum lipids, is to significantly reduce, or prevent, endothelial cell injury.

REFERENCES

Anti-atherosclerotic activity of lacidipine in hamsters


The calcium-channel blocker lacidipine reduces the development of atherosclerotic lesions in the apoE-deficient mouse
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\textbf{Background} Lacidipine is a widely used calcium-channel blocker, which has both long-lasting antihypertensive activity and also antioxidant properties. Previous studies have demonstrated the ability of lacidipine to reduce the development of atherosclerotic lesions in several animal models.

\textbf{Objective} The present study investigated the anti-atherosclerotic potential of lacidipine in the apoE-deficient mouse, an experimental model of atherosclerosis showing progressively complex and widespread lesions which closely resemble the inflammatory-fibrous plaques seen in humans.

\textbf{Methods} Lacidipine was administered daily by gavage for 10 weeks at dose levels of 0 (control), 0.3, 1.0 and 3.0 mg/kg.

\textbf{Results} Lacidipine administration reduces the extension of atherosclerotic lesions in the aorta of the apoE-deficient mouse without affecting plasma lipid levels. We also show that apoE-deficient mice have four-fold higher values of the proatherogenic peptide, endothelin, compared with the wild-type C57BL/6 mouse and that lacidipine administration reduced, in a dose-dependent manner, the concentrations of plasma endothelin.

\textbf{Conclusion} Lacidipine has anti-atherogenic effects in the apoE-deficient mouse, and reduces plasma endothelin concentrations. \textit{J Hypertens} 18:1429–1436 © 2000 Lippincott Williams & Wilkins.

Keywords: calcium channel blocker, lacidipine, atherosclerosis, plaque, apoE-deficient mouse

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well as lesions of atherosclerosis closely resembling, in appearance and distribution, those observed in man [20–24]. The role of the proatherogenic peptide, endotelin, was also examined, particularly with regard to its usefulness as a plasma marker of atherosclerosis progression [25]. Since lactidine has previously been demonstrated to affect endothelin-1 gene expression in stroke-prone hypertensive rats [26], we have also investigated the effect of lactidine administration on plasma endothelin levels.

**Methods**

**Animals and animal husbandry**

Homzygous, female, apoE-deficient mice were obtained from Glaxo-Wellcome Research and Development, Ware, UK. This colony was established from animals purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) which originated from apoE-deficient mice first engineered at the University of North Carolina in the laboratory of Dr Nobuyo Maeda (Chapel Hill, North Carolina, Bar Harbor, Maine, USA) [19,20]. The mice are on a C57BL/6 genetic background and females of this inbred strain (obtained from Glaxo-Wellcome Research and Development) were used as a control group in the present studies. Mice were approximately 13 weeks old at the beginning of the experiments. ApoE-deficient mice were randomly allocated to four groups of six animals each (one vehicle-treated control and three drug-treated groups), with a further group of six vehicle-treated control C57BL/6 mice.

Animals were housed three per cage on wood shavings, and fed a standard rodent diet containing 4.4% fat (wt/wt) and 0.2% cholestrol (wt/wt) (Altromin R; Altromin Rieper S.p.A., Vandoies, Bolzano, Italy). Diet and drinking water were available ad libitum. Animals were observed daily, and body weights and food intake were determined weekly. The research complied with national legislation and the work was performed under a Project Licence obtained according to Italian law (art.7, Legislative Decree n. 116, 27 January, 1992) with acknowledgement of the European Directive 86/609/EEC, with the Glaxo-Wellcome policy on the Care and Use of Animals, and with related codes of practice.

**Administration of lactidine**

Lactidine (Lacipil, Glaxo-Wellcome, Ware, UK) was administered daily by gavage for 10 weeks at dose levels of 0 (control), 0.3, 1.0 and 3.0 mg/kg body weight in 0.5% methyl cellulose (Sigma Chemical Co., Milan, Italy), at a standard dose volume of 10ml/kg body weight; C57BL/6 control mice received methyl cellulose vehicle.

**Biochemical measurements**

After the 10-week lactidine treatment, mice were fasted overnight and killed under pentobarbital anaesthesia (60 mg/kg, intraperitoneally) by withdrawal of blood from the vena cava. Blood was anticoagulated with tripotassium EDTA, centrifuged at 13000 × g for 3 min at 4°C. Total plasma cholesterol and triglyceride levels were determined on unfrozen plasma samples with an automated enzymatic technique (Boehringer Mannheim GmbH). Separation of total lipoprotein was performed on a pooled sample from each experimental group by sequential density ultracentrifugation with a TLA-100.2 rotor (Optima TL Ultracentrifuge, Beckman Instruments, Palo Alto, California, USA) in NaBr solution at a final density of 1.019, 1.063 and 1.210 for very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL), respectively, with a total centrifugation time of 5 h at 16°C. Plasma endothelin was measured by enzyme-linked immunosorbent assay (Cayman Chemical Co., Michigan USA) on plasma stored at −70°C, using a prolonged incubation time, as recommended.

**Tissue preparation**

At termination, after the removal of blood, animals were perfused with 10% buffered formalin for 10 min. The heart and aorta (ascending aorta, aortic arch with main arterial branches, thoracic descending aorta until the diaphragm) with surrounding adventitial tissues were excised under a stereo microscope. The aorta was separated from the heart at the base of the aortic arch, and stained with Oil Red O (ORO) by flotation. The aorta was opened longitudinally with microscissors along the ventral side, and along the inner curvature of the aortic arch, and pinned out on a wax block with fine pins. The extent of the atherosclerotic lesions in this (en face) preparation was quantified.

The heart was sectioned according to Tangirala et al. [21], namely it was sectioned transversely just below the level of the atria, postfixed in 10% buffered formalin overnight and embedded in paraffin. Sequential 7 μm sections were cut from the plane of sectioning, corresponding to the apex, towards the base of the heart until the aortic valve leaflets appeared. From this point, 17 sections of the aortic origin, representing every second serial section, over a distance of about 240 μm, were taken and stained with haematoxylin and eosin (H&E). The area of the atherosclerotic lesions in the aortic sinus was measured in the 17 sections using a computerized image analysis system.

**Quantification of atherosclerotic lesions**

The extent of the atherosclerotic lesions in the aortic en face preparations was quantified by morphometry on colour photographs taken with a camera (Olympus; OM 101) mounted on a stereo microscope (Olympus; SZ-PT). A Kodak Gold 100 film was used. For each aorta, two sets of photographs were taken. Firstly the entire aortic tree (ascending aorta, arch with branches, and
composed by an image analysis software and board verified by a second investigator who was ‘blind’ to the photographs, to obtain the lesion area extent, or the total extent of the manual tracing of the perimeter of formations using a computerized system composed by an image analysis software and board (Image Measure 3100, Microscience Phoenix Technology Inc, Washington, USA) installed on a HP computer (Vectra 486/33) and a digitizing board. Measurements consisted of the manual tracing of the perimeter of ORO positive areas and of the whole sample on the photographs, to obtain the lesion area extent, or the total extent of the image being observed.

The extent of the atherosclerotic lesion in aortic sinus sections was quantified according to Tangirala et al. [21]. The areas of the lesions were measured in 17 H&E sections using a computerized image analysis system composed by the image analysis software and board described above, and a black and white video camera (Jai; 711.00-CV) mounted on a Leitz Diaplan microscope. For each section, three images were captured, each comprising the area under one value leaflet (×4 original magnification). The outlines of the lesions were traced manually and the lesion size determined by the program. Lesion areas from the three images were summed to obtain the total lesion area for each section. Results are expressed as the average (μm²) of the data from the 17 sections analysed. All determinations were carried out by the same operator, in a ‘blind’ fashion.

Statistical analysis
Results are expressed as the mean ± SEM. One-sided Dunnet’s test was used to determine the differences between means. Analysis of covariance (ANCOVA) was used to test for differences between treatment groups in the lesion areas in en face aortic sections. Mixed model analysis of variance (ANOVA) was used to test for statistically significant differences between lacidipine groups and the apoE-deficient vehicle control group, with regard to lesion size measured on the aortic sinus preparations. Statistical tests were performed at the 5% level of significance. Regression analysis was performed on atherosclerotic lesions in the aortic sinus versus plasma endothelin values; the correlation coefficient (Pearson’s r) was calculated with P < 0.05.

Results
Biochemical measurements
During the 10-week period of treatment with lacidipine, the drug was well tolerated at all doses used (0.3, 1.0 and 3.0 mg/kg) and there were no clinical signs attributable to the compound. Body weight increase in apoE-deficient vehicle control mice, and in C57BL/6 control mice, was the same as in all lacidipine-treated groups. The apoE-deficient control group showed an increase of about 7.7 times the plasma cholesterol level of wild-type control, C57BL/6 animals (P < 0.01; Table 1). Mean levels of total cholesterol ranged from 11.75 to 13.49 mmol/l in the four apoE-deficient groups of mice, in comparison with 1.65 mmol/l in C57BL/6 control mice (Table 1). Mean plasma total cholesterol levels measured in apoE-deficient mice treated with lacidipine (0.3, 1.0, 3.0 mg/kg per day) were not sig-

| Table 1 | Plasma lipid profiles of apoE-deficient mice treated with lacidipine at 0.3, 1.0 and 3.0 mg/kg, and wild-type C57BL/6 control mice
<table>
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<tbody>
<tr>
<td>Mouse strain (Lacidipine dose)</td>
<td>Total cholesterol (mmol/l)</td>
<td>Triglyceride (mmol/l)</td>
<td>VLDL b cholesterol (mmol/l)</td>
</tr>
<tr>
<td>ApoE-deficient (control)</td>
<td>11.75 (0.26)</td>
<td>0.87 (0.03)</td>
<td>10.02 (1.52)</td>
</tr>
<tr>
<td>ApoE-deficient (0.3 mg/kg)</td>
<td>13.27 (0.31)</td>
<td>1.23 (0.07)</td>
<td>11.30 (1.72)</td>
</tr>
<tr>
<td>ApoE-deficient (1.0 mg/kg)</td>
<td>13.49 (0.22)</td>
<td>1.18 (0.04)</td>
<td>12.01 (1.43)</td>
</tr>
<tr>
<td>ApoE-deficient (3.0 mg/kg)</td>
<td>12.24 (0.33)</td>
<td>1.19 (0.03)</td>
<td>10.40 (1.59)</td>
</tr>
<tr>
<td>C57BL/6 (control)</td>
<td>1.65** (0.02)</td>
<td>0.90 (0.03)</td>
<td>0.07** (0.17**</td>
</tr>
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*Values are expressed as means (± SEM); n = 6 mice in each group.

Statistical analysis: data from the apoE-deficient vehicle-treated control mice was compared with mice in the three lacidipine-treated groups, and also with the C57BL/6 control mice; ** P < 0.01. VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. The level of total cholesterol in the three main lipoprotein classes (VLDL, LDL, and HDL) was quantified from pooled plasma from the mice in each group.
significantly different to the apoE-deficient control animals. Mean plasma triglycerides levels were comparable in C57BL/6 control animals and the four groups of apoE-deficient mice (control and lacidipine treated) (Table 1). Comparison of lipoprotein cholesterol profiles showed that in apoE-deficient mice the major cholesterol-carrying plasma lipoproteins were the VLDL and LDL fractions (98% of total); however, in the C57BL/6 animals, the main cholesterol-carrying plasma lipoprotein was in the HDL fraction (83.0% of total). The administration of lacidipine did not alter the lipoprotein profile in apoE-deficient mice, nor the cholesterol distribution in the different lipoprotein fractions (Table 1).

Plasma endothelin concentrations were evaluated. A value of 0.48 ± 0.03 pg/ml was detected in the C57BL/6 control mice; however, in the apoE-deficient vehicle control mice, values about four-fold higher were observed (2.067 ± 0.121 pg/ml; \( P < 0.01 \); Fig. 1). Administration of lacidipine in apoE-deficient mice induced a significant dose-dependent decrease in plasma endothelin levels; the values detected were 1.500 ± 0.050 pg/ml (NS), 1.149 ± 0.021 pg/ml \( (P < 0.01) \) and 1.147 ± 0.030 pg/ml \( (P < 0.01) \) in the lacidipine-treated groups (0.3, 1.0 and 3.0 mg/kg), respectively (Fig. 1).

**Quantitation of atherosclerotic lesions**

All apoE-deficient mice developed spontaneous atheromatous lesions in the aortic tract. No lesions were detected in the wild-type C57BL/6 mice. Under the stereo microscope, atheromatous lesions appeared as raised, ORO-positive deposits. Sites of predilection for lesion development were, in order of severity: the inner curvature of the aortic arch, the right common carotid artery, the left common carotid artery, and the left subclavian artery. In the thoracic tract, focal spotty lesions were seen at the branching of the arteries. The distribution of lesions was not significantly affected by the treatment with lacidipine.

Morphometry of en face aortic preparations (Fig. 2) in control apoE-deficient mice showed that the extent of lesions in the entire aorta (arch and thoracic segments) was equal to 2.18 ± 0.33% of the mean total surface area examined (corresponding to a mean area of \( 779 \times 10^7 \mu m^2 \) on a mean total surface area of \( 35.789 \times 10^7 \mu m^2 \)). In lacidipine-treated apoE-deficient mice, a reduction was observed in the total extent of lesions over the entire aortic surface examined, from 2.18 ± 0.33% (vehicle control) to 1.40 ± 0.33% (0.3 mg/kg), 1.24 ± 0.30% (1.0 mg/kg), 1.23 ± 0.33% (3.0 mg/kg), although these overall decreases did not reach statistical significance. However, in the thoracic segment, the extent of the atherosclerotic lesions was significantly reduced by all three dosages of lacidipine \( (P < 0.05) \); in the aortic arch there were also reductions at all three dose levels of lacidipine, and this reached statistical significance \( (P < 0.05) \) at the 3.0 mg/kg dose level of the drug (Fig. 3). The extent of atherosclerosis was also quantified in the aortic sinus (Fig. 4 and 5). In the apoE-deficient control group the average lesion area was 144 × \( 10^3 \mu m^2 \). Daily oral administration of lacidipine at 0.3, 1.0, 3.0 mg/kg, significantly reduced the mean lesion area in a dose-related manner by 10, 17 and 53%, respectively \( (P < 0.05, \text{ at } 3.0 \text{ mg/kg lacidipine).} \)

The relationship between plasma endothelin levels and the extent of the atheromatous lesions in control C57BL/6 and apoE-deficient mice was also examined. A statistically significant correlation was observed \( (P < 0.05; r = 0.81) \) in plasma endothelin values and the extent of the atherosclerotic lesions in the aortic sinus (Fig. 6).

**Discussion**

Transgenic apoE-deficient mice are a genetically defined murine model in which the pathogenesis of atherosclerosis can be investigated; in particular, the strain is considered to be of value because the mice
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Gross appearance of the atherosclerotic lesions in the aorta of an apoE-deficient mouse treated with vehicle (a), or lacidipine at 3.0 mg/kg daily (b). Lesions appear as raised red areas; large lesions are evident in the aortic arch and smaller deposits are present at the sites of arterial branching in the thoracic region. The edges of the lesions were traced manually and the lesion areas calculated by an image analyser system. Oil Red O stain; original magnification × 0.87.

Atherosclerotic lesion area measured in the thoracic (a), and arch (b), segments of the aorta in apoE-deficient mice after treatment with vehicle (control) or lacidipine at 0.3, 1.0 and 3.0 mg/kg daily. Graphs show the mean percentage of the aortic surface area covered by atherosclerotic lesions. n = 6 in each group; values are expressed as mean ± SEM. *P < 0.05, in lacidipine-treated mice compared with vehicle-treated controls.

Transverse sections of the aortic root at the level of the aortic sinus. In an apoE-deficient control mouse (a) extensive, advanced lesions with a well-defined fibrous cap and a necrotic core with nodular deposits of calcification are present. In the tunica media, no cells were stained with the anti-α-actin antibody, suggesting an abnormal differentiation of smooth muscle cells, this compares with descriptions of human atheromatous lesions. The volume of the lesions in an apoE-deficient mouse treated with lacidipine at 3.0 mg/kg (b) appears markedly reduced, and the lesion in less complex. H&E; original magnification × 4. Arrows indicate lesions.

Atherosclerotic lesion area measured in the aortic origin in apoE-deficient mice after treatment with vehicle (control) or lacidipine at 0.3, 1.0 and 3.0 mg/kg daily. Graphs show the average lesion area (× 10^4 μm^2) measured for each mouse in 17 cross-sections through the aortic origin, over a distance of 238 μm. n = 6 in each group; values are expressed as mean ± SEM. *P < 0.05, in lacidipine-treated mice at 3.0 mg/kg compared with vehicle-treated controls.
Correlation between the extent of atherosclerotic lesion areas in the aortic origin and plasma endothelin values in individual apoE-deficient mice, and in wild-type C57BL/6 control mice. Individual values show C57BL/6 control mice (C), apo-E-deficient control mice (□), and apoE-deficient mice treated with lacidipine 0.3 (○), 1.0 (+) and 3.0 mg/kg (△). Dashed lines indicate the 95% confidence interval of the regression line. The coefficient of correlation was \( r = 0.81; P < 0.05. \)

The present work demonstrates that the calcium antagonist lacidipine significantly reduced the development of atherosclerotic lesions in the apoE-deficient mouse. These results therefore provide further support to previous evidence of lacidipine-mediated anti-atherosclerotic activity, which has been reported in other animal models [6,14,18] and in in vitro studies [15,16]. Lacidipine is known to interfere with major processes of atherogenesis occurring in the arterial wall [15]. In particular, the drug inhibits smooth muscle cell migration and proliferation, and through the inhibition of acyl-Co A transferase (ACAT) activity, prevents cholesterol accumulation in macrophages in vitro as well as in the aorta of hypercholesterolemic rabbits [16]. Many components of plaque formation rely on oxidative stress for their expression and lacidipine is one of the most potent antioxidants, having activity similar to vitamin E [12].

The anti-atherosclerotic activity of lacidipine in the hypercholesterolemic apoE-deficient mouse was not associated with any reduction in the high levels of plasma cholesterol; this suggests a different mechanism of action to that described for other lipid-lowering drugs [35,36]. The reduction of atheromatous lesions, without accompanying changes in plasma lipids, has been reported in other studies on the suppression of atherogenesis by other calcium antagonists [6]. However, it is considered possible that lacidipine influences the atherogenic process by a specific mechanism, which is the result of a combined potent and long-lasting calcium antagonism in conjunction with the powerful antioxidant properties of the drug. These activities are further enhanced by the high lipophilicity of the drug [11,37]. In addition, it is unlikely that the anti-atherosclerotic properties of lacidipine could be related to the well-known antihypertensive effect of the drug, since 3.0 mg/kg per day has been shown not to affect blood pressure values in the apoE-deficient mouse [38]. How-
ever, on the basis of the present data, we cannot exclude that vasodilatation may have occurred, with a compensatory increase in cardiac output, and therefore that the effects seen may also be accounted for by vascular dilatation.

In the present work, lacidipine treatment significantly reduced both plasma endothelin levels and atherosclerosis in apoE-deficient mice. Other authors have demonstrated the effect of lacidipine on endothelin expression in the salt-loaded stroke-prone hypertensive rat, where a cardioprotective effect was observed which was not correlated with a detectable reduction in blood pressure [26]. Expression of the endothelin gene in endothelial cells is subject to a complex pattern of regulation pathways by numerous factors [39]. In particular, it is linked to the activation of a transcription factor called activated protein-1 (AP-1) [40]. Transcription factors are proteins typically located in the cytosol.

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Reduced progression of atherosclerosis in apolipoprotein E-deficient mice treated with lacidipine is associated with a decreased susceptibility of low-density lipoprotein to oxidation

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Summary

A study has been carried out in the apolipoprotein (apo) E-deficient mouse to investigate the activity of lacidipine (a calcium antagonist with antioxidant properties) in inhibiting the development of atherosclerotic lesions; of particular interest were changes in the susceptibility of low-density lipoproteins (LDL) to oxidation. Mice receiving a Western-type diet to accelerate the development of atherosclerosis were treated orally with vehicle or lacidipine at 3 or 10 mg/kg/day for 8 weeks. Lacidipine treatment (at 3 or 10 mg/kg) had no effect on the plasma lipid profile. However, a significant (P < 0.01) dose-related reduction of 43 and 50% of the aortic lesion area in respect to vehicle-treated mice was observed. Moreover, the resistance of mouse plasma LDL to undergo lipid peroxidation was significantly (P < 0.01) increased in apo E-deficient mice treated with lacidipine. The native LDL-like particle, derived from apo E-deficient mice treated with lacidipine, contained significantly lower concentrations of malonyldialdehyde than the vehicle-treated control group (P < 0.01). After exposure to human umbilical vein endothelial cells, LDL-like particle vitamin E levels (expressed as area under the curve; AUC), were significantly higher (P < 0.01) in both the 3 and 10 mg/kg lacidipine-treated groups, in comparison with the vehicle-treated control animals. We conclude that lacidipine reduced the extent of the atherosclerotic area in hypercholesterolaemic apo E-deficient mice, and that this reduction may be associated with the capacity of the drug to decrease the susceptibility of LDL to oxidation.

Keywords

antioxidant, apo E-deficient mice, atherosclerosis, dihydropiridine, high-fat diet, lacidipine, oxidized LDL

Several lines of evidence from both in vitro and in vivo studies suggest that the oxidation of low-density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis, although the reasons for this are not fully understood (Avogaro et al. 1988; Boyd et al. 1989; Steinberg et al. 1989; Rosenfeld 1991; Wiklund et al. 1991). Oxidatively modified
LDL is taken up by macrophages at an increased rate, in comparison to native LDL, and thus promotes cellular cholesterol accumulation and foam cell formation, which are the hallmarks of early atherosclerotic lesions (Steinberg 1997). Moreover, the oxidation process can induce the expression of adhesion molecules and facilitate transcription factor expression, mechanisms which play an important role in the further development of atherosclerosis (Cominacini et al. 1997a). LDL oxidation, occurring inside the artery wall, can be inhibited by many defence systems such as antioxidants in plasma, if these molecules are preserved, and able to carry out such activity (Steinberg et al. 1989). Because oxidized LDL cannot be detected in the circulation, considerable research has recently involved the measurement of the susceptibility of isolated LDL particles to in vitro oxidation (Esterbauer et al. 1987; Cominacini et al. 1991a). Antioxidant supplementation has been shown to reduce the progression of atherosclerotic lesions in Watanabe hyperlipidaemic rabbits (Mao et al. 1991) and to increase the resistance of LDL to oxidation in both healthy and diabetic subjects (Cominacini et al. 1991b; Dieber-Rotheneder et al. 1991; Babyi et al. 1992).

1,4-dihydropyridine (1,4-DHP) calcium channel blockers (CCBs), although markedly varied in their chemical structures and antihypertensive effects, contain aromatic rings capable of stabilizing oxygen radicals, and a hydrogen-donating reaction may also contribute to their antioxidant activity (Napoli et al. 1999). Of the several DHP CCBs, lacidipine has been demonstrated to have antioxidant properties in models of biological membranes by illustrating an activity comparable to the reference antioxidant compound vitamin E (van Amsterdam et al. 1992). In addition, lacidipine has been shown to reduce the extent of atherosclerotic lesions in cholesterol-fed hamsters (Cristofori et al. 2000a), in apolipoprotein (apo) E-deficient mice that fed on a conventional rodent diet (Cristofori et al. 2000b) and in man (Zanchetti et al. 2002). Apo E-deficient mice are characterized by a spontaneous and very pronounced hypercholesterolaemia and by several atherosclerotic features which are typical of lesions found in other animal models of atherosclerosis (Nakashima et al. 1994; Reddick et al. 1994). Furthermore, the LDL of apo E-deficient mice has been demonstrated to be highly susceptible to oxidation (Hayek et al. 1994). The apo E-deficient mouse model could therefore prove to be invaluable in assessing the atherogenic relevance of factors involved in the oxidative modification of lipoprotein (Breslow 1993).

The aim of the present study was to further investigate the effect of lacidipine on the development of atherosclerotic lesions in the apo E-deficient mouse challenged with a high fat (Western-type) diet and to evaluate the associated susceptibility of LDL to oxidation under conditions of oxidative stress.

Materials and methods

Animals and animal husbandry

Homozygous, female apo E-deficient mice (GlaxoSmithKline Research and Development, Ware, UK), approximately 6 weeks old at the beginning of the experiments, were used. This colony was established from animals purchased from the Jackson Laboratory, which originated from apo E-deficient mice first engineered at the University of North Carolina in the laboratory of Dr Nobuyo Maeda (Chapel Hill, NC, USA) (Piedrahita et al. 1992; Zhang et al. 1992). The mice were randomly allocated to three groups of 20 animals each (one vehicle-treated control and two lacidipine-treated groups).

Animals were housed three per cage on wood shavings and fed a Western-type diet (Adjusted Calories Diet, Harlan Tekland TD88137, Madison, WI, USA, containing 42% fat from milk fat and 0.15% cholesterol). Diet and drinking water were available ad libitum. Animals were observed daily, and body weights and food intake were determined weekly. The research complied with national legislation and was performed under a Project Licence obtained according to

Table 1 Plasma lipid profile in control (vehicle-treated) apo E-deficient mice and in mice treated with lacidipine at 3 and 10 mg/kg for 8 weeks

<table>
<thead>
<tr>
<th>Dose level of lacidipine (mg/kg)</th>
<th>Total cholesterol* (mmol/l)</th>
<th>Triglyceride* (mmol/l)</th>
<th>VLDL† cholesterol (mmol/l)</th>
<th>LDL† cholesterol (mmol/l)</th>
<th>HDL† cholesterol (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36.88 ± 0.28</td>
<td>0.87 ± 0.03</td>
<td>10.00 ± 0.07</td>
<td>1.52 ± 0.02</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>35.20 ± 0.31</td>
<td>1.23 ± 0.07</td>
<td>11.30 ± 0.05</td>
<td>1.72 ± 0.01</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>10</td>
<td>35.20 ± 0.22</td>
<td>1.18 ± 0.04</td>
<td>12.00 ± 0.03</td>
<td>1.43 ± 0.03</td>
<td>0.33 ± 0.01</td>
</tr>
</tbody>
</table>

*Values are expressed as means (±SEM); n = 20 mice in each group.
†VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein. The level of total cholesterol in the three main lipoprotein classes (VLDL, LDL and HDL) was quantified from six pooled plasma samples taken from mice in each dose-level group. Each pooled sample was the result of taking plasma from three or four mice.
Italian law (art.7, Legislative Decree number 116, 27 January 1992) which acknowledges European Directive 86/609/EEC. The studies are also in accord with the GlaxoSmithKline policy on the Care and Use of Animals, and also complied with related codes of practice.

**Administration of lacidipine**

Mice were orally dosed, by gavage, once daily for 8 weeks, with lacidipine (GlaxoSmithKline) at 3 or 10 mg/kg body weight, or vehicle (0.5% methylcellulose; methocel, Sigma Chemical Co., Milan, Italy) at a standard dose volume of 10 ml/kg body weight. In apo E-deficient mice, the 3 mg/kg lacidipine dose level induced plasma drug levels comparable to that achieved with the human therapeutic dose of 4 mg/kg (Zanchetti et al. 2002). The 10 mg/kg dose was chosen in this study to maximize any effect of the treatment.

**Biochemical measurements**

After the 8-week lacidipine treatment period, mice were fasted overnight and sacrificed under pentobarbital anaesthesia (60 mg/kg, i.p.) by withdrawal of blood from the vena cava. Blood was anti-coagulated with 1 mm tripotassium ethylene-diaminetetraacetic acid (EDTA) and centrifuged at 1900 g for 10 min at 4°C. Total plasma cholesterol and triglyceride levels were determined on fresh plasma samples using an automated enzymatic technique (Boehringer Mannheim GmbH, Monza, Milan, Italy). Separation of total lipoprotein was performed by sequential density ultracentrifugation with a TLA-100.2 rotor (Optima TL Ultracentrifuge, Beckman Instruments, Palo Alto, CA, USA) in NaBr solution at a final density of 1.019, 1.063 and 1.210 for very low-density lipoprotein (VLDL), LDL and high-density lipoprotein (HDL), respectively, with a total centrifugation time of 5 h at 16°C (Cristofori et al. 2000b).

LDL-like particles (d = 1.006-1.063) were isolated from pooled plasma by sequential-density ultracentrifugation, as previously described (Hayek et al. 1994). In particular, for each dose-level group, six pooled samples of 2.5 ml each were used. Each pooled sample was the result of mixing the plasma from three or four mice. To minimize LDL oxidation during the isolation process, all solutions were deoxygenated by bubbling with argon during the isolation. LDL was stored in the dark under nitrogen at 4°C, under sterile conditions, and used within 3 days. Immediately before the oxidation incubations, LDL was separated from EDTA and from diffusible low molecular mass compounds by gel filtration on PD-10 Sephadex G-25 m gel (Pharmacia, Uppsala, Sweden) in 0.01 mol/l phosphate-buffered saline at pH 7.4.

LDLs derived from apo E-deficient mice were oxidized either with copper ions or with human umbilical vein endothelial cells (HUVECs). The method for LDL oxidation with Cu²⁺ and for the evaluation of LDL susceptibility to oxidation (i.e. evaluation of the length of time of the lag phase) was based on development of fluorescence during copper-catalysed LDL oxidative modification, as previously described (Cominacini et al. 1991a). The hydroperoxide content of native LDL was determined by evaluating the level of malondialdehyde (MDA), as reported by Carbonneau et al. (1991). The extent of LDL oxidation was determined as the thiobarbituric acid adduct, using the high-performance liquid chromatography (HPLC) method of Carbonneau et al. (1991). Analytical separations were performed with a Hewlett-Packard 1050 HPLC connected to a reverse phase C18, 15 x 0.46 cm column (Bio-Rad, Hercules, California, USA); the system included a guard column (Microguard System, Bio-Rad) with a Hewlett-Packard spectrophotometric UV-VIS detector at 332 nm. An MDA standard was prepared by dissolving 220.3 mg of 1,1,3,3-tetraethoxypropane (Aldrich-Chemie, Steinheim, Germany) in 100 ml of deionized water to give a 10 mmol/l stock solution. Measurement of protein was carried out using the Pierce BCA protein assay reagent (Smith et al. 1985).

HUVECs were isolated from human umbilical veins as described by Cominacini et al. (1996) and used at passage 2-4. LDL oxidation was carried out by adding 1.5 ml of serum-free F-12 medium (Gibco, Life Technology, Rockville, MD, USA) containing 200 µg/ml protein to each well of HUVECs and incubating for 24 h at 37°C as previously described (Cominacini et al. 1996). Vitamin E was measured by HPLC with fluorescence detection as previously described (Cominacini et al. 1991a).

**Tissue preparation and quantification of atherosclerotic lesions**

At the end of the 8-week experimental period, blood was collected and the animals were perfused with 10% buffered formalin for 10 min. The hearts were rapidly dissected and sectioned according to the method of Tangirala et al. (1995a). Using this technique, the heart was sectioned transversely just below the level of the atria, postfixed in 10% buffered formalin overnight and embedded in paraffin. Sequential 7-µm sections were cut from the apex towards the base of the heart until the aortic valve leaflets appeared. From this point, 17 sections of the aortic root for each animal, representing every second serial section, over a distance of about 240 µm, were taken and stained with haematoxylin and eosin (H&E). The extent of the atherosclerotic lesions were measured using a computerized image analysis system.
made up of the image analysis software and board, as described previously (Cristofori et al. 2000b), and a black and white video camera (Jai; 711.00-CV) mounted on a Leitz Diaplan microscope (original magnification, x40). Results are expressed as the mean (±SEM) of the data from the 17 sections analysed for each animal. All assessments were carried out by the same operator in a 'blind' fashion.

Statistical analysis

Results are expressed as the mean (±SEM) ANOVA one-way analysis following Dunnett's test was used to compare the differences between the lacidipine groups and the vehicle-treated (control) group, and P < 0.05 was considered significant.

Results

During the 8-week period of lacidipine treatment at 3 and 10 mg/kg, the drug was well tolerated at both dose levels; there were no clinical signs in the animals attributable to the compound. Body weight increase in apo E-deficient vehicle control mice was the same as in the lacidipine-treated groups. The administration of lacidipine did not affect either the plasma total cholesterol triglyceride levels, or the cholesterol distribution in the different lipoprotein fractions (Table 1). Mean levels of total cholesterol were 36.88 ± 0.28 mmol/l in the apo E-deficient control group, and 35.20 ± 0.31 mmol/l and 35.20 ± 0.22 mmol/l in apo E-deficient mice treated with lacidipine at 3 and 10.0 mg/kg/day, respectively.

Quantification of atherosclerotic lesions

In control apo E-deficient mice, severe atherosclerosis developed during the 8-week period of the study and covered almost the entire aortic surface at the level of the aortic sinus (Figure 1). When the extent of the atherosclerosis was quantified at the aortic root of the heart, a significant dose-related reduction in aortic lesion development was found in mice that were given lacidipine at 3 and 10 mg/kg (P < 0.01 at both dose levels). The average lesion area was 305.5 ± 8.8 x 10^4 μm² in the apo E-deficient control group (Figure 2), 173.7 ± 6.7 x 10^4 μm² in animals treated at 3 mg/kg lacidipine and 152.6 ± 7.6 x 10^4 μm² in the 10 mg/kg lacidipine group (reductions of 43.1% and 50.1% of the control lesion area, respectively). All the results were expressed as mean ± SEM. Histological assessment of the aortic arch demonstrated advanced atherosclerotic disease with plaques showing foamy macrophages, calcification and fibrous caps, a morphology which closely resembles human atherosclerosis. No significant differences in the histological appearance were noted.

Figure 1

Atherosclerotic lesions in sections of the aortic root of the heart in a control apo E-deficient mouse (a), and in an apo E-deficient mouse receiving 3 mg/kg lacidipine (b) (haematoxylin and eosin-stained; original magnification, x40). In the control mouse (a) extensive, advanced lesions with a well-defined fibrous cap and a necrotic core with nodular deposits of calcification are present. The volume of the lesions in apo E-deficient mouse treated with lacidipine at 3 mg/kg (b) appears markedly reduced, and the lesion is less complex.

Susceptibility of mouse LDL to undergo lipid peroxidation

After 8-week treatment with lacidipine at 3 and 10 mg/kg, the susceptibility of mouse LDL to undergo lipid peroxidation following incubation with CuSO₄ was significantly reduced.
Figure 2  Effect of lacidipine at 0 (control), 3 and 10 mg/kg on atherosclerotic lesions measured in the aortic root of apo E-deficient mice after 8-week treatment. Results are expressed as mean ± SEM of the lesion area (x10^3 μm^2) measured in 17 cross-sections through the aortic root of each mouse, over a distance of 240 μm n = 20 in each group; **P < 0.01, in lacidipine-treated animals compared with the controls.

Figure 3  The susceptibility of the mouse low-density lipoprotein (LDL) to undergo lipid peroxidation following incubation with CuSO₄. LDL-like particles were derived from plasma of apo E-deficient mice after 8-week treatment with vehicle (control) or lacidipine at 3 or 10 mg/kg. Results are expressed as mean ± SEM of the lag phase (min); n = 6 pooled samples in each dose-level group; *P < 0.05 in comparison with the 3 mg/kg lacidipine group; **P < 0.01 in comparison with the control group.

Figure 4  Effect of lacidipine on the hydroperoxide content of native low-density lipoprotein (LDL). Malondialdehyde (MDA) content in LDL-like particles derived from apoE-deficient mice after 8-week treatment with vehicle (control) or lacidipine at 3 or 10 mg/kg. The results are expressed as mean ± SEM; n = 6 pooled samples in each dose-level group; **P < 0.01 in comparison with the control group.

(P < 0.01) in comparison with the control group. In the vehicle-treated control group, the lag time required for the initiation of CuSO₄-induced LDL oxidation was 100.3 ± 12.7 min (Figure 3). In contrast, in the two lacidipine-treated groups, the time required for LDL oxidation was increased, being 125.8 ± 15.2 min with lacidipine at 3 mg/kg and 146.8 ± 10.0 min at 10 mg/kg. This result paralleled the significantly (P < 0.01) lower concentrations of hydroperoxide content (MDA), detected in the LDL-like particles, derived from both lacidipine-treated groups, compared with the vehicle-treated controls (Figure 4). The mean MDA values, measured in vehicle and lacidipine-treated groups at 3 and 10 mg/kg, were 1.93 ± 0.31, 1.21 ± 0.30 and 0.93 ± 0.07 nmol/mg LDL, respectively. By plotting the lag-phase values detected in individual pooled samples from apo E-deficient mice treated with lacidipine or vehicle with the corresponding MDA values (Figure 5), a significant relationship was identified (R² = 0.89; P < 0.01).

**HUVEC-mediated oxidation of LDL-like particles**

Changes in MDA production and vitamin E content of LDL-like particles derived from apo E-deficient mice were also monitored and quantified during a 24-h incubation period with HUVECs. The amount of MDA formation in the cell-free medium (CFM) over the 24-h period was less than 1 nmol/ml. However, with HUVECs incubated with LDL-like particles from apo E-deficient mice treated with vehicle, a significant increase in MDA production was detected (P < 0.01). The formation of MDA was significantly (P < 0.01) reduced by lacidipine treatment, because lower quantities of MDA were detected from the fourth to the 24th hour of the incubation period. Expressing
Lacidipine in apoE knock-out mice

Figure 7 Vitamin E content, expressed as the area under the curve (AUC, in nmol/ml/h; mean ± SEM) during a 24-h incubation period with human umbilical vein endothelial cells (HUVECs). Low-density lipoprotein (LDL)-like particles were derived from the plasma of apo E-deficient mice after 8-week treatment with vehicle or lacidipine at 3 or 10 mg/kg. Dose-related increases in the AUC values were observed at both dose levels of lacidipine in comparison with the vehicle-treated control group; **P < 0.01; n = 6 for vehicle control; n = 11 for lacidipine at 3 mg/kg and n = 6 for lacidipine at 10 mg/kg.

Discussion

The present study demonstrates that lacidipine treatment significantly reduced the progression of aortic lesions in apo E-deficient mice that fed on a high-fat diet and that this effect was paralleled by a marked decrease in the propensity for the oxidation of plasma LDL, induced by different forms of oxidative stress. However, the anti-atherosclerotic activity of lacidipine in apo E-deficient mice was not caused by a reduction in plasma cholesterol levels. Therefore, the present results tend to reinforce the concept that mode of action of lacidipine on the atherosclerotic processes may be related to the known antioxidant properties of the compound (van Amsterdam et al. 1992). There is now growing evidence of a relationship between the susceptibility of LDL to oxidation and atherosclerotic risk (Regnstrom et al. 1992; Cominacini et al. 1993, 1994a, 1994b; Sasahara et al. 1994; Cominacini et al. 1997b; Sobal et al. 2001). Oxidatively modified LDL is taken up by macrophages at an increased rate, in comparison to native LDL, and this process can therefore promote cellular cholesterol accumulation and foam cell formation, which are the hallmarks of early atherosclerotic lesions (Steinberg 1997).

Several studies have demonstrated the important role of the inhibition of LDL oxidation in reducing the atherosclerotic process. Angiotensin-converting enzyme (ACE) inhibiting drugs such as captopril (Hayek et al. 1998) and fosinopril (Hayek et al. 1999) have been reported to inhibit LDL oxidation and the atherosclerotic process in apo E-deficient mice. Other potent antioxidant agents, such as vitamin E, are also reported to attenuate atherosclerosis in this animal model (Maor et al. 1997). Similarly, Tangirala et al. (1995b) also demonstrated anti-atherogenic effects in apo E-deficient mice using the antioxidant N,N'-diphenyl-1,4-phenylenediamine (DPPD).

Lacidipine belongs to the class of 1,4-DHP drugs, and an analysis of structural-functional relationships on the oxidative modification of LDL suggests an important role for the 2-substitution of the phenyl ring (Napoli et al. 1999). In addition, calcium antagonists of the DHP type are highly lipophilic and therefore their antioxidant effects could also
be explained by hypothesizing a direct interaction with plasma lipoproteins (Rojstaczer & Triggle 1996).

Vitamin E is the major lipid-soluble antioxidant present in blood. It acts synergistically with other circulating and cellular antioxidants to protect cells from damage and lysis induced by oxidative stress. Most of the vitamin E in blood plasma is present in the LDL fraction, and hence vitamin E is optimally placed to prevent free-radical-mediated modification of this lipoprotein. In the present study, the amount of vitamin E in apo E-deficient mice treated with lacidipine, and in vehicle-treated control groups, was measured. Significant differences in the basal LDL-like particle concentrations of vitamin E were found between the apo E-deficient mice treated with lacidipine and the vehicle-treated control group. The levels of vitamin E were maintained in animals treated with lacidipine at both the 3 and 10 mg/kg dose levels, because the vitamin E concentrations were significantly (P < 0.01) higher in both lacidipine-treated groups in comparison with the controls. This could mean that lacidipine may have reduced the progression of aortic atherosclerosis by lengthening the LDL-lag phase and working as a radical scavenger. This concept is consistent with the classic kinetic model proposed by Niki for lipid peroxidation (Niki 1987), where the length of the lag phase is directly related to the amount of antioxidant contained in the lipoprotein. Another possibility in explaining the effect of lacidipine on PDO is to catalyse the conversion of trace amounts of preformed lipid hydroperoxides to alkoxy and peroxy radicals, which in turn start another lipid peroxidation reaction. In our study, hydroperoxides were reduced in the LDL-like particles of apo E-deficient mice treated with lacidipine, in comparison with the vehicle-treated control group, and there was a direct correlation between the hydroperoxide concentrations in LDL and the length of lag phase. Therefore, lacidipine may have lengthened the LDL-lag phase also by reducing the LDL content of the lipid hydroperoxides.

In the present study, the antioxidative properties of lacidipine were also assessed in the in vitro model of LDL oxidation obtained by incubation with HUVECs, and interesting results were obtained. It is well known that the process of LDL modification induced by such endothelial cells in vitro closely resembles the corresponding process in vivo. Although cell-mediated oxidative modification of LDL has been the subject of several studies in recent years, the mechanism by which cells initiate LDL oxidation still remains unclear. Both extracellular superoxide radicals (Cathcart et al. 1989) and lipoxygenase activity (Cathcart et al. 1991; Chumulitrat et al. 1991) have been proposed. Attention has also focused on the reaction of superoxide with nitric oxide (Bruckdorfer 1993). Nitric oxide can react with superoxide to form the peroxynitrite anion. Decomposition of the peroxynitrite anion generates a strong oxidant with reactivity similar to the hydroxyl radical, which has been shown to initiate lipid peroxidation (Bruckdorfer 1993). Whichever mechanism of oxidation may be involved, this led to the initial hypothesis that the action of modifying cells is to accelerate the formation of lipid peroxides within the LDL particle. In the present study, the reduction in the content of hydroperoxides found in LDL-like particles of apo E-deficient mice treated with lacidipine may therefore also be the result of the action of the drug on endothelial cells. This conclusion is consistent with the demonstrated inhibitory effect of lacidipine on the generation of reactive oxygen species induced by pro-oxidant stimuli in endothelial cells (Cominacini et al. 1998). The results of the present study also show that LDL vitamin E consumption during HUVEC oxidation was reduced in apo E-deficient mice treated with lacidipine, in comparison with the vehicle-treated control group. Although the mechanism underlying this effect of lacidipine on vitamin E is not clear, one hypothesis was proposed by Thomas et al. (1995) which was that lacidipine acts as co-antioxidant.

In conclusion, in the present study, it has been demonstrated that lacidipine reduces the extent of the atherosclerotic lesion in the hypercholesterolaemic apo E-deficient mouse with a decrease in the susceptibility of LDL to oxidation. These effects of lacidipine may be related to the reducing property of the molecule, as well as the capacity of the drug to interfere in the cellular production of peroxidative products.

Acknowledgements

We thank Miss Sara Munaro and Mrs Monica Bonato, Daniela Spagnolo and Valentina Zantedeschi for their excellent technical assistance.

References

Lacidipine in apoE knock-out mice


Involvement of the Nitric Oxide System in the Anti-Atherosclerotic Potential of Lacidipine in the ApoE-Deficient Mouse: A Morphological, Functional, and Electrochemical Study

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ABSTRACT

The present study investigated the anti-atherosclerotic activity of lacidipine, a calcium antagonist with antioxidant properties in apoE-deficient mice. These mice show widespread vascular lesions which closely resemble the inflammatory-fibrous plaques seen in humans in atherosclerosis. Mice were fed a Western-type diet (WTD), and treated for 8 weeks with either vehicle or lacidipine at 3 or 10 mg/kg/day. In parallel with histological studies of atherosclerotic lesions in the aorta, functional studies on vascular acetylcholine (ACh) reactivity and analysis of voltammetric levels of nitric oxide (NO) were performed. Recent work has suggested that dihydropyridines (DHPs) modulate vascular relaxation via an increase in the release of NO. Lacidipine treatment had no effect on the plasma lipid profile. However, a significant (p < 0.01) dose-related reduction of 36.4% and 43.3% of the aortic lesion area in respect to methocel-treated mice was observed. Moreover, the aortic ring from control apoE-deficient mice fed a WTD for 8 weeks showed a lower relaxation in response to ACh in comparison to wild-type C57BL/6J mice; on the contrary, lacidipine-treated apoE-deficient mice lacidipine-treated displayed a response similar to that of wildtype C57BL/6J mice. Voltammetric analyses demonstrated a significant decrease of NO release in apoE-deficient mice, while lacidipine-treated mice showed enhanced activity of the NO system. We conclude that lacidipine reduced the extent of atherosclerotic area in hypercholesterolemic apoE-deficient mice, and this reduction may be associated with the capacity of the drug to maintain endothelial NO levels at concentrations useful to protect against vascular damage.

Keywords: Lacidipine; dihydropyridine; apoE-deficient mouse; atherosclerosis; nitric oxide; vasoprotection.

INTRODUCTION

Dihydropyridine (DHP) drugs such as amlodipine, lercanidipine, and lacidipine are anti-hypertensive agents capable of vascular protection via their activity as calcium antagonists. However, the ability of lacidipine to reduce the development of atherosclerotic lesions in several animal models has also been reported (Purchgott et al., 1984; Gaviiragli and Trist, 1998). Studies have shown that lacidipine is capable of vascular protection when administered both prophylactically and therapeutically at nonsustained anti-hypertensive dose levels to salt sensitive Dahl-S rats (Cristofori et al., 1991). Furthermore, lacidipine has been shown to reduce the extent of atherosclerotic lesions in cholesterol-fed hamsters (Cristofori et al., 2000a), in apoE-deficient mice (Cristofori et al., 2000b), and in humans (Zanchetti et al., 2002). Recent reports have suggested that DHPs are able to modulate vascular relaxation via an increase in the release of nitric oxide (NO) (Salomone et al., 1996; Crespi et al., 2001a). NO is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes (Moncada et al., 1991). NO was first characterized in 1987 as endothelial derived relaxing factor (EDRF) (Palmer et al., 1987), and has now been shown to play a role in a variety of biological processes. Recent findings indicate that the vasorelaxant action of calcium antagonists is facilitated by NO (Salomone et al., 1996). Furthermore, microdialysis experiments have shown that NO metabolites (NOx; i.e., nitrates + nitrates) can be monitored in vivo in the rat treated with N-methyl-d-aspartate (NMDA) (Luo et al., 1993). By means of the electrochemical technique of voltammetry applied with nafion and ortho-phenylenediamine (oPD) coated carbon fiber micro-electrodes (mCFE; 30 µm in diameter, 3 mm in length) used as biosensors, we have demonstrated that the NMDA-stimulated release of NO can be monitored in vivo in the rat corpus striatum (Crespi et al., 2001b). Furthermore, the same technique has been employed for the measurement of substance P-stimulated release of NO in rat aortic rings (Crespi et al., 2000, 2002).

Substance P is an amidated peptide known to have a vascular diluting function and has been associated with the calcium-dependent activation of the enzyme nitric oxide synthetase.
(NOS), and thus to an increased production of NO (Nava et al., 1996; Oldham et al., 1997).

The apoE-deficient mouse, created by the homologous recombination in embryonic stem cells, develops severe hypercholesterolemia and lesions of atherosclerosis (Piedrahita et al., 1992). The mouse shows progressively complex and widespread lesions that closely resemble the inflammatory-fibrous plaques seen in humans in atherosclerosis. However, the development of these lesions is exacerbated when the mouse is fed a high cholesterol, high fat, Western-type diet (WTD) (Breslow, 1996). Furthermore, the use of the WTD assures a consistency and reproducibility in the development of atherosclerotic lesions in individual animals. Therefore, with this in mind, the present study was designed to investigate the anti-atherosclerotic potential of lacidipine in apoE-deficient mice fed the WTD, and orally treated for 8 weeks with either vehicle (methocel) or lacidipine (3 or 10 mg/kg/day) by gavage. The dose levels of lacidipine were chosen on the basis of measurements of drug level in humans and in apoE-deficient mouse plasma (Cristofori et al., 2000b). The length of treatment was derived from previous studies in which it was demonstrated the time necessary to obtain a reliable and reproducible effect in mice at the dose levels chosen (Cristofori et al., 2004). Typically, humans receiving 4 mg/day of lacidipine have plasma concentrations of the drug equivalent to mice given a dose of 3 mg/kg/day. In addition, treatment of apoE-deficient mice with 3 mg/kg lacidipine did not affect the blood pressure of the animals (Cristofori et al., 2000b). In this way, a dose level of 3 mg/kg would permit the analysis and identification of the real and specific action of lacidipine on vascular morphology and functions. Furthermore, the higher lacidipine dose (10 mg/kg) was used in order to maximize treatment effects.

We therefore set out to conduct an investigation, and at the end of the lacidipine treatment period carry out, (i), histological assessment of atherosclerotic lesions in the aorta, (ii) functional ex vivo studies to examine the vascular properties of aortic ring preparations, and (iii) an electrochemical analysis of voltammetric levels of NO in the aortic rings.

These studies would allow the substantiation of the efficacy of lacidipine in protecting and maintaining vascular properties, and provide further supporting evidence on the interaction between lacidipine and the vascular NO system.

**MATERIAL AND METHODS**

**Animals, Study Design, and Drug Administration**

Homozygous, female, apoE-deficient mice were obtained from Glaxo Smith Kline Research and Development, Ware, UK. This colony was established from animals purchased from the Jackson Laboratory, USA, which originated from apoE-deficient mice first engineered at the University of North Carolina in the laboratory of Dr. Nobuyo Maeda (Chapel Hill, North Carolina, Bar Harbor, ME, USA) (Tangirala et al., 1995; Breslow, 1996). These mice are on a C57BL/6J genetic background and females of this inbred strain (also obtained from Glaxo Smith Kline Research and Development, UK) were used as wild-type control groups in the present study. Mice were approximately 6 weeks old at the beginning of the experiments.

ApoE-deficient mice were randomly allocated to 3 groups of 6 animals each (1 methocel-treated control and 2 lacidipine-treated groups). Animals were housed 3 per cage on wood shavings, and fed a WTD (Adjusted Calories Diet, Harlan Tekland TD88137, Madison, WI, USA, containing 42% fat from milk fat and 0.15% cholesterol) for 8 weeks. Additional "satellite" groups of apoE-deficient (n = 8) and wild-type C57BL/6J (n = 8) mice were fed a standard diet (Altromin R, Altromin Rieper S.p.A., Vandoies, Bolzano, Italy, containing 4.4% fat and 0.2% cholesterol) for 8 weeks. Some of these satellite animals (n = 5, naive apoE-deficient; n = 5 wild-type C57BL/6J) were sacrificed at the beginning of the study to assess vascular reactivity of the aortic ring before starting lacidipine treatment; another 6 animals (n = 3, naive apoE-deficient; n = 3 wild-type C57BL/6J) were euthanized at the end of the study to measure endogenous NO production. All diets and drinking water were available ad libitum. Animals were observed daily, and body weights and food intake were determined weekly. All research complied with national legislation. The work was performed under a Project Licence obtained according to Italian law (art.7, Legislative Decree n. 116, 27 January 1992) that acknowledges European Directive 86/609/EEC. The work also complied with the Glaxo Smith Kline policy on the Care and Use of Animals, and with related codes of practice.

Mice were dosed orally by gavage, daily, for 8 weeks with lacidipine (Glaxo Smith Kline S.p.A.) at 3 or 10 mg/kg body weight, or vehicle (0.5% methylcellulose; methocel; Sigma Chemical Co., Milan, Italy), administered at a standard dose volume of 10 ml/kg body weight. Lacidipine was initiated with the start of feeding the WTD at 6 weeks of age, for 8 weeks.

**Biochemical Measurements**

After the 8-week lacidipine or methocel treatment period, mice were fasted overnight and euthanized with carbon dioxide asphyxiation. Blood was withdrawn from the abdominal vena cava, anticoagulated with EDTA, and plasma prepared by centrifugation at 13,000 x g for 3 minutes at 4°C. Total plasma cholesterol levels were determined on plasma samples using an automated enzymatic technique (Boehringer Mannheim GmBH), as described previously (Cristofori et al., 2000b).

**Tissue Preparation and Quantification of Atherosclerotic Lesions**

After sacrifice and blood collection, the aortic tree was perfusion-fixed at a constant, near physiological pressure (80 mm Hg), via a cannula inserted in the left ventricle, with 10% buffered formalin, pH 7.4, for 10 min. The hearts were rapidly dissected out and sectioned according to the method of Tangirala et al. (1995). Using this technique, the heart is sectioned transversely just below the level of the atria, post-fixed in 10% buffered formalin overnight and embedded in paraffin. Sequential 7 μm sections were cut from the apex towards the base of the heart until the aortic valve leaflets appeared. From this point, 17 sections of the aortic origin, representing every second serial section, over a distance of about 240 μm, were taken and stained with haematoxylin and eosin (H&E). The extent of the atherosclerotic lesions were measured using a computerized image analysis system made
up of image analysis software and board, and a black and white video camera (Jai; 711.00-CV) mounted on a Leitz Diaplan microscope (X4 original magnification). Results were expressed as the mean (in \( \mu m^2 \)) of the data from the 17 sections analyzed. All assessments were carried out by the same operator, in a “blind” fashion. To further characterize the atherosclerotic lesions, some aortic tree samples (\( n = 2 \) /dose group) were postfixed in 2% glutaraldehyde solution in 0.1 M phosphate buffer at 4°C for 16 hours for scanning electron microscopy (S.E.M.). Specimens were dehydrated through graded acetone, critical point dried in a Balzers CPD 030, sputter coated with gold (MED 010; Balzers), and examined with a Zeiss S.E.M. operated at 10 kV.

Functional Studies: Vascular Reactivity

Noradrenaline (NA 0.1 \( \mu M \)) contracted aortic rings were prepared in Krebs solution at 37°C as described by Furchgott (1980, 1984). Acetylcholine (ACh), was added as a reference—relaxing compound at concentrations from 0.1 nM up to 1 \( \mu M \) (Zanchetti et al., 2002).

Voltammetric Analysis of NO

Amperometry, involving the use of NO sensitive mCFE (3 mm in length, 1.5 mm diameter), has been used to selectively monitor NO on the surface of the endothelial layer in rat aortic rings. This technique was used according to Crespi et al. (2000, 2002). Two different current values were determined. First, a basal amperometric current level (expressed in nanoAmperes, nA) resulting from the oxidation of basal endogenous NO; second, the stimulated release of endogenous NO by the local application of substance P. For this last technique, briefly, aortic rings were incubated for 5 hours at 37°C in vehicle (DMSO, 35%), then a single addition of 5 \( \mu L \) of substance P 1 mM (final concentration 25 \( \mu M \)) was made or a single addition of 5 \( \mu L \) of the substance P vehicle (PBS) was made to the lacidipine or DMSO incubated aortic rings at the 50th second of the amperometric measurements which lasted 300 sec.

Statistical Analysis

Results are expressed as the mean ± SEM. A 1-sided Dunnett’s test was used to determine the differences between means; the correlation coefficient (Pearson’s \( r \)) was calculated with \( p \) values < 0.05.

Results

Biochemical Measurements

During the 8-week period of lacidipine treatment at 3 and 10 mg/kg, the drug was well tolerated at both dose levels; there were no clinical signs attributable to the administration of the compound. Body weight increases in apoE-deficient methocel-treated mice (controls) were the same as in the lacidipine-treated groups. The administration of lacidipine did not affect the plasma total cholesterol level. Mean levels of total cholesterol were 36.88 ± 0.28 mmol/l in the apoE-deficient methocel-treated control group, and 33.20 ± 0.31 mmol/l and 35.20 ± 0.22 mmol/l in the apoE-deficient mice treated with lacidipine at 3 and 10 mg/kg/day, respectively.

Quantitation and Histology of Atherosclerotic Lesions

Histologic assessment of the aortic arch from control mice demonstrated advanced atherosclerotic disease with plaques showing foamy macrophages, calcification, and fibrous caps, a morphology that closely resembles human atherosclerosis. Treatment with lacidipine at 3 and 10 mg/kg did not change the histological appearance of the lesions, but S.E.M. examination of the inner curvature of the aortic arch demonstrated less extensive endothelial damage in mice treated with lacidipine at both dose levels than in the methocel-treated animals (Figure 1A, 1B). In particular, in apoE-deficient mice receiving lacidipine, the surface of the aortic arch had irregular endothelial cells with an increased number of microvilli; crater-like lesions were evident on endothelial surface and lympho-monocytic cells and platelets were frequently attached. ApoE-deficient methocel-treated mice receiving the WTD developed severe atherosclerosis during the 8-week period of treatment (Figure 2). The extent of the lesion area was 31.8 ± 31.7 \( \times 10^5 \) \( \mu m^2 \). In contrast, a significant dose-related reduction in atherosclerotic lesion development was found in mice given lacidipine at 3 and 10 mg/kg (\( * p < 0.05, ** p < 0.01 \), respectively); the extent of the lesion areas were 199.7 ± 23.4 \( \times 10^3 \) \( \mu m^2 \) and 178.0 ± 27.1 \( \times 10^3 \) \( \mu m^2 \) at 3 and 10 mg/kg lacidipine, respectively; this corresponds to reductions of 36.4% and 43.3% of the control lesion area, respectively.

Functional Studies: Vascular Reactivity

The results from the functional studies are presented in Figure 3. It should be noted that there is less relaxation in lacidipine-treated mice than control C57BL/6J and apoE-deficient mice at all doses of ACh except 100 and 1,000 nM. Moreover, the aortic rings from C57BL/6J wild-type mice had a high relaxation response to increasing levels of ACh. A similar response is presented in the aortic rings of the naive apoE-deficient mice sacrificed at the beginning of the study to assess basal vascular reactivity.

The aortic rings from methocel-treated apoE-deficient mice fed the WTD for 8 weeks resulted in lower relaxation in response to ACh at 0.1, 1, and 10 nM concentrations; no relaxation was recorded in the presence of higher concentrations of ACh (100 and 1000 nM). In contrast, the aortic rings from apoE-deficient mice receiving the WTD and lacidipine 3 and 10 mg/kg, displayed a response to increasing levels of ACh similar to that of the naive apoE-deficient mice. In particular the aortic rings obtained from the apoE-deficient mice treated with lacidipine 3 mg/kg displayed a response to ACh at 1000 nM very close to that of the control wild-type C57BL/6J aortic rings.

It is also of interest to note the low variability (i.e., small SEM) of the C57BL/6J wild type mice values. A similar degree of variability was also observed in the apoE-deficient mice treated with lacidipine, and in the naive apoE-deficient group. In contrast, high variability (i.e., large SEM) was seen in the apoE-deficient methocel-treated mice, when rings were challenged with high concentrations of ACh (100 and 1,000 nM). This finding further underlines the abnormal conditions of the aorta in this group of mice and tends to support the appropriateness of the range of concentrations of ACh selected for these studies.
Vollammetric Analysis of NO

Six-week-old wild-type C57BL/6J mice and naive apoE-deficient mice ("satellite" group, fed the standard diet) were used to measure the basal production/release of endothelial NO (Figure 4A). The aortic rings of these animals gave similar amperometric basal current levels (in nanoAmperes; nA) of endothelial NO (0.58 ± 0.02 nA and 0.53 ± 0.02 nA, respectively). A similar response to substance P-stimulated release of vascular endogenous NO was observed (Figure 4B) in the wild-type C57BL/6J mice and in the naive apoE-deficient mice groups (2.68 ± 0.34 nA and 2.79 ± 0.39 nA, respectively). In contrast, in the aortic rings of the apoE-deficient methocel-treated mice fed the WTD for 8 weeks, a significant increase in the basal endogenous NO levels was seen (Figure 4A), while the substance P-stimulated endogenous NO (Figure 4B) was significantly decreased. Conversely, in the aortic rings of the apoE-deficient mice fed the WTD and treated with lacidipine (3 and 10 mg/kg), both the basal NO related signals (Figure 4A) and the substance P-stimulated endogenous NO signals (Figure 4B) were significantly increased. To assess a possible effect of the WTD on NO production, these data were compared with voltammetric results from the aortic rings of wild-type C57BL/6J and naive apoE-deficient mice fed the standard diet (for 8 weeks) and treated with methocel or lacidipine 10 mg/kg (n = 3 each treatment) and a similar trend of increase in substance P-stimulated NO levels was observed after treatment with lacidipine. In particular, when observing data gathered following lacidipine 3 mg/kg, that's the most efficacious dose in humans, it appeared that endothelial NO levels were increased either in basal conditions or following substance P treatment (Figure 4A and 4B).

DISCUSSION

The present study demonstrated that lacidipine treatment significantly reduced the progression of atherosclerotic lesions in apoE-deficient mice fed a high-fat diet and that this reduction was mainly attributable to the effect of this compound on vascular function and NO levels. In fact, no histological differences were noted in lacidipine treated and non-treated mice. However, the anti-atherosclerotic activity of lacidipine was not associated with a reduction of plasma cholesterol levels. This would suggest a different mechanism of action for lacidipine in comparison with that described for other lipid lowering drugs (Tawara et al., 1986; Reckless et al., 1997). The data obtained in the present study provides further support for the previously accumulated evidence of a lacidipine-mediated anti-atherosclerotic activity, which has been reported in several other animal models (Nava et al., 1996; Ma et al., 2000; Crespi et al., 2001a), and also in in vitro studies (Piedrahita et al., 1992; Oldham et al., 1997). In particular, the findings of the present work appear to indicate that lacidipine "enhances" the activity of the endogenous NO system, both at basal levels and in response to substance P-stimulated NO release. This capacity of the drug would result in a facilitation of vasodilatation in the presence of atherosclerosis. This observation is supported by the present functional studies. These studies demonstrate that the aortic rings in vehicle-treated apoE-deficient mice fed the WTD (which developed extensive atherosclerotic lesions), showed an impaired relaxation in response to ACh. In contrast, the aortic rings from apoE-deficient mice fed the WTD and treated with lacidipine at 3 and 10 mg/kg, maintained vascular functional activities close to normal levels.
**Figure 2.**—A. Photomicrographs showing the cross-section of the aortic sinus in apo-E deficient mouse fed the Western type diet and methocel (control). The black line indicates the lesion area beneath each leaflet quantified by the software. Extensive lesions are visible beneath all 3 valve leaflets. Advanced lesions with fibrous cap and a necrotic core are present. (H&E, x40). B. Cross-section through the aortic origin of an apo-E-deficient mouse fed the WTD and treated with lacidipine 3 mg/kg/day for 8 weeks. Atherosclerotic lesions are markedly reduced in extent; the lesioned area appears well organized and is not different from controls. (H&E, x40). C. Extent of atherosclerotic lesions measured in the aortic origin of apoE-deficient mice fed a WTD and receiving methocel (vehicle control), or lacidipine at 3 and 10 mg/Kg/day; n = 6 in each group. Results are expressed as mean ± SEM of lesion area (×10^3 μm^2) measured for each mouse in 17 cross sections through the aortic origin, over a distance of 240 μm. *p < 0.05 and **p < 0.01 in lacidipine at 3, and at 10 mg/Kg groups, respectively, compared with the methocel-treated group.

**Figure 3.**—Vascular reactivity measured in noradrenaline-(0.1 μM) contracted aortic rings following exposure to increasing concentrations of ACh. The state of contraction (%) is shown in apoE-deficient mice (n = 6) fed a WTD and receiving methocel (0.5%) or lacidipine at 3 and 10 mg/Kg/day. In addition, vascular reactivity was measured in C57BL/6J control mice and in naive apoE-deficient mice (n = 5) at the beginning of the study to assess basal vascular reactivity. Results are expressed as the mean ± SEM.

In the voltammetric studies carried out in parallel with the investigations on vascular reactivity, other supporting evidence was forthcoming. The present results clearly demonstrate that voltammetry can measure basal, as well as substance P-stimulated NO release in vascular tissue of mice. In particular, in both wild-type C57BL/6J and naive apoE-deficient control groups, the values of basal and substance

**Figure 4.**—Measurement of the basal amperometric current levels of endothelial NO (nA) in aortic rings. A. wild-type C57BL/6J and naive apoE-deficient mice (n = 3) are compared to apoE-deficient mice fed the WTD and receiving methocel (vehicle control), or lacidipine at 3 and 10 mg/Kg/day; n = 6). Results are expressed as mean ± SEM; + p < 0.05 versus control mice (wild-type C57BL/6J and naive apoE-deficient); *p < 0.05 versus methocel vehicle control. B. substance P-stimulated amperometric levels of NO (nA): wild-type C57BL/6J and naive apoE-deficient mice are compared to apoE-deficient mice fed WTD and receiving methocel (control) or lacidipine 3 and 10 mg/Kg/day. Results are expressed as mean ± SEM; + p < 0.05 versus control mice (wild-type C57BL/6J and naive apoE-deficient); *p < 0.05 versus methocel.
P-stimulated release of NO were similar, suggesting no significant differences in the activity of their respective NO systems. These results correlate well with the lack of appreciable atherosclerotic lesions in the aorta of both mice and the evidence of similar functional reactivity. In contrast, in apoE-deficient methocel-treated WTD-fed animals, it appeared that basal NO levels were increased when compared to naive (6-week-old) apoE-deficient mice. This could indicate increased activity of the NO system with the development of atherosclerosis. This increase in the activity of the NO system could be either passive, i.e., simply a consequence of the presence of the lesion, or possibly an "active" response, i.e., a "protective" vasodilatory action of NO in presence of a damaged and malfunctioning vascular tissue. Moreover, the substance P-stimulated NO signal in apoE-deficient methocel-treated mice fed the WTD was reduced when compared to control mice (naive and wild-type). Substance P is known to have vascular dilating properties (Oldham et al., 1997) via the stimulation of the NO system (Crespi et al., 2000), and thus one can suggest that the NO system is adversely affected in the presence of abnormal, pathological vascular lesions (i.e., rapidly developing atherosclerotic plaques induced by the WTD). This effect appears to be related in particular to the severity of the lesion. The presence of substance P receptors within the endothelium has been described (Tawara et al., 1986; Reckless et al., 1997) and Oldham et al. (1997) reported that substance P acts as a vasodilator via NOS activation in endothelial cells. This indicates a reduced availability of endogenous NO in the methocel-treated mice fed the WTD, following the application of substance P. Therefore, the positive levels of substance P-stimulated NO release in lacidipine-treated mice fed the WTD, would indicate that lacidipine is able to maintain and possibly even increase the efficacy of the NO system, even in the presence of very large atherosclerotic lesions. Thus, lacidipine may be able to prevent the negative effects of atherosclerosis on the activity of the NO system.

Akopov (1996) has reported that the vasodilatory effect of lacidipine was significantly attenuated by the presence of the NO-synthase inhibitor, N-nitro-l-arginine. Bearing Akopov's (1996) work in mind, the present investigations show that lacidipine enhances the activity and the levels of endothelial NO. Therefore this may indicate that the factor responsible for the arterial endothelium modulating vascular relaxation, in the presence of DHPs, is probably a DHP-inducedincrease in the release of NO, rather than an enhancement of a calcium antagonist activity due to the basal release of NO at the level of vascular smooth muscle.

In conclusion, the reduced levels of atherosclerosis in apoE-deficient mice fed the WTD and treated with lacidipine at doses that do not control hypertension (Crespi et al., 2002), would indicate that the beneficial effects of this calcium antagonist in hypertensive patients are not simply restricted to the reduction of blood pressure. On the basis of the present findings it can be suggested that an additional property of lacidipine exists that may be related to the capacity of the drug to maintain endothelial NO levels (and consequently ONOO− levels) at "useful" (i.e., nanomolar) concentrations; this therefore results in protection against vascular damage. And finally, we consider that our studies further support the use of voltammetry with treated microbiosensors, as an appropriate technique for the analysis of rapid, and short lasting biochemical events.

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REFERENCES


