THE MECHANISM OF THE SYNERGISM BETWEEN NO AND H_2O_2 ON THE INHIBITION OF PLATELETS AGGREGATION

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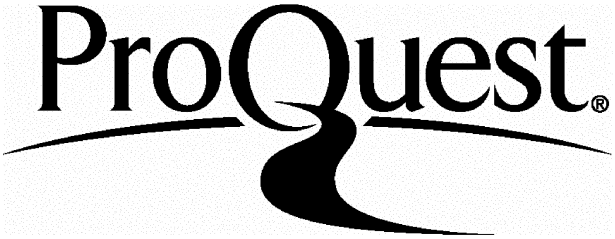
January 2004



Abstract

Nitric oxide (NO) is a potent inhibitor of platelets activation and its mechanism of action is to increase intracellular cyclic GMP level. A strong synergism has been shown to exist between low concentrations of hydrogen peroxide (H_2O_2) and nitric oxide in the inhibition of agonist-induced platelet aggregation. The aim of this thesis was to establish the mechanism of this interaction.

It was established that hydrogen peroxide in the presence of NO had no additional effect on the activity of pure soluble guanylyl cyclase or its activity in platelet lysates and cytosol. However, H_2O_2 was found to increase the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) a target for cGMP and cAMP. This occurs both in the presence and in absence of low concentrations of NO and at submicromolar concentrations of the peroxide. These actions of H_2O_2 were inhibited largely by an inhibitor of cyclic AMP-dependent protein kinase, even though H_2O_2 did not increase cyclic AMP. This inhibitor reversed the inhibition of platelets induced by combinations of NO and H_2O_2 at low concentrations. The results suggest that the action on VASP may be one site of action of H_2O_2, but that this event alone may not lead to inhibition of platelets.

Another modification of the proteins related to NO is the nitration of aromatic amino acids, particularly tyrosines through the formation of the reactive nitrogen species, peroxynitrite. I have now shown that H_2O_2 also increases the nitration of VASP and other platelet proteins and that the nitration is inhibited by the anti-oxidant epigallocatechin gallate. In addition to this founding, nitrated proteins were also found in the basal (non-stimulated) platelets immediately after separation of platelets from plasma.

Acknowledgements

First and foremost, I would like to thank my supervisor, *Professor K. Richard Bruckdorfer,* very much for all the support, advice and help I received from him and also for his patience when reading the drafts. He made fun of my English, he got angry but he made me to finish it. Without his encouragement and his support, I would never get to this point.

I would like to thank all my colleagues in the Biochemistry department, for being there for me and being active blood donors. I thank specially *Dr Vernon Skinner* for his technical support and also for his perfect platelets, which would never disappoint me.

Thanks to all my friends who never gave up, when I was giving up, especially during the writing.

Finally, special thanks to my sisters and my brother for all their support.

At last not least, thanks to my housband for his support durin the writing.

And to the memory of my parents and my lovely brother and to those who died with a sudden heart attack. I hope that my work will help us towards a better understanding of atherosclerosis and thrombosis.

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Abbreviations

AA	arachidonic acid
Ach	acetylcholine
ACD	acid-citrate-dextrose
ADP	adenosine di-phosphate
ATP	adenosine tri-phosphate
ABPs	actin-binding proteins
ALLN	Calpain inhibitor (MG101) N-Acetyl-Leu-Leu-Nle-CHO
ASA	Acetycsalicylic acid
BH_4	tetrehydrobiopterin
cAMP	cyclic adenosine mono phosphate
cGMP	cyclic guanosine mono phosphate
CP	creatine phosohate
CPK	creatine phosphokinase
CO	carbon monooxide
COX	cyclooxygenase
$CO_3^{\cdot-}$	carbonate radical
Carboxy-PTIO	2-(4-carboxypheyl)-4,4,5,5-tetramethylimidazoline-1-oxyl3-oxide
ddH_2O	double distilled water
dBcGMP	dibutyryl cyclic guanylyl monophosphate
DAG	diacylglycerol
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra acetate
EGF	endothelium growth factor
EGCG	epigallocatechin gallate
EPO	eosinophil peroxidase
EST	E64d (2S,3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane Ethyl Ester Loxistatin
FAK	Focal adhesion kinase
FGN	fibrinogen
FAD	flavin adenine dinucleotide

FMN	flavin adenine mono-nucleotide
sGC	soluble guanylyl cyclase
GTP	guanosine triphosphate
GSH-	glutathione
GSH-Px	glutathione peroxidase
GSSG	glutathione disulfide
IBMX	Isobutylmethylxanthine
L-NAME	N^G-nitro-l-arginine methyl ester
MAPK	myosin activated protein kinases
MG132	Carbobenzoxy-L-Leucyl-L-leucyl L-leucinal
MG101	Calpain I inhibitor
MLCK	myosin light chain kinase
MMP	matrix metalloproteases
MPO	myeloperoxidase
NO^+	nitrosonium ion
NO	nitric oxide
NO_2	nitrogen dioxide
$^{\cdot}NO_2$	nitrogen dioxide radical
N_2O_4	dinitrogen tetraoxide
N_2O_3	dinitrogen trioxide
NO_2^-	nitrite
$NO3^-$	nitrate
NADPH	nicotinamide adenine dinucleotide phosphate
cNOS	constitutive nitric oxide synthase
iNOS	inducible nitric oxide synthase
ODQ	oxadiazoloquinoxaline-1-one
ONOO	peroxynitrite
ONOOH	peroxynitros acid
O_2^-	superoxide
PDEs	phosphodiesterases
PEG	poly ethylen glycol
PKC	protein kinase C
PLC	phospholipase C

PLA_2	phospholipase A_2
PIP_2	phospho inositol biphosphate
IP_3	inositol triphosphate
PDGF	platelet derive growth factor
PGE_2	proctacyclin E_2
PGI_2	proctacyclin I_2
PGG2	prostaglandin G2
PGH2	Prostaglandin H2
PKA	protein kinase A
PKG	protein kinase G
PKC	protein kinase C
PRP	platelet rich plasma
PPP	platelet poor plasma
PP1	protein phoshatase1
PP2A	protein phosphatase 2A
PTK	protein tyrosine kinase
PTP	Protein tyrosine phosphatase
PVDF	polyvinylidene difluoride
PVP-10	polyvinyl pyrovidine (Mw10000)
ROS	reactive oxygen spices
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gels
SEM	sub-endothelial matrix
SNAP	S-nitroso-N-acetyl_DL-Penicillamine
SOD	superoxide dismutase
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-α	Tumour necrosis factor-α
TXA_2	tromboxane A_2
TXB_2	tromboxane B_2
TGF	transforming growth factor
vWF	von willibrand factor
VASP	vasodilator-stimulated phospho protein
XOD	xanthine oxidase

YC-1	([3-(5 hydroxymethyl-2'-furyl)-1-benzyl indazole])
UA	Uric acid

CHAPTER ONE:
GENERAL INTRODUCTION

1.1. Atherosclerosis and Thrombosis

Atherosclerosis is a multifactoral disease of the artery wall, which begins as a response to chronic injury to the endothelium and is characterised by the accumulation of lipids and various blood cells including macrophages leading to the formation of atherosclerotic plaques and narrowing of the arteries. Activated macrophages release mediators including growth factors and cytokines that evoke smooth muscle migration from the media to the developing fibrous cap in the intima, smooth muscle cell proliferation and production of collagen by smooth muscle cells (Lusis, 2000). The core of the plaque develops into a lipid-rich semi-liquid tissue, composed of products from apoptotic and necrotic foam cells. As the disease progresses the plaque enlarges and causes a reduction of blood flow and blockage of the arteries, leading to ischaemic events (Fig 1.1).

The disruption of an atherosclerotic plaque triggers a cascade of platelet-mediated events, which results in the formation of platelet-rich thrombus at the site of injury (Lusis, 2000). The initiation of athero-thrombosis involves a process of platelet adhesion, activation, and aggregation. The earliest event in the thrombotic process is the adhesion of platelets to the sub-endothelial matrix (SEM) at the site of vascular damage. The adhered platelets are activated, thereby releasing ADP and thromboxane A_2 (TXA_2). These molecules recruit additional platelets and amplify platelet activation and aggregation leading to formation of thrombosis.

Under normal physiological conditions, a well-defined distribution of NO, which is dependent on the laminar, turbulent, or pulsatile flow rate of blood, inhibits circulating blood elements from interacting with the vessel wall. Platelet adherence and aggregation as well as monocyte adherence and infiltration are opposed by NO.

Atherosclerosis and hypercholesterolemia disturb the endothelium-dependent nitric oxide mediated regulation of the vascular tone. An impairment of NO synthesis pathway may be one of the earliest events in atherogenesis. A reduction of NO synthesis and /or activity may contribute to the initiation and progression of atherosclerosis (Napoli and Ignarro, 2001).

Thus, understanding of the exact role of NO and all its pathways in cell metabolism will be useful in the future for the treatment of atherosclerosis, thrombosis and all other related disease.

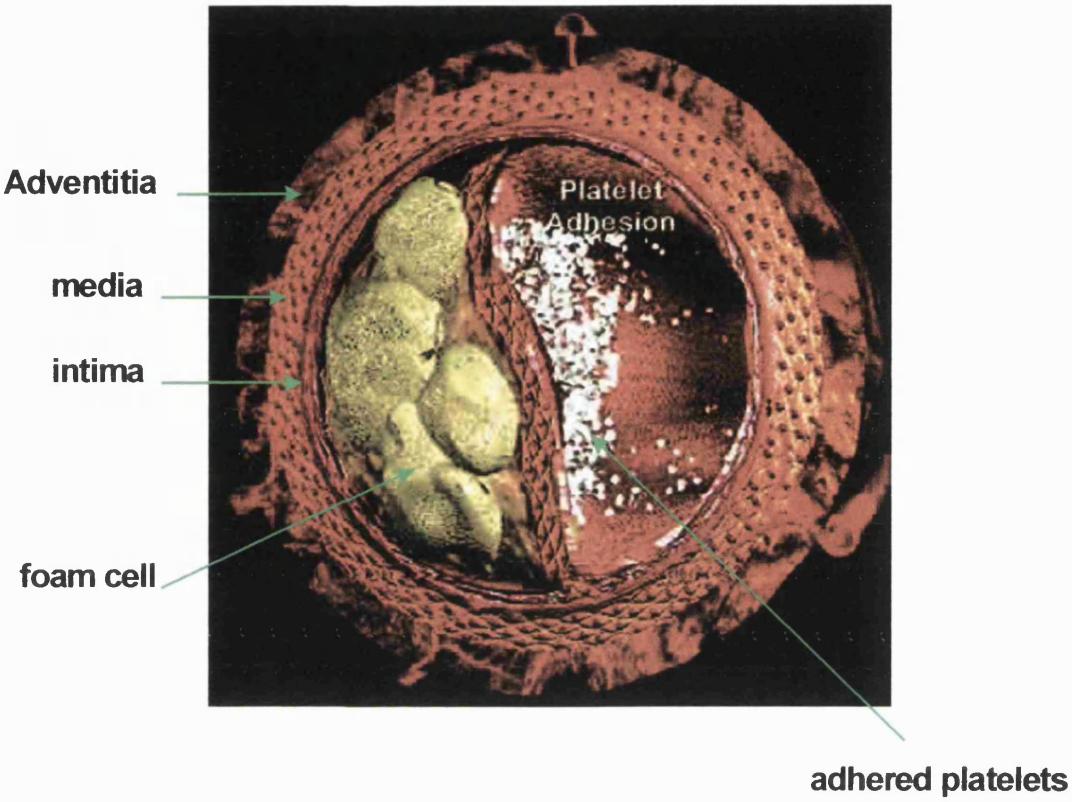


Fig 1.1: Atherosclerosis and thrombosis.

The development of atherosclerosis is primarily characterised by an accumulation of complex lipids, proteins and carbohydrates as well as a proliferation of cells, in the intima layer of an artery, which causes migration of the smooth muscle from the media to developing fibrous cap in the intima. Lumen of blood vessel becomes narrow and causes a reduction of blood flow and blockage of the arteries caused by a thrombus.

Picture adapted from www. Strokecentre.org

1.2 Platelets

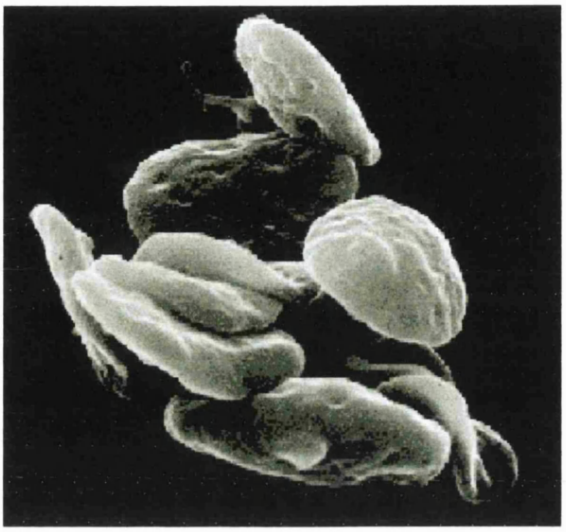
1.2.1 Description

Platelets are the smallest corpuscular components of human blood with a diameter of 2-4μm. Their physiological number varies from 150,000 to 300,000/mm^3 of blood. The origin of platelets is the bone marrow, where megakaryocytes, as the results of mitotic proliferation of a committed progenitor cell, liberate platelets as the end product of protrusions of their membrane and cytoplasm. The typical shape of resting platelets is discoid (Fig 1.2a) but upon activation they undergo a shape change to a globular form with pseudopodia up to 5μm long (Gordon, 1981) (Fig 1.2b).

1.2.2 Platelet production

The megakaryocyte, the parent cell of the platelet, is derived from pluripotential stem cells in the bone marrow. Individual megakaryocytes have been estimated to produce as many as 1000 platelets per cell, and apparently very efficient system facilitated by the absence of nuclei in platelets. There are two possible mechanisms whereby the platelet achieves the transition from being stationary constituents of megakaryocyte cytoplasm in the bone marrow to circulation cells in the bloodstream. One theory is that megakaryocytes themselves are released from the bone marrow and are carried to the pulmonary capillaries, where they fragment into individual platelet. Another is that the bone marrow endothelium has special properties that encourage formation of pseudopods extending from mature megakaryocytes to bone marrow sinuses and thereby directly release platelets into the blood. The life of platelet in the circulation is estimated about 6-12 days before the removal by the spleen (Gordon, 1981).

a)



b)

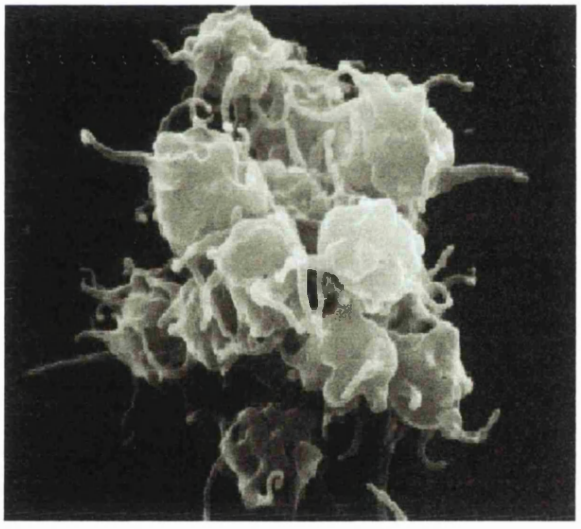


Fig 1.2: The presentation of human platelet during resting and activation period.

The typical shape of normal circulating platelet is smooth discoid (a). During activation in the present of agonist, platelets undergo a shape change from discoid to a more spiny spherical shape. At this stage, the pseudopodia are very pronounced (b).

Picture adapted from www. Platelet.com

1.2.3 Platelet structure

Platelets are composed of three principal components including:

Membrane structures, microtubules and granules and also non-granular organelles and surface glycoproteins.

1.2.3.1 Platelet membranes structure and cytoskeleton

The membrane structure of platelet consists of other structures including, the canalicular system, the dense tubular system and the cytoskeleton.

The platelet membrane, overlying glycocalyx and submembrane structures mediate responses to platelet stimulation and express specific antigenic characteristics. The surface glycoproteins serve as receptors, facilitate platelet adhesion and contraction, and determine expression of specific platelet antigens and antigens shared with other formed elements.

The platelet canalicular system is created by numerous invaginations of the platelet surface and, interspersed among these structures; a set of narrower channels termed the dense tubular system. The canalicular system provides a direct connection between the interior and the surface of the platelet, providing entrance of plasma ingredients into the platelet as well as exit of its own ingredients in connection with the release reaction.

The dense tubular system, on the other hand, is entirely enclosed and is the major site for storage of Ca^{2+} and the location of cyclooxygenase, the critical enzyme for conversion of membrane-derived arachidonic acid to unstable endoperoxide precursors of prostaglandin's and thromboxanes (Gerald et al., 1978).

The cytoskeleton provides a framework to anchor the platelet membrane and allow signal transduction to take place. In the resting platelet, the cytoskeleton maintains the discoid shape of the platelet. Furthermore, it is a framework against which the contractile proteins of the platelet can operate to initiate shape change and protrusion of pseudopodia at the onset of spreading.

The actin-rich cortex of cells lies directly under the plasma membrane (Fig 1.3). A variety of actin binding proteins (ABPs) link the cortex with the membrane both directly, and through other protein intermediates. These interactions are reciprocal, not only do membranous proteins immobilise cytoskeletal domains at adhesions, but the cytoskeleton immobilises inter-membranous proteins. Broadly, there are three types of ABP-membrane interactions.

These proteins either are integral membrane proteins or they bind to the surface of membranes by interacting with lipids or by binding to other membrane-associated proteins.

The integrin-mediated cell adhesion is thought be linked to focal adhesions through some of actin cytoskeleton via a series of interacting proteins including talin, vinculin, α-actinin, vasodilator-stimulated phosphoprotein and paxillin (Critchley, 2000).

1.2.3.2 Platelet microtubules

Platelet microtubules are the major inner structures of the platelet consisting of microtubules and contractile proteins.

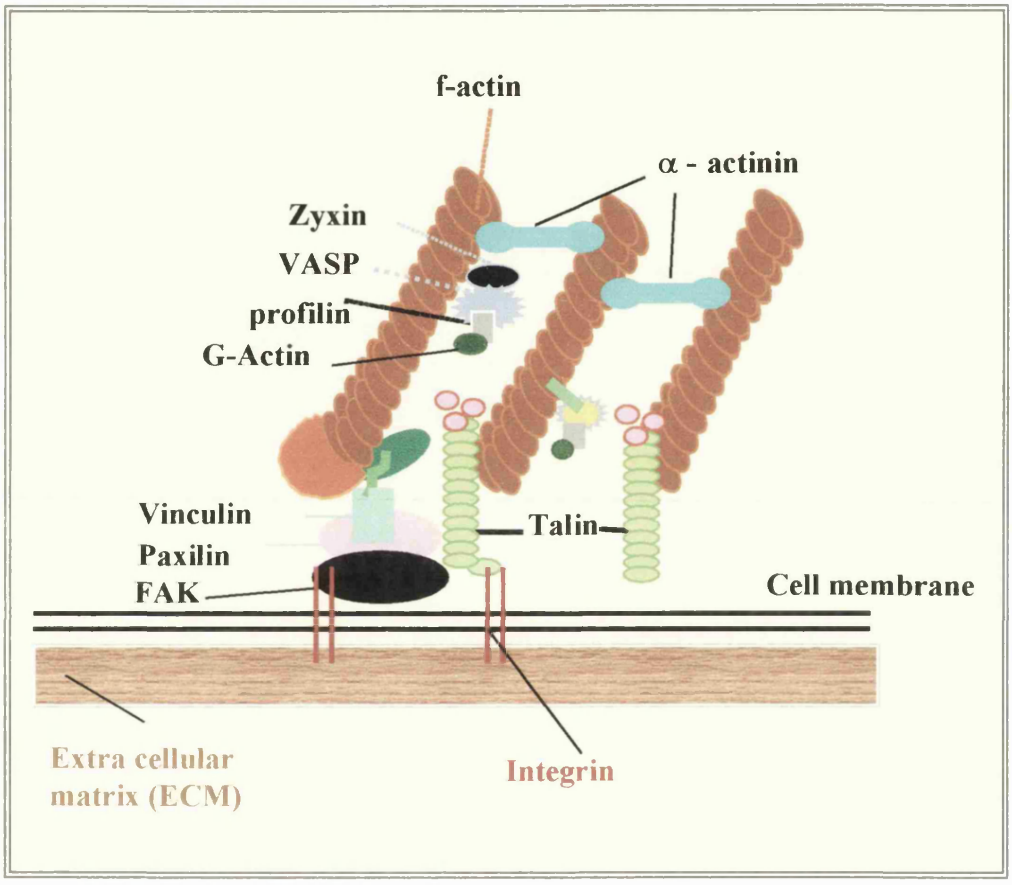


Fig 1.3: platelet cytoskeleton

Actin, actin-binding protein (ABP), vasodilator-stimulated phosphoprotein (VASP), talin, vinculin, paxillin, α-actinin and several membrane glycoproteins make up the cytoskeleton. In resting platelets, the cytoskeleton maintains the discoid shape of the platelet. (Picture adapted from Integrin-mediated cell adhesion (Critchley, 2000)

The microtubules are arranged in the form of an inner ring beneath the surface of the platelet and are distinct from the canalicular and dense tubular systems of the membrane zone. The microtubules provide structural support of the platelet, maintaining its discoid shape in the resting state, and influence the character of its contractile functions.

Contractile proteins largely consist of myosin and submembrane actin filaments that are anchored to the surface of the platelet by the transmembrane glycoprotein α-actinin. On stimulation of the platelet, the cytoplasmic concentration of Ca^{2+} activates calmodulin allowing it to combine with myosin light-chain kinase. This enzyme phosphorylates myosin, leading to the combination of myosin with actin to form contractile acto-myosin, which mediates the initial changes in shape of the platelet and ultimately, retraction of the formed clot.

1.2.3.3 Platelets granules

There are three kinds of granules in platelets:

α-granules are characterised by moderate electron density and are variable in size and content. These granules contain substances that are intrinsic to the platelet, including the following: platelet factor 4, ß-thromboglobulin, fibrinogen, vWf, PDGF, fibronectin, thrombospondin, IgG, factors V, VIII, XIII, EGF, TGFβ, and TFPI.

α-granule release appears to be an all or nothing event. In general, platelet aggregation *in vivo* is associated with release of α-granule content. P selectin is a component of the α-granule membrane. Release involves the granules nearest the platelet surface being transported to the platelet membrane and fusing with it so that a

small portion of the post-release external platelet membrane is made up of the inner membrane of the α-granule.

Dense bodies are granules characterised by high electron density and are fewer in number than α-granules. These structures serve as a depot for non-metabolic substances that are extrinsic to the platelet and may be picked up or released as indicated. On their release, these substances are particularly critical to platelet aggregation and include the following: ADP, ATP, serotonin, calcium, potassium, and catecholamines.

Lysosomal granules are also present in platelets, perhaps representing the original role of the platelet as a white blood cell. These granules contain at least seven acid hydrolases. These enzymes may contribute to the intracellular effects of phagocytosis of may create an uncertain amount of damage extracellularly at the site of platelet release. (Gordon, 1981).

1.2.3.4 Non-granular organelles

The contents and functions of the non-granular organelles of the platelet may be summarised as:

Mitochondria contain enzymes for oxidative metabolism and thereby provide a major source of energy through the generation of ATP. ATP is required, because the secretory phase of platelet activation needs energy.

Peroxisomes contain catalase, which protects the platelet from oxidative damage in connection with periodically intense metabolic activity.

Glycogen granules are the smallest and most numerous granules in platelets. Their abundance contrast with the paucity of platelet mitochondria and its distribution is

consistent with platelets metabolic energy being derived mainly by glycolysis rather than by oxidative phosphorylation. However, the burst of energy required to fuel the intracellular changes associated with platelets response to stimuli is provided by metabolic pool of ATP in the cytoplasm, which is in very slow equilibrium with ATP in the dense body. Platelets also contain occasional *ribosomal particles* and small amounts of *RNA* (Gordon., 1981).

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The various factors released from platelets and their intraplatelet sources.

A summary of different factors stored in platelets (Platelets in biology and pathology J.I.Gordon).

Type of granule and contents	**Function**
α-granules	
platelet factor 4	mitogen
platelet derived growth factor (PDGF)	mitogen
transforming growth factor (TGF)	mitogen
fibrinogen (FGN)	physical support of aggregation
von Willibrand factor (vWF)	physical support of adhesion
thrombospondin	reinforcement of FGN binding
fibronectin	involved in platelet adhesion
β-thromboglobulin	platelet specific anti- heparin protein
factor V,VII and XI	coagulation cascade proteins
protein S	coagulation pathway protein
plasminogen activator inhibitor 1	inhibition of fibrin degradation
Dense granules	
serotonin (5-HT)	vasoconstrictor and platelet agonist
ADP, ATP	platelet agonists
epinephrine	vasoconstrictor and platelet agonist
Ca^{2+}	reinforcement of platelet activation
Glycogen granules	
glycogen	provision of metabolic energy
Lysosomal granules	
Acid hydrolases	enzymatic digestion

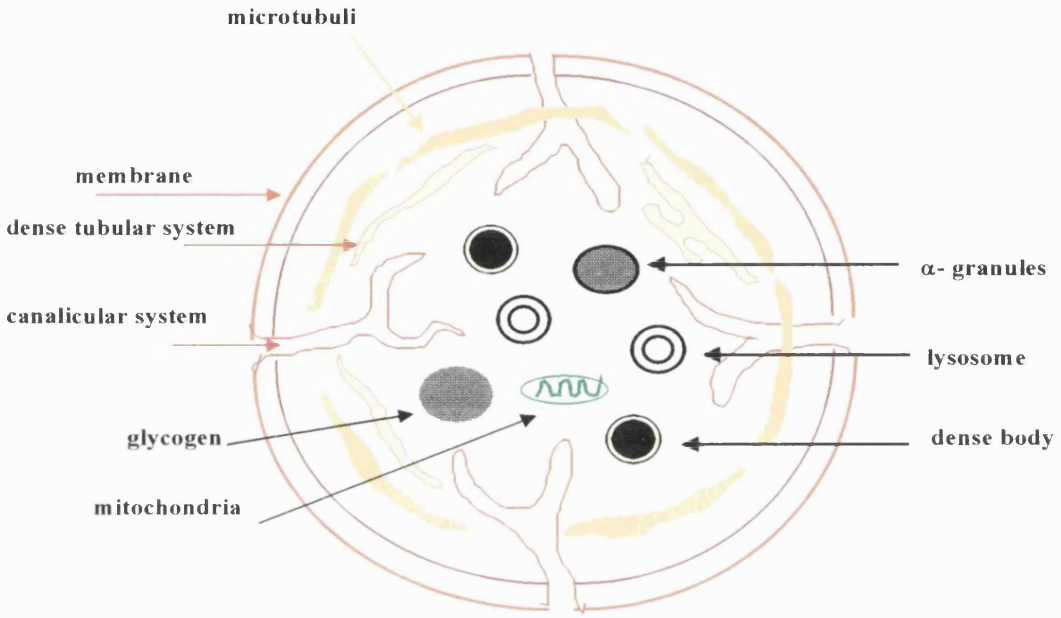


Fig 1.4: Ultrastructure of the human blood platelet.

Secretory granules and other subcellular organelles of the human platelet are presented in an equatorial plane. The surface connected canalicular system meanders around the granules, which discharge their contents to be exported to the extra-platelet space.

1.2.3.5 Platelet surface glycoproteins

Embedded in the phospholipid structure of platelet membrane are different kinds of glycoproteins, the receptors for activation and interaction with other cells. These receptors are termed integrins, and all consist of non-covalently associated α and β subunits common within the family.

On platelet surface the calcium-dependent glycoprotein IIb-IIIa (GP IIb-IIIa) complex is the most abundant glycoprotein. These glycoproteins are the primary mediators of platelet aggregation. Activation of the complex by strong agonists results in a poorly understood conformational change resulting in the expression of a fibrinogen-binding site. This includes a binding site on GPIIIa for the two Arg-Gly-Asp (RGD) sequences in the fibrinogen α chain as well as a separate dodecapeptide binding site on GPIIb (Gilbert C and White II., 1999). A single molecule of fibrinogen is bound for each GPIIb-IIIa "active site." Fibrinogen can bind to two contiguous platelets serving as a bridge between two platelets, resulting in the essential step in development of platelet aggregates. The numbers of GPIIb-IIIa sites (50-60,000/platelet) remain relatively constant. Following activation and aggregation, platelet contractility is mediated by the GPIIb-IIIa complex. This complex may be linked to the platelet cytoskeleton, perhaps via talin.

The glycoprotein Ib-IX (GPIb-IX) proteins are the primary mediators of platelet adhesion. In the presence of flow (arterial shear stress), adhesion is accomplished by the interaction of the von Willebrand factor (vWF) with subendothelial collagen and platelet the glycoprotein receptor (GPIb-IX). When the platelet GPIb-IX complex

interacts with thrombin, at lease a partial internal translocation of the GPIb-IX complex occurs, presumably mediated by the cytoskeleton. Therefore, the number of GPIb-IX complexes on the platelet surface is activation-sensitive and may vary considerably. There is no conformational activation of the GPIb-IX complex (Gilbert C and White II., 1999).

1.2.4 Platelet response

In response to a variety of agonists, including thrombin, collagen and ADP, platelets undergo a series of changes resulting in aggregation.

Thrombin: Thrombin is generated by activation of the coagulation pathways and is the most powerful of all the platelet agonists. Platelets have specific receptors for thrombin. The stimulation of platelets with thrombin can initiate extensive, granule secretion, independently of TXA_2 production, possibly through the hydrolysis of phosphatidyl inositol.

Collagen: When the vasculature is damaged, the removal of the endothelial cell layer exposes the vascular basement membrane, which is rich in collagen. Platelets have specific receptors for collagen. Also exposed is the large adhesive protein von Willebrand factor. This binds to platelet GPIb/GPIX/GPV complex.

ADP: Platelets have specific receptors for ADP, which acts as a weak agonist of platelets by inducing either little or no secretion of granules. Platelets themselves contain stores of ADP in the platelet dense granules. The release of these internal

stores of ADP acts as a positive feedback mechanism as this binds to surrounding platelets and accelerates the process of aggregation.

All these agonists stimulate platelets by initiating an influx of Ca^{2+} into platelet cytosol via activation of phosphatidylinositol. Once one of these agonists has bound to the platelet, internal signals are generated and platelet will respond to the stimuli.

The response of platelet include marked morphological and biochemical changes, which result in a series of events including:

a) *platelet activation*

b) *platelet secretion*

c) *platelet shape change*,

d) *platelet aggregation*

f) m*etabolism of arachidonic acid* (AA) and subsequent release of thromboxane A_2 (TXA_2).

1.2.4.1 Platelet activation

Platelet activation is the stage before platelet secretion and aggregation. During activation, some of the platelet adhesion molecules stored in the membranes of the α granules are relocated to the surface. The physiological activation of platelets involves the binding of specific agonists to their individual receptors on the surface of platelet. Platelet activation leads to an altered expression of already constitutively expressed surface glycoproteins. Increased numbers of GPIIb-IIIa complexes and reduced numbers of GPIb-IX complexes result from bi-directional trafficking of these glycoproteins between the cell surface, the surface-connected canalicular system and

intracellular storage. The activation of αIIbβ3, which is also called inside-out signalling leads to conformational changes of the complexes after which they develop a high affinity for their ligands, predominantly fibrinogen. The outside in signalling is associated with activation of tyrosine kinases and phosphorylation of the focal adhesion proteins.

It is fibrinogen that acts as the major "glue" which sticks platelets together during aggregation.

1.2.4.2 Platelet secretion and aggregation

Platelets normally circulate as flattened discs. At the same time as platelets undergo activation they undergo shape change. Discoid shape is lost and the cells become irregularly spherical with multiple pseudopodia (Fig 1.2). At this time, the platelets loosely associate with each other. This stage is called ***primary aggregation*** and is reversible. If the agonist is strong enough (like high concentration of thrombin) then these shape changes continue. The organelles are moved toward the cell centre, and the circumferential bundle of microtubules is shifted internally. The platelets become closely moulded to each other and the internal changes are more marked. The organelles become tightly packed and surrounded by a close-fitting band of microtubules and microfilaments. The channels of the open canalicular system become more dilated. Platelet pseudopodia are very pronounced. It is at this stage that platelet ***secretion*** is observed.

The platelet granules are secreted in a definite order depending on the strength of the agonist involved. The dense granules are released first (containing ADP, calcium and serotonin among others), followed by the α-granules (containing coagulation factors,

and adhesive proteins such as fibrinogen, fibronectin and thrombospondin) and finally the lysosomal granules (containing lysosomal enzymes). The secretion of these proteins accelerates aggregation and cements permanent attachments between platelets. The most important link between platelets is formed by the reaction of GPIIb/IIIa and fibrinogen. The fibrinogen is derived both from the plasma and from platelet internal stores.

After platelet secretion, the aggregation is irreversible and is known as ***secondary aggregation***. As secondary aggregation proceeds, the granules decrease in number and disappear as secretion takes place. Finally, the individual platelets lose their integrity and fuse with each other. Contraction of the platelet pseudopodia in the final stages coincides with clot retraction.

1.2.4.3 The mechanism of platelet activation

Activation of platelet by most stimulatory agonists involves an increase of cytosolic Ca^{2+} level through inostol triphosphate (IP_3)-dependent activation of phospholipase C (PLC) leading to release of Ca^{2+} from intracellular stores, as well as stimulation of the entry of extracellular Ca^{2+} (Verkerk and Jongkind, 1992).

These agonists stimulate a G-protein connected receptor, which lead to activation of phospholipase A_2 (PLA_2) and phospholipase C (PLC). PLC then splits apart PI-biphosphate (PIP_2), located in the plasma membrane lipid bilayer, into two products: inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG either releases arachidonic acid or activates protein kinase C, which can then activate different target proteins.

IP_3, on the other hand, detaches itself from plasma membrane and binds to IP_3- Ca^{2+} channel in the ER membrane in order to release Ca^{2+} into the cytosol. The increase concentration of Ca^{2+} acts as a positive feedback to release even more Ca^{2+}.

ADP as a platelet agonist activates a receptor operated cation channel, which leads to an immediate influx of extracellular Ca^{2+}.

The increase of intracellular free Ca^{2+} plays a key role during platelet activation through regulation of multiple Ca^{2+}-dependent enzymes, including the Ca^{2+}-dependent protein kinase C (PKC), the Ca^{2+}/calmodulin dependent myosin light chain kinase (MLCK), cytosolic phospholipase A_2 ($cPLA_2$), and the small GTPase Rap1 (Wolthuis et al 1998).

Both increased cytosolic Ca^{2+} and PKC activation are essential for granule secretion (Yang et al., 1996). The major target for PKC in platelet is the 47kDa protein, pleckstrin. Inhibition of PKC leads to decreased pleckstrin phosphorylation and inhibition of serotonin release.

Fibrinogen activates platelet by binding to its receptor, integrin $\alpha_{IIb}\beta_3$, and induces conformational changes of the receptor leading to turn over of actin filaments and re-organisation of actin cytoskeleton (Radomski and Moncada, 1993). The activation of integrin receptors is associated with activation of protein tyrosine kinases (PTK). Protein tyrosine kinases catalyse the activity of many enzymes, target proteins and membrane channels by phosphorylation, a common type of reversible covalent modification. Tyrosine kinases can be found as membrane-spanning receptors or as cytoplasmic forms.

The prostanoids (prostaglandins, prostacyclin and thromboxane) produced from arachidonic acid in enthotelial cells are involved in the regulation of platelet aggregation. Prostacyclin (PGI_2) is not produced in platelets, since the enzyme required for its synthesis (prostacyclin synthase) does not exist in platelets. PGI_2 produced in endotelial cells activates the surface receptors on platelets and is involved in the control of vascular tone through activation of adenylyl cyclase and an increase of intra cellular cAMP level. PGI_2 is also capable of penetrating through the cell membrane into platelet.

The most important enzymes for the production of prostanoids (including PGI_2) are cyclooxygenases (COX-1 and COX-2). Platelets are now known to have two isoforms of COX called COX-1 and COX-2 (Stuart et al., 1975). COX-1 is a membrane-bound enzyme that functions in all normal platelets, whereas COX-2 is a cytokine-inducible enzyme that appears in newly produced platelets and in other cells during inflammation.

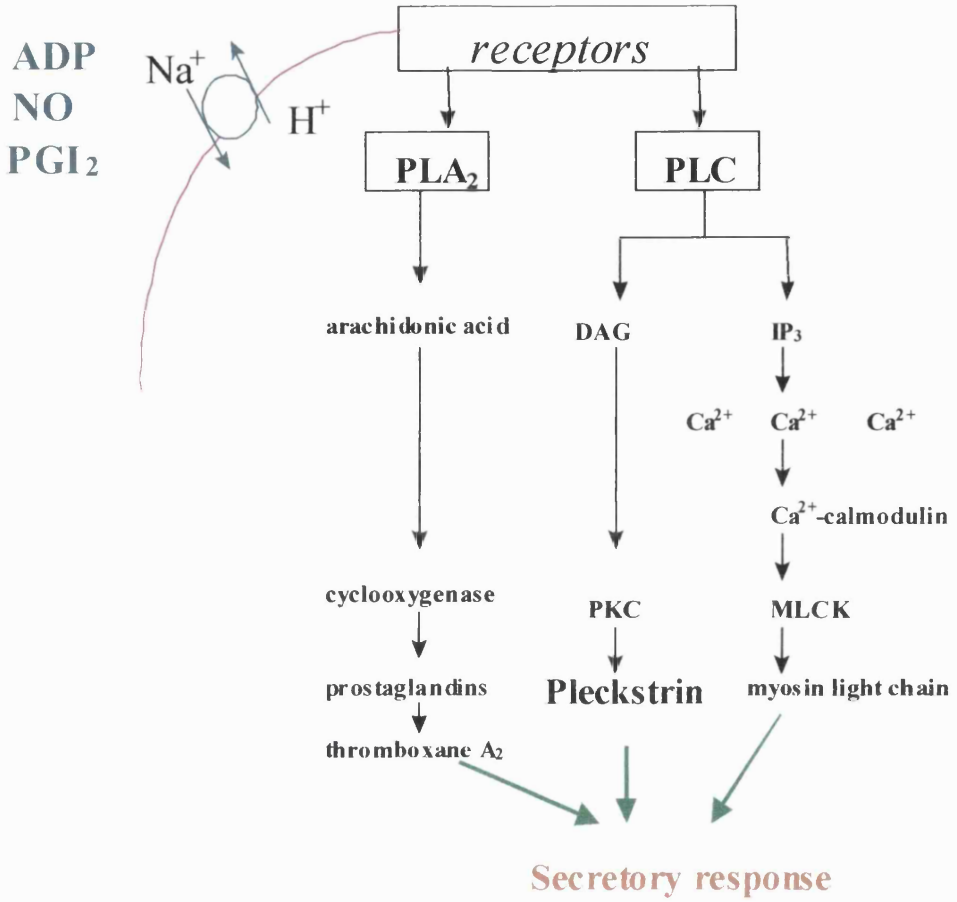


Fig 1.5: Schematic presentation of pathways involved in platelet activation.

Phospholipases and phospholipids (e.g. PLA2 and PLC) are involved in the processes of transmitting receptor-mediated signals from plasma membrane to intracellular proteins. Each of these processes are important in the regulation of platelets function.

1.2.4.3.1 The arachidonic acid pathway

Cyclooxygenase is a membrane-associated endoperoxide synthase with two catalytic sites. When ADP, collagen, epinephrine, or thrombin binds to its platelet membrane receptor site activates phospholipase A_2, a membrane-associated enzyme. Phospholipase A_2 frees arachidonic acid from its supporting membrane phospholipids, which then acts as a substrate for the COX pathway. COX rapidly modifies the free arachidonic acid in a two-step process. The first site converts arachidonic acid to the endoperoxide PGG_2. The second site converts the short-lived PGG_2 to PGH_2 (Fig 1.6). PGH_2 is then converted by the isomerase action of thromboxane synthase to thromboxane A_2 (TXA_2), which activates the neighbouring platelet. TXA_2 is rapidly hydrolysed to thromboxane B_2, a stable plasma product of the COX pathway. TXB_2, in turn, is converted to a variety of products, most of which are excreted via the kidney.

The products of arachidonic pathway are important in the maintenance of platelet function. Until now, there is no evidence for the presence of PGI_2 synthase inside platelet. PGI_2 is an important inhibitor of platelet inhibition in blood circulation.

1.2.4.3.2 The role of endothelium in platelets function

Vascular endothelium functions in a variety of important physiological processes. These will include interaction with neighbouring cells or extracellular matrix. For this interaction endothelial cells secret a number of mediators (factors), which may elicit biological responses by different signal transduction mechanisms. Such mediators are implicated in the regulation of endothelial cells as well as other cells including platelets. The production of prostanoids, NO and ROS as well as platelets agonists

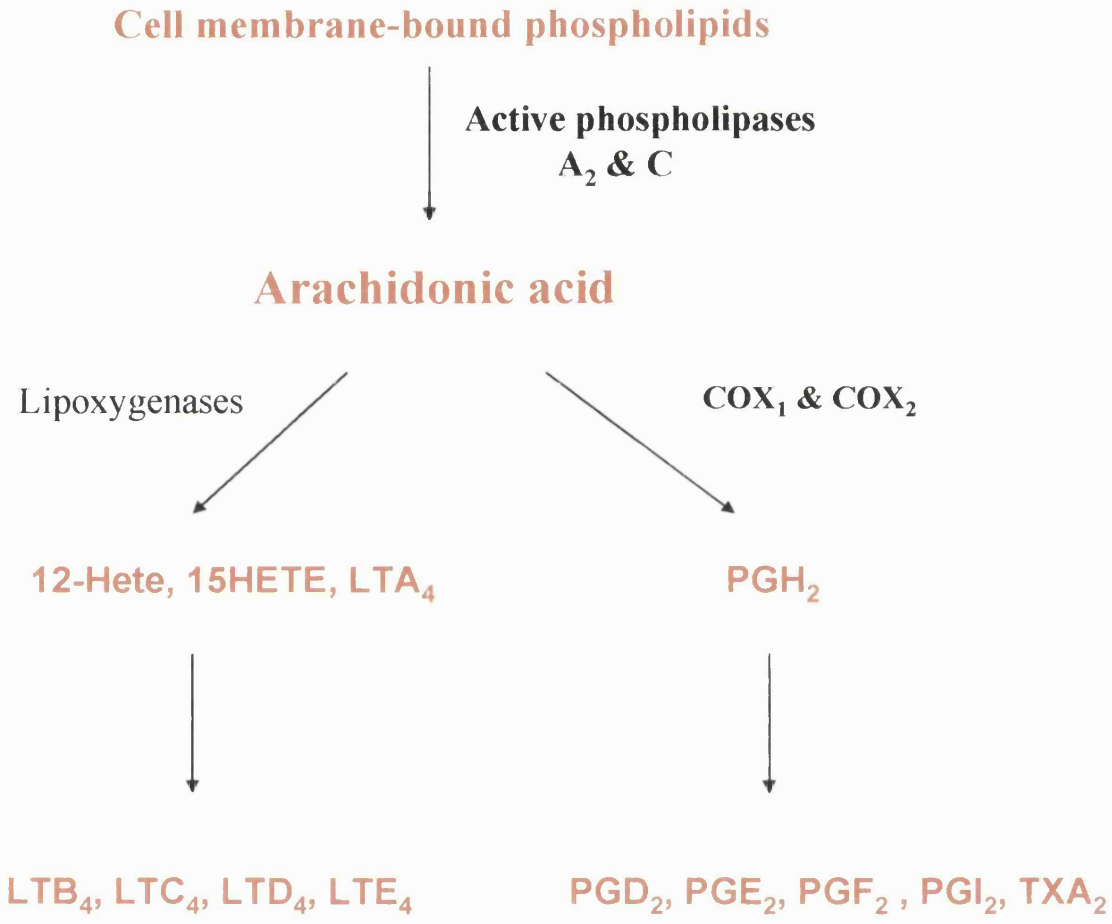


Fig 1.6: The arachidonic acid pathway

and coagulation intermediates by the endothelium is of great importance in the regulation of platelet function. The most relevant substance for this thesis produced by endothelium are PGI_2, nitric oxide, superoxide anions and hydrogen peroxide.

1.2.4.4 Inhibition of platelet activation

Platelet activation is tightly regulated under physiological conditions. Endogenous platelet antagonists such as PGI_2 and NO inhibit platelet aggregation by activation of adenylate cyclase and guanylate cyclase and increase of intracellular levels of cyclic nucleotides, cAMP and cGMP, respectively, which is the most potent endogenous mechanism of platelet inhibition. Major target enzymes of cyclic nucleotides involve protein kinases. Compared with other tissues and cell types, human platelets contains particularly high concentrations of protein kinases, which mediates their effects through phosphorylation of specific proteins including vasodilator-stimulated phosphoprotein (VASP), actin-binding protein (ABP), caldesmon and Rap1b. Phosphorylation of VASP, ABP and caldesmon inhibits platelet activation through suppression of the re-organisation of the cytoskeleton this is when phosphorylation of Rap1b inhibits of activation of platelets by reducing intracellular Ca^{2+} elevation (Schwarz et al., 2001).

An increase of cAMP and cGMP will also antagonise the activator-evoked release from intracellular store and the secondary store-mediated Ca^{2+} influx via phosphorylation of IP_3 receptors. However, the role of IP_3 receptor phosphorylation on inhibition of intracellular Ca^{2+}elevation is not clear.

cAMP-PK and cGMP-PK down regulate PKC activation either via Ca^{2+}level or through reduced DAG production which, besides Ca^{2+}, is necessary for PKC

activation (Radomski and Moncada, 1993).The inhibitory pathways in platelets exercise negative control imposed by cyclic nucleotides: cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). The levels of these cyclic nucleotides are elevated when inhibitors such as PGI_2 released from endothelium and adenosine interact with their receptors. Both cAMP and cGMP activate protein kinases, which are of key importance in cellular signalling. Protein phosphatases, which de-phosphorylate phosphorylated proteins, are also involved in regulation of cell signalling in different kind of cells including platelets.

1.3.1 Reactive oxygen species and free radicals

Reactive oxygen species (ROS) is a collective term used by biologists to include not only oxygen radicals ($OH^{\cdot}$, $O_2^{\cdot -}$ $^{\cdot}NO$) but also some derivatives of O_2 that do not contain unpaired electrons, such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2) and hypochlorous acid (HOCl).

A *free radical*, on the other hand, is an atom or group of atoms that contains at least one unpaired electron. Examples includes $OH^{\cdot}$, $O_2^{\cdot -}$ and $^{\cdot}NO$. Electrons are negatively charged particles that usually occur in pairs, forming a chemically stable arrangement. If an electron is unpaired, another atom or molecule can easily bond with it, causing a chemical reaction.

Due to high chemical reactivity of ROS, they will cause peroxidation of lipids and oxidation of proteins and DNA. To avoid the damage caused by ROS, the body has a defence system, which include antioxidants and an enzymatic repair system.

1.3.1 Superoxide radical ($O_2^{\cdot -}$)

$O_2^{\cdot -}$ is formed by the incomplete reduction of molecular oxygen.

$$O_2 + e^- \rightarrow O_2^{\cdot -}$$

The production of $O_2^{\cdot -}$ is mediated either by enzymes, including NADPH oxidase, lipooxygenase, cyclooxygenase, cytochrome p450 and xanthine oxidase or non-

enzymatically by redox reactive compounds such as the semi-ubiquinones, of the mitochondrial electron transport chain (Fig 1.6).

The production of ROS by NADPH oxidase is important in the regulation of the intracellular signalling cascade in various types of cells. Muscle cells and fibroblasts account for the most $O_2^{\cdot -}$ production in normal vessel wall. Thrombin, platelet-derived growth factor (PDGF) and tumour necrosis factor-α (TNF-α) stimulate NADPH oxidase-dependent Superoxide production in vascular smooth muscle cells and mechanical forces stimulate NADPH oxidase activity in endothelial cells (Arai et al., 1998a; Chatterjee, 1998a; Holland et al., 1998).

Apart from production of $O_2^{\cdot -}$ during cellular metabolic process, production of $O_2^{\cdot -}$ is increased during inflammation. Activated macrophages and neutrophils produce large amounts of $O_2^{\cdot -}$ and its derivatives via phagocytic isoform of NADPH oxidase during oxidative burst.

Xanthine oxidase generates $O_2^{\cdot -}$ by converting hypoxanthine into xanthine and xanthine into uric acid. Under normal conditions xanthine oxidase accounts for only a minor proportion of total $O_2^{\cdot -}$ production and NADPH oxidase is the major production of $O_2^{\cdot -}$ in our body (Droge, 2002).

Super oxide can be generated enzymatically during production of NO with either nNOS or iNOS.

However, $O_2^{\cdot -}$ may be reduce to form hydrogen peroxide (H_2O_2) in the presence of superoxide dismutase (SOD).

$$2\,O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

At present, three distinct isoforms of SOD have been identified in mammals, and their genomic structure, cDNA, and proteins have been described. Two isoforms of SOD have Cu and Zn in their catalytic centre and are localized to either intracellular cytoplasmic compartments (Cu/Zn-SOD or SOD1) or to extracellular elements (EC-SOD or SOD3). A third isoforms of SODs has manganese (Mn) as a cofactor and has been localized to mitochondria of aerobic cells (Mn-SOD or SOD2) (Weisiger et al., 1973).

1.3.2 Hydrogen peroxide (H_2O_2)

H_2O_2 is mainly produced by enzymatic reactions in most kinds of cells including endothelium, platelets, macrophages and muscle cells. These enzymes are located in microsomes, peroxysomes and mitochondria.

In plant and animal cells, superoxide dismutase, which dismutates $O_2^{\cdot -}$ to H_2O_2 , thus contributing to the lowering of oxidative reactions. Vascular endothelial cells generate additional H_2O_2 by the action of several oxidase enzymes, including xanthine oxidase (XOD), which makes both $O_2^{\cdot -}$ and H_2O_2 during its catalytic cycle (Jornot and Junod, 1993; Verkerk and Jongkind, 1992;).

H_2O_2 is able to diffuse easily through cellular membranes and at high concentrations is cytotoxic to all cell types. In the presence of transition metals (e.g., ferrous or cuprous ions) H_2O_2 can be converted into the highly reactive hydroxyl radical.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$

Alternatively, it can be converted into water and molecular oxygen in vivo by the reaction of the enzyme catalase or glutathione peroxidase. Catalase is generally found only in peroxisomes and catalysis very rapidly dismutation reaction.

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

$$H_2O_2 + 2GSH \xrightarrow{\text{GSHpx}} 2\ H_2O + GSSG$$

In platelets, the predominant enzyme is glutathione peroxidase. These enzymes are found in the cytosol and mitochondria.

1.3.3 Hydroxyl radical ($^{\cdot}OH$)

Except the iron-catalysed decomposition of hydrogen peroxide, which is considered the most prevalent reaction in biological systems, other reactions involving myeloperoxidase and Cl^- ions represent importance on the OH^- production process in neutrophils during phagocytosis.

$^{\cdot}OH$ is highly reactive and certainly capable of destroying isolated DNA, proteins or lipids *in vitro*.

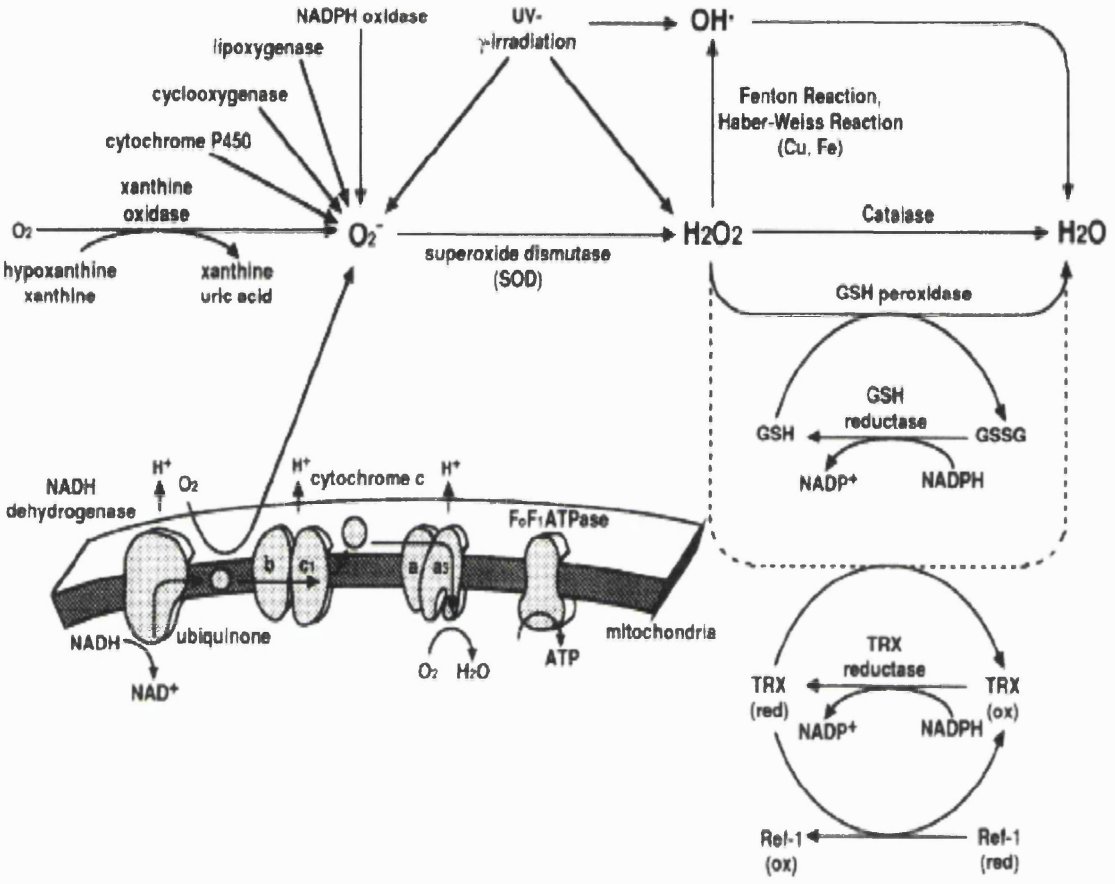


Fig 1.7: Metabolic pathways of reactive oxygen radicals.

Reactive oxygen species, such as H_2O_2, O_2 and ·OH, are generated in cells by several pathways. O_2 is generated by the leakage of electrons from mitochondria. It is also generated by the NADPH cytochrome P450 reductase, hypoxanthine/xanthine oxidase, NADPH oxidase, lipoxygenase and cyclooxygenase. Superoxide dismutase converts O_2 reducing activity as well as a function in refolding oxidised proteins. H_2O_2 is generated via dismutation of superoxide by SOD and is degraded into water by GSH peroxidase and catalase. Figure adapted from Hirata H (Kamata and Hirata, 1999)

1.3.4 Nitric oxide (NO)

Nitric oxide is produced in various types of cells including, the vascular endothelium. NO is an important mediator of physiological process such as vasodilatation and neurotransmission. It also plays a role in primary defence mechanism, produced during activation of macrophages, where it kills invading microorganisms. The disregulation of NO also has an important function in the pathology of many inflammatory disorders, including atherosclerosis. The importance of NO in platelets is considered in the next part after a short description of the history of NO.

1.4 The history of Nitric Oxide

Ascanio Sobrero synthesized the explosive nitroglycerin	**1846**
William Murrell established nitroglycerine as an angina treatment	**1879**
Ferid Murad found that NO activates guanylyl cyclase and relaxes smooth muscles	**1977**
Louis Ignarro found that NO causes relaxation response in artery	**1979**
Robert Furchgott discovered that the endothelium releases a factor, later called EDRF, which relaxes blood vessels	**1980**
Ignarro found that NO inhibits platelet aggregation and increases cGMP	**1981**
Steven Tannenbaum determined that mammals make nitrate	**1981**
Murad found that blood vessel relaxation was associated with increased cGMP	**1983**
Michael Marletta detected inorganic nitrite and nitrate made by mouse macrophages	**1985**
Ignarro and Furchgott independently speculated that EDRF is NO *at a conference.*	**1986**
Both Salvador Moncada and Ignarro independently published evidence that EDRF is NO	**1987**
Hibbs and Marletta found that arginine increases nitrite and nitrate formation in macrophages	**1987**
Moncada discovered that NO is made from L-arginine	**1988**
John Garthwaite detected NO made by nerve cells	**1988**
David Bredt and Solomon Snyder clone bNOS	**1991**
Furchgott, Murad, and Ignarro are awarded the Nobel Prize for Physiology or Medicine	**1998**

Fig 1.8: A summary of the history of Nitric oxide discovery.

1.4.1 Synthesis of NO in biology

NO is formed in all mammalian tissues by a family of enzymes termed NO synthases (NOS). Until now, three major isoforms of this family have been identified as:

NOS-I or nNOS (neuronal)

NOS-II or iNOS (the inducible form)

NOS-III or eNOS (endothelial)

These three isoforms were originally grouped into two forms:

constitutive (*cNOS = nNOS & eNOS*) = Ca^{2+} dependent

inducible *(iNOS)* = Ca^{2+} independent

NO synthase requires L-arginine, oxygen and cofactors including NADPH, FMN, flavin adenine dinucleotide (FAD) and tetrahydrobiopterin (BH_4). The rate of nitric oxide synthase is determined by the availability of the substrate L-arginine as well as the cofactors and results in the production of NO and citrulline (Fig 1.8) (Foerster et al., 1996).

Many tissues express one or more of these isoforms under physiological conditions.

1.4.1.1 The constitutive production of NO

Constitutive productions of NO (nNOS & eNOS) require calcium and calmodulin as cofactors and generate low (nanomolar) concentrations of NO. The activity of the enzyme is also regulated by the amount of available calcium. The basal NOS can be enhanced by increases in $[Ca^{2+}]i$ following receptor dependent cell stimulation like bradykinin, receptor independent like Ca^{2+} ionopore and physical stimuli such as shear stress. Acetylcholine (ACh) and bradykinin, which bind to a plasma membrane receptor on the endothelial cells, cause an influx of Ca^{2+} from the extracellular space. An intracellular change of Ca^{2+} from 100 to 500 nM changes the rate of NO synthesis from <5% to >95% of maximum (Knowles and Moncada, 1992). The influx of Ca^{2+} stimulates NOS to convert L-Arg to citrulline and NO (Figure 1.8). During last few years the existence of a mitochondria associated NOS have been discussed by different groups. This was originally based on cytochemical and immuno-ytochemical evidence (Bates 1995, Kobzik 1995) showing association of either NADPH diaphorase- or NOS antibody-binding to mitochondrial membranes.

1.4.1.2 The inducible NO synthase

The inducible form of NOS is expressed in a wide variety of cells, including macrophages, endothelial cells, fibroblasts, vascular smooth muscle cells and cardiac myocytes. This form of NOS is expressed by exposure to bacterial endotoxins and in response to inflammatory cytokines, including tumour necrosis factor, interferon gamma and interleukins 1&2. In many cells, the activity of this form is independent of Ca^{2+} level and once activated the enzyme continues to produce large amount of NO

for periods up to days in some instance (Vallance and Moncada, 1994). The expression of iNOS is regulated both at the level of transcription and at the level of iNOS mRNA stability.

In human platelets, the nitric oxide synthase is activated by the increase in intracellular calcium that occurs during platelet activation. The NO then may act as negative feedback system to limit the extent of activation.

The presence of both constitutive and inducible forms of NOS in platelet has been established (Mehta et al., 1995).

An oxidase activity role for different isoforms of NOS has been reported independently. Cofactor deficient NOS cannot catalyze the five-electron oxidation of L-argnine to NO, but it can receive electrons from NADPH and donate them for one electron reduction to molecular oxygen, which then produces superoxide radical. It has also been suggested by new studies that NOS cofactors and coenzymes might cause $O_2^{\cdot -}$ generation in the NOS-independent manner (Xu, 2000a; Xu, 2000b).

1.4.2 The role of nitric oxide in biology

Nitric oxide is a simple molecule, which is continuously produced in most mammalian cells, acting as a universal regulator of metabolism. It is now clearly recognised that NO participates in the control of vascular tone as an antagonist of the adrenergic regulatory system. It inhibits aggregation of platelets and their adhesion on a vascular wall. NO causes smooth muscle relaxation not only in the vascular wall (Wedel and Garbers, 1997), but also in the gastrointestinal tract (Brune and Ullrich, 1987). NO acts both on the central and peripheral nervous systems (Stone and Marletta, 1994). It regulates the activity of respiratory system organs, the digestive

tract and genito-urinary systems via efferent nerves. NO also influences the functioning of secretory tissues and cells.

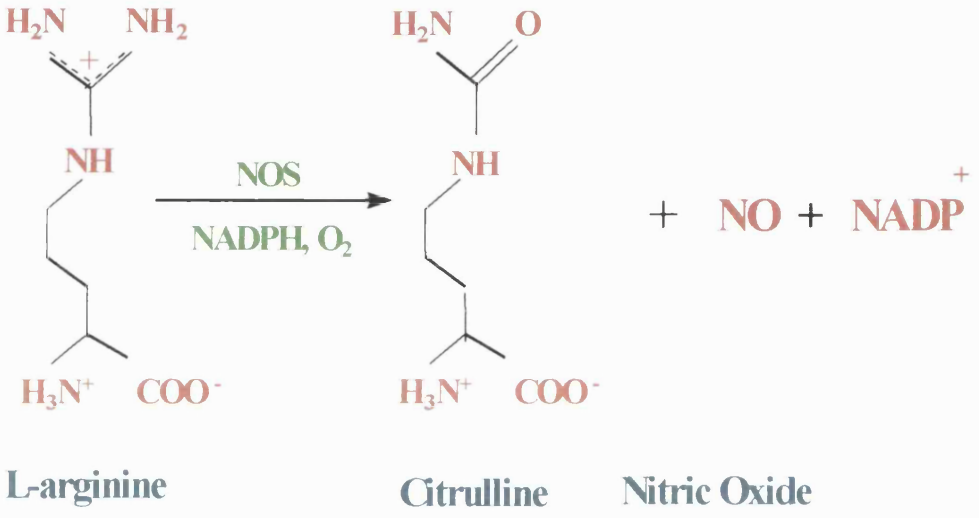


Fig 1.9: Synthesis of NO in biological system.

The reaction requires oxygen and cofactors, including flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH4) and haem as well as calmodulin. The NOS activity is dependent on the nicotinamide adenine dinucleotide phosphate (NADPH). Electrons (e-) are donated by NADPH to the reductase domain of the enzyme and proceed via FAD and FMN redox carries to the oxygenase domain. The electrons then interact with haem and BH4 at the active site to catalyse the reaction of oxygen with L-argenine generating citrulline and NO. The electron flow through reductase requires the presence of bound Ca^{2+}/ caM.

On the other hand, when released in large quantities for long periods, NO acts as a defence mechanism exhibiting cytotoxic/cytostatic activity and operating as one of the major effectors of the front line cellular immunity system.

1.4.3 The effects of NO

NO can undergo several fates within the cells. The effects of NO in general are divided to low concentration effects, which are mostly direct effects of NO and high concentration effects or indirect effects.

1.4.3.1 The direct effects of NO

The direct effects of NO most often involve the interaction with metal complexes. Most physiologically relevant effect on NO includes its interaction with soluble guanylyl cyclase, cyochrome P450, haemoglobin, cytochrome C oxidase and catalase. NO can also react with non-iron and zinc containing proteins.

This interaction of NO with Fe^{2+} ion of haem group produces nitrosyl-haem, a complex, which is stable for several minutes (comparing with the half life of NO which is about a few seconds (Kharitonov et al., 1997).

1.4.3.2 The indirect effects of NO

The indirect effects of NO are mostly related to the higher concentrations of NO. These effects are mostly produced through interaction of NO with O_2 or $O_2^{\cdot-}$ including nitrosation, oxidation or nitration.

These reactions of NO with proteins will modify the function of proteins. Therefore, these effects of NO mostly are considered as cGMP independent. The reaction of NO with molecular oxygen results in oxidation products, which can react with amino acids, low molecular weight peptides and protein-associated thiols such as cysteine, glutathione and albumin to form S-nitrosothiols. These products will increase the stability and bioactivity of NO and they may serve as transporters for NO. The nitrosations of proteins may be an important regulatory process in their bioactivity. NO-mediated smooth muscle relaxation has been attributed to activation of K^+ channels (Bolotina et al., 1994; Mistry and Garland, 1998). S-nitrosation of cysteine residues resulting from the addition of NO+ groups has been shown to modify the activity of several proteins. The nitrosylation is not relevant to this thesis, since we did not look at the nitrosylation of proteins in this work, but it would be worth investigation.

On the other hand, inactivation of NO occurs largely through oxidative reactions mediated by reactive oxygen intermediates, including $O_2^{\cdot-}$, H_2O_2 and lipid peroxyl radicals generated from lipid peroxides.

In human platelet, NO inhibits aggregation and activation of these cells via stimulation of sGC and an increase of cGMP. In platelets, inhibition of protein

phosphorylation, which is essential to calcium entry into platelets and inhibition of thromboxane A_2 receptor (Tsikas et al., 1999) have been implicated in cGMP-independent effects of NO. Other studies also suggested that the action of endothelium derived NO might be mediated primarily by cGMP-independent mechanism, which rely on its prior oxidation to nitrosating species in the plasma (Tsikas et al., 1999). Regarding this hypothesis activation of K^+-channels in smooth muscle cells and inhibition of protein phosphorylation in platelets is essential for Ca^{2+} entry into platelets and inhibition of thromboxane A_2 receptor (Tsikas et al., 1999).

A gelatinase A, matrix metalloproteinase (MMP) enzyme, activating pathway have been recently identified pathway for human platelets. SNAP (a NO donor) at concentrations between 0.1-10μM was shown to inhibit the secretion of gelatinase A induced by collagen or thrombin (Sawicki et al 1997). Human platelet express gelatinase A which causes platelet aggregation independent of thromboxsne or ADP. Regarding to this data NO inhibits the aggregation of platelets through inhibition of secresion of metalloprotease.

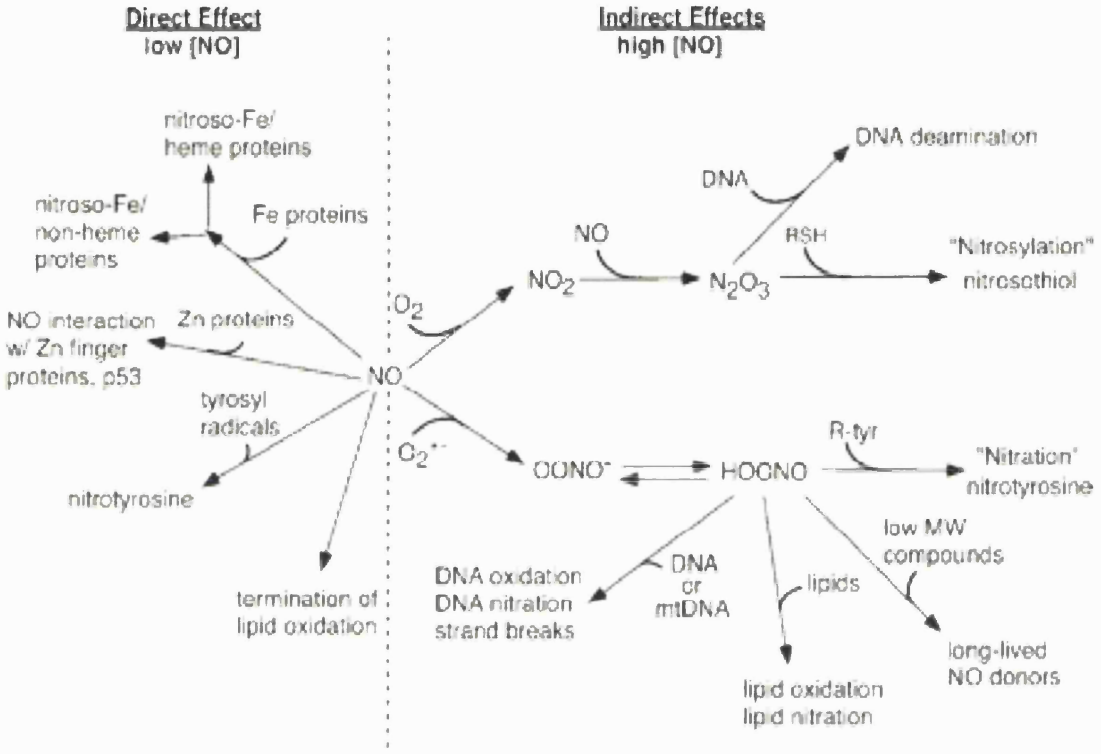
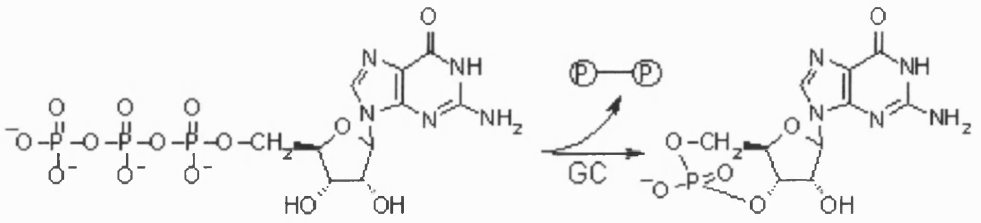


Fig 1.10: Summary of the chemistry of NO.

The direct and indirect effects of NO. Figure adapted from Davis et al 2001.

1.5.1Guanylyl cyclase

Guanylyl cyclases (GCs) are a family of enzymes, which catalyse the formation of the second messenger guanosine 3'5'-cyclic monophosphate (cGMP) from guanosine 5'-triphosphate (GTP).



GTP cGMP

According to their structural features and their regulation, GCs can be divided in two forms:

Membrane-bound and receptor linked GCs (particulate GCs)

Cytosolic GCs (soluble GCs)

The membrane-bound GCs belong to the group of receptor-linked enzymes with one membrane-spanning region and they are activated via interaction with peptide hormones (Koesling and Friebe, 1999; Wedel and Garbers, 1997).

Although all of these GCs share a conserved intracellular catalytic domain, they differ in their extracellular ligand-binding domains and are activated by different peptide hormones. The guanylyl cyclase A (GC-A) isoform acts as the receptor for the natriuretic peptides. Today there is no evidence that platelet contain this form of GC.

Cytosolic GCs (sGCs) is the only form of GC, which have been found in platelet. The sGCs are located in the cytoplasm and are activated directly via interaction with nitric oxide. sGC contains a 5-coordinated haem group with a His residue as the axial ligand for NO. Formation of the NO-haem complex leads to a conformational change, resulting in an up to 400-fold increase in catalytic activity of the enzyme (Koesling and Friebe, 1999).

So far two isoforms of the NO-sensitive heterodimeric enzyme have been identified, the ubiquitous $\alpha_1\beta_1$ isoform and the less broadly distributed $\alpha_2\beta_2$ isoform. Although both isoforms show the same regulatory properties, they appear to differ in their subcellular distribution. The N-terminals of the subunits are responsible for haem binding and haem coordination, whereas the cyclase catalytic domains are located in the C-terminal regions. The cyclase catalytic domain is conserved in the membrane-bound guanylyl cyclases as well as in the adenylyl cyclases (Garbers 1990).

Soluble GCs occur in relatively high concentration in vascular smooth muscle cells and platelets as well as in lung, kidney and brain tissues.

Activation of sGC by NO is initiated by binding of NO to the haem iron and proceeds via breaking of the histidine-iron bond and leads to the formation of a five-coordinated nitrosyl-haem complex. The coordinate chemistry of NO with ferrous haem is unique among diatomic ligands such as CO, O_2. Carbon monoxide (CO), like NO, binds to the haem group of sGC with high affinity, but only leads to a four-six fold activation of the purified enzyme (Brune and Ullrich, 1987; Stone and Marletta, 1994). In contrast to NO, binding of CO results in a six-coordinated haem complex with the histidine-iron bond remaining intact.

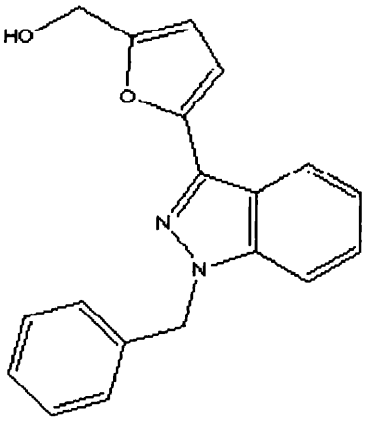
Studies on the dissociation of NO from sGC revealed that the enzyme has a high NO

dissociation rate comparing with other proteins (Foerster et al., 1996). NO dissociation was increased in the presence of the substrate MgGTP, yielding a half-life of about 5s at 37 °C for the NO-sGC complex (Kharitonov et al., 1997). This half-life is probably fast enough to rapid deactivation of the enzyme in biological system.

In recent years, a new non-biological activator of sGC has been identified. YC-1 ([3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole]) is a synthetic benzylindazole derivative, which activates sGC about ten fold in blood platelets in a NO-independent manner, since the activation was not blocked in the presence of NO scavenger, hemoglobin. YC-1 enhances the sensitivity of the enzyme to NO and CO and potentiates activation of sGC by NO or NO donors (Foerster et al., 1996; Friebe and Koesling, 1998; Ko et al., 1994). This latter effect was explained by the slower dissociation of NO from the enzyme. Apart from an increase in the formation of cGMP via the stimulation of sGC, the YC-1 also causes pronounced increases in cGMP levels by inhibition of phosphodiesterases (Galle et al., 1999). Thus, YC-1 may represent a new class of drugs that are of potential use in the treatment of cardiovascular diseases.

There is controversy regarding the haem dependent or independent activation of the sGC by YC-1. Friebe et al demonstrated that the interaction between YC-1 and sGC certainly requires the haem moiety group, since the haem deficient sGC was not stimulated by YC-1. However the binding of YC-1 to sGC does not change the Soret spectrum of the haem or the Raman spectrum of sGC (Denninger et al 2000), which certainly exclude the direct interaction between YC-1 and sGC. An independent study

by Martin et al and Murad (2001) later demonstrated that the haem moiety might be important but not necessary for activation of sGC by YC-1. In their study, YC-1 was capable of increasing the activity of haem deficient sGC, but the activity was substantially more when the haem moiety was present.



YC-1 ([3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole])

1.5.2 The mode of action of guanosine 3'5'-cyclic monophosphate (cGMP)

It has been established that cGMP plays an important role in the relaxation of smooth muscle cells, the inhibition of platelet aggregation and in retinal photo transduction. It also participates in signal transduction within the nervous system. Signalling by cGMP is mediated by different groups of cGMP effector molecules including cGMP-activated protein kinases and cGMP-gated ion channels. The level of cGMP is reduced by cGMP-degrading phosphodiesterases (cG-PDEs).

1.6 Phosphodiesterases (PDEs)

PDEs transform cyclic nucleotides into 5'-nucleotide monophosphates. The family of PDEs is large, consisting of complex proteins that differ in substrate affinity kinetic properties, subcellular localisation and Ca^{2+} sensitivity (Leroy et al., 1985). In mammalian cells, at least 20 genes and 50 different PDE proteins have been identified. They are grouped in seven gene families named PDE I-VII (Conti and Jin, 1999). Three types of PDEs are regulated by cGMP.

PDEI (cGMP stimulated PDE) hydrolyses both cAMP and cGMP with similar affinity and is stimulated only by the binding of cGMP to two allosteric regulatory sites. PDEIII (cGMP inhibited PDE) is preferred by cAMP and either inhibited or insensitive to binding with cGMP. PDE V (cGMP specific PDE) is specific for cGMP and the binding of cGMP will increase its allosteric activity.

The inhibitors of PDEs regulate platelet cAMP and cGMP level via inhibition of

degradation of cyclic nucleotides.

Cyclic nucleotide-dependent protein kinases are the major effector molecules mediating physiological effects initiated by cyclic nucleotide formation. Comparing with other tissues and cell types, human platelets contain particularly high concentrations of both cAMP and cGMP-PK (Eigenthaler et al., 1992).

1.7 Protein phosphorylation and protein Kinases

Protein kinases are enzymes, which phosphorylates proteins via transfer of a phosphate group from a donor substrate such as ATP and GTP. Protein kinases are either serine/threonine specific (*cAMP and cGMP dependent kinases*) or tyrosine specific (protein tyrosine kinases). Activation of protein kinases is the most common mode of signal transduction in biological system. More often phosphorylation of protein results in a change of protein conformation. In the case of enzymes, a change in conformation leads to a change in enzymatic activity.

Although expressed in many cell types, the serine/threonine protein kinases are found at high levels only in the lung, cerebellum, platelet and smooth muscle cells (Francis and Corbin, 1994), while tyrosine kinases are presence in all variety of cells.

1.7.1 Cyclic AMP dependent protein kinases

For cyclic AMP dependent protein kinases (PKA), the first messenger is frequently the hormone adrenaline. The second messenger, inside the cell, is cyclic adenosine monophosphate (cyclic AMP or cAMP), which is a ubiquitous second messenger in

all cells.

Cyclic AMP dependent protein kinases are tetrameric proteins, which consist of two catalytic sub-units (C) and two regulatory sub-units (R). The binding of cAMP to the R sub-units triggers the separation of R from C, which is the key step of activation. When cAMP molecules bind to the R sub-units, the PKA splits into three pieces and the two R sub-units and cAMP molecules stay together, but the two C sub-units are each freed to interact with other proteins.

1.7.2 Cyclic GMP dependent protein kinases

It is believed that the binding and activation of cyclic GMP dependent protein kinase (PKG) is responsible for most of the intracellular actions of cGMP.

PKG is a serine/threonine kinase, which is activated upon binding of cyclic GMP. There are two types of PKG: *PKGI and II* (Vaandrager and de Jonge, 1996).

PKGI is a cytosolic 76kDa homodimer that is presented in most tissues, but cerebellum, platelet and smooth muscle cells have particularly high levels of expression. Furthermore, there are two isoforms of PKGI, known as PKGIα and PKGIβ.

PKGII on the other hand is an 86kDa membrane bound protein that is less common and expressed only in intestinal epithelial cells (Lincoln and Cornwell, 1993) kidney and brain (Vaandrager and de Jonge, 1996), but absent from the cardiovascular system.

The known substrate proteins for these cyclic nucleotide kinases, which are involved in microfilament rearrangement in platelets, includes actin binding protein (ABP) (Chen and Stracher, 1989), caldesmon (Hettasch and Sellers, 1991), glycoprotein 1bβ (Wardell et al., 1989), heat shock protein 27 (Hsp 27) (Butt et al., 2001), myosin light chain kinase (MLCK) (Nishikawa et al., 1984), small GTPase protein Rap 1b (Franke et al., 1997) and vasodilator-stimulated phosphoprotein (VASP). VASP is a common substrate for both PKA and PKG (Eigenthaler et al., 1992).

1.8 Vasodilator-stimulated phosphoprotein (VASP)

VASP is a membrane-associated protein, which is localised, in the focal adhesion of well-spread cultured cells and periodically along stress fibres in a manner reminiscent of α-actinin and zyxin. In spreading cells, VASP is found in microspikes. VASP is also found in the ruffling membranes of migrating fibroblasts and the cell-cell contacts of epithelial cells and muscle cells. In most of these cells, VASP colocalises with mena, profilin, zyxin, vinculin and other focal adhesion proteins (Fig 1.3).

VASP is a member of proline-rich proteins family, which include the Drosophila protein Enabeled (Ena), mammalian orthologue (Mena) and the Ena-VASP like protein EV1 (Gertler et al., 1996). These proteins are composed of a central proline rich and N- and C-terminals also called EVH1 and EVH2 respectively (Fig 1.11 and 1.12). The central region of Ena/VASP protein harbours proline-rich stretches that are recognised by the G-actin-binding protein profilin (Gertler et al., 1996; Reinhard et al., 1995). VASP oligomerization and F-actin binding are confined to the C-terminal EVH2 domain (Bachmann et al., 1999; Huttelmaier et al., 1999).

VASP is believed to play an important role in the control of the cytoskeleton re-organisation. The inhibitory role of VASP might be through its interaction with actin cytoskeleton.

VASP may control the actin cytoskeleton by three different mechanisms 1) it may recruit G actin via its binding to profilin 2) it may stabilise and possibly organise newly formed filaments by direct binding to F-actin and oligomerization may potentiate both effects.

Vasodilator-stimulated phosphoprotein was originally identified as a major substrate for cAMP and cGMP kinases (Butt et al., 1994). It migrates in SDS/PAGE as a

46/50kDa-doublet. Three phosphorylation sites have been identified as serine^{157}, serine^{239} and threonine^{278}. The serine^{157} has been identified as the preferred site for cAMP-kinases and the only site, which will cause a shift of the molecular weight of VASP from 46 to 50kDa (Butt et al 1994).

VASP is found in a wide variety of cell types including platelets, vascular endothelial cells HL-60 cells, vascular smooth muscle cells, fibroblasts and cardio-myocytes (Grunberg et al., 1995; Pohl et al., 1994; Walter et al., 1995). VASP is highly enriched in platelet (Eigenthaler et al., 1992) and it is phosphorylated in response to vasodilators and platelet inhibitors, substances that raise intracellular cAMP and cGMP levels such as NO and prostacyclin (PGI_2) (Halbrugge et al 1990).

In the recent years, phosphorylation of VASP has been reported, to be correlated with the inhibition of platelet aggregation. Since the platelets of VASP-deficient mice show enhanced agonist-induced aggregation, indicating that in these cells VASP is indeed important as a negative regulator of actin polymerisation and aggregation (Aszodi et al., 1999).

Platelets also contain higher concentrations of cAMP and cGMP kinases compared with other cells, which makes the role of VASP even more important in platelet activation.

msetvicssr atvmlyddgn krwlpagtgp qafsrvqiyh nptansfrw 50

grkmopdppv vincaivrgv kynqatprifh qwrdarqvwg lnfgskedaa 100

qfaagmasal ea

legggppp ppalptwsvp ngpapeeveq qkrqqpgpse 150

hierrvsnag gppappaggp pppppgpppp gpppppglpp sgvpaaahga 200

gggpppappl paaqgpgggg agapg

laaai agaklrkvsk qeeasggpta 250

pkaesgrsgg gglmeermam larmkatqv gektpkdesa nqeepearvp 300

aqseavrrpw eknsttlprm kssssvttse tqpdpesssd ysdlqrvkqe 350

lieevkkelq kvkeeiieaf vqelrkrgsp 380

Fig 1.11: The amino acid sequence of VASP (Haffner et al., 1995)

Residues 1-113 represent the **EVH1** domain (N-terminals), residues 113-225 represent the **proline-rich domain** and residues 225-380 represent the **EVH2** (C-terminal) domain.

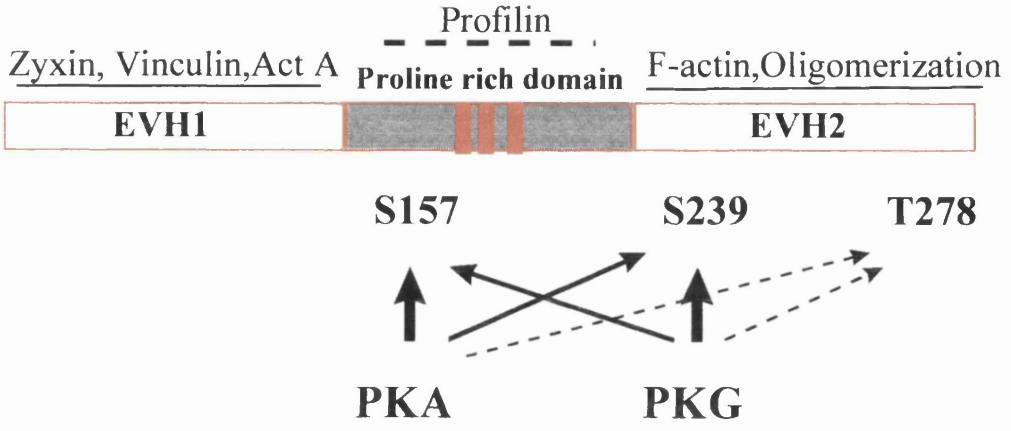


Fig 1.12: VASP structure, ligand binding and phosphorylation sites.

VASP consists o a central proline-rich domain and two Ena-VASP homology domains (EVH1 & EVH2). The EVH1 domain binds to zyxin, vinculin and bacterial surface protein ACT A and the EVH2 domain binds to filamentous actin (F-actin) and VASP oligomerization. The proline-rich domain on the centre binds to profillin. The preferences described for each of the kinases *in vitro* are indicated by the thickness of arrows. (Picture adapted from Harbeck B. et al 2000)

1.9 Synergism between nitric oxide and hydrogen peroxide

The role of H_2O_2 on the relaxation of smooth muscle and endothelial cells has been discussed for many years. For many years, it was suggested that H_2O_2 does activate sGC leading to relaxation of smooth muscle cells but the whole mechanism was not known. Now it is known that H_2O_2 induces the NO synthase by endothelial via a calcium dependent protein kinase activation (Shimizu et al., 1998).

The first study of the role of H_2O_2 on platelet function started in the late 60's. Since then a number of reports about the role of H_2O_2 on platelet activity have been published. Some of these results support the influence of H_2O_2 on activation of platelet aggregation and others indicated inhibition of platelet aggregation.

H_2O_2 have been reported to potentiate aggregation of platelets pre-exposed to ADP (Canoso et al., 1974). Later it was established that exposure of human platelet to micromolar concentrations of H_2O_2 increases platelet shape change, modifies aggregation and increases release reaction induced by ADP and thrombin (Rodvein et al., 1976). In a different study, H_2O_2 at 1μM significantly increased $[Ca^{2+}]_i$ release from human platelet and caused their activation (Del Principe et al., 1991).

In contrast, incubation of H_2O_2 with platelet rich plasma (PRP) decreased the non-metabolic ATP and ADP level, which lead to inhibition of granule release and inhibition of aggregation (Stuart et al., 1975; Stuart and Holmsen, 1977). Also incubation of human washed platelets with various concentrations of H_2O_2 (2-10mM) in the presence of 10 μM Fe^{2+} resulted in a decrease of the aggregating capacity maybe through lipid peroxidation (Ohyashiki et al., 1991).

However, a synergism role for H_2O_2 in the presence of NO was established in this laboratory (Naseem and Bruckdorfer, 1995). The simultaneous application of NO and

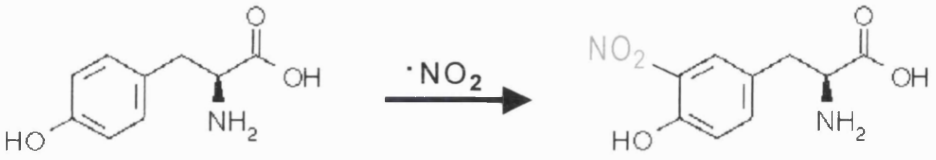
H_2O_2 to human platelets at physiologically relevant concentrations increased inhibition of platelet aggregation by NO almost to 100 fold. The similar effect was also observed when H_2O_2 was added with S-nitrosothiols. Addition of H_2O_2 after agonist (e.g., collagen) the peroxide enhanced aggregation of platelets. This effect of H_2O_2 was not reported by other peroxides. Other studies suggested that H_2O_2 inhibit the vascular smooth muscle tone via activation of sGC (Mittal and Murad, 1977; White et al., 1976), since H_2O_2 at micromolar concentrations stimulated NOS leading to increase concentration of NO. Other investigations showed that compound I (a form of catalase) play a critical role in activation of sGC by H_2O_2 (Burke and Wolin, 1987).

More recently, other targets except sGC have been identified for the inhibitory action of H_2O_2. A new publication discussed the action of H_2O_2 derived from endothelium, as an endothelium derived hyperpolarizing factor (EDHF) was proposed (Matoba et al., 2002). H_2O_2 acts as a mediator of ion channels on the vascular smooth muscle increasing K^+ conductance and causing hyperpolarisation and relaxation of smooth muscle.

The role of H_2O_2 as modulator of signal transduction has been discussed more since the discovery of its role in inhibition of protein phosphatases (especially protein tyrosine phosphatase) (Meng et al 2002). Inactivation of PTP by H_2O_2 was shown to regulate the activation of distinct MAPK pathways (Lee et al 2002). The exact role of H_2O_2 on the inhibition of platelet function is unclear and needs further investigation

1.10 Nitration of proteins

Nitration proceeds through the reaction of a nitro group with amino acids like tyrosine, cysteine, methionine, tryptophan and phenylalanine (Alvarez et al., 1999). In a biological system, molecules with aromatic rings are more susceptible to nitration than most other biological molecules. Nitrotyrosine is produced when the 3' position of a tyrosine residue is attacked by a nitrogen dioxide molecule.



Tyrosine **3-Nitrotyrosine**

The presence of nitrated proteins has been investigated by analytical and immunological methods using polyclonal and monoclonal anti-nitrotyrosine antibodies. Nitrotyrosine formation has gained increase attention since nitration of proteins have been observed in a number of human disease including, atherosclerosis, pulmonary and heart disease, acute and chronic kidney rejection, Alzheimer's disease, Parkinson's disease and amylotrophic lateral sclerosis as well as in un-stimulated cells and tissues (table 1.2).

1.10.1.1 Tyrosine nitration under physiological condition

RNS and ROS exist in biological cells and tissues under normal physiological conditions. A balance between their production and their clearance and also the availability of antioxidant system determines their importance and activity in redox reactions. The presence of these species increases the possibility of the production of nitrating agents and, as previously mentioned, the amino acids as tyrosine, tryptophan and phenylalanine are on great risk of oxidation and nitration.

Redox regulation under physiological condition has been associated with oxidative derivatization of proteins, including enhance activity of some signalling cascades. This activity would lead to the activation of protein kinases and inhibition of protein phosphatase. Protein nitration under physiological condition has been detected in numerous tissues (Greenacre and Ischiropoulos, 2001). In the cardiovascular system, basal nitration was found in all major types of cells, such as myocytes, endothelial cells, fibroblasts, and vascular smooth muscle cells (Davidge, 1998; Davidge and Zhang, 1998, Frustaci 2000, Kajstura2001). Basal protein nitration was also found in the plasma (Khan1998 Khalid). Some of the nitrated proteins have been identified. Structural proteins including mysoin heavy chain, α-actinin and desmin were found nitrated in control arterial myocytes (Mihm 2001). Heart succinyl-CoA (Turko 2001) and some proteins including myofibrillar creatine kinase (Mihm 2001a), prostacyclin synthase (Zou1996) were detected nitrated earlier.

1.10.1.2 Nitration of protein in pathological condition

Nitrated proteins have been found in non-stimulated cells under physiological conditions by a number of independent studies (Greenacre and Ischiropoulos, 2001). The significants of nitration under physiological condition is now

1.10.2 The nitrating agents

In 1994 Dr Joseph Beckman presented 3-nitrotyrosine as a footprint for peroxynitrite formation since then the presence of nitrated tyrosine has been used as a marker for detection of $ONOO^-$.

1.10.2.1 Peroxynitrite ($ONOO^-$)

Peroxynitrite, $ONOO^-$, is the product of a fast reaction between two free radicals NO and $O_2^{\cdot-}$.

$$NO + O_2^{\cdot-} \longrightarrow ONOO^-$$

The second orderrate constant for ONOO production was independently determined as 4.3, 6.7 and 1.9 x 10^9 M^{-1} S^{-1}. ONOO can also be formed by the reaction between nitroxyl anion (NO^-) and O_2 with a rate constant of 5.7 x 10^7 M^{-1} S^{-1}, much slower that of NO and superoxide. This reaction is of physiological relevance since NO^- is produced from NO catalysed by cytochrome C and concentrations of O_2 in vivo is many order of magnitude higher than $O_2^{\cdot-}$ (Beckman et al., 1992a).

System	Species	Location
CNS	Human	Spinal cord (Neurofilament L) Purkije cells of cerebellum, choroid plexus, cortical neurons
	Rat	Somata and dendrites of interneurons and spiny neurons of caudate-putamen nucleus, outer mitochondrial membranes, near plasma membranes in dendrites and within asymmetric synapses on dendritic spines, globus pallidus, astrocytes, small axons and synaptic vesicles in axon
	Murine	Spinal cord (Neurofilament L, Glial fibrillary acidic protein)
CVS	Human	Plasma (Albumin, 58kDa protein)
	Rat	Mesentric artery (60-65kDa)
GIT	Human	Basal cells of oral mucosa
Immune	Murine	Cortico-medullary junction & medulla of thymus
PNS	Rat	Sciatic nerves(Glial fibrillary acidic protein)
Renal	Human	Distal tubules, collecting ducts
	Rat	Proximal & convoluted tubules, endothelial cells of vas recta (40, 47, 58, 74, 80, 89, 102kDa)
	Murine	Kidney (66kDa)
Reproductive	Quail	Ovarian atretic follicles & post-ovulatory follicles
Respiratory	Rat	β_1-subunit of Na^+/K^+-adenosine triphosphate
Skeletal muscle	Rat	Diaphragm (50, 42,kDa proteins)

Table 1.2:
Detection of nitrated proteins under physiological condition (Greenacre and Ischiropoulos, 2001).

$ONOO^-$ is a strong oxidant capable of modifying most biological molecules, including such amino acids like tyrosine, tryptophan, cysteine and methionine (Alvarez et al., 1996; Mayer et al., 1995; Wolin, 1996). The specific reaction of $ONOO^-$ or its intermediates with protein tyrosine residues will cause nitration, which is readily reversible (Goldstein et al., 2000). $ONOO^-$ is not a free radical because the unpaired electrons on NO and $O_2^{\cdot-}$ combine to form the O-N bond. It acts as a nitrating agent, as well as a powerful oxidant, to modify proteins, lipids and nucleic acids. Several factors limit the rate of production and physiological effects of $ONOO^-$. Because of the short half-life for both NO and $O_2^{\cdot-}$ formation of peroxynitrite requires the presence of both components at the same time in the same place. Thus, due to both greater half-life and facile diffusion of NO compared to $O_2^{\cdot-}$, peroxynitrite formation will predominately occur nearer to the $O_2^{\cdot-}$ formation sites.

The formation of peroxynitrite is also limited by the presence of SOD. The micromolar concentrations of SOD can compete effectively for $O_2^{\cdot-}$ minimising the chance of $ONOO^-$ Production.

$$O_2^{\cdot-} \xrightarrow{\text{SOD}} H_2O_2 \qquad k = 2 \times 10^9\ M^{-1} S^{-1}$$

The influence of $O_2^{\cdot-}$ on the biological effects of NO has been known for years since the addition of SOD to physiological preparations did enhance the NO mediated effect (Beckman and Koppenol, 1996).

Peroxynitrite, when formed exists in protonation equilibrium with peroxynitrous acid. The ratio is depended on the local pH, since $ONOO^-$ is very unstable at physiological pH. At pH 7.4, 80% of peroxynitrite will be in anionic form (Crow et al., 1994). Unlike most all other molecules, which are more stable in their *trans* form, the *cis* peroxynitrite has been reported to be more stable (Symons, 2000; Worle et al., 1999). The *trans* configuration allows the hydroxyl group to attack nitrogen more easily, resulting in nitrogen dioxide and a hydroxyl radical (Fig 1.13). *Cis* peroxynitrite directly oxidises many important biological products like lipids, sulphydryls, iron-sulphur centre and zinc fingers. Consequently cis and trans peroxynitrite cannot directly intervert, because there is a significant energy barrier for isomerisation between the cis and trans anion, estimated to be 30 kcal mol^{-1} (Beckman et al., 1992b).

These reactions occur much faster than the formation of $OH^{\cdot}$ and $NO^{\cdot}_2$ and tyrosine nitration (see below). The *trans*-peroxynitrite is capable of acting as a two-electron oxidant in the present of a metal catalyst forming nitronium ion (NO_2^+).

As an alternative pathway $ONOO^-$ can also interact with CO_2 producing nitroso-peroxocarboxylate ($ONOOCO_2^-$, rate constant 5.8 X $10^4 M^{-1}S^{-1}$), which homolyses to carbonate radical ($CO_3^{\cdot-}$) and nitrogen dioxide ($^{\cdot}NO_2$), capable of nitrating the phenolic compounds (Squadrito and Pryor, 1998).The reaction of peroxynitrite with carbon dioxide is critical, because carbon dioxide is ubiquitous in biological systems.

Both peroxynitrite and peroxynitrous acid may cross biological membrane, via anion channels and passive diffusion, respectively (Fridovich et al 1995, Marla et al 1997, Dennicola et al 11998). The biological effects of peroxynitrite will be influenced by

its ability to permeate cell membrane and its diffusion distance. The biological half-life of peroxynitrite is estimated to be less than 100 ms, which is long enough for peroxynitrite to travel some distance (e.g. 5-20 μm) across extra and/or intracellular compartments (Marla et al., 1997a; Radi, 1998; Romero et al., 1999).

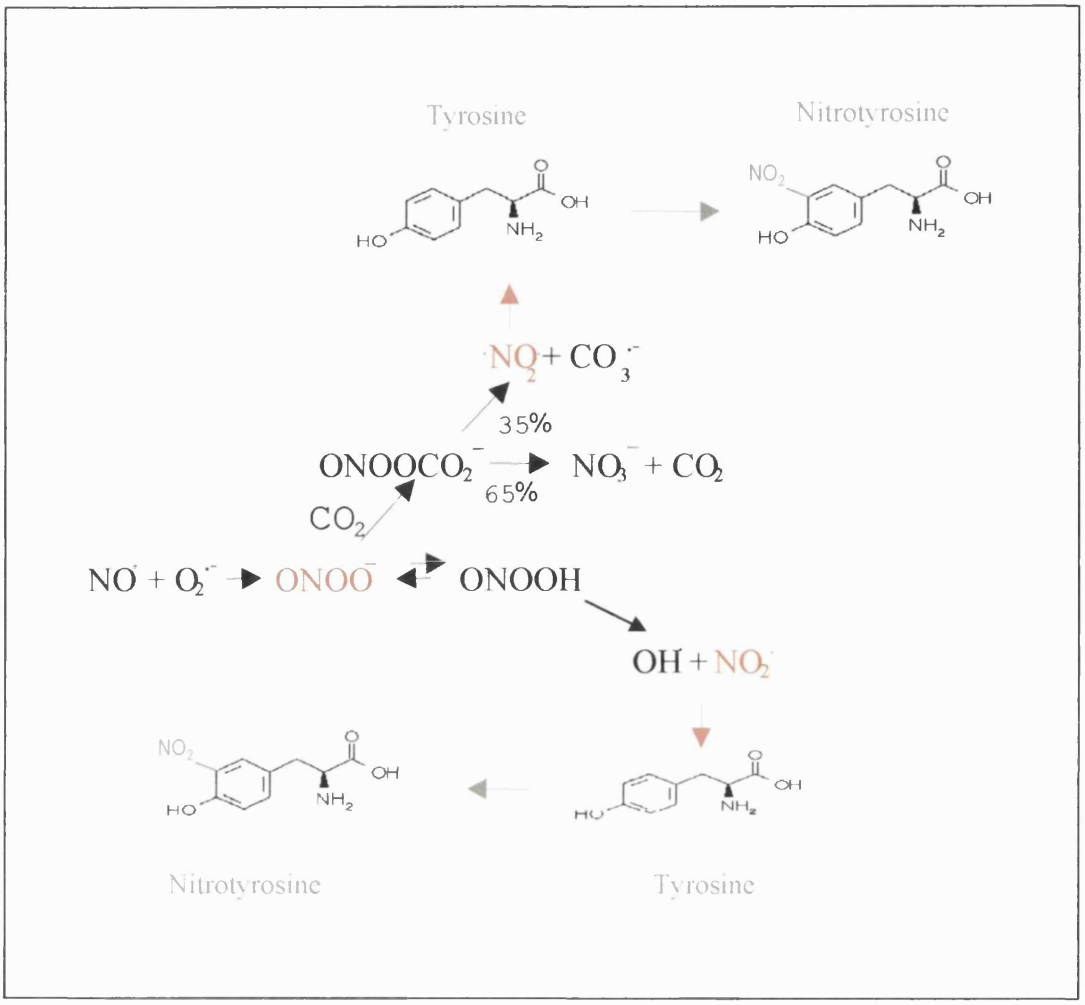


Fig 1.13: The pathways involved in metabolism of $ONOO^-$

$^{\cdot}NO_2$, the end product of $ONOO^-$ is the agent most capable for the nitration of tyrosine. The nitro group attaches to tyrosine residues and produces 3-nitrotyrosine. 3-nitrotyrosine is the marker for nitrated proteins *in vivo* and *in vitro*.

1.10.2.2 Other pathways for nitration of proteins

In contrast to Beckmans finding, Mayer and Pfeiffer demonstrated that the role of other nitrating agents, including nitrate on nitration of proteins is more important than $ONOO^-$, since they observed only little tyrosine nitration at physiological pH when NO (spermine NONOate) and $O_2^{\cdot-}$ (xanthine oxidase) were generated simultaneously to form $ONOO^-$ compared to treatment with pre-formed $ONOO^-$. They concluded that $ONOO^-$ might not be the most important nitrating agent *in vivo*. However Sawa and Reither published results, which suggested that accumulation of urate and the rapid consumption of oxygen by xanthine oxidase in the Pfeiffer and Mayer study might have caused an erroneous conclusion (Reiter et al., 2000; Sawa et al., 2000).

More recently, other publications revealed that $ONOO^-$ formation is not the only pathway by which, nitration of proteins may occur and some $ONOO^-$–independent pathways have also been suggested.

The role of certain haem-proteins such as different peroxidases on nitration of free and protein bound tyrosines has been established (Grzelak et al., 2001a; Kilinc et al., 2001).

1.10.2.3 Peroxidases as nitrating agents

The role of peroxidases on nitration of tyrosines has been established by different groups (Brennan et al., 2002; Gaut et al., 2002). Certain peroxidases catalyse the H_2O_2-dependent nitration of tyrosine. One of these enzymes is myeloperoxidase, which normally uses H_2O_2 and Cl^- to produce HOCl. Either HOCl or myeloperoxidase can then oxidise NO_2^- (formed from oxidation of NO) to form nitrogen species like NO_2Cl and NO_2 both capable of nitrating tyrosine residues.

Myoglobin also increases tyrosine nitration independently of $ONOO^-$ (Kilinc et al., 2001). Myoglobin catalyses the oxidation of nitrite and promoted nitration of tyrosine in the presence of H_2O_2. The presence of ferryl (Fe^{4+}) myoglobin and an acidic pH close to six was required for this reaction.

Eosinophil peroxidase also generates nitrotyrosine via oxidation of nitrite (NO_2^-) to form $^{\cdot}NO_2$. Using EPO and MPO-knock out mice, it was demonstrated that leukocytes peroxidase participates in nitrotyrosine formation in vivo and catalyse the generation of $^{\cdot}NO_2$ formation using H_2O_2 and NO_2^- as substrate (Brennan et al., 2002).

Another $ONOO^-$-independent mechanism, suggested for nitration is the direct reaction of NO with tyrosyl radicals. It has been shown that NO forms an unstable complex with the tyrosyl residues of PGH_2 synthase, which then can be oxidised to form nitrotyrosine.

1.10.3 Metabolism of nitrated proteins

Nitrotyrosine formation leads to modification of different enzymes, including inactivation of Mn-SOD, glutathione reductase (GR) and tyrosine hydroxylase or activation of cyclooxygenase (CO). Since these enzymes are important in cell signalling, a de-nitration mechanism seems to be crucial in biology.

Nitrotyrosine is a stable adduct involving a carbon-nitrogen bond that is difficult to remove chemically. Phosphotyrosine on the other side can be removed by addition of water across the phosphorus oxygen bond. This reaction will release 5-10 $kcalmol^{-1}$ of energy, whereas it requires 70-80 $kcalmol^{-1}$ to break a carbon-nitrogen bond. The routes of removing nitrotyrosine *in vivo* are not known yet, but proteolysis is likely to

be a major route of removal. Nitrotyrosine can be found in human urine as an amino acid and as decarboxylated and deaminated products (Ohshima et al., 1990).

There is possible that nitrated proteins are phagocytized by macrophages even that this has not been proved yet. It has also been suggested that nitration of a single tyrosine in a protein might accelerate the degradation of the protein by the proteasomes.

Recently the presence of a nitratase has been suggested in tissue homogenates of rat spleen, lung and heart by different investigators (Gow et al., 1996; Kuo et al., 1999).

1.10.4 Nitration of proteins in platelets

The influence of $ONOO^-$ on platelet functions have been investigated by different groups. This effect of $ONOO^-$ showed to be concentration-dependent. At high concentrations, in excess of 150μM, peroxynitrite acts as a platelet agonist by stimulating aggregation (Moro et al., 1994). At lower concentrations or in the presence of plasma, peroxynitrite acts as a platelet inhibitor, although much less effectively than NO (Brown et al., 1998). The mechanism of this inhibition has been proposed to be cGMP-dependent via an increase of formation of nitrosothiols with endogenous glutathione in the platelets (Mayer et al., 1995). Platelets may recover from this within minutes and then they might become activated again with collagen.

$ONOO^-$ causes a dose-dependent peroxidation of the lipids in platelets membrane, which changes the membrane structure (Radi et al., 1991). Lipid peroxidation will affect the exposure of specific receptors to platelet agonist and inhibits platelet adhesion or aggregation (Nowak and Wachowicz, 2001a).

1.10.5 The connection between tyrosine phosphorylation and nitration

The tyrosine phosphorylation has been well investigated during last decade. There are enough evidence to connect the tyrosine phosphorylation of proteins to the activation of platelets and other cells. Since many years, tyrosine phosphorylation has been known as an important regulator of signal transduction in different kinds of cells. A cross-reaction between nitration and phosphorylation in different proteins was reported. The idea that nitration may interfere with normal signal transduction through inhibition of tyrosine phosphorylation was studied by some groups (Gow et al., 1996). It has been reported that nitration of tyrosine in the presence of $ONOO^-$ is responsible for inhibition of tyrosine phosphorylation by 51%, suggesting that nitration does interrupt the signal transduction produced by tyrosine phosphorylation in endothelial cells (Gow et al., 1996). The interruption of tyrosine phosphorylation by tyrosine nitration can also be a kind of signal for the cell survival. More investigations need to be done to clear all hesitation in between tyrosine nitration and phosphorylation.

1.10.6 The role of nitration on the function of proteins

The exact role of nitration on the function of individual proteins is still not clear. There are some evidence that nitration of proteins down regulate their activities, including SOD and PGH_2 (Zou et al 1998, Mac-Milan et al 1999). In contrast, $ONOO^-$ does up-regulate the activity of the Src family in human pancreatic tumour tissues (Go et al., 1999; Jope et al., 2000; Li et al., 1998), tumour tissues (Mac Milan-Crow et al 1999) erythrocytes (Mallozzi et al., 1995) and synaptosomes (Di Stasi et

al., 1999). More investigations need to be done to clear the effect of nitration of individual proteins.

Nitration of proteins is a reversible phenomenon. It might up-regulate phosphotyrosine dependent signalling in cell-free systems and cell cultures.

There are different hypotheses about the mechanism of denitration of proteins *in vivo*. The increase of nitration might start the proteolytic degradation, since it has been shown that tyrosine nitration increases degradation of glutamine synthetase (Berlett et al., 1996). It has also been reported with the same group, that human plasma and rat tissue homogenate from brain, lung, and liver, had the ability to remove the nitrotyrosine epitope leading to decrease antibody binding (Gow et al., 1996). The human plasma is capable to remove nitrotyrosine in a time, concentration and temperature-dependent manner. These results raise the question that how nitrated proteins can still be detected in human plasma? In the same study Gow et al showed that in contrast to fresh plasma, the plasma, which was pre-incubated with $ONOO^-$ did not show any nitrotyrosine degrading activity suggesting that human plasma in the presence of $ONOO^-$ may loose its ability to remove the nitrotyrosine epitope.

It is possible that other mechanisms may be involved in denitration or breakdown of proteins in different cells and more investigations needs to be done.

1.11 Aim of the thesis

To investigate possible mechanisms of synergism between NO and H_2O_2 on the inhibition of platelet aggregations specifically two broad issues were considered:

1) The role of cGMP and the enzyme soluble guanylyl cyclase (sGC) in this synergism
2) To investigate the role of protein nitration

CHAPTER TWO:

MATERIALS AND METHODS

2.1 Equipment

Centrifuge Labofuge Model 400 (Heraeus, Germany)

Havertown, U.S.A.)

Electrophoresis equipment (Bio Rad, Amersham, Novex)

Multi-image camera (Flowgen, U.K.)

Platelet aggregometer (Chrono-Log Corporation, Model 490, optical aggregometer,

Semi-dry transfer (Bio rad, U.K.)

Spectrophotometer (MRX Microplate Reader, Dynex Technologies, Ashford, Middlesex, U.K.).

Transfer tank for wet transfer (Amersham, U.K.)

Thrombocounter Coulter Electronics Ltd, U.K.)

2.2 Materials used

Butterfly needles (sterile, 19 or 21 gauge; Venesystems, U.K)

Centrifuge tubes (Corning Ltd, U.K.)

C-bound nitrocellulose membrane (Amersham,U.K.)

Filter paper Wattmann 3 (Wattmanninternational, maidenstone, U.K.)

Gas sampling tubes (University College London glassblower, U.K)

Gas-tight syringes (Hamilton, U.S.A)

Micro magnetic stir bars (Pollution and Process Monitoring.,U.K.)

Platelet aggregometer cuvettes (Pollution and Process Monitoring.,U.K.)

Plastic universal collection tubes (B.D.H)

Polypropylene sterile syringes (Becton Dickinson Ltd, U.K)

Pre-cast 4-12% gels (Novex)

Rubber septa (Phase seperators, U.K)

Super signal West Dura Extended duration (Pierce, U.S.A.)

2.3 Reagents list

Acrylamide bis 30% (Bio Rad, CA, U.S.A.)

Acetylsalicylic acid (Sigma Chemicals Co., U.K.)

Anti- nitrotyrosine antibody (06-284, Upstate, Biotechnology, U.K.)

Anti-phosphoserine antibody (804-166-C100, Clone 7F12, Alexis Corporation, Ltd, U.K.)

Anti- phosphotyrosine antibody (PY20, CN Bioscience Company, U.K.)

Anti-Lyn antibody (Upstate, Biotechnology, U.K.)

Anti-PI3 kinase p85 (Upstate, Biotechnology, U.K.)

Anti-VASP antiserum (210-725-R100, Alexis Corporation, Ltd, U.K.)

Anti-Syk antibody (05-434, Upstate, Biotechnology, U.K)

Avidin-Horseradish Peroxidase Conjugate (Amersham, U.K.)

Anti-α-profilin antiserum (donkey) (Alexis Corporation, Ltd, U.K (Sigma Chemicals Co., U.K.)

Anti-rabbit Ig G, peroxidase-linked species (Amersham, U.K.)

Anti-mouse Ig G, peroxidase-linked species (Amersham, U.K.)

Ammonium persulfate (Sigma Chemicals Co., U.K.)

ALLN (Calbiochem, CA., U.S.A.)

Antioxidant (Invitrogen, Paisley, U.K.)

Bovin serum albumin , BSA (Sigma Chemicals Co., U.K.)

Biotinilated SDS-Page standard (Bio Rad, CA, U.S.A.)

Calpeptin (Calbiochem, CA., U.S.A.)

Creatine phosphate/Creatine phosphokinase (Sigma Chemicals Co., U.K.)

Cyclic guanosine monophosphate Kit (Alexis Corporation, Ltd, U.K.)

Cyclic adenosine monophosphate Kit (Alexis Corporation, Ltd, U.K.)

Epigallocatechine gallate (Sigma Chemicals Co., U.K.)

Human thrombin (Sigma Chemicals Co., U.K.)

Human collagen (Axis-shield Diagnostics, Dundee, U.K.)

Hydrogen peroxide 30% (w/w) (Sigma Chemicals Co., U.K.)

3-Isobutyl-1-methylxanthine (IBMX) (Sigma Chemicals Co., U.K.)

L-NAME (Alexis Corporation, Ltd, U.K.)

MG-132 (Calbiochem, CA., U.S.A.)

MOPS running buffer (Invitrogen, Paisley, U.K.)

Nu-page Transfer buffer (Invitrogen, Paisley, U.K.)

Nitric oxide gas (British Oxygen Company, U.K)

ODQ, oxadiazoloquinoxaline-1-one (Tocris Cookson, U.K.)

Protein kinase A inhibitor (CN Bioscience Company, U.K.)

Protein kinase G inhibitor (CN Bioscience Company, U.K.)

Prostaglandin I_2 (Sigma Chemicals Co., U.K.)

Phospho p38 MAP kinase anti-body (New England Biolabs, Inc, U.S.A.)

Saponin (Sigma Chemicals Co., U.K.)

S-nitrosogluthathione (Tocris Cookson, U.K.)

Thrombin (Sigma Chemicals Co., U.K.)

TEMED (B.D.H.)

Uric acid (Sigma Chemicals Co., U.K.)

YC-1 (Alexis Corporation, Ltd, U.K.)

Zaprinast (Tocris Cookson, U.K.)

2.4 Buffers and solutions

2.4.1 Platelet preparation:

- **Acid Citric Dextrose (ACD):**
  113.8mM glucose, 29.9mM tri-Na citrate, 72.6mM NaCl, 2mM citric acid pH 6.4

- **1 M Tris-HCl (pH 9.9)**
- **Modified Tyrode's buffer:**
  15mM NaCl, 5mM Hepes, 0.55mM $NaH_2PO_4.2H_2O$, 7mM $NaHCO_3$, 2.7 mM KCl, 0.5mM $MgCl_2$, 5.6 mM glucose pH 7.4

- **Washing buffer:**
  36mM Citric acid, 10 mM EDTA, 5mM glucose, 5mM KCl, 90mM NaCl pH 6.5

- **0.3M Citric acid**
- **3.8% Tri-Na-citrate**

2.4.2 Platelet aggregation

All stock reagents were prepared 100x more concentrated than the final concentration and diluted 1:100 with platelets. The maximum volume of each reagent added to 100µl of platelet was 5µl.

- NO solutions: stock solutions were made in different concentrations depended on the final concentrations required.

- H_2O_2 solution was prepared freshly, prior to each experiment from a 30% (w/w) stock solution and kept on ice. A series of concentrations were made X100 more concentrated than the final concentration, which were then diluted 1:100 with platelet samples. Each aliqute of H_2O_2 solution was only used once during experiments/sample preparation.

- Thrombin: 2U/ml
- Collagen: 50µg/ml

2.4.3 cGMP and cAMP measurements

- 3-Isobutyl-1-methylxanthine, IBMX: 20mM
- Saponin 300 mg/ml of platelet suspension.
- Diethyl ether: was saturated with water and mixed properly before use

2.4.4 Sample preparation:

- **Laemmeli's Sample buffer**:

0.125M Tris-HCl, 4% SDS, 20% v/v glycerol, 0.2M DTT, 0.02 % bromophenol blue (pH 6.8). The buffer was aliquted in 500µl quantities in eppendorf tubes and kept on -20 °C until use. Each aliqute was only defrosted once, and discarded after use.

2.4.5 Gel Electrophoresis:

- **0.5M Tris-HCl pH 6.8 (stacking gel buffer)**

  12.0 g Tris (FW 121.1) in 200 ml H_2O. Kept on 4◦C only for 1-2 months.

- **1.5M Tris-HCl pH 8.8 (running gel buffer)**

  36.3 g Tris (FW 121.1) in 200 ml H_2O. Kept on 4◦C only for 1-2 months.

- **SDS running buffer:**

  0.025M Tris-HCl, 0.192M glycine, 0.1 % SDS pH 8.3. This buffer was made fresly prior to each experiment.

- **Coomassie staining solution**

  0.25 g Coomassie Brilliant blue, 400ml methanol, 70 ml acetic acid, up to 1 liter in double disiled water. Kept in room temperature.

- **Destaining solution**

  400 ml methanol, 70 ml acetic acid, to final volume of 1 liter of ddH_2O

- **SDS transfer buffer:**

  0.025M Tris-HCl, 0.192M glycine, 0.1 % SDS, 20 % methanol. Was made freshly prior to each experiment.

- **Blocking solution**

  0.5% BSA, 1% PVP, 1 % PEG, 0.2 % Tween 20, 10mM NaF in 2 X PBS. Was made freshly prior to experiment.

- **Washing buffer**

  0.2 % Tween 20 in 1X PBS. Was made fresh prior to the experiment.

2.5 METHODS

2.5.1 Washed platelet preparation:

Whole blood taken from healthy volunteers was taken into a syringe containing the anticoagulant, acid-citrate-dextrose (ACD, Ratio 1:4 to blood), and the syringe was gently inverted to mix the anticoagulant with the blood. The blood was then transferred to universal tubes and centrifuged at 120g for 20 min at 20°C with no brake to produce platelet rich plasma (PRP). PRP was transferred into a sterile plastic centrifuge tube by sterile pasture pipettes, leaving at least 0.5 cm of PRP on the top of blood cells to reduce the risk of contamination by erythrocytes and neutrophils.

Washed platelets were prepared by addition of 50nM PGI_2 to PRP and centrifuging at 800g for 18 min at 20°C. The supernatant, platelet poor plasma, was discarded and the inside of the tube was wiped clean with tissues to remove any trace of plasma. Platelet pellet were then resuspended in 1 ml of modified Tyrode's buffer and the concentration of platelets were counted with a thrombocounter and diluted with the same buffer to give a final concentration of 3×10^8 platelets/ml (approximate plasma density). Platelets were left for 1h at room temperature for a full recovery of PGI_2.

PGI_2 increases the concentration of cAMP and is used to inhibit platelet activation during the isolation procedure. cAMP is normally to be broken down by endogenous phosphodiesterases during following 1-2 hours. During this time, the platelets are recovered and responsed to stimuli after this time.

2.5.2 Alternative method for preparation of washed platelet

Whole blood taken from healthy volunteers was taken into 3.8% tri-sodium citrate (citrate: blood 1: 9) inverted and centrifuged at 130g for 15 min at 20°C to produce PRP. The pH of PRP was then adjusted to 6.4 by the addition of 0.3M citric acid and the platelets re-centrifuged at 750g for 10 min at 20°C. The supernatant was discarded and the platelet pellet resuspended in EDTA-wash buffer. The pellet was then centrifuged again under the same conditions and the new supernatant was discarded, the platelet pellet was resuspended in modified Tyrode's buffer.

2.5.3 Preparation of NO solution

NO solutions were prepared from nitric oxide gas diluted in double distilled water (ddH_2O) according to the method of Palmer et al 1987. ddH_2O was boiled for 10-20 min and then allowed to cool down slowly to 70°C at room temperature. The water was then pulled into specially designed 100ml sampling glass tubes (made by the glassblower at University College, London), which was sealed by a Teflon-rubber septum at the end and subsequently de-oxygenated by N_2 gas for 45 min. The other end of the tube was then carefully sealed with a similar rubber septum to avoid the re-oxygenation of the water. NO was injected into the tube, through the septum using a gas-tight Hamilton syringe.

If the water has not been fully de-oxygenated, it will generate a yellowish brown colour following the addition of a high concentration of NO, which is due to the formation of NO_2.

The concentration of NO was calculated regarding Avogadro's Law: one mole of any gas at S.T.P. occupies approximately $22400cm^3$.

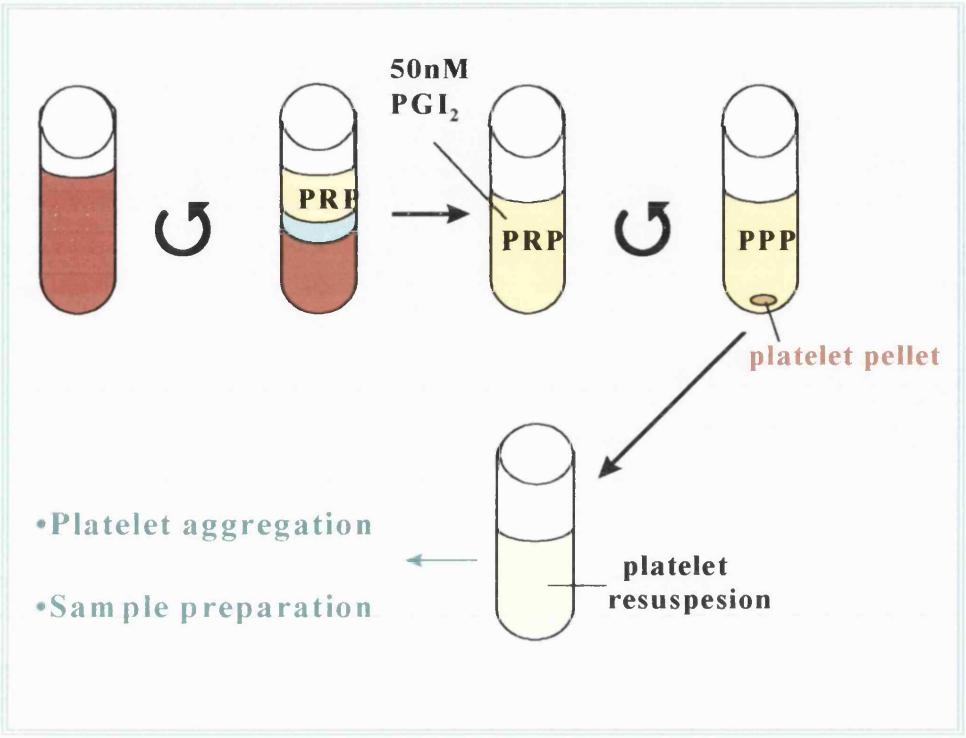


Fig 2.1:

Preparation of washed human platelet from plasma as described in the method.

2.5.4 Platelet aggregation in vitro

Platelet aggregation was performed using a dual channel aggregometer module, fitted with a dual pen chart recorder, based on the method of Born (Born et al 1962). The technique measures increases in light transmission of a stirred platelet suspension. Suspension of platelet has light scattering properties. Addition of an agonist, like thrombin, induces a reversible shape change of the platelets resulting in increased light transmission, which is detected by a photocell. Aggregation may be one of two types: *primary aggregation*, which is reversible and does not involve release of granule contents, and *secondary aggregation*, which is irreversible and is accompanied by granular secretion. A transitory phase between primary and secondary aggregation occurs.

The aggregometer was calibrated using either a WP suspension or PRP to give the baseline of 0% light transmission. Tyrode's buffer or PPP was used for the maximum reading (100% light transmission), as these would represent most closely an aggregated sample (Fig2.2). Aggregation tests were performed in duplicate, using 100 µl of WP in flat-bottomed glass cuvettes kept at 37°C with continual stirring (800rpm) by micro magnets. Platelets were stirred for 1 min prior to addition of the agonist to allow for temperature equilibration and to observe any spontaneous aggregation.

Aggregation was measured as percentage increases in light transmission through a stimulated platelet preparation, relative to the light transmission through a resting platelet preparation.

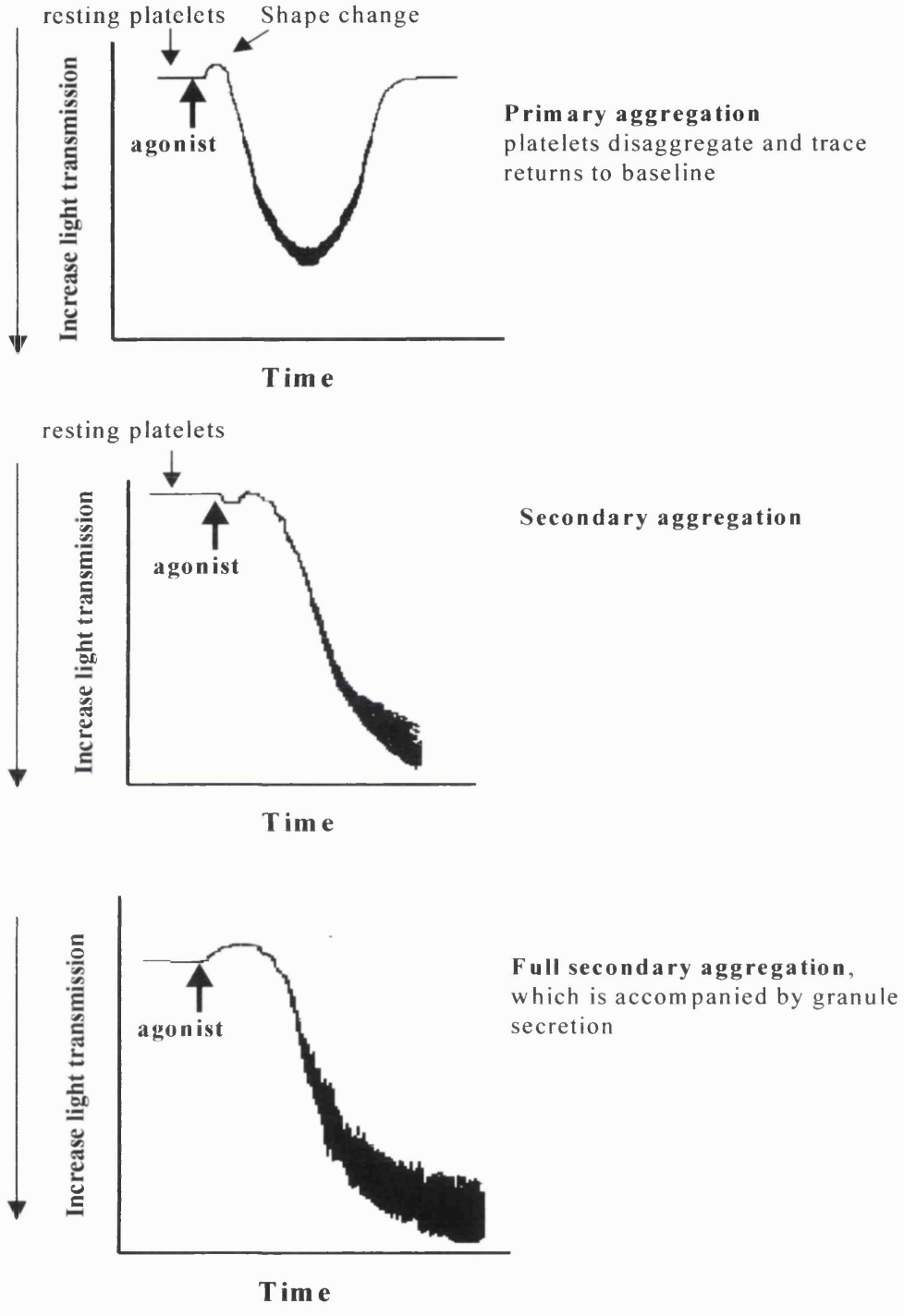


Fig 2.2:

Diagrammatic representation of different types of aggregation traces:

a) Primary or reversible aggregation, b) threshold response, c) secondary or irreversible aggregation.

The agonists used in this study were either 0.02 U/ml thrombin or 0.5 μg/ml collagen and they were added from stock solutions in volumes not more than 5μl and the aggregation was observed for 3 min after last addition.

The inhibitors of protein kinases or green tea extracts, epigallocatechin gallate (EGCG) were added to platelet samples for up to 30 min prior to addition of NO and H_2O_2.

2.5.6 Preparation of sGC samples for cGMP measurement

2 .5.6.1 Preparation of platelet lysate and post mitochondrial fraction

Washed platelets, resuspended in Tyrode's buffer and rested for 1 hour, were incubated with saponin 3mg/ml of platelet for 15 min, during which time the platelets were kept on ice. Saponin is a detergent, which produces pores in the cell membrane by breaking down the phospholipids bilayer. An alternative method of lysing platelets is repeated cycles of freezing and thawing of the cells, which was also used in some experiments and the results were compared.

For preparation of postmitochondrial fraction (cytosol containing supernatant), the cell lysate was centrifuged at 5000g for 15 min at 4°C. The supernatant, which contained cytosolic fraction was separated and kept on the ice. The pellet containing cell fragments and the membranes was also collected for looking at particular proteins.

2.5.6.2 Preparation of platelet samples for cGMP measurement

Washed platelet, cell lysates and cytosol were all pre-incubated either in the presence of a non-specific cGMP phosphodiesterase inhibitor (IBMX, 200μM) or the specific (Zaprinast, 50μM, Mcmahon et al 1989) for a period of 15-30 min before sample preparation. The samples were then stimulated with either NO or H_2O_2 alone or both together for 10 min, incubating on the platelet aggregometer at 37°C with stirring. The reaction was stopped with addition of 14% trichloroacetic acid (TCA) in equal volume to make a final concentration of 7.5%.

Purified soluble guanylyl cyclase was diluted in modified tyrode's buffer to make a concentration of 2.3ng/ml. The enzyme was then added into a buffer containing 3mM Mg^{2+}, 3mM DTT, 0.5mg bovine serum albumin/ml, 300μM GTP and 50mM triethylamine hydrochloride at the same time as NO ± H_2O_2 was added. The samples were then incubated on the platelet aggregometer at 37°C with stirring for 10 min.

The reaction was stopped with addition of 14% trichloroacetic acid (TCA) in equal volume to make a final concentration of 7.5%. The samples were kept on –20°C until use.

The buffer contains the chemicals that are required for conversion of GTP to cGMP.

$$\mathbf{GTP} \xrightarrow[\mathbf{10\ min.}]{\mathbf{37°C}} \mathbf{cGMP}$$

2.5.6.3 Measurements of cGMP

For the measurement of cGMP, an enzyme immuno assay kit was used. This assay is based on the competition between free cGMP and cGMP tracers, linked to the enzyme acetylcholinesterase, which compete for limited numbers of cGMP specific rabbit antiserum-binding sites. The concentration of cGMP tracer is always constant while the concentration of free cGMP (standards or samples) varies. This rabbit antiserum-cGMP complex (either free or tracer) binds to the mouse monoclonal anti-rabbit antibody that has been previously attached to the wells. The plate was washed to remove any unbound reagent. Addition of a substrate for acetylcholinestrase gives a distinct yellow colour and absorbs at 412 nm.

The absorbance read at this wavelength indicates the amount of bound cGMP tracer, which is inversely proportional to the amount of free cGMP present in the well.

Absorbance $\propto$ [Bound cGMP tracer] $\propto$ 1/ [cGMP].

2.5.6.4 Measurements of cAMP

Similar kit was used for the measurements of cAMP based on the same technique. In this assay, the competition is between free cAMP and cAMP tracers, linked to the enzyme acetylcholinesterase, which compete for limited numbers of cAMP specific rabbit antiserum-binding sites. The concentration of cAMP tracer is always constant while the concentration of free cAMP (standards or samples) varies. This rabbit antiserum-cAMP complex (either free or tracer) binds to the mouse monoclonal anti-rabbit antibody that has been previously attached to the wells. The plate was washed

to remove any unbound reagent. Addition of a substrate for acetylcholinestrase gives a distinct yellow colour and absorbs at 412 nm.

The absorbance read at this wavelength indicates the amount of bound cAMP tracer, which is inversely proportional to the amount of free cAMP present in the well.

2.5.7 Preparation of platelet samples for phosphorylation and nitration study

Washed human platelets were separated from plasma as described in 2.5.1 (page 73). Platelet resuspension was then rested on the lab bench for a period of 0 to 90 minutes dependent on the experiment. Specific information regarding sample preparation will be given in prior to results in each result chapter.

2.5.7.1 Protein assay (Modified Lowry assay)

Protein concentration was determined using modified micro-plate Lowry proteins assay.

Standard curve

A stock solution of 4mg/ml BSA was used for preparation of the standard curve.

Conc. BSA (µg)	*volume BSA (µl)*	*lysis buffer*
400	100	0
200	50	50
100	25	75
80	20	80
60	15	85
40	10	90
20	5	95
0	0	100

The total volume used of samples or standards was 100µl. 100µl of Lowry modified solution was pipeted to the wells containing either the standards or the samples. The plate was incubated for 20 minutes at room temperature. After this time, 50µl of Folin solution was added to each well and the plate was then incubated for 5-10 minutes. The plate was the read at 720nm or between 500-800nm.

2.5.7.2 Separation of platelet proteins

Proteins from platelets and platelet fractions were separated either on sodium dodecyl sulphate polyacrylamide gels 10 or 15 % (SDS-PAGE) or with pre-cast 4-12% gradient gels.

2.5.7.2.1 Preparation for SDS poly acrylamide gel electrophoresis

SDS gels were prepared according to the method of Laemmeli (1970).

The polymerisation was achieved by the addition of ammonium persulphate and TEMED. Therefore is important that APS and TEMED are added to the solution immediately before pouring of the gels *.

Chemicals	Separation Layer (ml)	
	10%	15%
bis acrylamide	10	15
1.5 M Tris-HCl pH 8.8	7.5	7.5
10% SDS	0.3	0.3
ddH_2O	12	7.1
ammonium persulphate (APS)*	0.150	0.150
TEMED*	0.010	0.010

ammonium persulphate (APS) and TEMED were added to the solution immediately before pouring of the gels. The tops of the gels were covered with either ddH2O or saturated isobutanol to avoid the dryness of the surface.

After polymerisation of the first layer the stacking layer is prepared and added on the top and the combs were sets.

Chemicals	**5% Stacking layer (ml)**
Bis acrylamide	0.44
0.5 M Tris-Hcl pH6.8	0.83
10% SDS	0.033
ddH_2O	2.03
ammonium persulphate (APS)*	0.017
TEMED*	0.0017

2.5.7.2.2 Preparation of the samples for both systems of electrophoresis

Prior to electrophoresis platelet samples were centrifuged at 3000 rpm for 5 min at 4°C. Aliquot of each sample equivalent to 50-100µg protein was boiled at 70°C for 10 mins in the presence of Laemmeli sample buffer. Samples were then loaded and electrophoresed until the dye front was reached the bottom of the gel.

2.5.7.2.3 NOVEX pre-Cast Gels (Nu-PAGE 4-12% gradient gels)

50-100 μg of protein was loaded per well and the gels were run with constant voltage at 200V for 60 minutes.

2.5.7.2.4 Coomassie Brilliant Blue staining

After electrophoresis the stacking gels were removed and the resolving gels were stained with Coomassie blue for 30 minutes. The gels were then destained either with ddH2O over night or in destainer solution for 1 –2 hours with constant shaking.

2.5.7.2.5 Western blotting

After electrophoresis, proteins were blotted onto either PVDF or high bound nitrocellulose membranes using semi-dry transfer for 45 min with a constant current at 125mA.

The membranes were cut in dimensions 8x9 cm. prior to blotting PVDF membranes were soaked in 100 % methanol for 10 minutes and nitro-cellulose membranes were soaked in the transfer buffer for 30 minutes. 12 pieces of Whatman No.3 filter papers were cut in dimension 9x10 cm pre-soaked in transfer buffer for a few minutes prior to transfer. The filter papers, membranes and the gels were set together by Sandwich model with following order, anode (+), filter papers, membrane, gel, filter papers and catode (-). The proteins were transferred for 1 hour at 120 volts or 1 hour at 2 mAmps/cm^2 of membrane.

2.5.7.2.6 Pancake S staining

In all experiments after transfer, the membranes were rinsed in PBS and stained by Ponseaue S for 5 minutes. This staining technique is an easy and quick way to check if the gels were running all right and if the transfer was fine.
The membranes were then rinsed in water 3 time and then washed in 20 ml of ddH_2O for 5 minutes.

2.5.7.2.7 Immunobloting

The membranes were blocked in the blocking solution for 2h at room temperature. After blocking the membranes were probed with primary antibodies overnight at 4°C. The membranes were later washed 3x5 minutes and 1x15 minutes in 0.5% PBS tween and then incubated with secondary antibodies and Streptavidin Horseradish Peroxidase for 1h for a maximum period of 1h. After incubation with secondary antibody the membranes were washed 3x5 minutes and 1x 15 minutes. Both primary and secondary antibodies were diluted in the blocking solution in the appreciate dilution.

2.5.7.2.8 Antibodies

The phosphoserine antibody was used at a dilution of 1 in 500. When the phosphotyrosine antibody because of a better specificity was used in 1 in 1000 dilution. Both anti-nitrotyrosine antibodies were used in a dilution of 1 in 500. Anti-VASP antiserume, which always showed very good specificity, was always used in 1:2000 dilutions. The anti-nitrotyrosine antibodies used in this study are the best

available antibodies in the market, but still the specificity of both of them are not good enough. Some differences in the binding of antibodies were detected in different experiments.

2.5.7.2.9 Detection of protein modification (Visualisation of the bands)

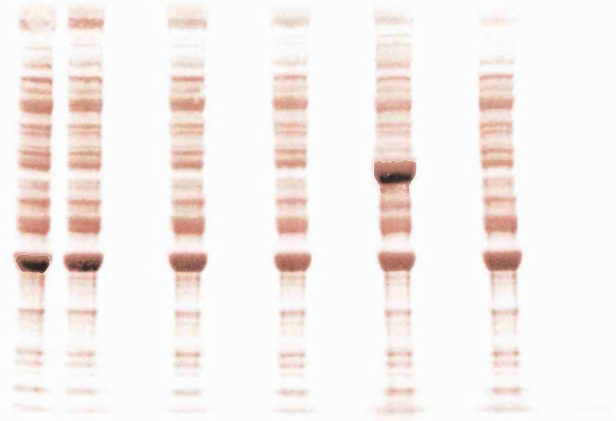
To detect any modification of proteins, membranes were incubated for 5 min with either ECL or West Dura super signal. The substrate reacts with the peroxidase and produce light, which is catch with the Multi-Image Camera.

2.5.7.2.10 Comparison between SDS-PAGE and pre-cast (NU-page) gradient gels

In the beginning of the project, Nu-PAGE gels and the NOVEX Tris- Glysine gels were used. Neither of these commercial gels were able to separate the double bands of our interested protein. One explanation can be that linear gels are better for the separation of proteins in this range of molecular weight. May be in gradient gels the bands are not separated as well as the 10% gel. The buffer system for the running and transfer, which are purchased from the Invitrogen are different from the traditional SDS-PAGE system. It is not clear if the buffers will make different. However, because of this problem we choose to use the pre-cast gradient gels when we are looking at total proteins modification, because of a better separation and a better transfer of all proteins. We preferred to prepare our own tris-glycine gels when

looking at the double 46 and 50kDa band. These gels were more reliable and more economic.

a)



b)

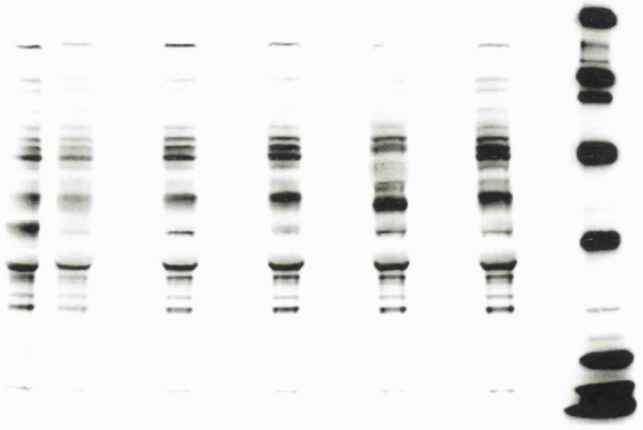


Fig 2.3:

a) membrane stained with Ponceau S after transfer of proteins

b) same membrane after Immunobloting with antibody

2.6 Preparation and characterization of polyclonal antibody (RF1256) to nitrated proteins

The polyclonal antibody was raised in a similar way to the commercial polyclonal antibodies. For this aim, the rabbits were immunised with peroxynitrite-treated Keyhole Limpet Hemocyanin (NT-KLH) using a schedule designed to produce high titre IgG antibodies. Antibody-positive serum was treated with a sequence of 50% saturated ammonium sulphate, protein G-agarose chromatography and affinity purification. The last step was performed using a synthetic peptide containing glycine and 3-nitrotyrosine (Gly, Ntyr) was synthesised. Poly (Gly, Ntyr) was immobilised to a 5ml HiTrap (Amersham Pharmacia Biotech) NHS-Sepharose affinity column and immune rabbit IgG chromatogrammed. Retained protein was eluted with Glycine/HCl buffer, pH 2.5. Each fraction was tested for its ability to bind to peroxynitrite-treated bovine serum albumin immunised to plastic wells, using a semi-quantitative ELISA developed specifically for this purpose (Barabanli et al 1999). Antigen binding was fractions were pooled, dialysed against a total of 1000 volumes of 200mM phosphate-buffered saline, pH 7.4 (PBS) and finally to 10mM, before being concentrated ten-fold to a protein concentration of 92μg/ml. This affinity-purified antibody was characterised for specificity, primarily using the ELISA that utilised the commercially available (Upstate) anti-nitrotyrosine polyclonal antibody. RF1256 showed similar sensitivity against nitrated bovine serum albumin as the commercial polyclonal. In this study, RF1256 will be used parallel to the commercial polyclonal antibody in the most nitration experiments.

2.7 Statistical analyses

All experiments were performed at least three times. Averages were expressed as mean ± SEM. Statistical significance was analysed by paired Student t-test. Values of of $P<0.05$ were considered statistically significant.

CHAPTER THREE:

THE ROLE OF cGMP IN THE SYNERGISM BETWEEN NO AND H_2O_2

3.1 Introduction

Nitric oxide is a physiological inhibitor of the activation of platelets by agonists such as collagen and thrombin (Radomski et al., 1987). NO may act as an anti-thrombotic agent as well as a vasodilator. The mean target for physiological effects of NO *in vivo* is soluble guanylyl cyclase (sGC). NO activates sGC by binding to the prosthetic ferrous haem with high affinity to form a ferrous-nitrosyl-haem complex resulting in subsequent changes in protein conformation and an increase of cGMP (Ignarro, 1989).

H_2O_2, formed by dismutation of superoxide anion, is produced in large quantities by activated neutrophils, but also by most cells during their normal respiration, including platelets. H_2O_2 is released by platelets at rest and after activation. Platelets may also be exposed to H_2O_2 prior to activation at endothelium surface (Kinnula et al., 1991; Maresca et al., 1992). Addition of exogenous H_2O_2 has been reported to both enhance and inhibit platelet aggregation as described in general introduction. Hydrogen peroxide has also been reported to have direct effect on other tissues. It induces the relaxation of arterial smooth muscle (Burke-Wolin et al., 1991b), which may result from an increase in the production of NO by increase activity of NOS (Valen et al., 1996). Others have proposed that the mechanism may be related to the actions of the hydrogen peroxide by formation of compound I in catalase in smooth muscle (Burke and Wolin, 1987; Wolin and Burke, 1987), although this enzyme is not present in platelets in large amounts. H_2O_2 also showed to increase the activity of sGC (Burke and Wolin, 1987; White et al., 1976).

Previous work in our laboratory showed that hydrogen peroxide, at concentrations between 2 and 25μM, which were well below those normally used to cause oxidative

stress (mM), enhanced the inhibitory action of NO and nitrosothiols on platelets by lowering the IC_{50} for NO up to one hundred fold (Naseem et al., 1995; Naseem and Bruckdorfer, 1996). The effect of H_2O_2 on NO induced inhibition of platelet aggregation was studied carefully by addition of the peroxide both before and after addition of NO. Addition of hydrogen peroxide, either 1min before or 1min after NO did stimulate the inhibition of aggregation, but the greatest inhibition was obtained when NO and H_2O_2 were added simultaneously. In addition, H_2O_2 had no effect on the inhibition of aggregation if added 30 min before NO (Naseem and Bruckdorfer, 1995). The peroxide alone had no inhibitory action, except in the presence of plasma, which contained endogenous nitrosothiols (Naseem et al., 1996).

Addition of a NO scavenger, carboxy-PTIO, to washed platelet only a few minutes before addition of NO and H_2O_2 partly inhibited the synergism. This observation supported the fact that presence of NO is necessary for observed inhibitory action. The presence of sGC inhibitor (ODQ) abolished the synergism between NO and H_2O_2 confirming that at least low concentrations of cGMP is essential to the synergism, even if it is not the major initiator of the synergism. Addition of dibutyryl cGMP together with H_2O_2 only inhibited the aggregation partly and much less effectively than NO and H_2O_2 in combination. These results are summarised below (Fig 3.1).

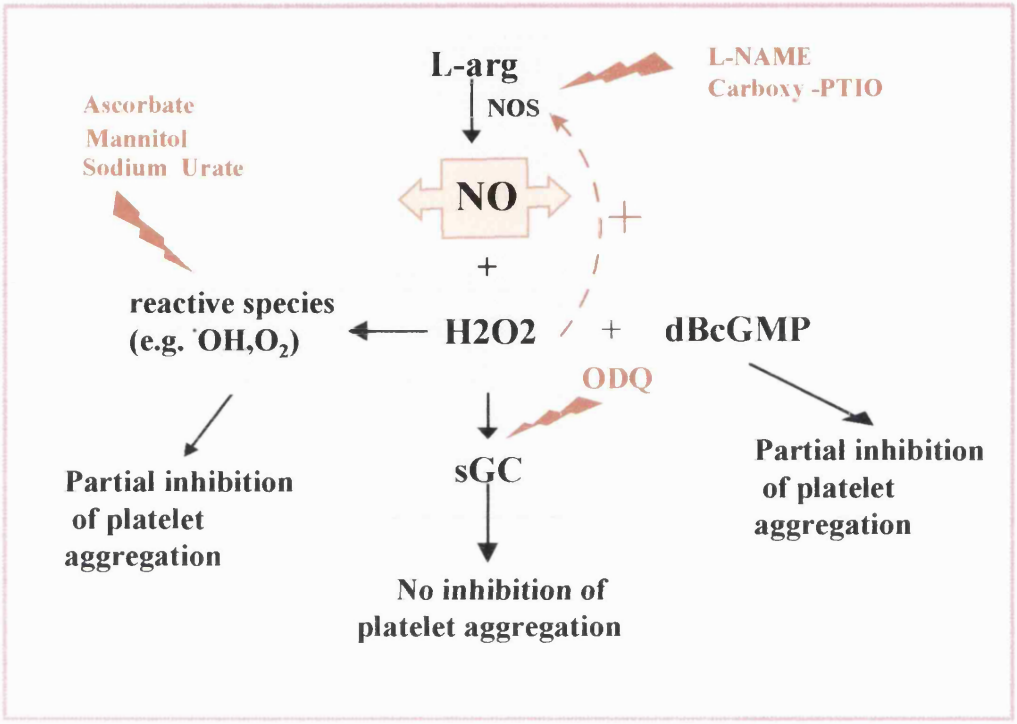


Fig 3.1: The summary of the published results

(Naseem et al., 1993; Naseem and Bruckdorfer, 1995)

L-NAME =NOS inhibitor, carboxy-PTIO= NO scavenger, ODQ= sGC inhibitor

Ascorbate, mannitol and sodium urate = scavengers of reactive oxygen species.

3.1.1 Aim

The aim of this study was to investigate the importance of soluble guanylyl cyclase (sGC) on the synergism between NO and hydrogen peroxide and to establish, whether the effect of hydrogen peroxide is exclusively through activation of sGC.

3.2 The effect of NO and H_2O_2 on platelet aggregation of washed human platelets

The effect of H_2O_2 on platelet aggregation and platelet sensitivity to NO was investigated, initially by using solutions of NO. The plasma environment is rich in antioxidants and free radical scavengers, such as ascorbic acid, superoxide dismutase, catalase and glutathione peroxidase (GSH-Px), which may have important effect on experiments with exogenous NO and H_2O_2. By using washed platelets the effect of NO and H_2O_2 could be studied in the absence of these and even other plasma constituents.

3.2.1 The influence of hydrogen peroxide on inhibition of NO-induced platelet aggregation

The synergism between NO and H_2O_2 on platelet aggregation was confirmed by repeating the reported results by Naseem et al (Naseem and Bruckdorfer, 1995), which was obtained in this laboratory. In the previous study as well as in this study the maximum inhibition was observed when 25μM H_2O_2 and 10nM NO were added into platelets simultaneously.

Addition of H_2O_2 simultaneously with NO into human washed platelets decreased the aggregation response of platelets to thrombin and to a less extent to collagen. The addition of 25 μM H_2O_2 increased the inhibition of aggregation by 10nM NO up to 50 folds (Fig 3.2). The IC_{50} for NO was calculated as 80±5.6nM, which was decreased in the presence of 25μM H_2O_2 to 8±2.9nM NO. Either NO or H_2O_2 at this concentrations separately only inhibited the aggregation between 2-5%. These results agreed with previous results reported in this laboratory (Naseem and Bruckdorfer, 1995).

The volunteers used for all these experiments were chosen from healthy members of the laboratory who denied taking any medication for at least a week. A variation in platelet responses to different agonists, including thrombin was observed between different blood donors. This may depend on the diet or life style or genetic differences among the donors.

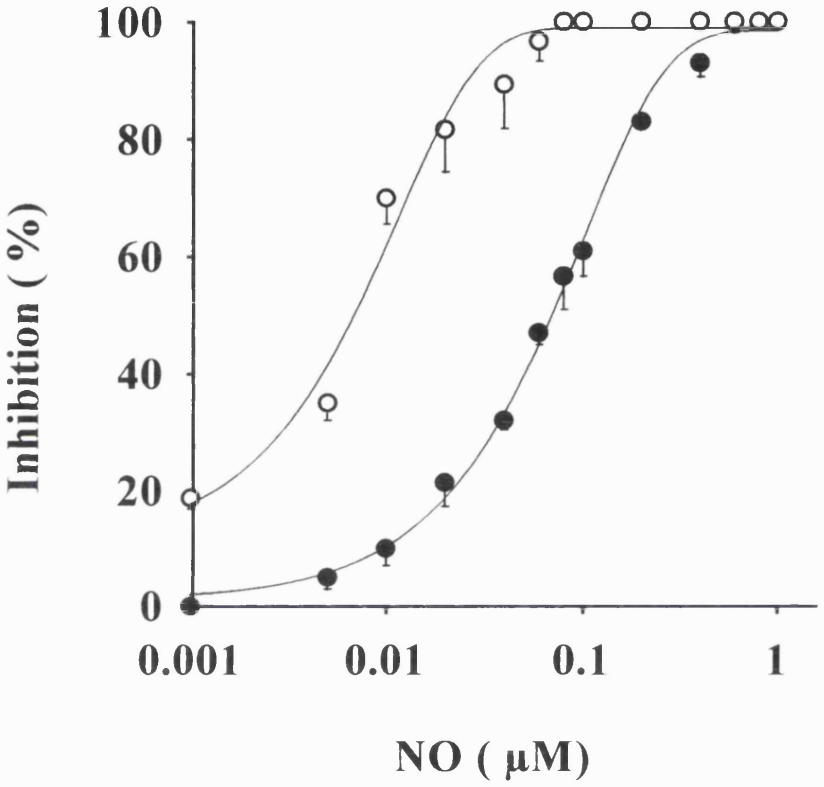


Fig 3.2: Inhibition of platelet aggregation by NO in the presence and absence of H_2O_2.

Isolated washed human platelets were incubated with varying concentrations of NO in the absence (●) or the presence of 25μM H_2O_2 (○) for 1 min before addition of thrombin (0.02 units/ml) and then incubated for 3 min at 37°C with stirring. The results are calculated as a percentage inhibition of the maximum aggregation induced by thrombin and presented as the mean ± S.E.M of 3 independent experiments.

3.2.2. The influence of YC-1 and hydrogen peroxide on inhibition of platelet aggregation

To establish whether the synergism between NO and H_2O_2 was unique to NO, platelet aggregation was studied in the presence of the single concentration of H_2O_2 (25μM) and different concentrations of YC-1. YC-1, as described in general introduction, has been reported to inhibit platelet aggregation independent of NO pathway (Wu et al., 1995). YC-1 also appeared to act independent of activation of the protoporphyrin IX complex of soluble guanylyl cyclase (Friebe et al., 1996; Friebe and Koesling, 1998). Synergism between YC-1 and H_2O_2 was reported previously, but at higher concentrations of hydrogen peroxide than used in this study (Wu et al., 1999a). The reported synergism effect between YC-1 and H_2O_2 by Wu et al was much less comparing with the synergism effect between NO and H_2O_2.

In this study, the synergism between YC-1 and H_2O_2 was studied using a range of concentrations of YC-1 (between 5 to 100μM) together with a single concentration of H_2O_2 (25μM). YC-1 inhibited platelet aggregation in the presence of 25 μM H_2O_2 simultaneously but only to a limited extent (Fig 3.3). The IC_{50} for YC-1 alone was calculated as 10 ± 19.1μM, which in the presence of H_2O_2 was only decreased to 8 ± 13.2 μM.

The synergism effect observed between YC-1 and H_2O_2 was much less compared with a combination of NO and H_2O_2. This result suggests that the synergism effect might be unique to NO and different mechanisms independent of cGMP are involved.

However a three fold increase of cGMP in the presence of NO and H_2O_2 was observed in intact human washed platelet (Naseem and Bruckdorfer, 1995), which require further investigation.

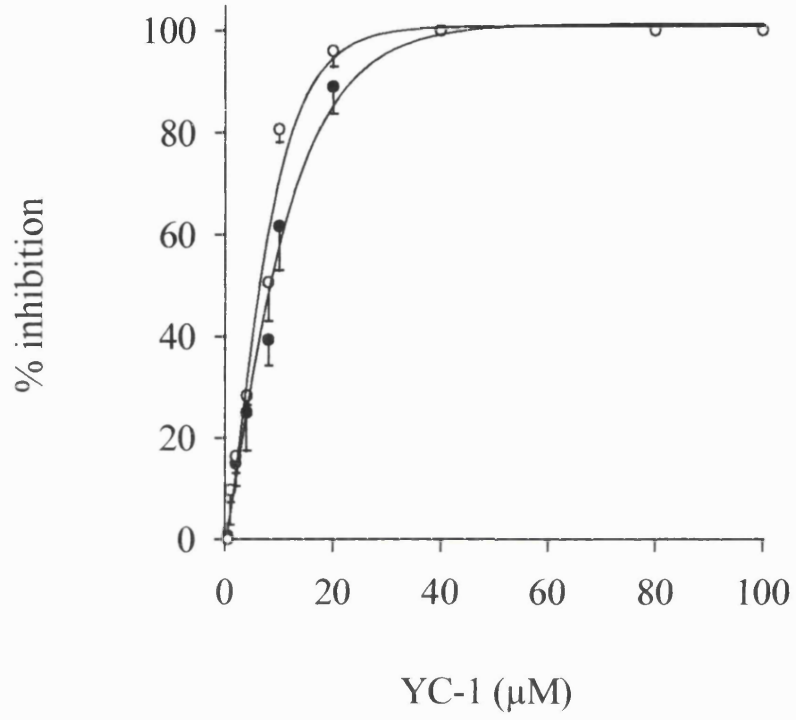


Fig 3.3: The synergism between YC-1 and H_2O_2 on the inhibition of platelet aggregation.

Isolated washed human platelets were incubated with varying concentrations of YC-1 in the absence (●) or the presence of 25μM H_2O_2 (○) for 1 min before addition of thrombin (0.02 units/ml) and then incubated for 3 min at 37°C with stirring. The results are calculated as a percentage inhibition of the maximum aggregation induced by thrombin and presented as the mean ± S.E.M of 3 independent experiments.

3.3 The mechanism of action of hydrogen peroxide on the stimulation of soluble guanylyl cyclase

A small increase of cGMP in intact platelet was observed associated with synergism between NO and H_2O_2. It was therefore necessary to investigate whether H_2O_2 has any action on the activation of the sGC. To achieve this, the synthesis of cGMP was measured in the presence of H_2O_2, using either purified enzyme or cell fractions of human platelets.

3.3.1 The effect of NO and H_2O_2 on the purified sGC

The purified enzyme was prepared on the laboratory of Dr D. Koesling (University Bochum Germany). The final concentration of the enzyme used in the all experiments was 2.3ng/ml (as recommended by Dr Koesling). To reach this concentration, the purified enzyme was diluted in modified Tyrode's buffer prior to the experiment. The enzyme was then diluted further 1:1 with a special buffer containing 3mM Mg^{2+}, 3mM DTT, 0.5mg/ml bovine serum albumin (BSA), 300 μM GTP and 50mM triethylamine hydrochloride to optimise the activity of the enzyme (Burstyn et al., 1995). Addition of sGC to the buffer would initiate the conversion of GTP to cGMP and therefore, it was important that sGC should be added to the buffer simultaneously with NO or NO/H_2O_2. The samples were then prepared at 37°C with stirring for 10min and the reaction was stopped by addition of 15% TCA as described in the methods. The cGMP concentration was then measured using commercial cGMP kits.

Since NO is known as the activator of sGC, the stimulation of the enzyme with only NO could also act as a control to show that the purified sGC and the assay was

working. Increased concentrations of NO clearly activated the sGC and resulted in an elevation of the cGMP level. Addition of H_2O_2 either alone, or in the presence of NO did not change the activity of the enzyme, since no significant changes on the cGMP concentrations were observed (Fig 3.4).

It was possible that the lack of effect was due to absence of other factors in the cells. Therefore, the next step of this study will be to investigate the activation of the cytosolic form of sGC by using the cytosolic fraction of washed platelet. The possible importance of the platelet membranes in this synergism was determined using platelet lysates.

3.4 The influence of H_2O_2 on sGC using lysate or the cytosolic fraction of platelet

Post mitocondrial cytosolic fraction:

To study the activity of cytosolic sGC in the presence of NO and H_2O_2, the human washed platelets were lysed in the presence of saponin, a detergent, and the cytosolic fraction was separated by centrifugation at 4°C (as described in methods).

Addition of NO to cytosolic fraction of washed human platelets increased the cGMP level significantly, confirming that the sGC is responding (Fig 3.5). H_2O_2 did not increase the cGMP either in the presence or in absence of NO. In fact at a higher concentration of NO (1μM), H_2O_2 seemed to have an inhibitory effect on the stimulation of sGC by NO, but the differences were not significant.

One final possibility was that membrane associated co-factors were required, despite the fact that the main sGC activity was in the cytosol. Therefore cell lysates were used to repeat the experiments.

Cell lysates:

The effect of NO and H_2O_2 on stimulation of sGC was investigated in platelet lysate. The cGMP concentration was increased in the presence of different concentrations of NO. H_2O_2 alone did not change the levels of cGMP in platelet lysates. At 1μM NO, the cGMP level was significantly decreased in the presence of H_2O_2 (Fig 3.6) ($P < 0.001$).

These results suggest that for the synergism to occur at the level of sGC the whole cell system is required. Alternatively other mechanisms may to be involved in this synergism, which do not involve activation of sGC.

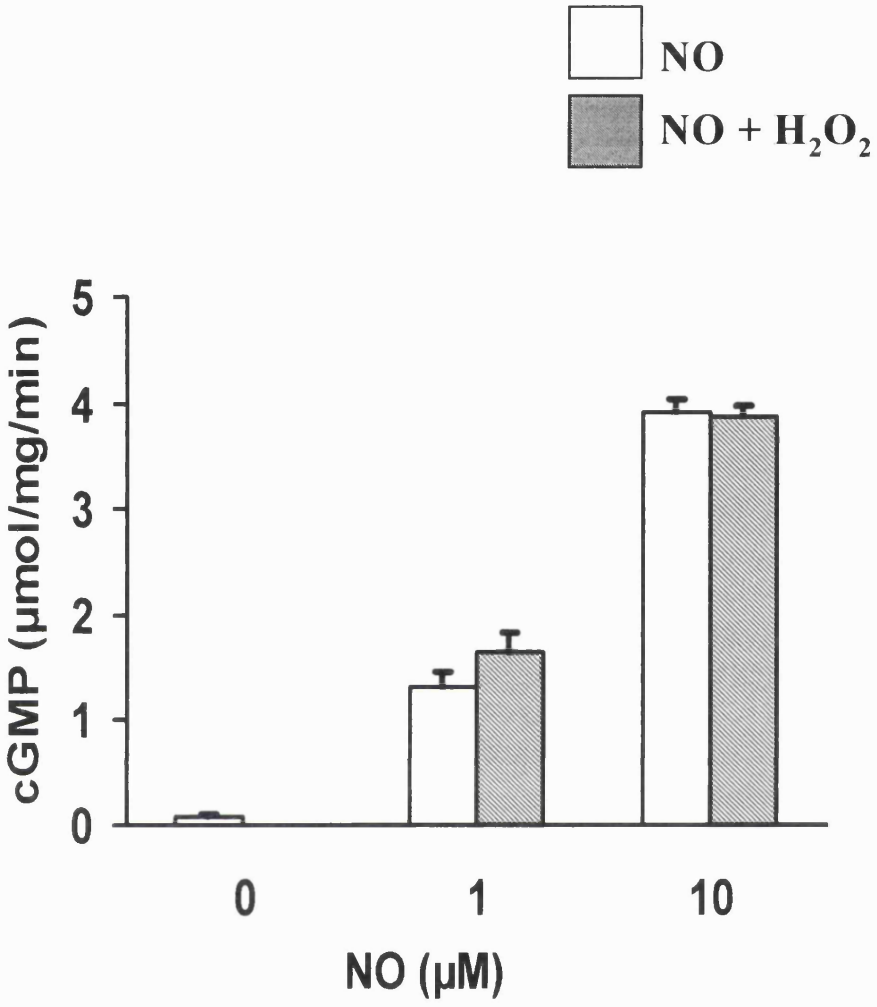


Fig 3.4: Interactions of H_2O_2 and NO the activity of purified soluble guanylyl cyclase.

The purified enzyme was diluted in a buffer as described in method and incubated with different concentrations of NO alone(open bars) or together with 25µM H_2O_2 (Hatched bars)for 10 min at 37 °C with stirring. The reaction was later stopped with addition of 7.5% TCA. The cGMP contents were determined by using ELISA. Values are presented as mean ± S.E.M of 3 independent experiments.

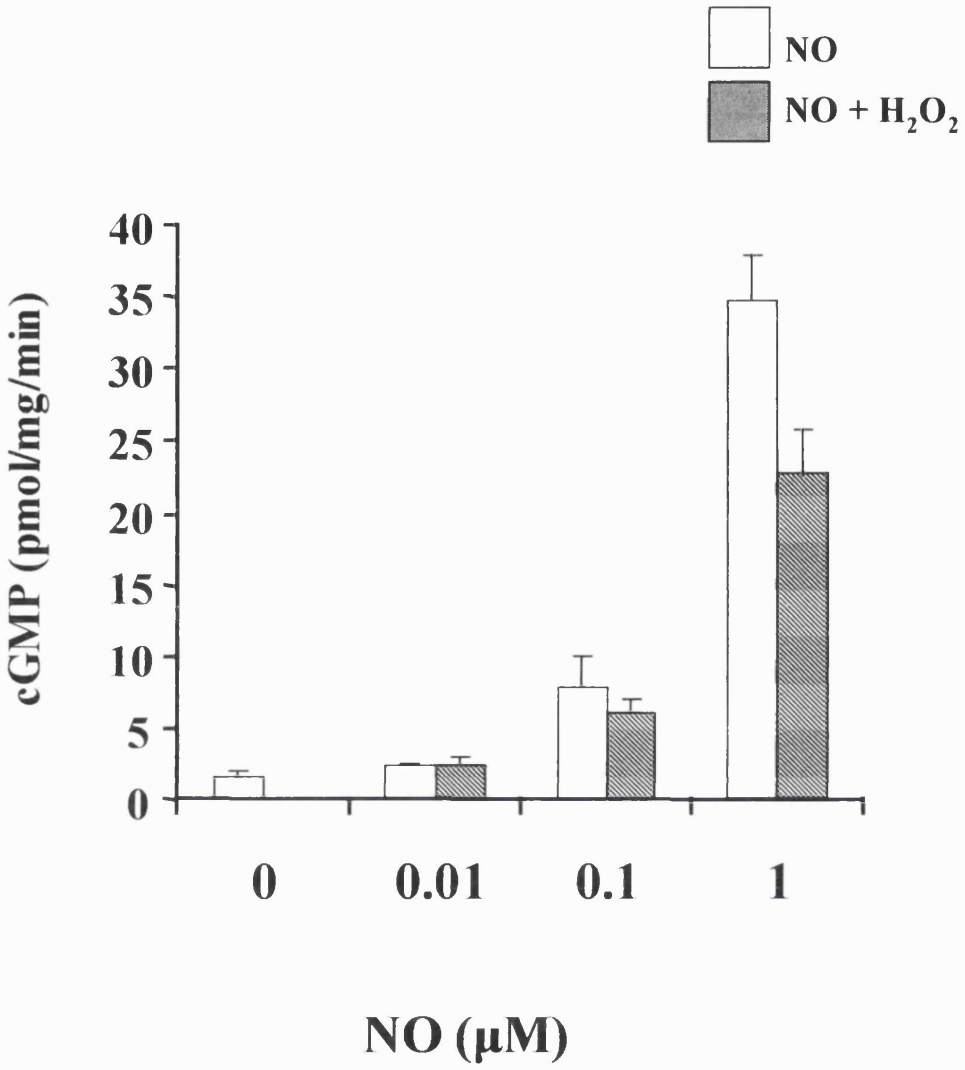


Fig 3.5: Interactions of H_2O_2 and NO on biosynthesis of cGMP in cytosolic fractions of platelets

The cytosolic fraction of human washed platelets were incubated with different concentrations of NO alone (open bars) or together with 25μM H_2O_2 (Hatched bars) in the presence of IBMX (200μM) for 10 min at 37 °C with stirring. The reaction was later stopped with addition of 7.5% TCA. The cGMP contents were determined by using ELISA. Values are presented as mean ± S.E.M of 3 independent experiments.

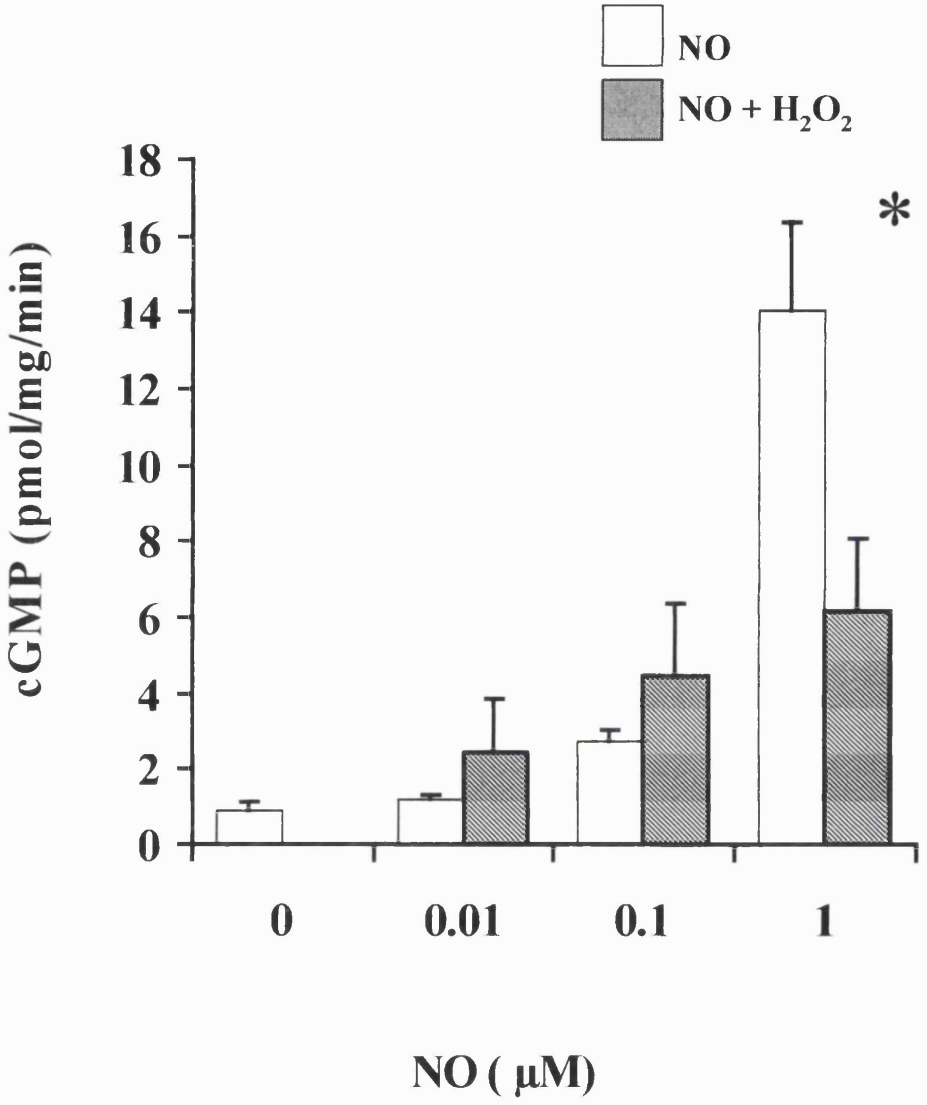


Fig 3.6: Interactions of H_2O_2 and NO on platelet lysates

The lysate of human washed platelets were incubated with different concentrations of NO alone (open bars) or together with 25μM H_2O_2 (Hatched bars) in the presence of IBMX (200μM) for 10 min at 37 °C with stirring. The reaction was later stopped with addition of 7.5% TCA. The cGMP contents were determined by using ELISA. Values are presented as mean ± S.E.M of 3 independent experiments. $P< 0.001$

3.5 The role of H_2O_2 and NO on the stimulation of purified guanylyl cyclase mixed with cell lysate of platelets

To confirm whether cytosolic or membrane proteins are required for activation of soluble guanylyl cyclase reproduced by NO and H_2O_2, sGC at concentration used as in the previous experiments was added to platelet lysates. The activity of sGC was then investigated in the presence of NO and H_2O_2. No increase of cGMP was detected as in other experiments (results not shown). Up to this stage, almost all evidence pointed against an involvement of sGC in the mechanism of the synergism. The veracity of the results was further investigated in the presence of YC-1 and H_2O_2.

3.6 The effect of YC-1 on the stimulation of sGC

The synergism between YC-1 and H_2O_2 was also investigated looking at activation of purified sGC and the platelet cytosolic fraction.

Purified enzyme:

Addition of YC-1 at two different concentrations (10 and 100μM) increased the enzyme activity, measured by changes of cGMP synthesis. This increase of cGMP in the presence of YC-1 was on the levels expected regarding the previous publications (Friebe et al., 1996; Friebe and Koesling, 1998). The presence of H_2O_2 did not increase further the formation of cGMP significantly, either when added alone nor in the presence of YC-1 (Fig 3.7).

Post mitochondrial cytosolic fraction:

The cGMP level in the cytosolic fraction of washed platelet in the presence of 5, 10, and 50μM of YC-1 was increased to 10.3 ± 0.67, 16.13 ± 4.12 and 40 ± 5 pmol/mg/min respectively (Comparing with control 1.53 ± 0.54). A lower concentration of YC-1 (5μM) + H_2O_2 increased the activity further: this did not occur at higher concentrations of YC-1 (P = 0.016) (Fig 3.8).

In conclusion, H_2O_2 did not enhanced the activity of sGC in the presence of YC-1. Thus, only addition of 5μM YC-1 into the cytosol showed a significant increase of cGMP. These paralleled the aggregation results, since YC-1 showed minimal synergism with H_2O_2 at concentrations between 4 -20μM. At these concentrations, only a 1-2% synergism between YC-1 and H_2O_2 was observed.

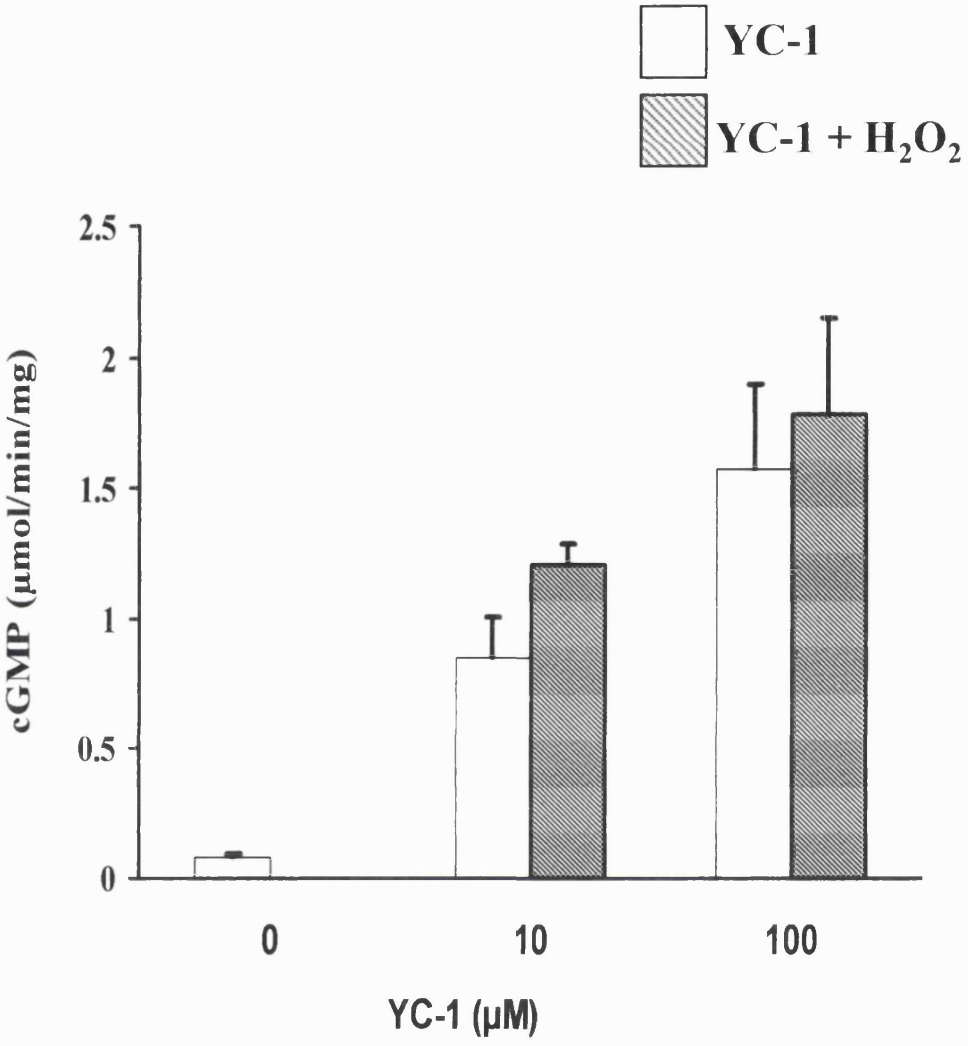


Fig 3.7: Interaction between H_2O_2 and YC-1 on the increase activity of pure soluble guanylyl cyclase.

The purified enzyme was diluted in a buffer as described in method and incubated with different concentrations of YC-1 alone (open bars) or together with 25µM H_2O_2 (Hatched bars) for 10 min at 37 °C with stirring. The reaction was later stopped with addition of 7.5% TCA. The cGMP contents were determined by using ELISA. Values are presented as mean ± S.E.M of 3 independent experiments.

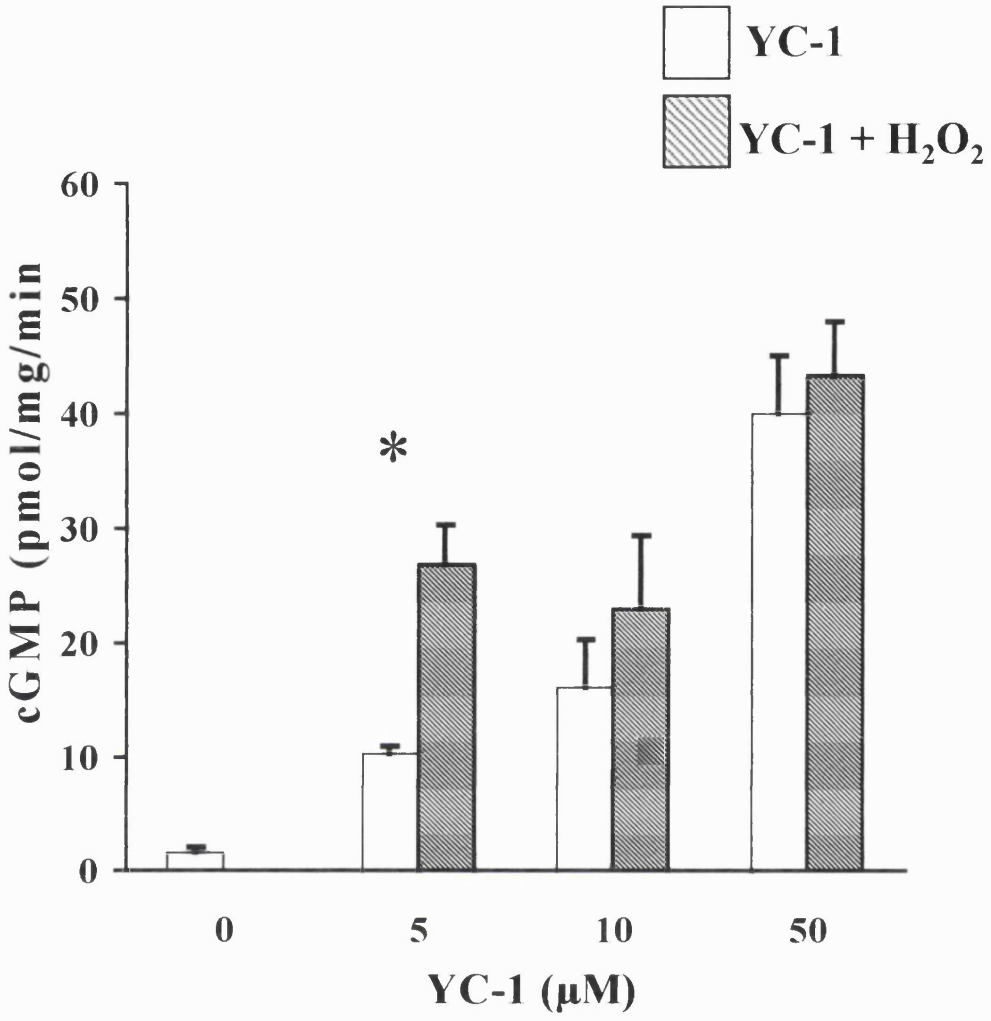


Fig 3.8: Interactions of H_2O_2 and YC-1 on biosynthesis of cGMP in cytosolic fraction of platelet

The cytosolic fraction of human washed platelets were incubated with different concentrations of YC-1 alone (open bars) or together with 25μM H_2O_2 (Hatched bars) in the presence of IBMX (200μM) for 10 min at 37 °C with stirring. The reaction was later stopped with addition of 7.5% TCA.
The cGMP contents were determined by using ELISA. Values are presented as mean ± S.E.M of 3 independent experiments. * P = 0.016

3.7.1 The recovery of platelets response to thrombin from the effects of nitric oxide or nitric oxide/hydrogen peroxide

Another approach to this investigation was to determine whether H_2O_2 changed the duration of the response to NO, which was also reported in an earlier paper (Naseem et al., 1995). To achieve this, the recovery of platelet response to thrombin by NO alone or NO and H_2O_2 was studied over a period of 5 to 60 minutes.

Platelets in general show a 98% aggregation response to thrombin at a concentration of 0.02 U/ml. In this experiment a typical synergism effect between NO and H_2O_2 was observed as in previous experiments.

Addition of 10nM NO only inhibited platelet response to thrombin up to 8%. 25μM H_2O_2 had exactly the same effect on aggregation as 10nM NO, therefore they overlap each other on the curve (Fig 3.9). In the presence of 25μM H_2O_2 with 10nM NO, 100% inhibition was achieved, which remained near maximum for up to 15 minutes, reaching 30% after 30 minutes and back to normal response after 60 minutes (Fig 3.9). In the presence of 0.1μM NO only 60% inhibition of the aggregation was detected.

A 100% inhibition was detected in the presence of 1μM NO, which was remained near the maximum for 15 minutes and declined after 30 minutes. After 60 minutes, 70% aggregation response was detected in this sample (Fig 3.9).

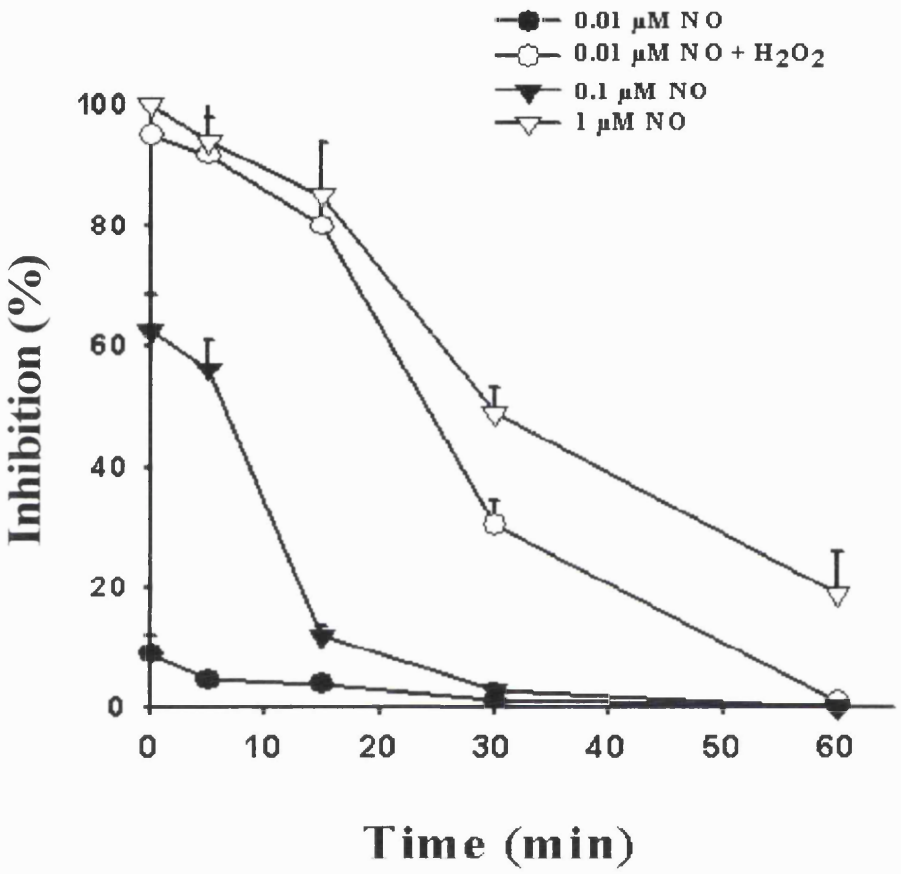


Fig 3.9: The time course of the inhibition of Platelet aggregation induced by NO and hydrogen peroxide.

Isolated washed platelets were incubated with 0.01μM NO (●), 0.1μM NO (▼) and 0.01μM NO + 25μM H_2O_2 (○) and 1μM NO (□) for an interval between 0 -120 sec.
Values are presented as mean ± S.E.M of 3 independent experiments.

3.7.2 The stability of cGMP in the absence of phosphodiesterase inhibitor

The effect of H_2O_2 on the duration of synergism was interested. To be able to connect these results to the increased cGMP level in the presence of NO and H_2O_2, it was important to study the role of H_2O_2 on the duration of cGMP. In cGMP studies, the PDEs were inhibited by addition of 200μM of IBMX (a PDEs inhibitor). Since in the aggregation experiments no phosphodiesterase inhibitor was presented, for a more acurrent comparison the cGMP level was measured in the presence of NO and H_2O_2 and the absence of IBMX. Since cGMP is breaking down by phosphodiesterase during seconds, the cGMP level was only measured during a period of 15-120 sec.

For this study, washed human platelets two hours after separation from plasma were incubated with 10 and 100nM of NO (same concentrations as previous experiment) in the presence and absence of 25μM H_2O_2. The samples were incubated at 37 °C with stirring for a period up to 120 seconds. The reaction was then stopped with addition of TCA in 1:1 dilution.

The cGMP level increased in response to different concentrations of NO (10 and 100nM) to 1.88 and 10.90 pmol/10^8platelets respectively in less than 15 seconds. In the presence of H_2O_2 with the same concentrations of NO (10 and 100nM) the level of cGMP was significantly increased to 3.03 and 18.50 pmol/10^8 platelets respectively. H_2O_2 alone increased the cGMP level to 1.65 ± 0.03 pmol/10^8 platelets (Fig 3.10).

In figure 3.10 the levels of cGMP in the presence of 10nM NO and 25μM H_2O_2 is overlapping each other therefore difficult to distinguish between them.

In the absence of PDEs inhibitors, the cGMP levels were declined very quickly. The decrease of cGMP was significant for all concentrations of NO. A fall of cGMP concentration was recorded 15 s after addition of NO ± H_2O_2. 15 s after addition of NO the cGMP levels were declined and back to basal level after 80 s. In the presence of H_2O_2 the cGMP level was also declined after 15 s, but much slower and it was not back to basal after 120 S (Fig 3.10).

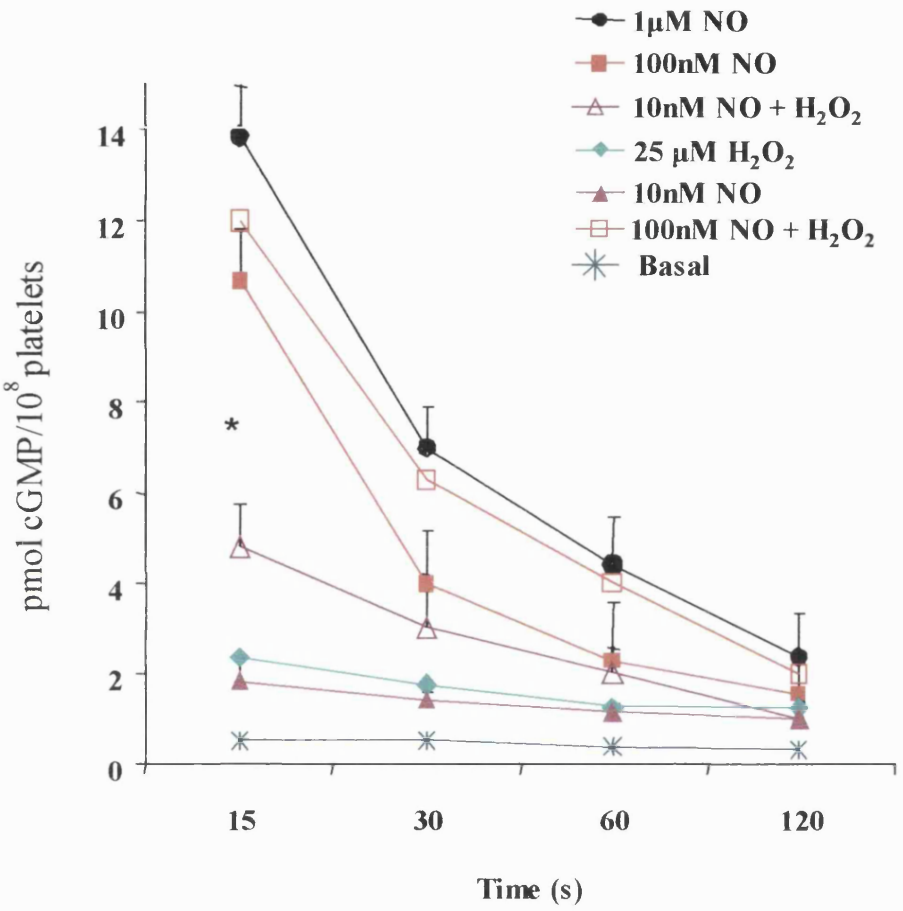


Fig 3.10: Platelet cGMP changes induced by NO and hydrogen peroxide in the absence of a phosphodiesterase inhibitor.

Isolated washed platelets were incubated with 0.01μM NO, 0.1 and 1 μM NO or 0.01μM NO and 0.1μM NO +25μM H_2O_2 μM or 25μM H_2O_2 alone for an interval between 0 -120 S in the absence of IBMX. The reaction was stopped with addition of 7.5% TCA. The cGMP was measured by ELISA. The results are typical of at 3 independent experiments.
*P< 0.002 (10 nM No + H_2O_2)

3.8 DISCUSSION

NO is clearly of key importance in the enhanced inhibition of platelet aggregation by H_2O_2, since carboxy-PTIO a NO scavenger and the inhibitor of NOS (L-NAME) completely blocked the synergism between NO and H_2O_2 (Naseem and Bruckdorfer, 1995). The possibility that other reactive oxygen and nitrogen species (e.g. hydroxyl radical and singlet oxygen) may be produced during reaction between NO and H_2O_2 was investigated in the previous study (Naseem and Bruckdorfer, 1995). The production of these species may contribute to the action of NO and the final inhibitory effect. The addition of scavengers such as sodium urate, ascorbate or mannitol did partly reversed the inhibition by NO/ H_2O_2, but had no effect on inhibition by higher concentrations of NO (Naseem et al., 1993).

An increase of the production of OH radical in the presence of NO and H_2O_2 was reported independently of the presence of iron or other transition metals (Weidauer et al 2002).

The effects of biological concentrations of H_2O_2 was studied in various vascular cells by different groups. H_2O_2 at 100μM was reported to cause activation of sGC (Burke and Wolin, 1987), activation of protein kinase C (Packer et al 1992, Rao et al 1995), prostaglandin production (Burke-Wolin et al., 1991b, Gurtn er et al 1991, Wolin et al 1987, Omar et al 1992) and hyperpolarisation (Weir et al, 1995) in various vascular cells. H_2O_2 appears to cause an endothelium-dependent vasodilatation of rat skeletal muscle in vivo, mediated by PGE_2 and PGI_2 (Wolin et al, 1987). H_2O_2 $_{also}$ causes endothelium-independent contraction of human placental arteries mediated by thromboxane A_2. The vasorelaxing effects of H_2O_2 on rat aorta and canine basilar artery, appeared to be induced by H_2O_2 itself and required formation of cGMP (Yang

et al., 1999; Yang et al., 1998). The mechanism of action of H_2O_2 on the stimulation of sGC was reported to be the result of its metabolism by catalase. Catalase stimulates sGC through its compound I intermediate (Wolin et al., 1998).

In this study, H_2O_2, on its own, was not capable to stimulate purified sGC. Thus, the mode of action of hydrogen peroxide in the synergism is unclear, but appears to require the presence of activated sGC, since the inhibitor of sGC, ODQ, completely blocked the synergism (Naseem and Bruckdorfer, 1995). In addition, a modest rise in cGMP was observed, in the presence of a phosphodiesterase inhibitor, which appeared to account for the observed synergism. Addition of exogenous cGMP, dibutyryl cyclic GMP, (dBcGMP) exhibited synergism with H_2O_2, but were much less effective than with GSNO or NO, suggesting that an additional mechanisms than that of the cGMP system is required, which may occur before or parallel to the formation of cGMP (Naseem and Bruckdorfer, 1995).

Addition of NO, 30 min before addition of H_2O_2 did not show any synergism (Naseem and Bruckdorfer, 1995), suggesting that simultaneously addition of NO and H_2O_2 or at least addition of them with only seconds delay is essential for maximum synergism. This may be due to the possibility that may be other reactive species are produced during the reaction of NO and H_2O_2.

Similar synergism effect was also evident, but to a lesser extent, between hydrogen peroxide and CO, another ligand for sGC, which activates the enzyme to a much smaller extent (Burke-Wolin et al., 1991b; Deinum et al., 1996). A synthetic compound, YC-1 activates the enzyme independently of NO and CO (Ko et al., 1994; Wu et al., 1995), but acts synergistically with both NO and especially with CO to

increase sGC activity (Wolin and Burke, 1987). YC-1 is thought to decrease the rate of dissociation of NO from the haem moiety, resulting in longer periods of activation. YC-1 has also been reported to act as inhibitor of cyclic nucleotide phosphodiesterases (Galle et al., 1999). However addition of YC-1 simultaneously with H_2O_2 did not enhance inhibition of the platelet aggregation, as was the case with NO and H_2O_2. This result was further evidence, in addition to the presence of L-NAME, that the synergism is NO-dependent.

H_2O_2 alone does not inhibit aggregation of washed platelets, but it is conceivable that the peroxide may induce a rise in endogenous NO synthesis by activating either eNOS (Drummond et al 2000, Cai et al 2001) or iNOS (Milligan et al., 1998). This is unlikely to be an important mechanism in this case, since synergism between GSNO and H_2O_2 was only slightly impaired by the presence of L-NAME. H_2O_2 does not increase the stability of NO in solution (Naseem et al., 1996). The extent of synergism between the H_2O_2 and YC-1 was limited to low concentrations of the YC-1 in platelet cytosol, but no synergism was observed using purified sGC. Wu et al (1999) have reported that H_2O_2 does enhance platelet inhibition by YC-1, but the effect was relatively small at low concentrations of the peroxide (Wu et al., 1999a) as was found in this study. This suggests again that there may be other mechanisms involved that are not simply regulated by cyclic nucleotide concentrations and are unique to NO.

In this work, the possibility that the site of synergism between NO and H_2O_2 was at the level of sGC was investigated. Surprisingly no increases in cGMP synthesis were observed when NO and H_2O_2 were added to purified bovine sGC over those seen with NO alone. It is clear now that the mode of action of H_2O_2 in this synergism is not

directly through sGC but an indirect effect can be possible. The possibility that other proteins in cytosol would participate in this synergism was considered. This was studied by addition of sGC to platelet lysates again no synergism was observed between NO and H_2O_2. These observations are consistent with previous work, which showed little synergism between H_2O_2 and dibutyryl cyclic GMP with respect to platelet inhibition (Naseem and Bruckdorfer, 1995). An increase of cGMP might be required in this synergism, but other mechanisms seem to be essential to complete the action of H_2O_2. The fact that the breakage of the cell membrane with saponin seems to interrupt the synergistic activation of sGC make clear that the connection of the membrane is essential for the synergism between NO and H_2O_2. Further investigation is needed to clarify the role of the cell membrane proteins on this synergism.

H_2O_2 has been reported to increase significantly the levels of cGMP in intact endothelial cells (Zembowicz et al., 1993) and aortic smooth muscle (Marczin et al., 1992). However, in all of these studies phosphodiesterase inhibitors were present throughout the experiments. The possibility that H_2O_2 itself may inhibit phosphodiesterases and so increases the duration of the cyclic nucleotides in the platelets has been eliminated in this study, since we did not observe any increase of cGMP or cAMP in the presence of H_2O_2.

In the absence of IBMX, the cGMP level fell after a few seconds and H_2O_2 was not capable of prolonging the stability of cGMP, not even in the presence of higher concentrations of NO (100nM and 1μM). Under these conditions, H_2O_2 caused only minor and transitory increases in cGMP at all concentrations of NO tested compared with the previous data, which showed significant increases of cGMP in the presence of IBMX. In the absence of PDE inhibitors, the increased cGMP disappeared only

within seconds. This observation raises the question whether this short time will be enough for cGMP to activate other systems.

In conclusion, the mechanism of the synergism is more complicated than expected. H_2O_2 on its own did not show any direct effect on the activation of sGC. This also suggests that hydrogen peroxide does not increase NO synthases. In separate studies attempt to measure NO production using a WPI detector, no increase in the concentration of NO could be detect in the presence of H_2O_2.

The activation of other pathways may be necessary other than the direct activation of sGC, since the simultaneously addition of NO and H_2O_2 clearly did not cause activation of the purified enzyme.

In the absence of IBMX, cGMP was only stable for a few seconds. This increase of cGMP may activate other pathways, which are essential for the final effect. The down stream effect of cGMP is protein modification including phosphorylation of specific proteins. In next part of this study the role of NO and H_2O_2 on the modification of proteins was studied.

Recent studies suggested, that low levels of ROS modulate signal transduction pathways in mammalian cells. Sonda et al (2000) showed that tyrosine phosphorylation of FAK by H_2O_2 acts as a suppressor of apoptosis. H_2O_2 stimulates protein phosphorylation (Scieven et al 1997), activates protein kinases (Abe et al 1996, Barchowsky et al 1995, Guan et al 1996, Hout et al 1997) inhibits tyrosine phosphatases (Caselli et al 1998), stimulates phospholipases and regulates transcription factors such as activator protein-1 (Schulze-Osthoff et al 1997). In most of these studies much higher concentrations of H_2O_2 was used comparing with our synergism study.

In the next chapter the downstream effect of cGMP were investigated, particularly the cytoskeletal proteins. Figure 3.11 represents the summary of the relevant information on the synergism between NO and H_2O_2 as understood at this stage of the project.

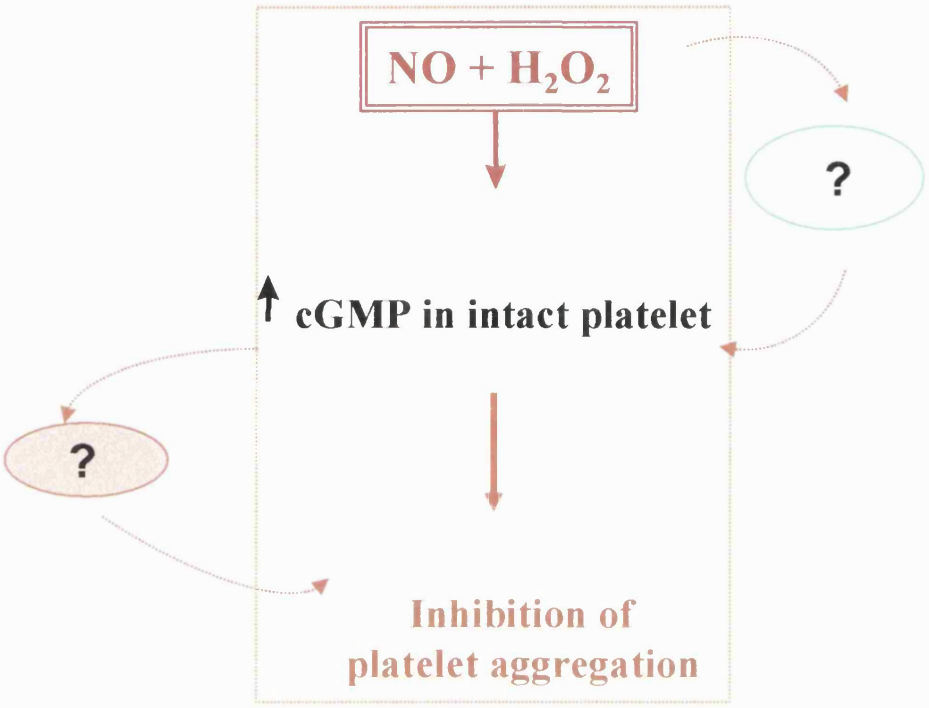


Fig 3.11: The conclusion of the result chapter 1

H_2O_2 was not capable of increasing the activity of sGC when was added directed to purified enzyme. Neither did the addition of both NO and H_2O_2 simultaneously. An increase of cGMP might be required in this synergism, but other mechanisms are likely to be essential to complete the action of H_2O_2 on the activation of sGC.
The activation of other pathways is necessary before the activation of sGC and the cell membrane seems to be essential in these pathways, since the synergism effect was only observed in intact cells.
Still an increase of cGMP is not the major mechanism behind the synergism, since addition of H_2O_2 and dibutyryl cyclic GMP (an analogue of cGMP) caused only partial inhibition of platelets aggregation. A lot more works needs to be done to complete the gaps in this investigation.

CHAPTER FOUR:

THE EFFECT OF NO AND H_2O_2 ON THE PHOSPHORYLATION OF PLATELETS PROTEINS

4. INTRODUCTION

The conclusions from the previous chapter made it important to investigate other major mechanisms that interact with inhibition of platelet function. This will include the downstream effect of sGC and cGMP, which includes the cytoskeleton proteins and the cell signaling.

The cGMP and cAMP are involved in different parts of cell signalling pathways, which will end up in the phosphorylation of proteins including the cytoskeletal proteins through activation of different protein kinases. Of the cytoskeleton proteins, a few have already been associated, with the inhibition of platelet shape change and platelet activation. One of these proteins is the vasodilator-stimulated phosphoprotein, which is known as a substrate for both PKA and PKG (Halbrugge et al., 1990).

The actin cytoskeleton controls the cell shape, which leads to aggregation of platelets and the movement of other cells like macrophages. In platelets, the cytoskeleton proteins control polymerisation via a direct or indirect interaction through actin binding proteins.

One of the major proteins involved in the actin polymerisation in platelet is VASP. The interaction between VASP, platelet cytoskeleton and integrin receptors are of importance in regulation and inhibition of platelet aggregation, via inhibition of in and out signaling (Horstrup et al., 1994).

4.1.1 The interaction of integrin receptor with VASP on the inhibition of platelet activation

The major transmembrane components of focal adhesions are integrins, a large family of adhesion molecules that binds both extracellular matrix proteins and counter

receptors. When platelets are activated by agonists, such as collagen, collagen-related peptides, prostaglandins, or ADP, integrin switches from a resting to an activated conformation, including the binding to fibrinogen. Fibrinogen binding is important for cross-linking platelets at the site of vascular injury and promotes the aggregation of platelets and clot formation. Although agonist-induced activation of platelets occurs via several signal transduction pathways, the final activation occurs most probably through altered interactions between the cytoplasmic domain of αIIbβ3 and components of the focal contacts. Experimental evidence suggests that the membrane proximal region of both α and β subunit of αIIbβ3 have a crucial role for modulating the conformational changes leading to activation or inactivation of the fibrinogen receptor (Zamarron et al., 1991).

The presence of VASP in cytoskeleton, its ability for binding to vinculin, zyxin, and the fact that phosphorylation of VASP in platelets correlates with inhibition of fibrinogen binding to the fibrinogen receptor, αIIbβ3 integrin, suggests a role for VASP in regulating fibrinogen receptor activation.

4.1.2 VASP

The highest levels of VASP are observed in platelets, but stomach, intestine, spleen, lung and Endothelial cells of blood vessels are also rich sources of VASP (Eigenthaler et al., 1992). VASP is phosphorylated in response to vasodilators and platelets inhibitors, substances that raises intracellular cAMP and cGMP levels. Its interaction with actin and integrin receptors is of importance in the control of platelet activation. Phosphorylation of VASP in the presence of nitric oxide donors has been reported to be related with the inhibition of platelet aggregation via the inhibition of fibrinogen receptor (integrin $\alpha_{IIb}\,\beta_{III}$). The phosphorylation of VASP is reversible and

it occurs at three separate sites (two serines and one threonine) that are dependent upon kinase activation (Abel et al., 1995). This post-translational modification of VASP may impede its interaction with focal adhesion proteins and actin binding proteins and its direct interaction with actin via the C-terminal domain of VASP. This mechanism is considered important in the re-organisation of the platelet cytoskeleton during the course of platelet shape change and the subsequent aggregation process that is inhibited by NO.

Phosphorylation of VASP occurs at three different sites, with phosphorylation of ser^{157}, as the preferred site for PKA, which cause a shift in the apparent molecular mass from 46-50kDa (Halbrugge et al., 1990).

VASP protein consists of three different domains, including EVH1 domain, profilin binding domain and EVH2 domain (Fig1.12). One of the phosphorylation sites, $serine^{157}$ is located in the proline-rich domain, which is involved in the interaction between VASP and profilin-actin complex. The two other phosphorylation sites are located in EVH2 domain (Fig 1.11), which is involved in the actin polymerisation and granulation. VASP binds to vinculin, zyxin and Act A via its EVH1 domain (Harbeck et al., 2000). Phosphorylation of $serine^{157}$ showed only a minor effect on the actin polymerisation, additional phosphorylation of $serine^{239}$ is necessary to prevent filament formation (Harbeck et al., 2000).

The de-phosphorylation of proteins is achieved by serine/threonine phosphatase 1 and 2. H_2O_2 has been reported to inhibit the activity of protein phosphatase1 (PP1), protein phosphatase2A (PP2A) and calcineurin (PP2B) with IC 50s of 12, 25 and 45μM respectively. While at higher concentrations up to100mM, it stimulates all these enzymes in cell lysates (Sommer et al., 2002).

Hydrogen peroxide at low concentrations, below those concentrations known to produce oxidative stress, may have a function as a signalling molecule. H_2O_2 also induces the relaxation of arterial smooth muscle (Burke-Wolin et al., 1991b), which may result from an increase in the production of NO (Marczin et al., 1992; Zembowicz et al., 1993). However, in platelets there is no significant rise in cyclic GMP or cAMP following exposure to hydrogen peroxide (Naseem et al., 1996; Sabetkar et al., 2001). Recently, H_2O_2 has been proposed to be one of the endothelium-dependent hyperpolarizing factors, which induce relaxation in small blood vessels (Matoba et al., 2000; Matoba et al., 2002) and may be formed by dismutation of superoxide anion synthesised by eNOS.

The phosphorylation of VASP was investigated using SDS/PAGE and western blotting. Using anti-VASP antibody, which recognises an epitope present in both phospho and dephospho forms (Abel et al., 1995), it was possible to detect both phosphorylated (46kDa) and non-phosphorylated (50 kDa) forms of VASP.

4.2 Aim

The aim of this study was to investigate the role of H_2O_2 on events downstream of sGC activation by looking at serine phosphorylation of platelet proteins. The direct effects of the hydrogen peroxide on the protein phosphorylation were studied, using the vasodilator- stimulated phosphoprotein as a marker.

4.3 VASP in non-stimulated human platelet after separation from plasma

VASP is known to exist in both phosphorylated and non-phosphorylated forms in *in vitro* studies. To investigate the role of the phoshorylation of VASP on the synergism between NO and H_2O_2, it was important to know in which form VASP exist in the normal circulating platelets and to be able to follow the changes during the recovery of platelets. For this aim, washed human platelets were prepared in the same way as for aggregation and cGMP experiments by addition of 50nM prostacyclin into plasma. Platelets were then separated from plasma by centrifugation at 800g for 20 mins at 20°C.

PGI_2 is known as an activator of adenylyl cyclase (AC). Activation of AC increases the concentration of cAMP leading to activation of protein kinases including protein kinase A and protein kinase G. These protein kinases then phosphorylate a series of proteins including the vasodilator-stimulated phosphoprotein. The phosphorylation of VASP with protein kinase A, as described previously, increases its apparent molecular weight from 46 to 50kDa.

As in the aggregation and cGMP studies, after separation from plasma platelets were rested for 2 h before addition of NO or NO/H_2O_2. During this 2 hours time, platelets recover from the effect of PGI_2 and thereafter the cAMP level decline to the basal level. For study of VASP, samples were prepared during this time.

In washed platelet samples, prepared by addition of PGI_2 (Fig 4.1a) immediately after separation of platelets from plasma (lane 1), VASP only exists in 50kDa form. This is due to the presence of cAMP after addition of PGI_2 and activation of PKA, which leads to phosphorylation of serine[157]. After 30 -60 minutes, the 46kDa form appears

(lane3). After 2 hours (recovery or resting time), VASP will be mostly detected in the 46kDa form (lane 4). The addition of PGI_2 to washed platelets after 2h recovery time was capable of increasing the phosphorylation of VASP to its 50 kDa form (lane 5, Fig 4.2 a).

The phosphorylation of VASP was also studied in the absence of PGI_2 (Fig 4.1b) using anti vasp antiserum (1:2000 dilution), which will recognize both phosphorylated and non-phodphorylated forms of VASP. To achieve this, platelets were separated by lowering of pH instead of addition of PGI_2 (method described in chapter 2). In these samples, in contrast to previous samples, VASP exists only in the 46kDa immediately after separation of platelets from plasma (lane 1) and also after 2 hours recovery time (lane 4). The addition of PGI_2 to these samples 2h after separation of platelets from plasma also increased the phosphorylation of VASP to 50 kDa form (Fig 4.2b, lane 5).

In conclusion, VASP exists in its 46 kDa phosphorylated or non-phosphorylated form in normal platelets during normal blood circulation. An addition of PGI_2 to platelets either during the preparation procedures or after separation of platelets from plasma is capable to increase the phosphorylation of VASP. This is due to an increase of cAMP concentration and activation of PKA. The extent of phosphorylation and also dephosphorylation of VASP during separation of platelets from plasma was different in different blood donors.

4.4 The effects of H_2O_2 on the phosphorylation of VASP

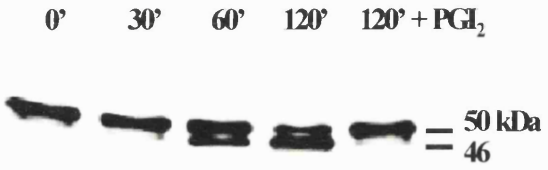
To investigate the effect of H_2O_2 on the phosphorylation of VASP, platelets were rested for at least 2h, after separation from plasma, during which time the cAMP concentration decreases. At this time point, VASP was mostly present in 47kDa form (detected with anti-VASP antiserum, Cont., Lane 4, Fig 4.2a). Using anti-phosphoserine antibody, the presence of 47 kDa forms of VASP was detected after 2 h resting period (lane 1, Fig 4.3). Addition of 25μM H_2O_2 for 1 min to platelet after 2 h recovery time increased the phosphorylation of VASP in its 50kDa form (Lane 5, Fig 4.2). The increase of phosphorylation caused by H_2O_2 was less compared to the effects of PGI_2 (Lane 1, Fig 4.1 and 4.2) when it was added initially to platelets or 2 h after separation of platelets from plasma (Fig 4.1 Lane 5). In both of these cases, PGI_2 shifted VASP to its 50kDa form by 90-100%. H_2O_2 increased the molecular weight of VASP partially and to a maximum up to 60%.

Addition of NO, at a low concentration of 10nM, did not increase the phosphorylation of VASP. The simultaneously addition of 10nM NO with 25μM H_2O_2 increased the phosphorylation of VASP detected by phosphoserine antibody. An increase of 50kDa form of VASP was detected using anti-phosphoserine antibody (Fig 4.2). The increase in phosphorylation of VASP in the presence of NO appears to be an additive effect rather than real synergism. The maximum phosphorylation of VASP observed with addition of H_2O_2 was at concentration ranges of 25 to 100μM. Higher concentrations of H_2O_2 decreased the amount of phosphorylation detected by the antiphosphoserine antibody (Fig 4.2).

NO alone at concentrations above 10nM increased the phosphorylation of VASP gradually from 47kDa to 50kDa again the simultaneous addition of NO and H_2O_2 increased the extent of the phosphorylation, but was additive.

Indeed a decrease in VASP phosphorylation was observed in the presence of 10nM NO and 250μM H_2O_2 (Fig 4.3).

a)



b)

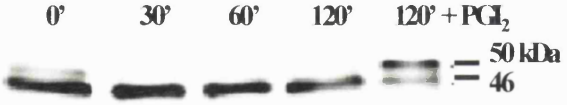


Fig 4.1: VASP in non-stimulated platelets after separation of plasma

Platelet samples were prepared by a) addition of prostacyclin to plasma or b) lowering of pH.
Samples were collected after 1, 30, 60, 120 minutes incubation at room temperature. PGI_2 (50nM) was added to one sample after 120 minute. Proteins were separated using 10% SDS-PAGE. The presence of different forms of VASP was then detected using anti-VASP anti-serum (dilution 1:2000). The results are typical of three independent experiments.

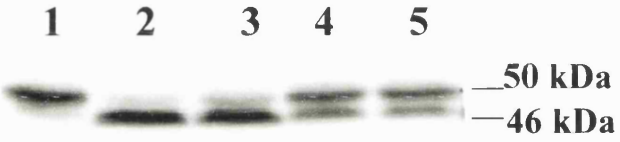


Fig 4.2: The effect of NO and hydrogen peroxide on the apparent molecular weight of VASP.

Control samples were collected immediately after separation from plasma (lane 1) and also after 2h recovery time (lane 2). Platelets were then stimulated for 1 min with either 0.01 μM NO (lane 3) or 25 μM H_2O_2 alone (lane 5) or both together (lane 4). Platelets proteins were separated using 10% SDS-PAGE. VASP phosphorylation was analysed using anti-VASP antibodies (dilution 1:2000). The data are typical of those found in three independent experiments.

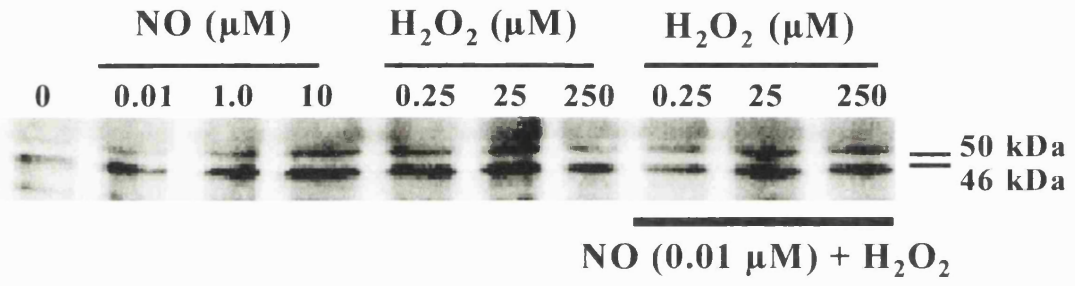


Fig 4.3: VASP serine phosphorylation in intact human platelets in the presence of different concentrations of NO and H_2O_2

Washed human platelets were stimulated for 1 min with different concentrations of either NO or H_2O_2 alone or both together at a range of concentrations. VASP phosphorylation was analysed using monoclonal phosphoserine antibodies (dilution 1:500) following immunoblotting from a 15% SDS gel. The data shown are typical of those in three independent experiments.

4.5 The reversibility of VASP phosphorylation in the presence of H_2O_2 and NO

In order to discover whether the phosphorylation of VASP was related to the synergism of NO and H_2O_2, it was essential to discover whether the time of phosphorylation and dephosphorylation parallels that of the inhibition.

It is known that dephosphorylation of VASP is essential for the subsequent actin polymerisation and activation of platelet. In this study, the time required for dephosphorylation of VASP in the presence of NO and hydrogen peroxide (Fig 4.4a) or only H_2O_2 (Fig 4.4b) was determined.

10nM NO and 25μM H_2O_2 were added simultaneously to washed platelet, after 2 hours recovery time, and then incubated for a periods of 0.5-120 min. The samples were then run on 10% SDS gels and phosphorylation of VASP was investigated by western blotting using anti-phosphoserine antibody.

The amount of phosphorylation of VASP was increased after only 0.5-1 min of addition of NO+H_2O_2. The maximum phosphorylation was achieved between 2-15 minutes (with 15 minutes as highest phosphorylation point). Phosphorylation was then diminished reaching the basal level after a period of 60-90 minutes, varying in different blood donors (Fig 4.4).

This time course paralleled the time required for the recovery from inhibition of platelet aggregation in the presence of NO and H_2O_2, which also returned to normal after 60 minutes (Fig 3.8).

In the presence of H_2O_2 alone the pattern of phosphorylation was very similar to the one with NO+ H_2O_2. The dephosphorylation of VASP was increased after 1 minute reaching the maximum after 15 minutes. VASP was then dephosphorylated after 30

minutes up to 60 minutes. This observation was detected using anti-VASP antibody (results not shown).

4.6 The effect of specific protein kinase inhibitors on thrombin-induced platelet aggregation

In order to confirm the role for cyclic nucleotides and protein kinases on the synergism between NO and H_2O_2 the effect of protein kinase A and G were investigated by using inhibitors for both protein kinases on the aggregation of platelets.

For this aim washed platelets after 2 hours recovery were incubated with the presence of cyclic GMP–dependent protein kinase inhibitor, KT5823, (Grider et al., 1993 and Ziolo et al., 2003) or with the cyclic AMP-dependent protein kinase inhibitor (myristoylated PKA inhibitor 14-22 amide, Niisato et al., 1999) for 30 min, prior to addition of NO/H_2O_2. The platelets response to thrombin 0.02 U/ml was then investigated as the percentage aggregation in the presence and absence of the inhibitors.

In the presence of 234nM KT5823, the normal synergism between NO and hydrogen peroxide was detected (Fig 4.5). In contrast, in the presence of PKA inhibitor at 36nM (reported K_i) this synergism was almost completely reversed (Fig 4.5).

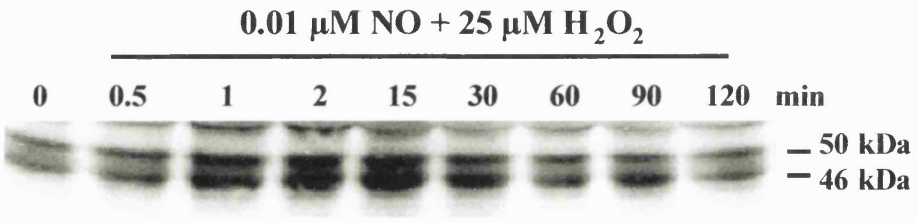


Fig 4.4: The time course of phosphorylation of VASP in intact human platelets in the presence of NO + H_2O_2

Washed human platelets were incubated in the presence of 10nM NO + 25μM H_2O_2 for a period of 0-120 min. VASP phosphorylation was analysed using monoclonal phosphoserine antibodies following immunoblotting from a 15% SDS/PA gel.

The data shown are typical of three independent experiments.

This result confirmed the importance of both protein kinases on the mechanism of synergism. The presence of PKA seems to be more important since the PKA inhibitor almost inhibited the synergism between NO and H_2O_2. The possibility that the role of PKA in this synergism might be through pathways others than VASP needs to be considered.

In conclusion, the observation that phosphorylation of VASP by H_2O_2 is through phosphorylation of serine[157] and the fact that the synergism of H_2O_2 and NO is inhibited by a PKA inhibitor are consistent. This still leaves the paradox that H_2O_2 is not capable of inhibiting platelet aggregation by its own.

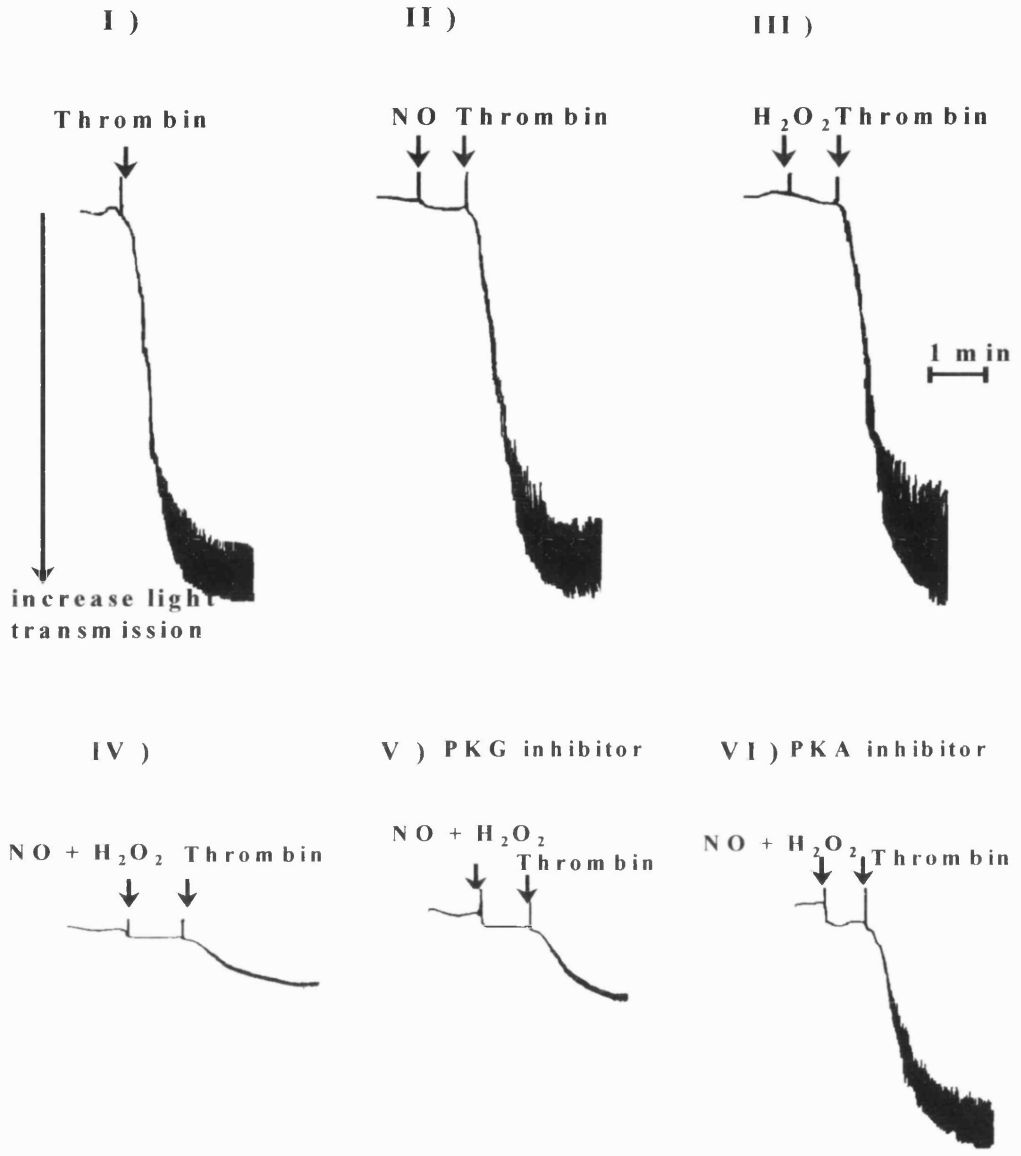


Fig 4.5: The influence of inhibitors of protein kinases, NO synthesis and soluble guanylyl cyclase on inhibition of platelet aggregation induced by NO and hydrogen peroxide.

Washed platelets were activated with 0.02 U thrombin /ml and after pre-exposure to (1) buffer, (2) 10nM NO (3) 25μM H_2O_2 (4) 10nM NO + 25μM H_2O_2 (5) 10nM NO + 25μM H_2O_2 in the presence of myristoylated 14-22 peptide protein kinase A inhibitor 36nM (30 min prior exposure) and (6) 10nM NO + 25μM H_2O_2 in the presence of KT5823 234μM and the extent of aggregation measured by conventional aggregometery. The results are typical of three independent experiments.

4.7 The effect of different pathways inhibitors on the phosphorylation of VASP

The inhibitors of protein kinase A and G and also ODQ and L-NAME were added separately into washed platelet after 2 hours recovery and 30 minutes prior to addition of NO and H_2O_2. The NO (10nM) and H_2O_2 (25μM) were added simultaneously and incubated with platelets for 1 minute. The reaction was then stopped by addition of sample buffer and the samples were then run on 10% SDS-PAGE gels and phosphorylation of VASP was investigated by western blotting and using anti-phosphoserine antibody.

The NOS inhibitor (L-NAME at 800μM) and sGC inhibitor (ODQ at 10μM) both in the presence of NO and H_2O_2, slightly inhibited the phosphorylation of VASP at 46kDa form but there were no changes of 50kDa form was observed (Fig 4.7). The inhibition of phosphorylation in the presence of these two inhibitors indicates that both endogenous NO and the soluble guanylyl cyclase are essential to the phosphorylation of VASP. This data agree with previous data when addition of L-NAME and ODQ to platelets partially inhibited the synergism between NO and H_2O_2. The addition of dibutyryl cGMP also inhibited aggregation partially (White et al., 1976).

The PKG inhibitor, KT5823, had little effect on phosphorylation induced by simultaneous addition of NO and H_2O_2 and then only in the 46kDa form, indicating that PKG is activated by the addition of NO and H_2O_2, which to some extent leads to phosphorylation of VASP at serine[239]. In contrast, the myristoylated PKA inhibitor (14-22 peptides) at 36nM reduced the serine phosphorylation of both 46 and 50kDa

VASP. The PKA inhibitor showed a very significant decrease of phosphorylation, which agrees with all previous findings in this chapter. These results confirms the importance of PKA in the synergism between NO and H_2O_2 also that the complete mechanism is still not clear.

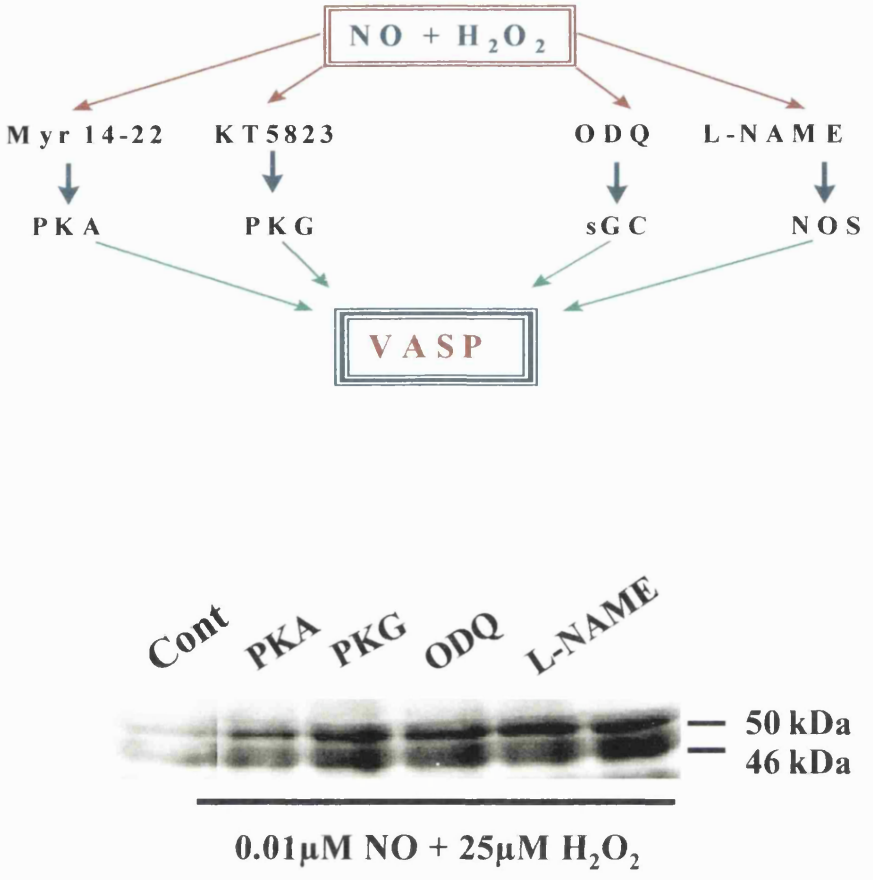


Fig 4.6: Phosphorylation of VASP in the presence of the inhibitors of different pathways.
Washed human platelets were exposed for 1 min to 0.01μM NO + 25μM H_2O_2 (Lanes 2-6) after pre-incubation for 30 mins with either myristoylated 14-22 peptide protein kinase A inhibitor 36nM (Lane 2), PKG-inhibitor KT5823 234μM (Lane 3), ODQ 10μM (Lane 4) and L-NAME 800μM (Lane 5) or buffer (Lane 1). VASP phosphorylation was analysed using monoclonal phosphoserine antibodies (dilution 1:500) following immunoblotting from a 10% SDS gel The results are typical of two independent experiments.

4.8 Discussion

Earlier studies suggested that the mechanism underlying the synergism between NO and H_2O_2 was not primarily at the level of soluble guanylyl cyclase. The actions of the hydrogen peroxide on events downstream of cGMP formation may be of importance. The complete series of events mediating NO-dependent inhibition of platelet aggregation has not been fully elucidated. However, cGMP is known to activate cGMP kinase 1 (cGK1), which reduces $[Ca^{2+}]_i$ and inhibits platelets, and initiates the phosphorylation of VASP (Reinhard et al., 1992), the IP_3 receptor (Haug et al., 1999) and the thromboxane receptor (Butt et al., 1994). The importance of VASP in platelet function was indicated by the modification of platelet function in VASP knockout mice (Aszodi et al., 1999). At low concentrations of NO, the phosphorylation of ser^{239} of VASP occurs, whereas PGI_2 acts via the phosphorylation of ser^{157} leading to an apparent change in molecular weight (Horstrup et al., 1994). NO at higher concentrations also leads to the phosphorylation of ser^{157} and a decrease in the exposure of fibrinogen binding sites (Butt et al., 1994; Horstrup et al., 1994). Indeed, the synergism often observed between NO and PGI_2 may in part involve the enhancement by NO of PGI_2-induced phosphorylation of ser^{157} and an additional phosphorylation of Ser^{239} at the same time. The role of different domains of VASP on actin polymerisation was studied by Harbeck et al. The EVH2 domain, which consists of two phosphorylation sites, including Ser^{239} and one nitration site (Fig 5.2) is reported to be the important part of this protein in the control of actin polymerisation. In contrast the proline-rich domain which consists of Ser^{157} did not show any effect on the reorganisation and polymerisation of actin filaments (Harbeck et al., 2000) therefore there is no evidence until now to suggest that this phosphorylation site is

directly involved in the inhibition of platelets activation. The proline-rich domain is important in the binding of profilin to actin and the productions of the actin-profilin complex. There is no evidence confirming the involvement of this complex on the activation or the cell shape of platelets.

In the current studies, it was revealed that H_2O_2 induced phosphorylation of both the 46 and 50kDa bands in the absence of NO and without a rise in ambient cyclic GMP or cAMP concentrations. This provides further evidence that the basis of their synergism is more complex and the small elevation of cGMP or at least the presence in low concentrations is a small part of the whole mechanism behind this synergism. When NO and H_2O_2 were added simultaneously, there was again increased serine phosphorylation at both 46 and 50kDa, but the effects were little more than additive.

Hydrogen peroxide alone does not inhibit platelet aggregation, but still enhances VASP phosphorylation even at sub-micromolar concentrations. This suggests that other events are essential to connect VASP phosphorylation with inhibition of platelet activation, events that may be dependent on NO or activated by low concentrations of NO. The phosphorylation of Ser^{157} by hydrogen peroxide is not enough to cause inhibition of aggregation, since H_2O_2 was not capable to inhibit platelet aggregation on its own. The fact that no synergism was observed between PGI_2 and H_2O_2 may reflect the greater potency of PGI_2 in initiating the phosphorylation of Ser^{157} or that this eicosanoid has further actions required for inhibition, not shared by the peroxide.

Previous studies have shown that H_2O_2, at least at concentrations of 1mM, induces tyrosine phosphorylation of focal adhesion protein (Vepa et al., 1999) and that hydroxyl radical, probably produced by the Fenton reaction, were the active mediator of phosphorylation. We have also found that hydroxyl radical scavengers, mannitol and urate, can partially reduce the synergism between NO and H_2O_2 (Naseem et al.,

1996; Naseem and Bruckdorfer, 1995). Thus, it is reasonable to speculate that other radical species may be involved in this phenomenon. Hydrogen peroxide has been reported to activate the $p70^{S6k}$ signalling pathway in mouse epidermal cells (Bae et al., 1999) using an enzyme generating system. Similarly reports of the enhancement of tyrosine phosphorylation of protein kinase C by hydrogen peroxide was achieved at a concentration of 5mM (Konishi et al., 1997). Sub-micromolar concentrations of H_2O_2 did inhibit the inward rectifying potassium current in endothelial cells (Bychkov et al., 1999).

The mechanism of action of H_2O_2 is unclear, although many mechanisms have been reported on known signal transduction pathways, mainly at concentrations that stress cells (Suzuki et al., 1997b). The evidence provided here by the inhibitors of the relevant protein kinases suggests that, both in the case of inhibition of aggregation and with VASP phosphorylation, cAK may be one pathway for the action of H_2O_2. At low concentrations, the peroxide had no effect on ambient cyclic AMP or cyclic GMP concentrations, but nevertheless appears to activate the cAK. H_2O_2 may inhibit phosphatase activity and intensify the phosphorylation of VASP or other proteins. This is worthy of further investigation since H_2O_2 has been reported to have such effects, but usually at much higher, cytotoxic concentration (Guy et al., 1993).

VASP is associated with the inhibition of agonist-induced aggregation, but not calcium regulation and degranulation, in NO or PGI_2 treated platelets. The phosphorylation of ser^{157} is the key to inhibition of fibrinogen binding (Horstrup et al., 1994), and it may be that H_2O_2 enhances the ability of NO to modify VASP, thus enhancing the effectiveness of the latter as a platelet inhibitor. However, although H_2O_2 induces the phosphorylation of ser^{157} independently of the addition of low doses of NO, this does not preclude the possibility that the observed synergism involves

VASP. The peroxide may simply substitute for higher concentrations of NO with respect to VASP phosphorylation and the low dose NO may complete the process of inhibition by some other mechanism in which H_2O_2 has no influence.

The precise function of VASP is uncertain; it may act as an adapter protein, linking the extracellular matrix, via integrin-associated proteins such as vinculin, to the actin polymerisation mechanism of the platelet.

The effect of H_2O_2 on the phosphorylation of VASP was not due to an increase of cAMP, since H_2O_2 shown not to increase the amount of cAMP in intact platelet samples (results not shown). H_2O_2 may activate PKA directly independently of cAMP present. The phosphorylation of VASP in its 50kDa form in the absence of intracellular cAMP was also detected in the cells stimulated by PGE_1 (Weber et al 1999).

The anti-phosphoserine antibody, which would only recognise VASP in its phosphorylated form, was not able to detect any significant increase of VASP phosphorylation in the samples stimulated with 10nM NO alone. In contrast, NO at concentrations 1 and 10μM increased the phosphorylation significantly in both 46 and 50kDa form (Fig 4.4). The phosphorylation of VASP in the presence of different NO donors, including SNP and S-nitrosoglutathione has been studied by other groups. The results presented here agreed with these studies (Hallbruge et al 1990). However, addition of NO and H_2O_2 simultaneously only increased the phosphorylation of VASP in the same extent as H_2O_2, when added alone (Fig 4.3 and 4.4).

There are different views concerning cytotoxic concentration of H_2O_2. In some publications, the millimolar concentration of H_2O_2 is still at physiological range (Beckman, 1996).

In this study the addition of 250μM, H_2O_2 in contrast to 0.25 and 25μM did not increase phosphorylation of VASP (Fig 4.4). This concentration of H_2O_2 is generally considered to be at the border of cytotoxicity.

Hydrogen peroxide alone was not able to increase the cAMP or cGMP levels (chapter 2), but was still capable of causing phosphorylation of VASP, unless a very high concentration of cGMP is present. That was not the case as we reported in the previous chapter. The possibility that PKA may cause phosphorylation of VASP in the absence of cAMP been suggested by other groups (Weber et al 1999). The existence of any cross-reaction between PKA and PKG and the role of both kinases on the phosphorylation was studied in following experiments.

As described previously, VASP phosphorylation has been reported to correlate directly with the inhibition of platelet aggregation either through actin polymerisation or inhibition of integrin receptors (Walter et al 1993, Horstrup 1994). In this study, it was observed that H_2O_2 alone does increase the phosphorylation of VASP causing an increase of its molecular weight to 50kDa. Therefore, H_2O_2 at this concentration should have been able to inhibit platelet aggregation in the absence of NO. As described in the previous chapter, H_2O_2 alone does not inhibit the aggregation of platelet induced by thrombin at this concentration. The mechanism of inhibition of platelet aggregation induced by phosphorylation of VASP is not fully understood and more investigations need to be done. There is evidence that phosphorylation of serine^{157} had only a small effect on the inhibition of actin polymerisation. Additional phosphorylation of serine^{239} does prevent filament formation (Butt et al 1994). There is no evidence that the phosphorylation of both serine residues are required happen simultaneously or with a limited time difference or if it will amplify the final effect.

Still PGI_2 is capable of inhibiting platelet aggregation and as described here it also phosphorylates VASP on the serine[157].

CHAPTER FIVE:

THE NITRATION OF PLATELET PROTEINS

5.1 Introduction

In the view of the fact that studies on synergism between NO and H_2O_2 in platelets could not be completely explained by activation of sGC or by phosphorylation of VASP, others aspects of the interaction between NO and reactive oxygen species were considered. NO is not known to react directly with H_2O_2, but does form peroxynitrite when interact with superoxide anion (Beckman et bal 1992). As indicated in the introduction, peroxynitrite formation leads to the nitration of aromatic amino acids in proteins, particularly tyrosine. The nitration of proteins was primarily detected in tissues from different kinds of inflammatory disease. Beckman and co-workers (1992) proposed that $ONOO^-$ was the most probable nitrating agent in these disease, leading to hundreds of publications. Later peroxynitrite was also suggested as a common nitrosating agent of proteins. More evidence of involvement of this agent in different kind of protein modifications is available now. New studies on nitration revealed that $ONOO^-$ may not be the only nitrating agent and may not be the most common route of nitration either. There is now sufficient evidence to suggest that nitration of proteins may play an important role in the normal cell signalling, since nitration of important enzymes including glutathione peroxidase, prostacyclin synthase and superoxide dismutase have been detected even under physiological conditions (Greenacre and Ischiropoulos, 2001).

The availability of both superoxide anions and NO in platelets raises the possibility that peroxynitrite may be formed endogenously in platelets. This oxidant may also be released from the endothelium by agonist-stimulated process (Kooyet al 1994). $ONOO^-$ and ONOOH both cross the biological membranes, via anion channels and passive diffusion, respectively. The biological half life of peroxynitrite is less than

100ms, which is enough for ONOO to travel some distance between 5-20μm across extra and/or intracellular compartment (Denicola et al., 1998; Marla et al., 1997b). This is enough for this oxidant to penetrate the adjacent cells causing nitration of cytosolic or membrane proteins.

Moro et al found that higher concentrations of peroxynitrite (50-150 μM) activate isolated platelets, when at lower concentrations it inhibits platelet activation. In the presence of plasma, only the inhibitory action of peroxynitrite was observed (Moro et al., 1994).

Naseem (1997) found that IC_{50} for peroxynitrite to be at 5μM and that this could lead to nitration of tyrosine residues in platelets proteins. Recently it was also discovered that nitration may be the results of reactions others than those involving $ONOO^-$. Various peroxidases were reported to be able to cause nitration of proteins independently of peroxynitrite. Incubation of human haemoglobin with nitrite and hydrogen peroxide was found to induce auto-nitration of haemoglobin and nitration of other proteins including bovine serum albumin. This effect of haemoglobin was reported to be due to the pseudoperoxidase activity of haemoglobin (Grzelak et al., 2001a). Similar effect was detected by myoglobin in a different study (Kilinc et al., 2001). Incubation of whole erythrocytes with nitrite and hydrogen peroxide induces nitration of erythrocyte membrane proteins much stronger when the cellular catalase was inhibited with azide (Grzelak et al., 2001b). Eosinophil peroxidase and myelo-peroxidase also increased the nitration of proteins in the presence of hydrogen peroxide and nitrite (Sampson et al., 1998, Gaut et al., 2002).

In this study we also investigated the possibility of increase thromboxane release during bleeding. To eliminate this possibility we asked our volunteers to take one or

two doses of 600mg aspirin 8 to 16 hours before experiment. Alternatively the aspirin was added to the anticoagulant buffer (acid citrate dextrose). We also investigated the possibility that maybe the release of ADP from platelet or erythrocytes during bleeding is causing activation of platelet. For this aim a mixture of creatine phosphate (CP) and creatine phospho kinase (CPK) was added to anticoagulant, which was taken into the syringes before bleeding.

An increase in protein nitration might be the result of an increase in the formation of nitric oxide or that of superoxide anions, or both, as components of the peroxynitrite leading to nitration of tyrosine and modification of other amino acids. Alternatively there may be other mechanisms at work, which are unrelated to peroxynitrite but require the formation of other nitrating species such as nitrogen dioxide radical as described in general introduction (chapter one).

The amino acid sequence of VASP includes four tyrosine residues. There is possible that nitration of VASP as well as its phosphorylation may cause conformational changes of the protein and as described in the first part of this chapter the location of each nitrated tyrosine residues can be important in the binding ability of VASP to its different targets. Tyrosine nitration may facilitate the serine phosphorylation of VASP, which then can cause inhibition of platelet aggregation.

Yet there is no report of tyrosine phosphorylation in VASP despite the location of key tyrosine residues in EVH1 and EVH2 regions.

If nitration is to be an important physiological process regulating protein activity it would need to show some selectivity for specific proteins. Furthermore, this process should also be reversible.

5.2 Aim

The aim of this study was to investigate spontaneous nitration of VASP and other proteins during the preparation of washed platelets under physiological conditions, by using freshly prepared platelet samples.

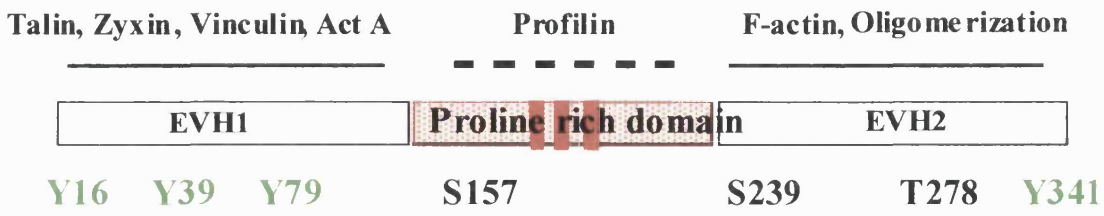


Fig 5.1: Model of connections between VASP and different cytoskeletal proteins through its domains and the distributions of different tyrosine and serine residues.

The diagram represents three different domains of VASP. The EVH1 domain binds to its target proteins, including talin, zyxin, viculin and Act A. Three of four tyrosine residues are present in this end of protein. The middle part of VASP, known as prolin rich domain connect this protein to profilin, which also binds to actin cytoskeleton. The only phosphorylation site, which causes mobility of protein in SDS-gel, is located in this domain. There is no observation showing any connection between phosphorylation of this site and inhibition of actin polymerisation. The serine[157] phosphorylation does not inhibit binding of VASP to profilin (Harbeck et al., 2000). The end part or c-terminal of VASP, known as EVH2 domain, connects VASP to F-actin. Two phosphorylation sites in this domain are important in the inhibition of actin polymerisation and oligomerization, which leads to platelet shape change and inhibition. One of the four-tyrosine residues is present at the end of this domain.

5.3 Preparation of platelet samples for nitration study

Platelets are sensitive cells and can be readily activated during the process of isolation. Care must be taken, even during withdrawal of blood, when wide gauged needles should be used, and the collection done in acid/citrate/ dextrose buffer pH 7.4. The blood is then centrifuged gently to prepare platelet - rich plasma (PRP). The gentlest way of isolating the platelet from plasma is by addition of low concentrations of prostacyclin and a double centrifugation after re-suspension in buffer to remove traces of plasma. The platelets are then rested for 90-120 min to allow them a full recovery of platelet activity following the return to normal of cyclic AMP concentrations. Alternatively, platelet can be isolated by lowering of the pH in the PRP, which also inhibits platelet activation during centrifugation.

5.3.1 Spontaneous nitration of platelet proteins

The initial aim of this study was to determine the extent to which protein nitration occurs in rested platelets and the reason for the variation observed by Naseem et al (1997). For this aim, platelets were separated from plasma with addition of 50nM PGI_2 as in earlier work. After separation of the platelets from plasma, samples were collected immediately and up to a period up to 120 minutes, the time normally allocated for platelet recovery. Platelet proteins were then separated on a 4-12% gradient pre-casting gel. The extent of the nitration was studied by either using the commercial polyclonal anti-nitrotyrosine antibody (Upstate) or using RF1256 polyclonal anti-nitrotyrosine antibody, which was developed in our laboratory.

In this study, we demonstrated that some of platelet proteins are nitrated after separation of platelets from plasma. The possibility that platelets may have been

exposed to activation or cell stress during isolation procedures was studied by comparing two different isolation protocols.

Immediately after isolation of platelets from plasma, a significant amount of nitration was observed in certain proteins with molecular weights of 14, 33, 35-38, 46, 50, 53, 55, 68 and 72kDa. Using polyclonal antibodies some of these proteins have been identified: the 38kDa protein was identified as MAPKinase P^{38} MAPK and the 46/50kDa protein as VASP. The 14kDa protein, 53-55, 68 and 72kDa proteins were identified as profilin, lyn, paxillin and anti syk respectivelyusing immunoprecipitation (Sabetkar & Low unpublished data).

An important observation was that nitration was transitory for some proteins, but apparently irreversible for others. Nitration of 33kDa protein was irreversible and did not change through out the 2h period. In contrast, nitration of certain other proteins was changed by time (Fig 5.1a). The amount of nitration diminished during the first 15 minutes and then rapidly decreased after 30-60 min, which varied in different blood donors. The 14 kDa protein (profilin) normally is much more nitrated than it is in the presented picture (Fig 5.3). The explanation is that, this particular gel was stopped earlier and profilin is on the borderline very close to the end of the gel. It is possible that the contact between the antibody and the ages of the membrane was poor as well.

A positive control for anti-nitrotyrosine antibody was also purchased from Upstate. This control is a mixture of four nitrated proteins. These proteins include 16 and 32kDa forms of SOD, 66kDa BSA and 215kDa myosin. Regarding to the instruction all these 4 proteins should appear with anti-nitrotyrosine antibody. In this study only at very high concentrations was possible to get four of the proteins and if high

concentration of the control was used then the 66kDa protein would be very strong, which would almost destroy the whole blot.

5.3.2 The specificity of anti-nitrotyrosine antibody

The specificity of nitration observed in these proteins was investigated by eliminating anti-nitrotyrosine antibody from blotting procedures, when all other steps were kept exactly as the original protocol.

In the absence of the nitrotyrosine antibody, no nitration at all was detected. This observation indicates that the positive signal is only observed in the presence of the nitrotyrosine antibody binding to its epitope and that these signals are specific binding of nitrotyrosine antibody to nitrated proteins (Fig 5.3b).

In a separate experiment, the specificity of nitrotyrosine was studied by blocking the antibody binding with dithionite (Low et al, unpublished data).

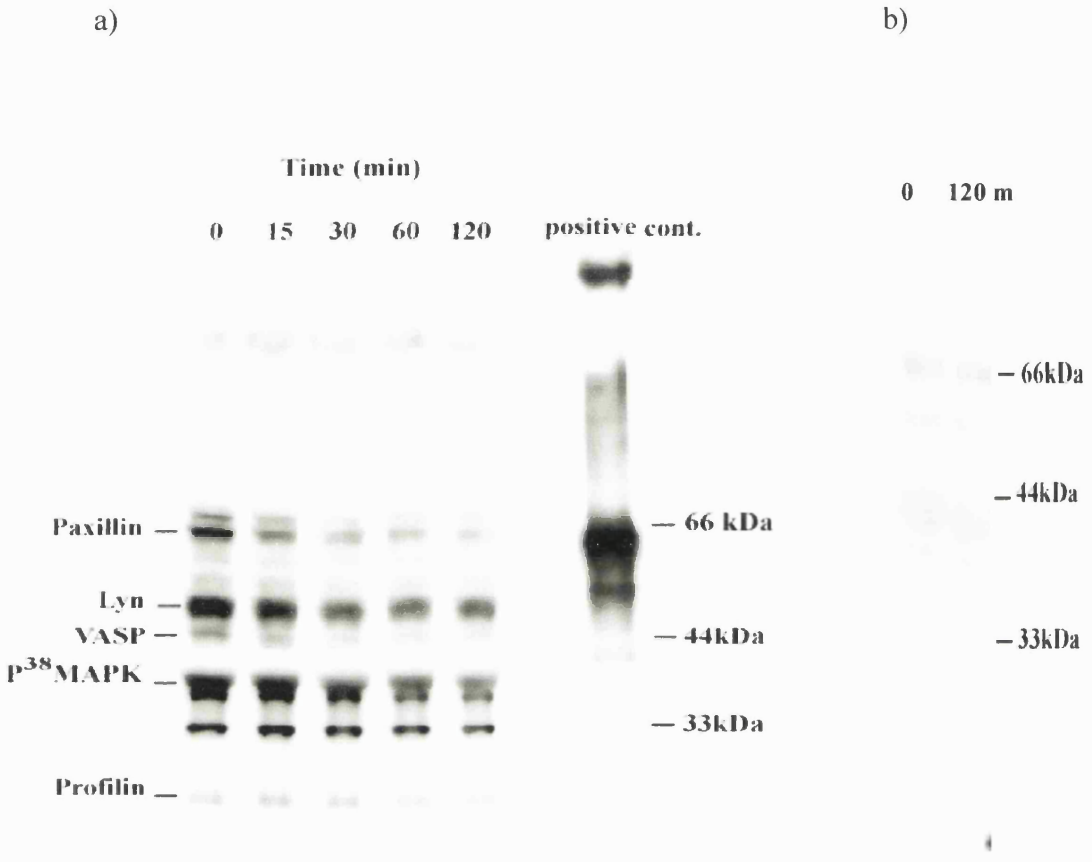


Fig 5.2: Nitration of proteins in freshly isolated platelet

a) Washed human platelets were separated from plasma by addition of 50nM PGI_2 and samples were collected immediately after separation of platelets and up to a period of 120min.
b) The specificity of the nitration was studied by eliminating the nitrotyrosine antibody from the procedure.
The platelet proteins were separated using 4-12% pre-cast gradient gels and blotted with anti-nitrotyrosine antibody (upstate antibody, dilution 1: 500). The positive control was a mixer of four nitrated proteins including 16 and 32kDa SOD, 66kDa BSA and 215kDa myosin.

5.4 Influence of isolation procedures on the nitration of proteins

In section 5.2, nitration of proteins was found in freshly centrifuged platelets, prepared in the presence of PGI_2, a platelets protectant. It was possible that PGI_2 itself has a specific effect on the nitration, therefore an alternative technique was employed. Washed platelets were separated from plasma either by *addition of PGI_2* or by *the lowering of pH* to 5.5 as described in the method section (chapter two). Furthermore, it was possible that the initial syringe puncture may damage the endothelial cells or perturb platelets, in either case causing activation of the platelets. In order to reduce this, the first 5ml of blood was taken into a separate syringe and then discarded so that a midstream sample was used. During the procedure, care was taken to minimise the risk of activation of the cells.

Parallel samples of blood were then processed by each of the two washing methods and platelets were isolated from plasma. The platelet samples were then treated in exactly the same ways for investigation of nitration. Platelet samples were collected immediately after separation of platelets from plasma and up to 60 minutes after separation. PGI_2 was then added to one platelet sample again 60 minutes after separation and incubated for only 1 minute. This sample was made to check if PGI_2 is capable to cause a second phosphorylation of VASP in washed platelets in the absence of plasma.

In both preparations of samples, the extent of nitration of VASP was the same (Fig 5.4). The nitration could therefore not be attributed to prostacyclin because when it was added to platelets that had rested for 60 min, there was no further increase in protein nitration (Fig 5.4a). After zero time in the presence of PGI_2, the 50kDa VASP was nitrated. The phosphorylated 50 kDa form was absent in platelets separated by

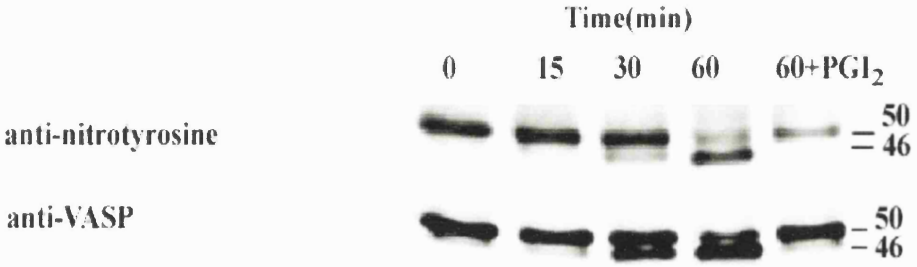
the low pH method, but the 46 kDa form was nitrated. This suggests that nitration just follows the pattern of serine phosphorylation and has no influence on the apparent molecular weight.

The serine phosphorylation and tyrosine nitration of VASP were compared using anti-nitrotyrosine and anti-phosphoserine antibodies. Both forms of VASP appear to be capable of being nitrated and nitration of VASP was reversible (Fig 5.4b). The extent of the nitration then was decreased to the basal level after 60 min varying between different blood donors and different experiments.

5.5 Inhibition of platelet activation and cyclooxygenase by aspirin and nitration of platelet proteins

To eliminate the possibility that the activation of arachidonic pathway during isolation was involved in basal nitration of platelet proteins, the basal nitration was investigated in the presence of aspirin. Aspirin either was added to the anticoagulant or was taken by the blood donors at least 16 hours before the experiments. The blood was then centrifuged as usual and platelets were separated by addition of PGI_2. Platelet samples were collected after separation of platelet from plasma (Fig 5.5 lane 1&2) and after 60 minutes of recovery time (Fig 5.5 lane 3&4).

a)



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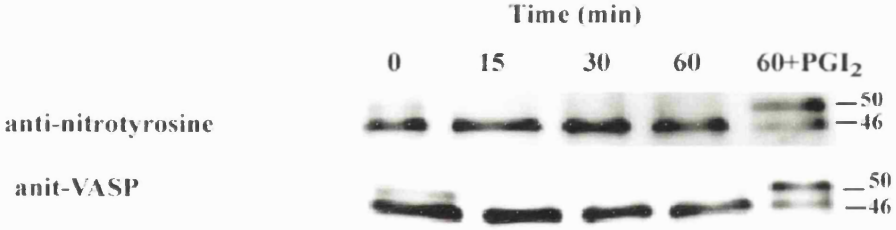


Fig 5.3: Nitration of VASP in platelet separated by addition of PGI_2 or by lowering of pH.

Washed platelets were separated from plasma by addition of 50nM PGI_2 to plasma (a) or by lowering of pH to 6.4 (b), during separation procedure. Samples were collected immediately after separation up to a period of 60 min. 50nM PGI_2 was then added into platelet after 60 min and incubated with platelets only for 1min. Platelet proteins were separated using 10% SDS-PAGE and membranes were blotted with either anti-VASP or anti-nitrotyrosine antibodies (upstate 1:500).

Aspirin is a well-known inhibitor of cyclooxygenase pathway (Patrignani et al 1983), which is essential to normal platelet activation and to the prevention of systemic hemorrhage. Activation of this pathway begins when a platelet agonist such as ADP, collagen, epinephrine, or thrombin binds to its platelet membrane receptor site. This activates phospholipase A_2, liberates arachidonic acid from membrane phospholipids, and then acts as a substrate for the COX pathway. Platelets (and other cells) are now known to produce two isoforms of COX called COX-1 and COX-2. COX-1 is a membrane-bound enzyme that functions in all normal platelets, whereas COX-2 is a cytokine-inducible enzyme that appears in newly-produced platelets and in other cells during inflammation, but is less than 10% of total COX activity.

Aspirin irreversibly acetylates both COX-1 and COX-2 at serine 529. For the COX-1 enzyme, the attached acetyl group sterically hinders the access of arachidonic acid to the reactive site. Acetylation does not appear to hinder the activity of COX-2. Several clinical studies establish that aspirin, at doses between 30 and 325 mg/day, reduces the incidence of arterial thrombotic events.

Administration of aspirin had no significant effect on the basal nitration. Therefore COX –1 and its metabolites can not be considered important in the cause of the nitration.

nitration and tyrosine phosphorylation was detected in these samples regarding VASP. By the increase of nitration a decrease of tyrosine phosphorylation was observed (Fig 5.9). There is enough literature to confirm the cross-reaction between tyrosine phosphorylation and tyrosine nitration (Gow et al 1996).

5.10 Nitration induced by collagen

The nitration of protein is a process, which seems to occur in part in circulating platelets, but is also strongly enhanced during the process of isolation from other blood cells. Furthermore, the nitration does not appear to be permanently present and is accelerated by the presence of calcium ions. During the initial stage of the isolation of platelet from blood the concentration of calcium ions is high and may prevent the maximum acceleration of nitrated proteins. The question arises whether during activation or stimulation of proteins by other agents a second wave of nitration can be evoked. Collagen is an important physiological activator of platelets and among the main activators. Collagen is a unique platelet agonist that acts both as an adhesive surface protein and as a strong activator. Collagen-induced signal transduction in platelets leads to a series of reactions including the activation of GPIIb/IIIa, formation of TXA_2, secretion of activators like ADP upon the release of α- and dense granules, and formation of the procoagulation phospholipids surface.

The role of collagen on cell signalling through activation of proteins like P38 MAPKinase, Syk and other proteins has been established.

In this study the role of collagen on the nitration of platelet proteins was investigated by addition of a series of concentrations of collagen (0.2, 2, 20 μg/ml) for 1 min in washed and recovered platelets. The extent of tyrosine nitration or phosphorylation was studied using the commercial anti- nitrotyrosine or anti-phosphotyrosine antibodies.

The extent of nitration was increased with increase of concentration of collagen (Fig 5.8). The increase of nitration in this case might be caused by the increase production of endogenous NO in the presence of collagen. A cross-reaction between tyrosine

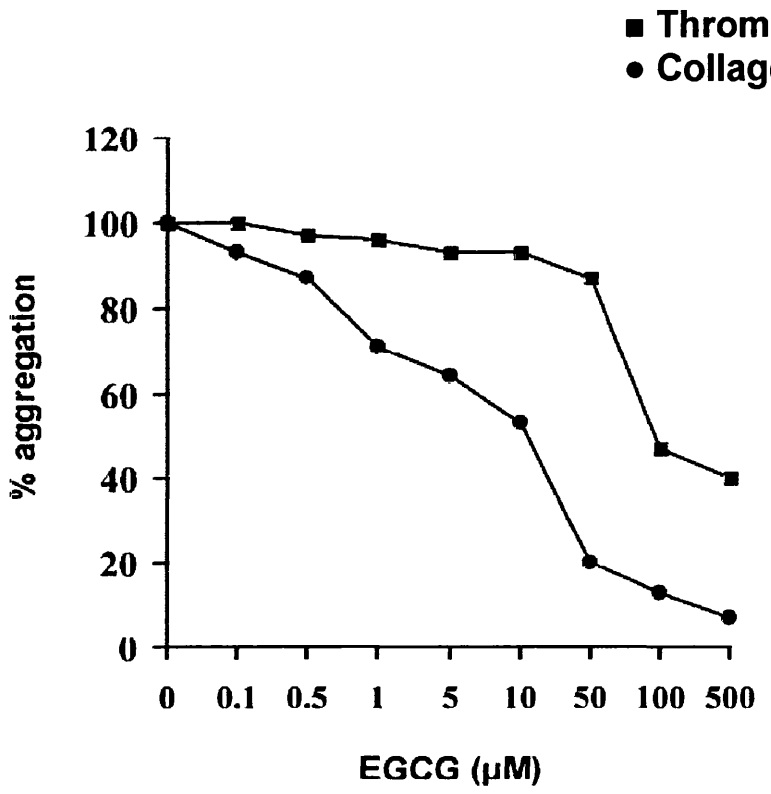


Fig 5.8: The effect of EGCG on the inhibition of platelet aggregation

Washed platelets after recovery were incubated with EGCG for 20 min prior to addition of different concentrations of either collagen or thrombin. The aggregation was measured as increased light transmission for 3min.

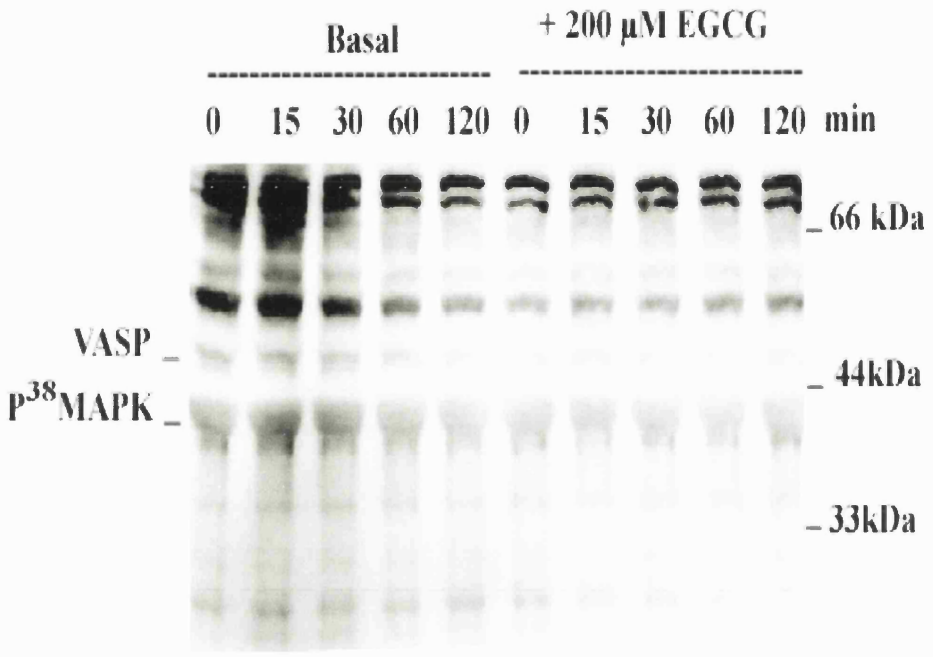


Fig 5.7: Inhibition of basal nitration in the presence of EGCG

200µM EGCG was added into anticoagulant and blood was treated as described in the original protocol. Samples were prepared immediately after separation of washed platelets from plasma and up to a period of 120 min. Platelet proteins were then separated using 4-12% pre-cast gradient gels and membranes were blotted with anti-nitrotyrosine antibody (upstate 1:500).

able to reduce the basal nitration significantly in several independent experiments. The inhibition of collagen-induced aggregation by EGCG was also reported by other group previously (Sageska-Mitane et al 1990). The real mechanism of the action of EGCG on the nitration is not known yet, but it is worth investigations. Different independent studies supports the inhibitory roles of EGCG on the nitration. The role of the flavonoids on different kinases was also investigated by different groups. An inhibitory action of EGCG on protein kinases has been established separately by Bain (2003) and Singh et al published very recently (2003). EGCG inhibits mitogen activated protein kinase sub group c-Jun kinase in human osteoarthritis chondrocytes.

effects in cultured cells. Alternatively, it may be argued that low-levels of nitration may be of importance in cell signalling processes.

5.9 The effect of EGCG on the inhibition of platelet aggregation

The nitration results raised the question if the compound would have any effect on inhibition of platelet aggregation induced by different platelet agonists. Therefore, the role of epigallocatechin gallate on the activation of platelets was studied by measuring the aggregation response induced by constant concentration of either thrombin or collagen in the presence of a series of concentrations of EGCG.

For this aim the EGCG was added to platelet samples after full recovery from PGI_2 (2 hours after separation of platelets from plasma) and the samples were incubated for 30 min at room temperature. Platelet samples were then incubated for one min in aggregometer and after 1 min either collagen (0.5μg/ml) or thrombin (0.02 U/ml) was added and aggregation was measured for 3 min after addition of the agonist.

The IC_{50} for EGCG was calculated in the presence of both agonists. EGCG significantly inhibited the aggregation induced by collagen even at low concentrations.

The IC_{50} for EGCG in the presence of collagen was 10 ± 2μM. The IC_{50} for EGCG in the presence of thrombin was 100 ±10μM, which is ten times more than the IC_{50} for collagen (Fig 5.8).

The observed inhibitory effect of EGCG on the nitration (Pannala et al., 1997) raises the question if the effect of this flavanoid on the inhibition of platelet aggregation is induced by its effect on the nitration or vice versa. What is obvious is that EGCG was

5.8 The effect of EGCG on the basal nitration of platelet proteins

To investigate further the basal nitration a known inhibitor of ONOO-induce nitration was used. EGCG was shown to inhibit nitration induced by ONOO, but not oxidation (Schroeder et al., 2001). It was also reported that EGCG inhibits NOS via inhibition of iNOS (Chan et al 1997). Therefore, it was interested to see if this compound had any effect on the basal nitration of platelet's proteins. This would help to ascertain the involvement of ONOO or NOS on the basal nitration of platelet proteins.

For this aim EGCG was added to anticoagulant (ACD) prior to withdrawal of blood. In the presence of this polyphenol a significant reduction in the nitration of a wide spectrum of the platelet proteins, including VASP was detected. Some of the higher molecular weight proteins were still nitrated (Fig 5.7).

In order to investigate the possibility of involvement of any proteosome activityin nonstimulated cells, a cocktail of different proteosome inhibitors (50 μM calpeptin, 50μM MG-132 peptide, 100μM ALLN and 1mM EST) was prepared and added to anti coagulant (ACD) prior to withdrawal of blood. The simultaneous addition of a cocktail of protease and proteosome inhibitors had no effect on the rate of disappearance, suggesting that the loss of nitration was not simply due to normal proteolysis (picture not shown). Therefore, it is possible that, even during the process of isolation, losses in nitrated proteins may be occurring, which are accelerated by the presence of intra-platelet calcium and that epigallocatechin gallate prevents further nitration occurring. These results may have implications for the presence of nitrated proteins in other cell types. The nitration of these proteins may be attributed to stress

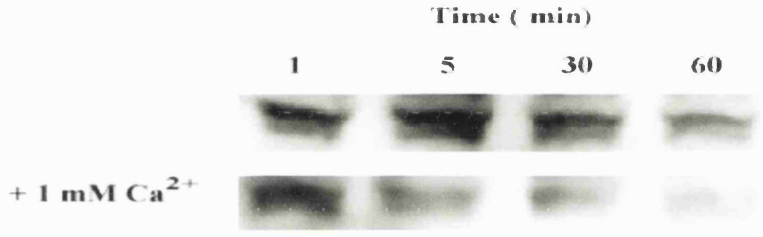


Fig 5.6: De-nitration of VASP in the presence of calcium

Washed platelet immediately after separation were incubated at 37 °C for up to 1 hour in the presence and absence of exogenous Ca^{2+}(1mM). Platelet proteins were then separated using 4-12% pre-cast gradient gels and membranes were blotted with anti-nitrotyrosine antibody (RF1256 dilution 1:500).

no explanation for this individual variation, but it may be related to the activity of calcium-activated proteases such as calpain.

The stripping of membrane and re-probing with anti-VASP anti-body showed that concentrations of VASP in each lane were similar.

The possibility of the proteolysis was investigated by using a mixture of four different protease inhibitors either by addition to anticoagulant or by adding it to platelet samples after separation of platelet from plasma. In the presence of these inhibitors no significant changes on basal nitration was detected (results not shown).

5.7 The effect of calcium on nitration

The disappearance of nitrated proteins from the platelets was a consistent founding, but the rates varied between different platelet preparations. It has been suggested that nitration of platelets may render them more susceptible to proteolysis. Some proteolytic enzymes are calcium dependent. The concentration of calcium in the isolation buffer used was low and may have been a source of variation. Calcium is also a key extracellular messenger during platelets activation. In platelets, an addition of exogenous calcium will inhibit the release of intracellular calcium, which is necessary for granule release. Calcium is a key second messenger that activates a broad range of signalling enzymes. These enzymes are functionally linked to integrin $\alpha_{IIb}\beta_3$ activation, cytoskeletal reorganisation and granule secretion. One of these enzymes is calpain a calcium-dependent thiol protease enzyme, which is highly presented in platelets. Subsequent integrin $\alpha_{IIb}\beta_3$-dependent calcium influx and phosphoinositid in turnover promotes activation of calpain, which then causes proteolysis of its substrates including FAK, Src kinases, paxillin, talin, α-actinin and PTP-1B. The activation of calpain by calcium requires activation of other proteins including vWF.

Addition of 1mM of calcium to freshly isolated platelets did not decrease the extent of nitration immediately after isolation (Fig 5.6). However, the presence of the calcium accelerated the disappearance of nitration after 5 minutes and when incubated for 1 hour. Sometimes (as shown in Fig 5.6) this occurred within 1-5 minutes, whereas in other samples it occurred over 30 minutes. At the present there is

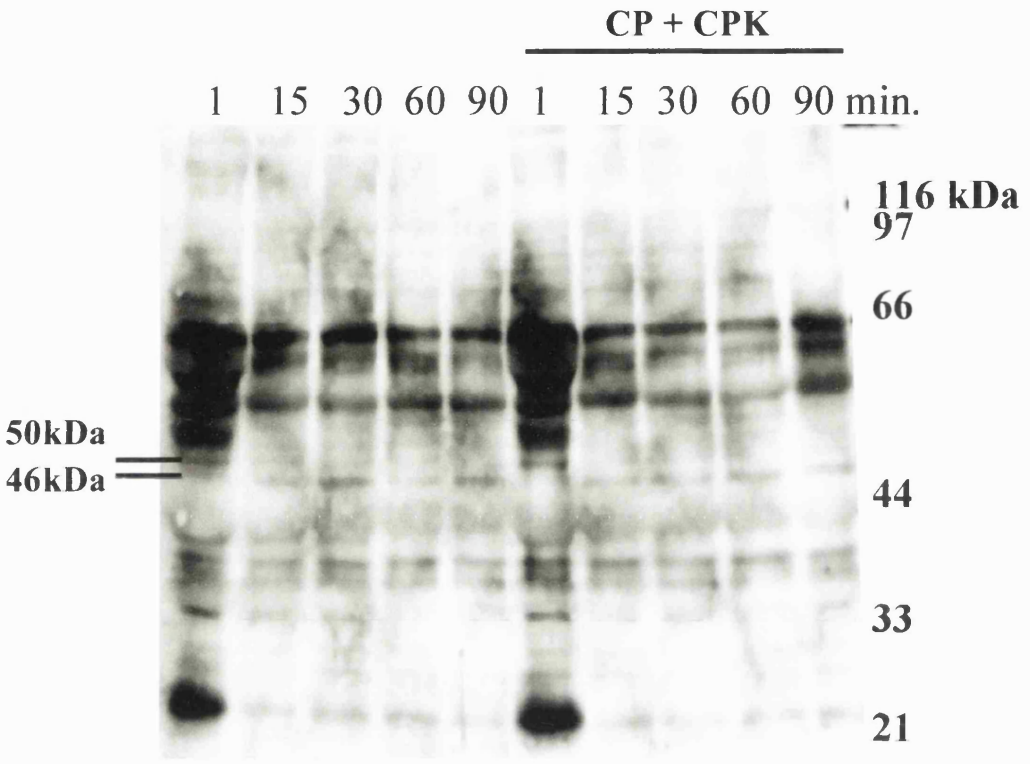


Fig 5.5: Nitration of platelet proteins in the presence of Creatine/Creatine phosphate.

Washed platelets were separated from plasma by addition of 50nM PGI_2 to plasma, during separation procedure in the presence and absence of creatine phosphate/creatine phospho kinase this reagent was added to anti coagulant, which was taken into syringe before the blood was taken. Samples were collected immediately after separation up to a period of 60 min. Platelet proteins were separated using 10% SDS-PAGE and membranes were blotted with anti-nitrotyrosine antibodies (polyclonal RF1256 dilution 1:500). The experiment was typical of two independent experiments.

5.6 Scavenging of ADP in the plasma

The protection of platelets with aspirin will not prevent damage to erythrocytes during blood withdrawal and processing. Minor injury may release of adenine nucleotides from erythrocytes. In addition, platelets contain stores of ADP in their dense granules. The release of ADP acts as a positive feedback mechanism as this binds to surrounding platelets and accelerates the process of aggregation. Addition of creatine phosphate and creatine phospho kinase (CPK) during the anticoagulation of blood scavenges ADP.

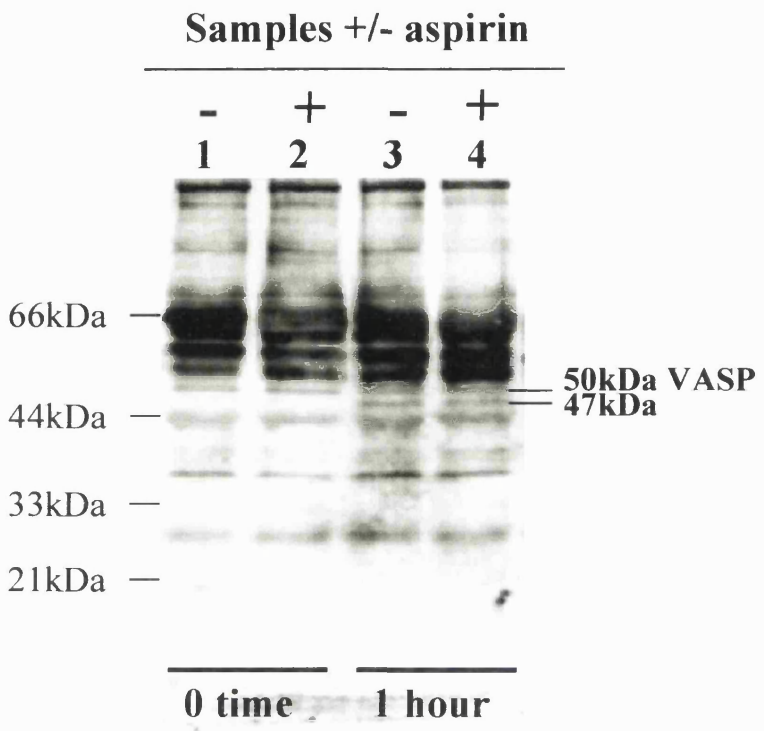
CPK catalyses the following reaction to remove the traces of ADP:

$$\text{ADP} + \text{phosphocreatine} \xleftarrow{\text{CPK}} \text{ATP} + \text{Creatine}$$

For this study both creatine phosphate and creatine phosphokinase were prepared in stock solution x1000 more concentrated than final concentration. The reagents were then added to anticoagulant (ACD) prior to blood collection (4µl/4ml of ACD). The platelet suspension was then prepared as usual.

The nitration of platelet proteins was investigated in the presence and absence of CK/CPK (Fig 5.5). No differences were observed one minute after isolation. In both sets of samples, the extent of nitration declined when incubated for a period up to 90 minutes.

a)



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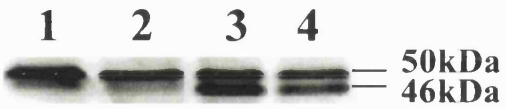

Fig 5.4: Nitration of platelet proteins in the presence of Aspirin.

Washed platelets were separated from plasma of either normal volunteers (-) blood or the volunteers who has been taken aspirin 16h before the blood was taken (+). The platelets were then separated by addition of 50nM PGI_2 to plasma, during separation procedure. Samples were collected immediately after separation (1 & 2) and after 60 min (3 & 4). Platelet proteins were separated using 10% SDS-PAGE and membranes were blotted with either anti-nitrotyrosine antibodies (upstate 1:500) (a) or with anti-VASP anti-serum (1:2000)(b).

It is clear that nitration of proteins may occur during normal physiological processes and this has been clearly demonstrated to be the case in platelets. In other cell types there are examples of spontaneous nitration of proteins - e.g. NH_2 terminal C-Jun kinase and prostacyclin synthase in endothelial cells during activation of the cells (Go et al., 1999; Zou et al., 1999). Furthermore, peroxynitrite appears to activate the PI_3-kinase/ Akt pathway (Klotz et al., 2000). The question is whether these modifications are of biological significance or just an epi-phenomenon. It is clear that excessive nitration may cause extensive protein damage, as may occur in certain disease states. However, specific targeting of tyrosine or other residues on proteins may be the basis for a signalling system comparable to that of tyrosine phosphorylation. In platelets some of the proteins exhibit tyrosine phosphorylation even at rest (*src* family) and resting platelets do have some nitrated proteins. Of course, for this to be subject to regulation, it would be preferable that nitration is under enzymatic control. It has now been clear that certain enzymes, such as superoxide dismutase, myeloperoxidase and possibly xanthine oxidase or nitric oxide synthase, enhance nitration either from peroxynitrite or other nitrogenous substrates, but these may not be directly involved in the nitration processes described above. In a recent publication, it was shown the nitrotyrosine could replace phosphotyrosine in SH2-specific peptides that bind to the SH2 domain of the *src* family tyrosine kinase, *lyn* (Mallozzi et al., 2001), a kinase that is also important in platelet activation (Gross et al., 1999). In this study we found that *lyn* itself may be nitrated in fresh platelets (Fig 5.3). Clearly a great deal of work needs to be done in the isolation of individual signalling proteins and the identification of changes in nitration or other modification which can be linked with specific cellular events.

It is always tempting to assume that nitration of proteins has many parallels with the signalling mechanisms afforded by tyrosine phosphorylation, but the evidence for this is still scant. Even though nitration of proteins occurs during activation of platelets, the process appears to involve radicals (nitric oxide and hydrogen peroxide) and indeed peroxynitrite, all of which are inhibitory to platelet activation. From this it appears that nitration is more likely to have a role in limiting the activity of platelet activation mechanisms.

Nitration may occur in the circulation as a result of shear stress, which is also known to activate phospholipase C and fibrinogen binding. It is possible that nitration is a protective mechanism, which is reversible when the cells are no longer stressed. This may prevent unwanted activation from occurring. In pathological states overproduction peroxynitrite may lead to excessive nitration, so that the nitro groups are not amenable to enzymatic reduction or the proteins are less readily cleavage by proteolytic enzymes. The least appealing proposition is that nitration is of no importance in normal physiology or in platelets and that neither of the enzyme proposed are common in platelet. There is possible that other peroxidases present in platelet will be capable of nitrating proteins in the presence of endogenous concentrations of nitrite and hydrogen peroxide under physiological condition. The search for a clear role continues.

It will be of great interest to identify the nitrated proteins and also to study the role of the nitration of these proteins on the platelets function. More studies need to be done to clarify the cause of the nitration in the basal or to ascertain that these proteins are originally nitrated in circulation.

The incubation of platelet samples with proteasome and proteolysis inhibitors did not show any significant differences in the basal nitration. Since platelets do not have

nuclei only small amount of protein synthases will occur in platelet. These will raise the question that if all these proteins need to be removed by proteolysis, will the cell survive after that? Alternatively, the cell will go through a cell dead. Will cell signalling mean a suicide death in platelets? Only limited numbers of references exists about the proteolysis in platelet. There is possible that the proteolysis in platelet is not of the same importance as it is in other cell. It will be understandable that the existing proteins have to go through a recycling process of nitration and denitration or phosphorylation and dephosphorylation in their cell signalling process. The mechanism of denitration or dephosphorylation in on these cells might be of the same importance that the proteolysis system is in other cells. However these need to be investigated. The presence of some proteasome and proteolysis enzymes in platelets has been established in different studies, but no comparison between platelets and other cells exists.

Since the major core of this thiese is based on the western blotting, the speceificity of the antibodies used will be of great importance. In this study, different antibodies have been used. The anti-vasp antibody was one of the good and reliable antibodies and the best one used in the whole project. Two different anti-nitrotyrosine antibodies were in general similar, but in total veriable in different experiment.

CHAPTER SIX:
THE NITRATION OF PLATELET PROTEINS AND THE INTERACTION BETWEEN H_2O_2 AND NO

6.1 Introduction

At the beginning of this investigation there was no apparent reason why protein nitration should be part of mechanism of synergism between NO and H_2O_2. However, as will be discussed below, further evidence became available, which suggested this idea. Beckman et al proposed that superoxide dismutase could perform the reverse of its conversional reaction and form superoxide anion from hydrogen peroxide. Since it was also suggested that this enzyme might generate peroxynitrite and enhance nitration of proteins, the link between nitration and hydrogen peroxide seemed at least a distinct possibility.

More recently, the nitration of proteins was reported in the presence of different peroxidases, including myeloperoxidase and eosinophil peroxidase as an alternative pathway for peroxynitrite. Hydrogen peroxide is essential for this reaction. In this kind of reactions nitrite (NO_2^-), at physiological or pathological levels, is a substrate for the mammalian peroxidases and that formation of $NO_2^{\cdot}$ via peroxidase-catalyzed oxidation of NO_2^- in the presence of H_2O_2 may provide an additional pathway contributing to cytotoxicity or host defence associated with increased $NO^{\cdot}$ production (Vliet A et al 1997). However these enzymes do not exist in platelets, and until know there are no evidence of the involvement of any other peroxidases on the nitration of platelet proteins.

The presence of nitrated proteins in non-stimulated platelets, immediately after the separation of these cells from plasma was established in this study as described in the previous chapter. The aim was to investigate the possibility that NO and hydrogen peroxide at physiological concentrations, may lead to the nitration of platelet proteins.

On this basis, the effect of NO and H_2O_2, on nitration was investigated, particularly with respect to VASP, but also other proteins.

6.2 The effect of hydrogen peroxide on the nitration of platelet proteins

Washed platelets were isolated from platelet rich plasma and allowed to rest for 90-120 min before use. Hydrogen peroxide, over a range of concentrations between 0 and 250μM, was added to the platelets for a period of 1 minute. Platelet proteins were then isolated on 10% SDS/PA gels and transferred to nitrocellulose by western blotting. The blots were probed with a commercial polyclonal anti-nitrotyrosine antibody (Upstate).

The first samples were collected immediately after separation of platelets from plasma as the first control. The rest of samples were then prepared after a full recovery of the platelets (2h after separation of platelets from plasma).

In the control samples, clear nitration of VASP in its 50kDa form was detected as shown in the previous chapter. Also, a 33kDa protein was observed to be nitrated immediately after separation of platelets from plasma. No nitration of VASP was detected in the samples prepared 2h after separation of platelets from plasma. During the same period of time, nitration of a 53-56 kDa protein was increased. This 53-56 kDa protein was later identified by anti lyn antibody as described previously.

Addition of H_2O_2 (0.25 and 25μM) after 2h recovery time, induced a clear increase in the extent of nitration of VASP (Fig 6.1). The nitration of proteins was increased in the presence of 25μM H_2O_2, at which we have previously shown that H_2O_2 alone

neither activates or inhibits platelet activation when added before the platelet agonist (Naseem et al., 1996; Naseem and Bruckdorfer, 1995). At high concentrations of H_2O_2 (250μM), the increase in nitration was less compared to that seen at lower concentrations of H_2O_2 (Fig 6.1).

In the presence of H_2O_2 the nitration of a 72kDa protein was also increased. With increasing concentration of H_2O_2 the extent of nitration also decreased as in the case of VASP. Addition of H_2O_2 did not increase the nitration of a 33kDa protein.

6.3 Nitration of VASP in the presence of H_2O_2

Immediately after separation of platelets from plasma, as described in the previous chapter, VASP exists in a nitrated 50kDa form. Two hours after separation of platelets from plasma VASP was only detected in its 46kDa form with anti-VASP antiserum and no nitration was detected in this sample (Fig 6.2). In the presence of 25μM H_2O_2, VASP was detected in both 46 and 50kDa forms. However, the anti-nitrotyrosine antibody only detected the 46kDa form of VASP in these samples.

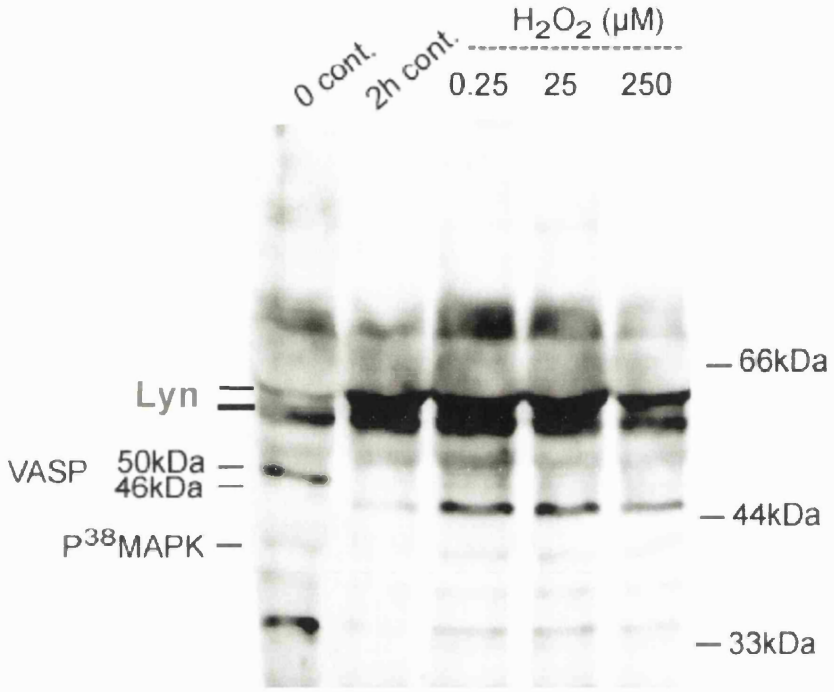

Fig 6.1: Nitration of platelet proteins in the presence of hydrogen peroxide

Washed human platelets were incubated for 2h after separation from plasma and then incubated with a series of concentrations of H_2O_2 for 1 min. One control sample was collected immediately after separation of platelets (as the 0-control). The platelet proteins were separated using 10% SDS-PAGE and blotted with anti-nitrotyrosine antibodies (RF1256 1:500). Results typical of 3 independent experiments.

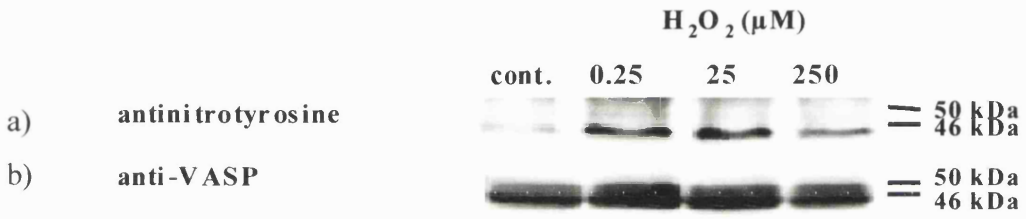

Fig 6.2: Nitration of VASP in non-stimulated platelets (a) and the presence of hydrogen peroxide (b)

Samples were prepared 2h after separation of platelets from plasma and then incubated with a series of concentrations of H_2O_2 for 1 min (Fig 6.2).
The platelet proteins were separated using 10% SDS-PAGE and blotted with anti-nitrotyrosine (RF1256 1:500) and anti-VASP antibodies.

6.4 The nitration induced by hydrogen peroxide is not permanent

For further investigation of the effect of the nitration of VASP induced by H_2O_2 on the platelet function, it was important to study the reversibility of the nitration.

As described previously, addition of 25μM H_2O_2 to washed platelet increased the nitration of proteins, including VASP, within 1 min. In this study, the maximum nitration of VASP was detected after 1-30 min. After 30 min a significant decrease in the amount of the nitration was observed and after 60 mins no nitration was detected with the anti-nitrotyrosine antibody (Fig 6.3). The disappearance of nitration in the presence of H_2O_2 (Fig 6.3) does correlates well with the disappearance of serine phosphorylation induced by both NO and H_2O_2 (Fig 4.4).

The nature of the denitration of proteins is not known yet. It is possible that nitration of a protein will act as a signal for the degradation of a protein as part of a normal cellular response or that the proteolytic enzymes may become activated by nitration. In this study the possibility of the degradation of VASP after nitration was investigated. For this aim the same samples as in Fig 6.3 were immunoblotted with antiVASP antiserum. In a different experiment the same membrane, which was first probed with antinitrotyrosine antibody was stripped and reprobed with anti-VASP antiserum.

The degradation of VASP following nitration is unlikely since stripping and re-probing the same membrane with anti-VASP antibody showed the clear presence of VASP in all lanes. Even in the sample where no nitration was detected with anti-nitrotyrosine antibody, VASP was present and the concentrations seemed homogenous in all lanes (Fig 6.3).

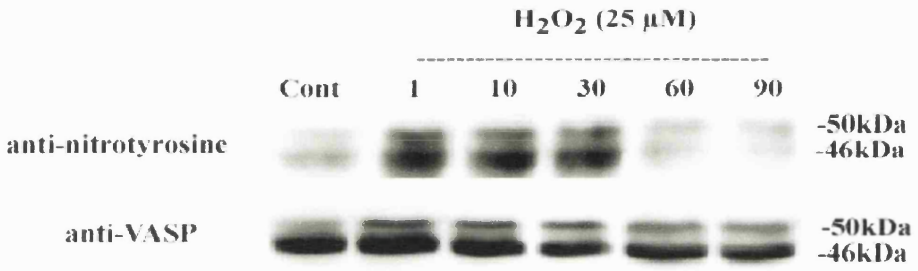

Fig 6.3: The disappearance of nitration following exposure of hydrogen peroxide

Washed human platelets were incubated for 2h after separation from plasma and then incubated with 25μM of H_2O_2 for a period of 1-90min. The platelet proteins were then separated using 10% SDS-PAGE and blotted with either anti-nitrotyrosine (upstate 1:500) or anti-VASP antibodies.

6.5 Influence of the inhibition of proteolysis on nitration

To establish whether the disappearance of the nitration was simply the result of proteolysis, a cocktail of proteolysis and proteosomal inhibitors (final concentrations: 50 μM calpeptin, 50 μM MG-132 peptide, 100μM ALLN and 1mM EST) were added to the platelets for 30 min, before the addition of H_2O_2. (Cooray et al., 1996, Adachi et al., 2003, Allen et al., 2002, Inomata et al, 1996)

These included inhibitors for calpain, which is a calcium-activated thiol protease, an important proteolytic enzyme in platelets. These inhibitors were also selected because they were membrane permeable.

An increase of protein nitration or inhibition of the denitration may have been anticipated in the presence of the proteosome inhibitors. Surprisingly the inhibition of proteolysis did not block the denitration of the proteins. Unexpectedly in the presence of these inhibitors, the nitration of platelet proteins including VASP was less. The denitration was accelerated in the presence of these inhibitors (Fig 6.4).

In this experiment, as in all other experiments, the membrane was stained with Ponceau S (a water-soluble staining solution) before blotting procedure. The total protein content of each lane (stained by ponceau) was identical. The same membrane was then stripped and reprobed with antiVASP antibody (figure not shown). VASP was present in all the samples.

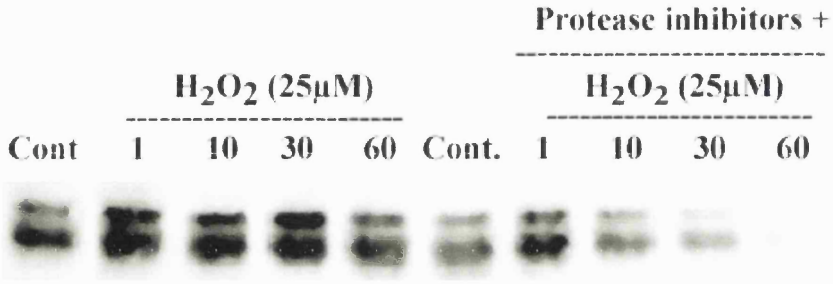

Fig 6.4: Inhibition of nitration of VASP in the presence of protease inhibitors

Platelet samples were prepared in the presence and absence of a cocktail of proteolysis (and proteosomal) inhibitors, 30 min prior to addition of 25μM H_2O_2 for 1 min. The cocktail included 50μM calpeptin, 50 μM MG-132 peptide, 100μM ALLN and 1mM EST. The platelet proteins were separated using 10% SDS-PAGE and blotted with anti-nitrotyrosine antibodies (upstate 1:500).

6.6 Investigation of the mechanism of nitration of VASP induced by H_2O_2

The nitration of VASP by H_2O_2 was investigated in the presence of different inhibitors, including aspirin, uric acid, calcium, ODQ, L-NAME, and EGCG. Washed platelets after 2 h recovery time were incubated with each of these inhibitors separately for 30 min, prior to addition of H_2O_2.

Aspirin as described previously (5.5) is an inhibitor of cyclooxygenases. Inhibition of these enzymes by aspirin will block the production of thromboxane A2, a vasoconstrictor and activator of platelet. In a previous chapter, no changes in the basal nitration were observed, when aspirin was administrated by the blood donor before the blood was taken. It is interesting to see if the mode of action of H_2O_2 on the induced nitration is at the site of the COX enzymes.

Uric acid, on the other side is known as a scavenger of free radicals including $ONOO^-$ and NO_2^-. EGCG in previous chapter did block the nitration of proteins in non-stimulated platelets (basal); therefore it was used in this experiment to see if it would have any effect on the nitration caused by H_2O_2. L-NAME and ODQ as described previously are inhibitors of NOS and GC enzymes, respectively.

The presence of 1mM aspirin did not change the extent of the nitration induced by H_2O_2, which indicate that COXs were not involved in this mechanism. The activity of sGC was necessary for synergism between NO and H_2O_2 on the inhibition of platelet aggregation, but not for the nitration of VASP by H_2O_2, as the presence of 10μM ODQ did not change the amount of the nitration. Calcium had no effect on the nitration induced by H_2O_2. Uric acid, an antioxidant, decreased the extent of the

nitration slightly, but not to the same extent as L-NAME or EGCG. Much more significant inhibition of nitration was observed in the presence of 800μM L-NAME or by addition of 2μM EGCG. Therefore generation of NO seems to be crucial for the nitration caused by H_2O_2.

EGCG, as described in the previous chapter is an effective inhibitor of peroxynitrite formation via scavenging of superoxide (Yang et al., 1994; Yin et al., 1994). It also causes the inhibition of $ONOO^-$ mediated tyrosine nitration (Pannala et al., 1997) Addition of EGCG to platelets at concentrations as low as 2μM completely blocked the nitration of proteins induced by H_2O_2 (Fig 6.5).

EGCG was also reported to inhibit lipid oxidation and lipooxygenase activity and also increasing the activity of SOD in the red cells (Li et al 2000).

The possibility of a scavenging effect of EGCG on H_2O_2 is excluded, since EGCG has been shown to increase the production of H_2O_2 (Sakagami et al 2001, Yang et al 2000).

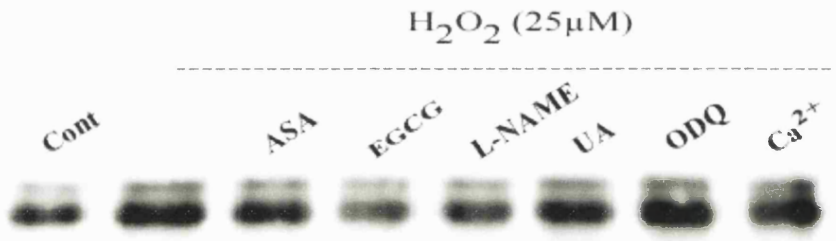

Fig 6.5: Inhibition of nitration of VASP in the presence of specific inhibitors

Washed human platelets were incubated for 2h after separation from plasma and then incubated with the presence of 1mM Aspirin, 2μM EGCG, 800μM L-NAME, 200μM uric acid, 10μM ODQ, and 1mM calcium for 30 min prior to addition of 25μM H_2O_2 for 1 min. The platelet proteins were separated using 10% SDS-PAGE and blotted with anti-nitrotyrosine antibodies (upstate 1:500).

6.7 Investigation of possible synergism between NO and H_2O_2 on the nitration of platelets proteins.

In order to investigate the effect of NO on the nitration of platelet proteins, a NO donor, GSNO was added to platelet samples at different concentrations. The synergism effect between NO and H_2O_2 on protein nitration was also studied by simultaneously addition of them to platelet samples.

The addition of low concentrations of GSNO (0.01 and 0.10 μM), did not change the nitration of proteins significantly comparing with the control. In contrast, addition of higher concentrations of GSNO to the samples as much as 1 and 10μM, decreased the nitration of VASP.

In the presence of hydrogen peroxide, decreased nitration of most of the proteins including p38 MAPK, VASP and Lyn was observed, when higher concentration of GSNO was added. Only the nitration of profilin was not changed significantly.

There is controveris about the exact concentrations of NO released by GSNO. According to the published data of Mayer et al (Mayer et al 1995), 10μM of GSNO will release around 20nM NO. Regarding to this group, the concentrations of GSNO, used in this experiment (0.01 and 0.10 μM) were may be to low to have any effect on protein nitration. The 1 and 10μM GSNO may release NO just in the physiological range, equal to the concentration of NO that we used in aggregation and sGC experiments. On the other hand, it is possible that the effect of exogenous NO on the nitration might be different to that of endogenous NO.

a) b)

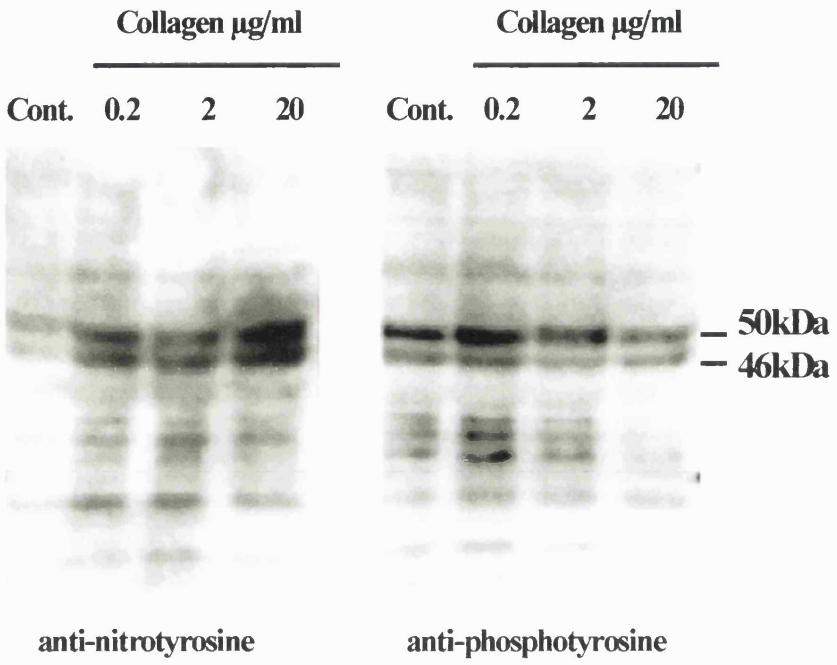

Fig 5.9: Tyrosine phosphorylation and nitration of platelet proteins in the presence of different concentrations of collagen

Washed human platelets, which were allowed, to rest for 2 hours after isolation(lane 1, control) were incubated with 0.2, 2 and 20 µg/ml of collagen for 1 min (lane 2,3 &4). The proteins were then separated on 10% SDS gel and transferred into nitrocellulose membranes. The membranes were then blotted with anti-nitrotyrosine (Upstate 1:500) (a) and anti-phosphotyrosine (b).

5.11 Discussion

In this study the presence of nitrated proteins in non-stimulated human platelets were established. It has to be mension that the patent of the nitration, presented in different figures in this chapter, may be different, which is due to the use of different electrophoresis equipmen, different type of gels or different nitrotyrosine antibodies. However, this variation does not affect the final conclusion. The presence of nitration under physiological conditions means that either nitration is a constant feature of platelet proteins in the circulation or that it is due to the handling of the platelets during isolation. We also showed that a second wave of nitration could be induced by addition of collagen after the first nitration had subsided.

The nitration of proteins seems to occur as the result of a range of stimuli, which are not mutually exclusive, but may arise through different mechanisms, which may or may not directly involve nitration by peroxynitrite. The nitration of protein was reported to be modulated in the Rat's Retina by light (Miyagi M., 2002). In addition, nitration of other proteins have been reported during normal physiological condition (table 1.2, Greenacre., 2001).

In this study, nitration of proteins showed to be stimulated by normal physiological processes, such as the activation of platelets by collagen. as well as the presence of low concentrations of hydrogen peroxide (will be discussed in next chapter). Mechanical stress on the platelets, during the isolation of the cells from blood, is sufficient to induce a transitory nitration. The fact that protein nitration occurs during cell activation by collagen suggests that the process may be linked to the associated cell signalling processes. This may be due to an increase in NO production following rises in intracellular calcium or the production of superoxide anions by either mitochondrial oxidation or from NADPH oxidase. Certainly L-NAME is inhibitory,

indicating a requirement for endogenous NO, but nitration was not enhanced by the addition of 100 μM S-nitrosoglutathione (next chapter).

The biological significance of protein nitration is still not clear, although nitrating species such as peroxynitrite does seem to influence the phosphorylation of tyrosine in important signalling proteins. These effects may be indirect and not due to direct competition on specific tyrosine residues for nitration or phosphorylation. There is much to be done to ascertain whether nitration of proteins is an important cellular process or just an inconsequential epiphenomenon.

Exposure to extracellular sources of peroxynitrite may be important in the regulation of platelet function, but there may also be a role for protein nitration caused by the endogenous processes within the platelet. Proper investigation needs to be done to ascertain the cause of nitration. This will be the base for future work to be done.

In this study it was demonstrated that the nitration of proteins at least in human platelet is a reversible process, as different nitrated proteins seemed to be de-nitrated after a period of time. The nitration of proteins during physiological conditions is now more acceptable than it was decays ago. For a chemist it would be difficult to accept the theory of the denitration, because of the requirement of huge amount of energy for the removal of the nitro group. Access to that amount of energy in biological system is difficult to imagine. Today a few processes are suggested for the denitration of the proteins.

We have strong evidence that proteins can become de-nitrated spontaneously in platelets. Significant decrease of nitration was observed in all of the experiment done in this study. The nitration of proteins appears to be reversible although the nature of the products of "denitration" cannot be ascertained using antibody technology. The

rate of denitration seems to be determined by cellular regulators such as the concentration of calcium.

The denitration of proteins was discussed independetly by different group. The nature of denitration is not clear yet, but the existence of a denitratase enzyme in different tissues was suggested by independent studies.

The concept that the nitration of protein tyrosine residues may not be permanent has been proposed in recent years, by Murad and co-workers (Kamisaki et al., 1998). They found that homogenates of rat tissues decrease the extent of nitration over time when exogenous nitrated albumin is used as substrate. They have provided further evidence that the loss of the stable nitro group may be by way of reduction of hydroxylamine's (Balabanli et al., 1999). The existence of mammalian nitro-reductases is well established for non-peptide drug (Gillette, 1969), but to date evidence for the existence of such enzymes for protein denitration has not been established.

In platelets, there is clear evidence of a decrease in the extent of nitration in specific proteins over time, although we have found in recent studies that not all of them are so variable. This is certainly the case with VASP where there is a clear decrease, which can be enhanced by the addition of calcium (see above). This may be due to calcium-activated proteases. The addition of exogenous calcium may be activates calpain, which causes disconnection of cytoskeleton proteins and also proteolysis of these proteins, leading to decrease of the extent in nitration. In all of these experiments the presence of VASP up to one hour after addition of calcium was examined using anti-VASP anti-serum. The concentration of this protein was constant during this time.

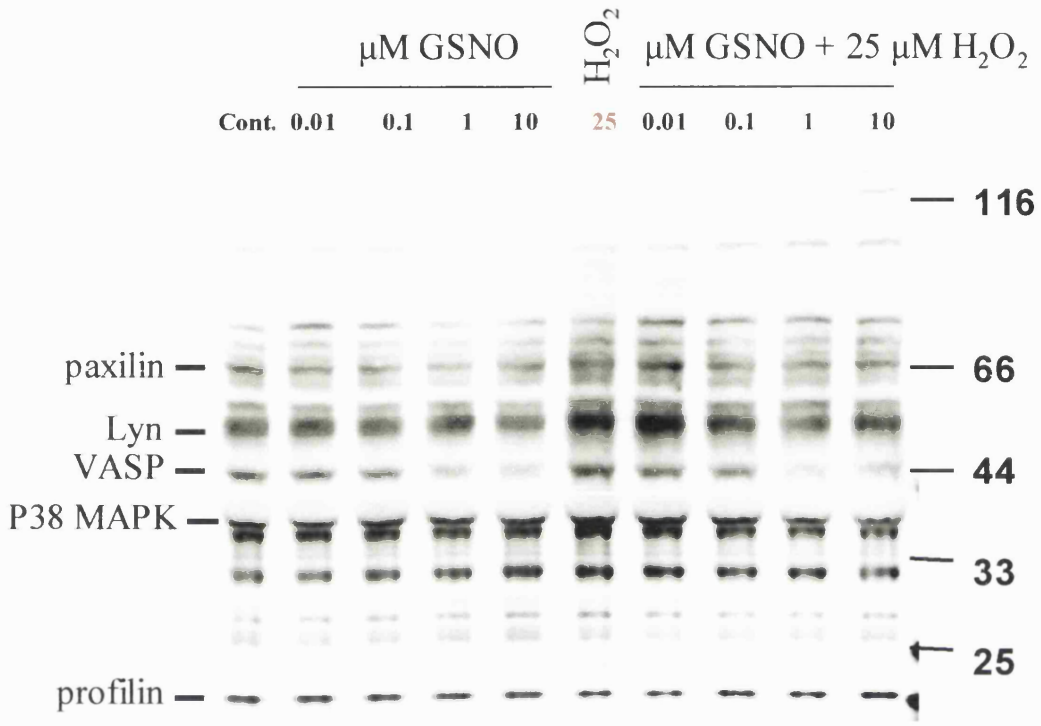

Fig 6.6: Nitration of platelet proteins in the presence of GSNO and hydrogen peroxide

Washed human platelets were incubated for 2h after separation from plasma and then incubated with the presence of a range of concentrations of GSNO alone or in the presence of 25μM H_2O_2 for 1 min. The platelet proteins were separated using 4-12% Nu-PAGE gradient gels. The membranes were then blotted with anti-nitrotyrosine antibodies (upstate 1:500).

6.8 DISCUSSION

In this study, H_2O_2 on its own was capable to increase the nitration of VASP and other proteins. The presence of endogenous NO was required for this action of H_2O_2, since L-NAME blocked the nitration enhanced by H_2O_2. EGCG, an iNOS inhibitor, did also block the nitration of proteins induced by H_2O_2, even more effectively than L-NAME. This increase in nitration may result from an increase in NO-formation or $O_2^{\cdot-}$ production, or both, as components of peroxynitrite leading to nitration of tyrosine and modification of other amino acids. Alternatively, there may be other mechanisms at work, which are unrelated to peroxynitrite but require the formation of nitrating species such as the nitrogen dioxide radical.

Although there is evidence in the literature that, in smooth muscle cells, H_2O_2 increased NO production (Burke-Wolin et al., 1991b; Zembowicz et al., 1993), we have not found this to be the case in platelets. As described previously (chapter 3), exposure of platelets to H_2O_2 alone (in the presence of IBMX) only increase the cyclic GMP concentration to a very minor extent. Direct measurements of [NO] by chemiluminescence's using a Sievers luminescence detector failed to show any increase in NO production on addition of H_2O_2 from a concentrated platelet suspension ($1x10^9$ platelets/ml). H_2O_2 may increase the extent of nitration by either increasing the concentration of $O_2^{\cdot-}$ or by inhibition of SOD, although no direct evidence exists for these mechanism. There appeared to be a requirement for basal levels of NO to be formed because nitration was significantly reduced by the presence of L-NAME (800 μM), but ODQ at 10μM did not prevent nitration. Surprisingly, addition of exogenous NO in forms of NO donor (GSNO) did not increase nitration and in some cases also decreased the extent of nitration in the presence of H_2O_2. The

addition of exogenous NO might influence the activity of NOS and as a consequence affect the production of NO.

A more recent proposal is that $O_2^{\cdot-}$ might arise by a reversal of the activity of SOD to generate $O_2^{\cdot-}$ from H_2O_2 (Brunelli et al., 2001). The $O_2^{\cdot-}$ would then be available to form peroxynitrite with NO and subsequently cause the nitration.

In this study, EGCG completely prevented nitration by H_2O_2 at concentrations as low as 2μM, (Fig 6.6). This was not achieved by 200μM uric acid (Fig 6.6), or by 100μM ascorbate. Whereas urate and ascorbate have small effects on the activation of washed platelets at the concentrations employed, little was known of the effects of EGCG. In independent studies, EGCG was shown to increase the production of H_2O_2 (Miura et al., 1998, Roques et al., 2002). Therefore, the possibility that the compound might scavenge H_2O_2 is excluded. This antioxidant was known to decrease iNOS activity, for which there is little convincing evidence of this occurring in platelets, but also the nitration of proteins, by peroxynitrite (Pannala et al., 1997, Nakagawa et al. 2002). Schroeder et al. have shown that a related compound, epicatechin, inhibits nitration caused by peroxynitrite (less effectively than EGCG), but it does not inhibits its other oxidation activity (Schroeder et al., 2001). This may mean that these compounds selectively inhibit $^{\cdot}NO_2$ arising from the spontaneous breakdown of peroxynitrite, but do not quench the hydroxyl radicals formed simultaneously. Therefore this cannot be taken as proof that the nitration is derived from the prior formation of peroxynitrite, but may arise through generation of $^{\cdot}NO_2$ by other routes (Brennan et al., 2002; Gaut et al., 2002; Sampson et al., 1998; Wu et al., 1999b). To connect this founding to the inhibition of platelets aggregation by H_2O_2, the nitration of proteins may therefore have some indirect regulatory effects that alter the sensitivity of the cells to either agonists or antagonists (e.v. thrombin or NO). It has been demonstrated that

enzymatic mechanisms may be involved in nitration, which do not directly result from the formation of peroxynitrite (Halliwell, 1997).

Protein nitration can also occur via trapping of NO by tyrosyl radical (Gunther et al., 1997) and during oxidation of nitrite, by H_2O_2 catalysed by different peroxidases including myeloperoxidases and eosinophil peroxidases (Brennan et al., 2002). In this system NO_2 seems to be the reactive intermediate responsible for the nitration (Brennan et al., 2002; Sampson et al., 1998; Wu et al., 1999b).

As described in the previous chapter (regarding basal nitration), nitration of VASP was primarily detected in the 46kDa form, but this was changed to an apparent molecular weight of 50kDa in the presence of prostacyclin (Fig 5.1). There is no evidence that tyrosine nitration of VASP will cause a molecular shift from 46 to 50 kDa (as the case with serine phosphorylation, even in this case only one serine residue is capable of increasing the molecular weight). In the presence of prostacyclin serine phosphorylation, rather than nitration, is likely to be the principal determinant of the apparent molecular weight. In the presence of H_2O_2, VASP was nitrated and that was dependent upon which form of VASP was present in the sample. Only that form of VASP was nitrated and this was indicated by re-probing the membrane with anti-VASP antibody (Fig 6.2).

In this study, we also showed that during activation by collagen, platelet proteins (particularly cytosolic proteins such as VASP) become nitrated and this was dependent upon the formation of endogenous NO as shown previously in Naseem et al., 2000.

After an increase in nitration at 1 minute after addition of H_2O_2, the amount of nitration was diminished in the samples incubated with H_2O_2 for more than 30 minutes. This parallels, in time, the waning of the synergism in between NO and H_2O_2 on platelets aggregation.

Tyrosine nitration has been proposed to be linked with tyrosine phosphorylation, a known cell signalling process, and may itself be a part of normal cell signalling. If this is so, the nitration of proteins needs to be reversible. Alternatively, one might speculate that the removal of modified proteins can be considered as a part of defence mechanism against oxidative or nitrative stress. As indicated in chapter 5, de-nitration of proteins has been investigated intensively by some groups in the past few years. Davis and co-workers have provided extensive evidence that oxidative modification of proteins increases their susceptibility to proteolytic degradation as well as the presence of de-nitratase activity in human plasma and rat brain and liver homogenate has also been suggested (Davies, 1987; Davies et al., 1987a; Davies et al., 1987b; Davies and Delsignore, 1987).

More controversially, others have suggested that there may be enzymes present in tissues, which can remove or modify the nitro groups by a mechanism that may involve its reduction to an amine (Balabanli et al., 1999; Nolte, 1998). The possibility that the nitro group may be reduced to amino group was also proposed by Murad et al. but not yet proven.

These kinds of enzymes may be present in platelets, but no evidence for these exists at the presence. The disappearance of nitrated protein was also discussed as a result of degradation of protein by proteases, but again in this study this was not the case as VASP was present in equal concentrations in nitrated and non-nitrated samples.

The disappearance of the nitration may be due to calcium-activated proteases, but we have been unable to block the disappearance of the nitrated proteins, in the presence of different proteosome and protease inhibitors. Nevertheless, more direct proof is required to demonstrate the origin of this enzymatic activity.

In conclusion, H_2O_2 , along with other reactive oxygen species, may have the ability to modify the function of VASP at least in platelets, but that other events are required (provided by NO) to complete the process of inhibition of platelet aggregation by synergism. The possibility of H_2O_2 acting as a signalling molecule involved in the nitration of proteins, which leads to the cell signalling, was investigated. The nitration of VASP in particular was used as a model of nitration along with other platelet proteins. The extent of nitration varied in different blood donors and different experiments, but a significant amount of nitration was detected in all experiments.

The role of H_2O_2 on the nitration of other proteins, and especially VASP, was interesting, but the link between nitration of VASP and inhibition of platelet aggregation is not known yet. It is possible that nitration caused by H_2O_2 is a signalling mechanism, since the nitration caused under this condition was reversible and only persisted for short period of time. In the case of nitration of VASP, the possibility that denitration is caused by degradation of proteins appeared to be unlikely, since the presence of different proteolytic inhibitors did not change either the rate or the extent of denitration and the amount of the proteins in each lane was equivalents. This was indicated by re-probing of the same membrane with anti-VASP antibody. In the samples where no nitration was detected still VASP was present, sometimes in both forms.

In conclusion, the nitration of VASP by H_2O_2 may be important signalling event and connected to inhibition of platelet aggregation by H_2O_2.

CHAPTER SEVEN:

GENERAL DISCUSSION

The original aim of this thesis was to investigate the nature of the synergism between nitric oxide and hydrogen peroxide. During the time of thesis, new questions have arises and also new information about the possible physiological significance of hydrogen peroxide as a signalling molecule has been made available. In this concluding discussion, the initial and the subsequent area of interest are highlighted and summarised.

7.1 Synergism between NO and H_2O_2 on inhibition of platelet aggregation

In earlier work, the simultaneous addition of NO and H_2O_2 increased the inhibition of platelet aggregation up to 100% at concentration of NO, which had almost no inhibitory effect when added alone. If NO was added 30 min before or after H_2O_2 no synergism would be observed. The synergism was, however still evident if H_2O_2 was added 30s before or after the NO and was still significant even with a 1 minute separation. This may have been due to the production of other reactive species during a fast-reaction between NO and H_2O_2. This possibility was suggested when addition of uric acid, mannitol and sodium urate partially inhibited the synergism effect of NO and H_2O_2 on the inhibition of aggregation. However, such reactions seem to be unlikely from the knowledge of the chemistry. Alternatively, hydrogen peroxide itself may have independent actions on platelet function that enhance the effects of NO.

As expected the addition of the NO scavenger carboxy-PTIO to washed platelets inhibited synergism between NO and H_2O_2, but so did inhibition of soluble guanylyl cyclase with ODQ.

7.2 The role of sGC in the synergism reaction between NO and H_2O_2

The mechanism of action of NO on the inhibition of platelet aggregation maybe either direct or indirect through activation of sGC and increases in cGMP concentration, but at this concentration NO alone has no significant action on either of these. H_2O_2 alone also did not stimulate the sGC enzyme either in purified cell extract or in cell lysate. A small but non-significant increase of cGMP concentrations, in the presence of NO and H_2O_2 was only detected in whole platelet preparations in the presence of PDEs inhibitor (Naseem et al. 1995 and data presented in chapter 3). However in the absence of PDE inhibitors, a small but significant rise was observed at a time point 15s after addition of NO and H_2O_2. Therefore, it is uncertain that a connection between sGC and the cell membrane is essential for the action of H_2O_2. In this study, it was shown that the synergism between NO/H_2O_2 on the increase in cGMP concentrations was only found with intact platelets. This may mean that another intermediate event connected to the cell membrane is required for activation of sGC by H_2O_2 and the increase of intracellular cGMP concentrations. In endothelial cells, H_2O_2 was shown to increase the levels of cGMP through the activation of catalase (Burke and Wolin et al 1987). In this study, H_2O_2 was not capable of increasing the activity of sGC in either the cell lysate or cytosolic fraction. In agreement with this, Mittal and Murad (1977) also showed that activation of guanylate cyclase (partially purified from rat liver) could not be achieved by the addition of H_2O_2 alone.

cGMP is involved in many pathways in all mammalian cells and in several cellular signalling pathways. In this study, the activation of sGC and an increase in cGMP concentration was expected to be central to the synergism between NO and H_2O_2 relating to the inhibition of platelet aggregation. In the presence of the sGC inhibitor,

ODQ, no synergism was detected, confirming that indeed sGC activity was necessary. The nitric oxide synthase inhibitor, L-NAME, also inhibited the synergism between NO and H_2O_2 confirming the importance of endogenous NO in this synergism.

As indicated above, in the absence of PDE inhibitor, a small but significant increase of cGMP was observed with addition of NO, H_2O_2 or both simultaneously, but only at a single time point after 15sec. The levels of cGMP were then decreased, shortly after (between 15-30sec) (Fig 3.10). It is possible that the levels of cGMP increase only seconds after addition of NO or H_2O_2 and we may miss the earlier incubation as 5 sec. This short incubation times are more difficult to measure in practice. It is possible that the cGMP is only stable for a short amount of the time, but may will be enough for the signalling process to occur. However, this tight time scale differs significantly from the separation of addition of NO and H_2O_2 needed for the synergism in inhibition of aggregation to occur.

In conclusion, the mechanism of the synergism between NO and H_2O_2 is much more complicated than we expected. sGC may not be the major mechanism behind the synergism, but it is necessary for this process. Furthermore, NO synthases is also necessary for the synergism, but NO may operate through a mechanism that may involve sGC only indirectly or other reactions such as the nitrosylation of key proteins may play a role.

Towards the end of this study, data from other laboratories described H_2O_2 as an endothelial hyperpolarizing factor in normal physiological conditions. This role of H_2O_2 is through activation of the ion channels on the vascular smooth muscle,

increasing K^+ conductance and causing hyperpolarisation and relaxation of the vascular smooth muscle (Matoba et al 2000, Vanhoutte et al 2001). This effect of H_2O_2 may be other important role of the oxidant involved in the inhibition of platelet aggregation by NO and H_2O_2, since the K^+ conductance channels are present in platelets. These K^+ channels (Ca^{2+} regulated) are known to have free SH groups, which may suits for nitrosylation by NO and oxidation by H_2O_2.

7.3 The role of YC-1 on the inhibition of platelet aggregation in the presence of H_2O_2

YC-1 is a synthetised activator of soluble guanylyl cyclase. A small, but significant increase on the inhibition of platelet aggregation was observed in the presence of YC-1 and H_2O_2, but to a much lesser extent compare to NO and H_2O_2. This synergism was reported previously by Wu et al (1999), but at much higher concentrations of H_2O_2 than used in this study. At the lower peroxide concentration, they showed little evidence

YC-1 is known to cause activation of sGC and increases the concentrations of cGMP through a NO-independent way by binding to the allosteric site on sGC.

In this study, the shape of the inhibition curve for NO+H_2O_2 and YC-1+H_2O_2 was different. A more cooperative interaction was observed in the presence of NO, when the shape of the curve for YC-1 would represent a typical non-cooperative interaction. NO is known to interact with the haem moiety in sGC, which then causes conformational changes and activation of the enzyme through a three steps reaction. Other binding sites (un-identified non-haem sites) on the enzyme was suggested for

NO (Stone et al 1996). YC-1 was also reported to increase the cGMP concentrations, in the presence of NO, through the sensitisation of sGC by inhibition of phosphodiesterases, but the exact mechanism of the interaction between YC-1 and sGC is not clear.

In the presence of YC-1, an increase of the cGMP was observed by stimulating either purified sGC or cytosolic fraction of platelet in the presence and absence of purified sGC. No significant changes of cGMP was observed in the presence of H_2O_2 (as the case of NO).

7.4 Phosphorylation of VASP in the presence of H_2O_2

H_2O_2 increased the phosphorylation of VASP and increased the apparent molecular weight of this protein from 46 to 50kDa. No increase in cAMP concentration (nor cGMP) was observed in the presence of H_2O_2 alone, indicating that the increase of phosphorylation of VASP on serine^{157}, (which was thought only to be possible through activation of PKA), was independent of cyclic nucleotide concentrations. Phosphorylation of VASP controls the activation of actin polymerisation and inhibits platelet aggregation. H_2O_2 is incapable of inhibiting platelet aggregation on its own, but increases the phosphorylation of VASP. H_2O_2 may only phosphorylate VASP on Ser^{157} (the preferred phosphorylation site for PKA), but this needs to be verified. There have been no reports regarding the specific involvement of Ser^{157} in the control of actin polymerisation and remodelling and inhibition of platelet aggregation. This action of H_2O_2 is then completed by NO through phosphorylation of Ser^{239} (the preferred phosphorylation site for PKG) by increasing cGMP concentration. It may then be postulated that synergism between them will cause the final inhibition of

aggregation. Neither NO nor H_2O_2 at these low concentrations (10 nM and 25 μM) are capable of inhibiting platelet aggregation separately, but each one does exert an effect on VASP phosphorylation (Fig 4.3).

H_2O_2 enhances the inhibition of platelets in the presence of NO, whereas other peroxides appear to be unable to do this or even attenuate the effects of NO (Naseem et al 1999). A similar synergism was also shown between H_2O_2 and insulin (Schmid et al 1999). In this study, physiological concentrations of H_2O_2 (< 0.1μM) were not sufficient to trigger the autophosphorylation of the insulin receptor in the absence of insulin, But did enhance the response to 100nM insulin. Further work is needed to understand the mechanism of action of hydrogen peroxide on VASP phosphorylation.

7.5 Nitration of proteins in platelets

The nitration of VASP and some other proteins were detected even in the non-stimulated basal platelet samples immediately after rapid separation from plasma. Changing the platelet isolation techniques, or addition of aspirin and creatine phosphate to anticoagulant buffer, to reduce activation during the isolation process, did not inhibit the extent of this nitration. This indicates that these proteins is nitrated during circulation of platelets in blood. The cause of the nitration is not yet clear, but the nitration of some of these proteins are reversible and the extent of the nitration is significantly diminished an hour after separation from plasma. The transient nature of the nitration of VASP indicated that this is a dynamic process that may possibly have a physiological function. Other platelet proteins also exist in nitrated form in normal circulating platelets. In this study, the significance of the nitration of platelet proteins

under physiological condition was demonstrated in contrast with the usual association of nitration with pathological conditions.

7.6 Nitration of proteins in the presence of H_2O_2

In this study we found the H_2O_2, even at low concentrations well below that needed to induce oxidative stress, can bring about the nitration of proteins (i.e: VASP) within the space of one minute. Increased protein nitration in the presence of millimolar concentrations of H_2O_2 has been observed immunocytochemically in renal epithelial cells, but no specific proteins were identified (Persleni et al 1996).

It is not known, whether tyrosine nitration or serine phosphorylation of VASP happens first, or if any of these modifications facilitate each other. There is a possibility that VASP is nitrated first in the presence of H_2O_2 and nitration then changes the conformation of the protein and makes it more available for serine phosphorylation (there is no supporting evidence for this suggestion at present).

There are some indications of the possible mechanisms by which nitration by H_2O_2 occurs. It has been reported recently that nitration may occur by processes independent of the formation of peroxynitrite, since it has been shown in simple systems that nitration may be promoted by myoglobin, although it had a requirement for the presence of H_2O_2 (Klinic et al 2001). Indeed other authors have shown the haem-containing peroxidases are also active in this respect, but again the presence of H_2O_2 was required (Abu-Soud et al 2000, Gaut et al 2002, Sampson et al 1998). In a recent study, Ulrich et al showed that H_2O_2 increases the nitration of cyclooxygenase in the presence of arachidonic acid (Published abstract 2003). The formation of ferryl

radicals was suggested as a prelude to the oxidation of NO to $NO_2^{\cdot}$, which could then nitrate the proteins.

In this study, it was shown that NO synthesis is required for the nitration to occur, Since the process is almost completely inhibited in the presence of L-NAME. The decrease of nitration in the presence of L-NAME may be due to reduction of $O_2^{\cdot-}$ production, as a result of the inhibition of NOS by this inhibitor. In contrast, no further nitration of platelet proteins arises as a result of the addition of exogenous NO, in the form of S-nitrosoglutathione (GSNO). Indeed a decrease of nitration was observed at higher concentrations of this NO donor.

No increase in NO synthesis or cGMP was detected in platelets by the addition of these low concentrations of H_2O_2. It is possible that H_2O_2 does increase the endogenous concentrations of NO in the physiological range, but such levels are not easy to detect due to the detection limits of the methods available.

H_2O_2 at low concentrations, at least lower than that required for inducing oxidative stress, may act as a signalling molecule e.g. as endothelium dependent hyperpolarizing factor (Matoba et al 2000). There is evidence that H_2O_2 may influence signal transduction pathways, but the published articles often use concentrations of H_2O_2 in the millimolar range. H_2O_2, at concentrations of 1mM, induces tyrosine phosphorylation of focal adhesion proteins (Vepa et al 1999). In this study, an increase in serine phosphorylation of VASP at concentrations of peroxide comparable to those that induce nitration of VASP has been demonstrated. The relationship in between these two events is unclear. Whereas serine phosphorylation is accompanied by an increase in the apparent molecular weight of VASP, nitration seems to occur in whichever form is determined by the extent of serine phosphorylation (i.e. 46 and 50 kDa forms). Under certain conditions, peroxynitrite is

able to increase tyrosine phosphorylation (Mandoro et al 1997). Therefore, more work is required to evaluate the relationship between protein phosphorylation and nitration in known signalling pathways and its time dependence and outcomes on cellular activity. The role of H_2O_2 in terms of cellular activity seems to be passive, requiring another antagonist or agonist to complete the action. It is therefore possible that nitration is a permissive event, which facilitates further activation or inhibition of cellular processes.

Another explanation of this effect of H_2O_2 on nitration can be that it may stimulate the reverse action of superoxide dismutase to produce the superoxide anion (Mc Bride et al 1999, Brunnelli et al 2001). However, H_2O_2 also stimulates the formation of higher oxidation states of haem containing proteins. It was proposed by Hazen and colleagues (Abu-Soud et al 2000) that, in the presence of ferryl radicals, $NO_2^{\cdot}$ may be generated in the presence of NO. From this, we may infor that nitration of tyrosine residues on platelet proteins, may results from the interaction of H_2O_2 with haem containing proteins such as peroxidases.

In addition, Alvarez and Radi (Alvarez et al 2001) have shown that H_2O_2 was able to inhibit the decay of peroxynitrite, which again would enhance the effect of the endogenously produced oxidant.

Alternative mechanisms may be considered. The auto-nitration of tyrosyl radicals has been proposed for prostaglandin H synthase (Gunther et al 1997). The peroxidase activity may be enhanced by the presence of H_2O_2 or other peroxidases, although this may not lead to the nitration of other proteins. The consumption of NO by lipoxygenases has also been demonstrated (O' Donnell et al. 1999). This process may

result in the formation of peroxynitrite (or NO_2.) if 12-lipoxygenase, (for example in platelets) is activated by peroxides (Fujimoto et al 1998).

7.7 The reversibility of nitration

The nitration of VASP is not a permanent event and disappears at rates comparable to those reported for platelet total proteins after the addition of peroxynitrite to platelets (Low et al 2002). The mechanism for the disappearance of the nitration is also of interest. The nitration of VASP appears to be reversible, or at least the nitrated tyrosine residues are converted to a form not recognised by selective antibodies raised against nitrated proteins. The loss of nitrated VASP was still evident in the presence of membrane permeable protease inhibitors. A decrease of nitration could be achieved using L-NAME, the nitric oxide synthase inhibitor, but not by ODQ, the inhibitor of guanylyl cyclase that inhibits VASP serine phosphorylation. The antioxidants uric acid and ascorbate were ineffective in reducing the extension of nitration. In contrast, the flavanoid epigallocatechin gallate completely suppressed nitration and was also shown to inhibit the activation of platelets by other agonists. The role of nitration and the mechanism of its induction by H_2O_2 on platelet function are not clear, but may have a role in modulating other processes that occur during platelet activation.

In the case of the addition of H_2O_2, the duration of the nitration corresponds approximately to the duration of VASP phosphorylation and the synergism between NO and H_2O_2 (30-60 mins). The denitration of proteins accured both in freshly prepared cells and also after nitration was induced by H_2O_2. The denitration was

accelerated by calcium ions. A second wave of nitration can then be induced by the addition of H_2O_2 and this again subsides. The loss of nitrated proteins may be achieved by proteolysis (possibly by calcium activation), but the addition of inhibitors of proteolysis did not change the rate of loss. However, further evidence is required to show that specific enzymes are present that reduce and remove the nitro group from nitrotyrosine. The existence of such enzymatic activity has been proposed by Murad and co-workers (Kamiasaki 1998, Balabanli 1999), but still no "denitrase" enzyme has yet been isolated. There is evidence that nitro- groups on non-peptide compounds may be reduced to hydroxylamines using a cytochrome P450.dependent enzyme system. More work is required to verify that this can be achieved with proteins, for example by identification of the aminotyrosine products, which are not recognised by nitrotyrosine antibody.

It is possible that a series of events are required for synergism to occur and that more investigations are needed to clarify the role of each event on platelet functions.

The increase of nitration in the presence of H_2O_2 was further evidence of production and involvement of reactive species, which could act as nitrating agents. Surprisingly addition of uric acid or ascorbic acid to platelets samples did not inhibit the nitration caused by H_2O_2. Both uric acid and ascorbic acid showed partial inhibitory action on nitration caused by peroxynitrite but in this work, antioxidants did not have any effect on the nitration caused by the endogenous mechanisms.

7.8 The significants of nitration in physiological systems

In this study, the significance of nitration was studied in the presence of normal physiological agonists of platelet functions like calcium and collagen. Also some significant amount of nitration was found following isolation of platelets from plasma , the extent of the nitration was decreased thereafter. Addition of 1mM calcium to washed platelets accelerated the denitration of VASP immediately after separation of platelet. This effect of calcium might be important, since during the isolation of platelet from blood the concentration of calcium ions is high. Calcium may prevent the maximum acceleration of nitration of the proteins thereby preventing nitration reaching the toxic levels. In contrast, addition of collagen increased the amount of tyrosine nitration and decreased the tyrosine phosphorylation of VASP. Collagen is an important physiological activator of platelets, which induces signal transduction in platelets leading to a series of signalling reactions. The increase in nitration caused by collagen may be through an increase of NO synthase. However, the nitration of VASP in the presence of collagen might also be of physiological importance in cell signalling processes.

In conclusion, these observations, but are novel and of possible importance. The mechanism of the interaction between different nitration process on each other and their final effect on the maintenance of the haemostasis and platelet function needs to be investigated further.

Although new information on the interactions between NO and H_2O_2 has been found during the course of this work, it is clear that the mechanism involved are complex and would provide the subject matter for another thesis.

Further work to be done

Nitration of proteins with simultaneous addition of NO and H_2O_2

We found a few proteins to be nitrated in the presence of H_2O_2 it is important to investigate the nitration of these proteins in the presence of H_2O_2 in different conditions. In this project we did not look at the changes in the nitration with simultaneous addition of both NO and H_2O_2. It is interesting to determine whether the presence of more endogenous NO would change the extent of the nitration caused by H_2O_2. It is important to investigate the mechanism of phosphorylation of VASP in the presence of H_2O_2.

GSNO at higher concentrations reduced the extent of the nitration of VASP. It is also interesting to investigate why this occur.

Nitration of VASP

Since the findings of nitration of VASP (e.g. in the basal and during the exposure of platelets to H_2O_2) are novel more investigations are needed to establish:

- which tyrosine residues are nitrated
- whether tyrosine nitration affects other modifications of this protein, including tyrosine phosphorylation and serine phosphorylation
- what influence would nitration have on the function of this protein and on its interaction with other proteins
- would actin polymerisation be affected by nitration

- is modification of VASP by nitration involved in the synergism between NO and H_2O_2

Identifications of nitrated proteins

With help of modern proteomic techniques, it is hope to identify which other proteins are modified by H_2O_2 or NO either by nitration or by serine and tyrosine phosphorylation. The sequence of these events may help to establish the relationship between them.

The role of H_2O_2 on the ion channels

Since the involvement of H_2O_2 on the stimulation and also inhibition of ion channels has been discussed (Matoba et al 2000, Bychkov et al., 1999; Vanhoutte, 2001), it would be interesting to determine the activity of these channels in platelets in the presence of NO and H_2O_2.

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Presentations of work

- 3re international peroxynitrite meeting, Asilomar, California, may 27-31, 2001(poster)
- NO meeting, San Francisco, California, June 3-7, 2000 (Poster)
- 3rd platelet meeting, Univerity of Bristol, Sep 21-22 , 2000 (Oral)
- Departmental meetings (Oral presentations)

Meetings attended

- 2nd platelets meeting, University of Leicester, July 19-20 (1999).
- NO meeting, Glaxo Wellcome, Dec.18 (2000).
- NO meeting, Astra zeneca, Loughbrough, (2001).
- NO meeting, wolfson institute, UCL, London (2003)

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FEBS 23624 FEBS Letters 473 (2000) 119–122

The nitration of platelet cytosolic proteins during agonist-induced activation of platelets

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Received 28 March 2000

Edited by Barry Halliwell

Abstract The nitration of protein tyrosine residues by peroxynitrous acid has been associated with pathological conditions. Here it is shown, using a sensitive competitive enzyme-linked immunosorbent assay and immunoblotting for nitrotyrosine, that spontaneous nitration of specific proteins occurs during a physiological process, the activation of platelets by collagen. One of the main proteins nitrated is vasodilator-stimulated phosphoprotein. Endogenous synthesis of nitric oxide and activity of cyclo-oxygenase were required for the nitration of tyrosine. The nitration was mimicked by addition of peroxynitrite to unstimulated platelets, although the level of nitrotyrosine formation was greater and its distribution among the proteins was less specific.

Key words: Platelet; Collagen; Protein nitration; Vasodilator-stimulated phosphoprotein

1. Introduction

Platelet aggregation, induced by specific agonists, leads to both biochemical and morphological changes in the cell. Platelets release nitric oxide (NO) during platelet aggregation, but not at rest [1,2]. NO, which activates soluble guanylyl cyclase leading to the formation of cGMP, inhibits platelet activation through the action of cGMP-dependent kinases [3] and their phosphorylation of vasodilator-stimulated phosphoproteins (VASPs) [4]. The platelet-derived NO limits the activation of neighbouring platelets [5] to avoid excessive recruitment of these cells into thrombi. Differences in the potency of platelet agonists to elicit NO biosynthesis have been reported: collagen and arachidonic acid are the most potent, while thrombin has a much smaller effect [1,6]. During activation, platelets also form superoxide anions ($O_2^{\bullet-}$) and hydrogen peroxide [7,8]: the peroxide strongly enhances the ability of NO to inhibit platelet aggregation [9].

NO reacts with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$), a potent oxidant [10], which has been shown to be released by endothelial cells [11]. Peroxynitrite inhibits platelet aggregation, but less effectively than NO [9,12–14]. The mechanism of this inhibition has been proposed to be both dependent [12,14] and independent [13] of cGMP. Peroxynitrite is extremely reactive and oxidises, hydroxylates and nitrates phenol groups on amino acids [15] and this may account for the presence of nitrated proteins in normal plasma [16]. Furthermore, nitrotyrosine has been found in inflammatory conditions, e.g. atherosclerotic plaques [17] and synovial fluid in rheumatoid arthritis [18], and has been used extensively as a marker of peroxynitrite formation in vivo.

Since both the reactants required for peroxynitrite formation, NO and $O_2^{\bullet-}$, are produced during the platelet activation/aggregation process, we examined the possibility of the nitration of protein tyrosine residues. The spontaneous nitration of proteins was shown to arise as part of a normal cellular process, in this case platelet activation, and that this nitration may target specific proteins in these cells.

2. Materials and methods

Prostacyclin (synthetic sodium salt), thrombin (human), bovine serum albumin (BSA), N^G-nitro-L-arginine methyl ester (L-NAME), acetylsalicylic acid (ASA) and (3-[3-cholamidopropyl) dimethylammonio]-1-propane-sulphonate (CHAPS) buffer were purchased from Sigma (Poole, UK), collagen Type I (equine) from Hormonchemie (Munich, Germany) and oxadiazoloquinoxaline-1-one (ODQ) from Tocris-Cookson Chemicals (Southampton, UK).

Biotinylated goat anti-rabbit IgG antibodies and ECL reagents were obtained from Amersham-Pharmacia (Hertfordshire, UK), avidin–biotin horseradish peroxidase from Dako (Beaconsfield, UK), anti-nitrotyrosine was from TCS Biologicals plc (High Wycombe, Buckinghamshire, UK) and anti-VASP antibodies from Alexis (Nottingham, UK). Pre-cast 10% were prepared in the laboratory and nitrocellulose (pore size 0.45 μm) were purchased from Novex (San Diego, CA, USA).

2.1. Platelet preparations

Venous blood was taken with informed consent from healthy volunteers, who denied taking any medication in the previous 14 days, placed in acid-citrate-dextrose anticoagulant and centrifuged for 20 min at $150 \times g$ to yield platelet-rich plasma (PRP). Washed platelets (WP) were prepared from PRP in the presence of prostacyclin as described elsewhere [9,19], suspended in buffer (NaCl 137 mM, NaH_2PO_4 4.2 mM, $NaHCO_3$ 11.9 mM, KCl 2.7 mM, pH 7.4) and diluted to a count of 3×10^8 platelets/ml. The platelets were allowed to rest for 1 h at room temperature and used within the following 2 h.

To prepare cytosolic and membrane fractions, WP were sonicated at 4°C (Soniprep, M.S.E., UK) for 2×15 s bursts separated by a 15 s pause and the cytosol separated from the membrane fractions by centrifugation at $1500 \times g$ for 10 min. The pellet, which contained the membrane fraction, was solubilised in 0.1% CHAPS buffer [20].

2.2. Preparation of peroxynitrite

Sodium peroxynitrite was prepared as described previously [9]: con-

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Abbreviations: ODQ, oxadiazoloquinoxaline-1-one; L-NAME, N^G-nitro-L-arginine methyl ester; CHAPS, (3-[3-cholamidopropyl) dimethylammonio]-1-propane-sulphonate; NOS, nitric oxide synthase

PII: S0014-5793(00)01490-3

trols were prepared by the same procedure, except that the solutions were passed directly into water instead of NaOH, leading to immediate decomposition of the oxidant. NaOH was then added to restore the pH to 10. The concentration of stock peroxynitrite solutions was determined spectrophotometrically ($\varepsilon_{302\,nm} = 1760\ M^{-1}\ cm^{-1}$) using decomposed peroxynitrite as the blank.

2.3. Measurement of nitrated proteins

The nitrotyrosine content of proteins in the platelet samples was estimated using a competitive enzyme-linked immunosorbent assay (ELISA) developed in this laboratory [16] using nitrated (NT)-BSA as a standard. The results were extrapolated from a semi-log plot of the standard curve and were expressed as nmol BSA equivalents/mg protein. The protein concentrations were analysed using a modified Lowry method [21].

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

After various treatments, the reactions were stopped by addition of an equal volume of Laemmli's buffer [22]. Proteins were separated by SDS–PAGE on 10% gels. The separated proteins were transferred to nitrocellulose membranes using wet transfer techniques. Membranes were blocked with BSA (0.5%) dissolved in phosphate-buffered saline (PBS)/Tween (1% PVP-10, 1% PEG, 0.2% Tween and 10 mM NaF) for 60 min. Membranes were probed with either, anti-nitrotyrosine (1:800) or anti-VASP (1:2000) for 60 min. The membranes were washed several times with PBS/Tween, followed by incubation with horseradish peroxidase-linked goat anti-rabbit IgG for 60 min. The protein bands were visualised using ECL reagents.

In some experiments, membranes were stripped by washing with a stripping buffer (100 mM mercaptoethanol, 2% SDS and 62.5 mM Tris–HCl; pH 6.7) at 50°C for 30 min. The membranes were washed and probed with anti-VASP antibodies. After washing, the membranes were incubated with horseradish peroxidase-linked goat anti-rabbit IgG.

2.5. Statistical analysis

All data are presented as the mean ± S.E.M. of at least three independent experiments, unless otherwise stated. Statistical analysis was performed using Student's unpaired *t*-test.

3. Results

3.1. Nitration of proteins following agonist-induced activation of platelets

Low levels of nitrated proteins were found in most, but not all unstimulated platelets (Fig. 1) as measured by ELISA. In the cytosolic fraction 0.18 ± 0.08 nmol nitrotyrosine/mg protein were found, but the basal level of nitration in the membrane fraction was higher at 0.31 ± 0.08 nmol/mg protein. The CHAPS buffer used to solubilise the membrane fraction was found to have no effect on the reproducibility of the ELISA.

The activation of WP by collagen (0.2–20 μg/ml) for 1 min led to a concentration-dependent increase in the formation of nitrotyrosine residues on platelet proteins in the absence of exogenous peroxynitrite. The greatest effect was found with collagen (20 μg/ml) where nitrotyrosine was increased to 0.75 ± 0.15 nmol nitrotyrosine/mg protein, an almost four-fold increase above basal levels ($P < 0.01$) (Fig. 1). Collagen (2 μg/ml) increased nitrotyrosine levels on cytosolic proteins from a basal level of 0.18 ± 0.08 nmol to 0.56 ± 0.1 nmol nitrotyrosine/mg protein ($P < 0.05$) after 1 min. However, at lower concentrations of collagen (0.2 μg/ml) the elevation in nitrotyrosine did not reach statistical significance. There was some variation between platelet preparations, from a two- to a five-fold increase in nitrotyrosine, which may reflect the varying responsiveness to collagen of platelets taken from different donors. No increase in the levels of nitrated proteins was detected in the membrane fractions at any concentration of collagen. In experiments where thrombin (0.02–0.2 U/ml) was used to stimulate platelets, it was less effective in causing platelet nitration and the apparent increase was not statistically significant (not shown). The presence of EGTA (1 mM) abolished the collagen-induced formation of nitrotyrosine (not shown).

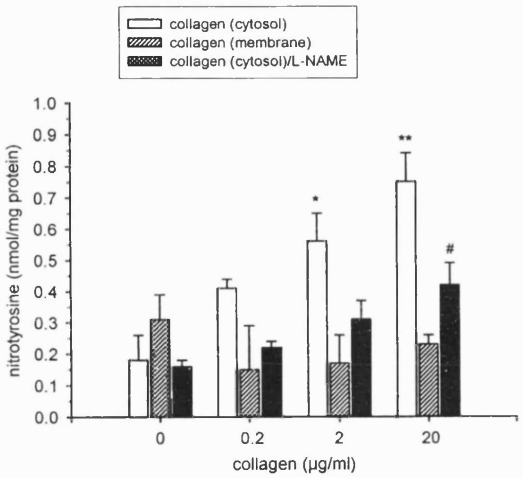

Fig. 1. Protein bound nitrotyrosine formation in collagen-stimulated platelets. WP were incubated with collagen (0.2–20 μg/ml) for 1 min with stirring at 37°C. The membrane and cytosolic fractions were prepared as described in Section 2. Nitrotyrosine was measured by a competitive ELISA. The data shows the nitrotyrosine formed in the cytosol after treatment of collagen (no fill) and membrane (hatched fill). In some experiments platelets were preincubated with L-NAME for 30 min prior to the addition of collagen (black fill). The results are expressed as nmol nitrotyrosine/mg protein and represent the mean ± S.E.M. of four independent experiments: $^{*}P < 0.05$ or $^{**}P < 0.05$ for nitration compared to basal levels, $^{\#}P < 0.05$ for the effects of L-NAME.

The experiments were repeated in the presence of L-NAME, a competitive inhibitor of nitric oxide synthase (NOS), used at submaximal concentrations (100 μM). Collagen (20 μg/ml) induced 0.75 ± 0.15 nmol nitrotyrosine/mg protein, while in the presence of L-NAME this was reduced to 0.42 ± 0.09 nmol ($P < 0.05$) (Fig. 1). The reduction in nitrotyrosine suggests that endogenously synthesised NO was required for the formation of a nitrating species and subsequently nitrotyrosine.

The presence of nitrotyrosine in specific platelet proteins was determined by immunoblotting with an anti-nitrotyrosine antibody. Low levels of nitrotyrosine were detected in the proteins of resting platelets (Fig. 2A, lane 2) particularly those with molecular weights of 46 to 50 kDa. However, stimulation of the platelets with collagen led to a marked increase in the extent of nitration in these proteins. The greatest effect was again observed with 20 μg/ml collagen (Fig. 2A, lane 5), confirming results described above with the ELISA on total platelet proteins. The presence of L-NAME (100 μM) also reduced the signal for nitrotyrosine in the bands of nitrated proteins. Furthermore, the pre-incubation of platelets with ASA (1 mM) returned the extent of protein nitration of VASP to those of resting platelets or lower. ODQ (10 μM), a specific inhibitor of soluble guanylyl cyclase [23], had no effect on the amounts of nitrotyrosine detected.

The proteins which were most strongly nitrated following activation of platelets by collagen, molecular weights of 46

and 50 kDa, were shown to be VASP, representing the phospho (50 kDa) and de-phospho (46 kDa) forms of the protein, by stripping and re-probing the membrane with anti-VASP antibody (Fig. 2B). This was confirmed by immuno-precipitation of VASP with this antibody, re-running on a gel and probing with anti-nitrotyrosine antibody to reveal nitrated protein only at the 46 and 50 kDa bands (not shown).

3.2. Nitration of platelet proteins by exogenous peroxynitrite

The extent of nitration of proteins induced by collagen was compared to that in platelets exposed to solutions of authentic peroxynitrite. This led to a concentration-dependent increase in the formation of nitrotyrosine residues on both membrane and cytosolic proteins (Fig. 3). Nitrotyrosine levels were greater than those previously induced by agonist-stimulated platelet activation. However, as with spontaneous nitration, nitrotyrosine was found to be higher in the proteins from the cytosolic fraction. Peroxynitrite (3 μM) induced 12.6 ± 3.2 nmol nitrotyrosine/mg protein in the cytosolic fraction and 1.6 ± 0.3 nmol/mg protein in the membrane fraction (Fig. 3). The presence of exogenous glutathione (1 mM) protected platelet proteins, causing an almost complete abolition of nitration (not shown). Decomposed peroxynitrite did not raise nitrotyrosine concentrations significantly above the levels found basally (Fig. 3).

Immunoblotting of the peroxynitrite-treated samples showed that the oxidant caused the nitration of a broad spectrum of different proteins of which VASP was only a minor contributor (not shown). This is in contrast to collagen, which induced a more selective process of nitration.

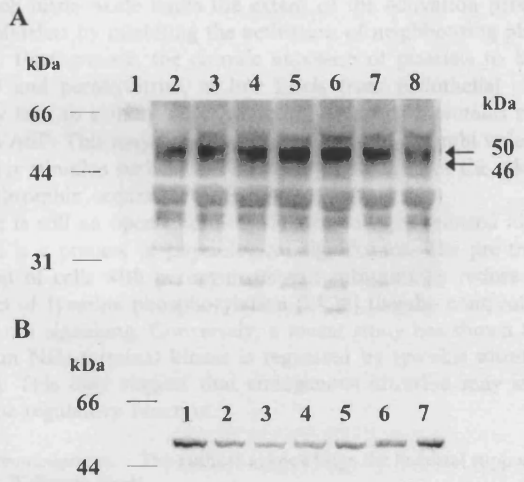

Fig. 2. A: Protein-bound nitrotyrosine formation in collagen-stimulated platelets. WP were incubated with collagen (0.2–20 μg/ml) for 1 min with stirring at 37°C. In the same experiment the platelets were incubated with L-NAME (100 μM), ASA (1 mM) or ODQ (10 μM) for 30 min, prior to addition of collagen. Platelets were lysed with Laemmli's buffer and subjected to SDS–PAGE and immunoblotting as described in Section 2. Lane 1, NT-BSA; lane 2, resting platelets; lane 3, collagen 0.2 μg/ml; lane 4, collagen 2 μg/ml; lane 5, collagen 20 μg/ml; lane 6, collagen 20 μg/ml+ODQ 10 μM; lane 7, collagen 20 μg/ml+L-NAME (100 μM); lane 8, collagen 20 μg/ml after pre-incubation with 1 mM ASA for 30 min. The blot is representative of four independent experiments. B: Identification of VASP as a possible target for endogenous nitration. Experimental protocol was identical to that of (A), except the membranes were stripped and re-probed with anti-VASP antibody. Lane 1, resting platelets; lane 2, +collagen 0.2 μg/ml; lane 3, collagen 2 μg/ml; lane 4, collagen 20 μg/ml; lane 5, collagen 20 μg/ml+ODQ (10 μM); lane 6, collagen 20 μg/ml+L-NAME (100 μM); lane 7, collagen 20 μg/ml after pre-incubation with ASA 1 mM. The blot is representative of four independent experiments.

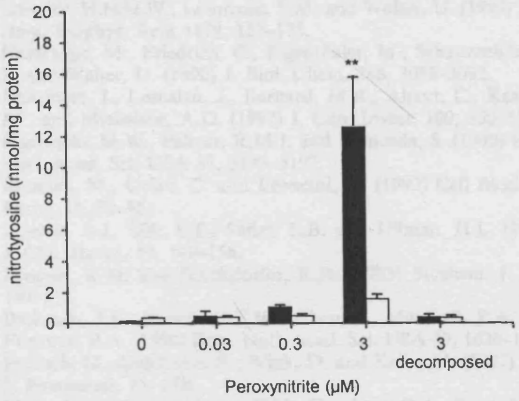

Fig. 3. Peroxynitrite-induced nitration of platelet proteins. Peroxynitrite was added to WP and incubated for 1 min at 37°C with continuous stirring. The platelets were separated into cytosolic and membrane fractions as described in the Section 2. Nitrotyrosine was then measured using a semi-quantitative ELISA. Results are expressed as nmol nitrotyrosine/mg protein and represent the mean ± S.E.M. of five independent experiments. $^{**}P < 0.01$ for nitration compared to basal levels.

4. Discussion

In the present study we investigated whether the nitration of proteins could be formed as part of a normal physiological process. Low levels of nitration were detected in the majority of, but not all, samples of resting platelets and has been observed previously in unstimulated cell lysates of cultured endothelial cells [24] and in human plasma [16]. The basal levels of nitration found in platelets suggested that they were exposed to a nitrating species either in vivo, or possibly by activation of the cells during the isolation process.

The activation of platelets with collagen, but not thrombin, led to an increase in the levels of cytosolic nitration. This is direct evidence that nitration may occur as part of a normal physiological process. The reduction in nitrotyrosine formation in the presence of L-NAME indicates that endogenously synthesised NO is a key requirement for the formation of nitrating species and subsequently nitrotyrosine. The lower rates of synthesis of NO in thrombin-stimulated platelets [1,6] probably accounts for the inability of thrombin to induce significant nitration. ASA was also found to be an inhibitor of the nitration process. Activation of prostaglandin H synthase-2 leads to the formation of a tyrosyl radical within the enzyme complex that may react with NO to form nitrotyrosine leading to autonitration of this enzyme and possibly other proteins [25]. Alternatively, the formation of thromboxane A_2 during platelet activation will be inhibited by ASA. This eicosanoid acts primarily to increase platelet $[Ca^{2+}]_i$ leading to the activation of platelet NOS. Thus, in the presence of ASA no nitrating species would be formed.

The nitration of platelet proteins following activation by collagen is not uniform across all platelet proteins. Firstly, nitration occurred mainly among the cytosolic proteins. Fur-

thermore, specific proteins appear to be targeted for nitration during the activation process. One of these proteins was identified as VASP, a protein involved in cytoskeletal rearrangement during platelet aggregation [26]. VASP is a primary target for both cGMP- and cAMP-dependent kinases leading to the phosphorylation of two serine and one threonine residues [27]. The phosphorylation of serine 157 leads to a change in the apparent molecular weight from 46 to 50 kDa, but both forms appear to be capable of being nitrated. VASP has four tyrosine residues and their functional significance is unknown. The consequences of the nitration of VASP on its function require further investigation. The identity of the other protein bands that appear to be nitrated has not been established.

The exposure of platelets to low concentrations of authentic peroxynitrite, capable of the inhibition of platelet activation [10], caused extensive nitration of platelet proteins again mainly in the cytosolic fraction (Fig. 3), a phenomenon already observed both in platelets [28] and neuroblastoma cells [29]. This suggests that peroxynitrite formed extracellularly, e.g. by the endothelium, may diffuse into platelets (or other cells) and modify their cytosolic proteins [30]. The nitration occurred over a broad spectrum of proteins, appearing to be indiscriminate, but much more intense than after stimulation with collagen. This indicates that when platelets are exposed to collagen, the formation of endogenous NO and peroxynitrite is not sufficient to inhibit their activation. It is possible that the nitration process may be part of a mechanism by which nitric oxide limits the extent of the activation process in platelets by inhibiting the activation of neighbouring platelets. Furthermore, the chronic exposure of platelets to both NO and peroxynitrite, at low levels from endothelial cells, may lead to a more selective action on specific proteins such as VASP. This may prevent the formation of thrombi unless a major stimulus such as the exposure of collagen or the release of thrombin occurs.

It is still an open question whether collagen-induced nitration is a process of physiological significance. The pre-treatment of cells with peroxynitrite can substantially reduce the level of tyrosine phosphorylation [24,28] thereby compromising cell signalling. Conversely, a recent study has shown that c-Jun NH_2-terminal kinase is regulated by tyrosine nitration [31]. This may suggest that endogenous nitration may serve some regulatory function.

Acknowledgements: The authors acknowledge the financial support of The Wellcome Trust.

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NITRIC OXIDE: Biology and Chemistry
Vol. 5, No. 3, pp. 233–242 (2001)
doi:10.1006/niox.2001.0343, available online at http://www.idealibrary.com on IDEAL®

Synergism between Nitric Oxide and Hydrogen Peroxide in the Inhibition of Platelet Function: The Roles of Soluble Guanylyl Cyclase and Vasodilator-Stimulated Phosphoprotein

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Received August 30, 2000, and in revised form January 29, 2001; published online May 7, 2001

In previous studies, a strong synergism between low concentrations of hydrogen peroxide and nitric oxide in the inhibition of agonist-induced platelet aggregation has been established and may be due to enhanced formation of cyclic GMP. In this investigation, hydrogen peroxide and NO had no effect on the activity of pure soluble guanylyl cyclase or its activity in platelet lysates and cytosol. H_2O_2 was found to increase the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), increasing the amount of the 50-kDa form that results from phosphorylation at serine[157]. This occurs both in the presence and in the absence of low concentrations of NO, even at submicromolar concentrations of the peroxide, which alone was not inhibitory to platelets. These actions of H_2O_2 were inhibited to a large extent by an inhibitor of cyclic AMP-dependent protein kinase, even though H_2O_2 did not increase cyclic AMP. This inhibitor reversed the inhibition of platelets induced by combinations of NO and H_2O_2 at low concentrations. The results suggest that the action on VASP may be one site of action of H_2O_2 but that this event alone does not lead to inhibition of platelets; another unspecified action of NO is required to complete the events required for inhibition.

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Key Words: **nitric oxide; hydrogen peroxide; platelets; PKA; PKG; soluble guanylyl cyclase.**

NO is a physiological inhibitor of the activation of platelets by agonists such as collagen and thrombin (1). It activates the enzyme soluble guanylyl cyclase (sGC), by binding to the prosthetic ferrous heme with high affinity to form a ferrous–nitrosyl–heme complex resulting in subsequent changes in protein conformation (2). The activated enzyme converts GTP to cyclic GMP, leading to the activation of cGMP-dependent kinase (cGK) and the phosphorylation of specific target proteins in platelets and in other tissues (3). These include vasodilator-stimulated phosphoprotein (VASP), a target for both cGMP-dependent (cGK) and cAMP-dependent (cAK) kinases, and therefore are sensitive to increases in both cyclic GMP and cyclic AMP (4). The phosphorylation of VASP is reversible (5), and it occurs at three separate sites (two serine and one threonine) that are dependent upon kinase activation. This post-translational modification of VASP may impede its interaction with focal adhesion proteins and actin-binding proteins (4) and its direct interaction with actin via the C-terminal domain of VASP (6). This mechanism is considered important in the reorganization of the platelet cytoskeleton during platelet shape change and the subsequent aggregation process that is inhibited by NO.

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Hydrogen peroxide is released by platelets at rest and after activation. Platelets are also exposed to this peroxide prior to activation at the endothelium surface, where there is active extracellular superoxide dismutase (7, 8). In previous work we have shown that hydrogen peroxide, at concentrations that are below those normally used to cause oxidative stress (2–25 μM), enhanced the inhibitory action of NO and nitrosothiols on platelets, lowering the IC_{50} for NO up to 100-fold (9, 10). The peroxide itself had no inhibitory action, except in the presence of plasma, which contained endogenous nitrosothiols (10). This synergism was evident to a lesser extent with CO (11), another ligand for sGC, which activates the enzyme to a much lesser extent (12). Hydrogen peroxide has been reported to have a direct effect on other tissues. It induces the relaxation of arterial smooth muscle (13), which may result from an increase in the production of NO (14). Others have proposed that the mechanism may be related to the actions of the hydrogen peroxide by formation of compound I in catalase in smooth muscle (15), although this enzyme is not present in platelets in large amounts. Thus, the mode of action of the peroxide is unclear, but it appears to require the presence of activated sGC, since the inhibitor ODQ completely blocked the synergism (10). In addition, a modest rise in cyclic GMP was observed, in the presence of a phosphodiesterase inhibitor, which appeared to account for the observed synergism (9, 10).

Purification of sGC revealed that the enzyme is a heterodimer consisting of an α- and a β-subunit and contains stoichiometric amounts of ferro-protoporphyrin IX, a five coordinate heme with histidine as the axial ligand (2, 16). The enzyme activity may also be increased by a synthetic compound YC-1 (17), which is thought to activate the enzyme independent of NO and CO, but which acts synergistically with both gases to increase sGC activity, especially in the case of CO (18). YC-1 also inhibits platelet activation (19).

The aim of the study was to ascertain the mechanism by which the H_2O_2 exerts its effects. The direct effects of the peroxide on sGC were investigated, using both cell lysates and purified enzyme. Alternatively, the effects of H_2O_2 on events downstream of sGC activation were studied, using the phosphorylation of VASP as a marker.

METHODS

Materials

Prostacyclin, H_2O_2, thrombin, and isobutylmethylxanthine were all purchased from Sigma UK, Poole, Dorset. Collagen (type 1 equine) was obtained from Hormonchemie GmbH (Munich, Germany), and NO gas from Lynde Gas (Stoke on Trent, Staffs., UK). 2-(-4-Carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1oxyl 3 oxide (carboxy-PTIO), myristoylated 14-22 peptide protein kinase A inhibitor, and protein kinase G inhibitor (KT5823) were from Calbiochem-Novabiochem (Nottingham UK). Oxadiazoquinoxalin-1-one and *S*-nitrosoglutathione were from Tocris-Cookson Chemicals (Southampton, UK). ELISA kits for cyclic GMP and cyclic AMP, diethylamine-NO (DEA-NO), and YC-1 ([3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole]) were from Alexis Corp. (UK) Ltd (Nottingham, England), as were antiserum against VASP and monoclonal antibody against phosphoserine (7F12).

Preparation of Platelets and Subcellular Fractions

Venous blood was collected with informed consent from healthy donors who denied taking medication in the previous 14 days, into acid/citrate/dextrose anticoagulant. Platelets were isolated in the presence of 30 nM prostacyclin and tested for 2 h for full recovery of platelet function (9). Platelets were exposed to saponin (10 mg/ml) for 15 min at 4°C to prepare the lysates and then centrifuged for 15 min at 3000 rpm to separate the cytosolic and membrane fractions.

Platelet Aggregation

Isolated platelet suspensions (final concentrations 3×10^8 platelets/ml) were incubated in 300-μl cuvettes for 1 min at 37°C with continual stirring at 1000 rev/min (with magnetic stirrer bar) in a Payton aggregometer before the addition of agonists and antogonists. Platelet aggregation was induced by the addition of thrombin (0.02 U/ml) and was mea-

sured as previously described (9). Briefly, the aggregation was measured as the percentage of maximum aggregation after 3 min from the changes in light transmission. NO and/or H_2O_2 were added simultaneously 1 min before the thrombin using separate syringes. The pH of the platelet suspensions was monitored for each condition.

Preparation of NO and Other Solutions

Solutions of NO gas were prepared as previously described (9); those of other NO donors and H_2O_2 were prepared freshly on each occasion.

Assay of Cyclic GMP/Soluble Guanylyl Cyclase Activity

The assay of enzyme activity was determined in intact platelets, lysates, and cytosol in the presence of the following: 3 mM Mg^{2+}, 3 mM DTT, 0.5 mg of bovine serum albumin/ml, 300 μM GTP, and 50 mM triethylamine hydrochloride. NO or NO donors were incubated with the samples for 10 min, and then the reaction was terminated by the addition of trichloroacetic acid (final concentration, 7.5%). The concentrations of cyclic GMP and cyclic AMP were then measured by using a commercial ELISA method, as was cyclic AMP. In some cases, the platelets were preincubated with 200 μM isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, for 30 min prior to experimentation.

When purified soluble guanylyl cyclase was used in the experiments, the activity was also measured from the conversion of [α-^{32}P]GTP to [^{32}P]GMP as described previously (20). sGC was purified from bovine lung to apparent homogeneity by the immunoaffinity procedure as described previously (20).

Gel Electrophoresis and Western Blotting

After treatment of platelets with the various reagents, the reactions were stopped by addition of an equal volume of Laemmli's buffer (0.125 M Tris–HCl, 40 g of sodium dodecyl sulfate/L, 200 ml of glycerol/L, 0.2 M dithiothreitol, and 0.2 g/L bromophenol blue, pH 6.8) (21). Proteins from platelets and platelet fractions were dissolved in SDS and 50 μg of protein separated on 10 or 15% SDS–PAGE. Proteins were then blotted onto PVDF membranes using semi-dry transfer. Membranes were blocked with BSA (0.5%) dissolved in phosphate-buffered saline (PBS) and 1% polyvinylpyrrolidone (PVP-10), 1% polyethylene glycol (PEG), 0.2% Tween-20, and 10 mM NaF for 60 min. Membranes were then probed either with VASP antiserum (diluted 1:2000) followed by peroxidase-labeled anti-rabbit IgG antibody or with the monoclonal anti-phosphoserine antibody (diluted 1:1000 followed by labeled anti-mouse IgG). Antibody binding was detected using the ECL system (Amersham International, Bucks, U.K.). The optimum conditions, amounts of proteins, and ratios of primary and secondary antibodies were established to ensure linearity of the intensities of those observed on the bands.

Statistical Analysis

All data are presented as the mean ±SE of at least three independent experiments. Statistical analysis was performed using Student's unpaired *t* test.

RESULTS

The Influence of Hydrogen Peroxide on Nitric Oxide-Induced Platelet Inhibition

The synergism of H_2O_2 with NO on the inhibition of platelets activated by thrombin was demonstrated (Fig. 1A) as shown previously (9). There was little synergism of H_2O_2 on platelet inhibition by YC-1 (Fig. 1B). No synergism occurs between the peroxide and prostacyclin (not shown).

The Role of Soluble Guanylyl Cyclase in the Actions of Hydrogen Peroxide

H_2O_2, in the presence of IBMX, was previously shown to increase the formation of cGMP by NO (10 nM) to a greater extent than NO alone in intact platelets (9, 10). In the absence of IBMX, the initial level of cyclic GMP induced by NO (at 15 s) was increased significantly to a limited extent by H_2O_2, when added simultaneously with 100 nM NO, but not with 10 nM NO (Fig. 2). H_2O_2 alone had no independent action of cyclic GMP levels or aggregation. Addition of authentic NO, in the presence of IBMX, gave rise to a substantial increase in cyclic

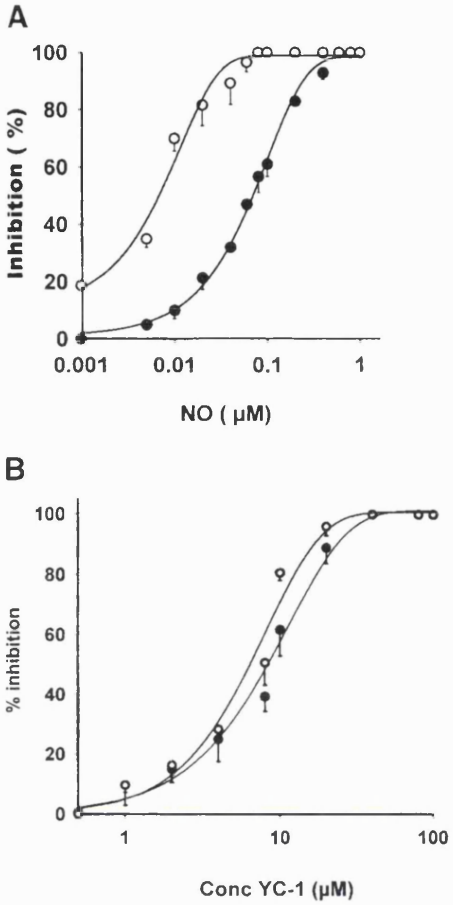

FIG. 1. Dose–response relationship between H_2O_2 and (A) NO and (B) YC-1 in inhibition of platelet aggregation by thrombin. Isolated washed human platelets were incubated with varying concentrations of NO (A) or YC-1 (B) in the absence (●) or in the presence of 25 μM H_2O_2 (○) for 1 min before the addition of thrombin (0.02 units/ml) and then incubated for 3 min at 37°C with stirring. The results are calculated as a percentage inhibition of the maximum aggregation induced by thrombin and are presented as means ± SE of 3 independent experiments.

GMP in preparations of platelet cytosol, up to 20-fold with 1 μM NO (Fig. 3A). However, in the presence of H_2O_2 (20 μM) no synergism was seen. Indeed, at high concentrations of NO the peroxide appeared to be inhibitory. Similar data were obtained using platelet lysates and with NO donors, *S*-nitrosoglutathione and DEA-NO (not shown). There were no significant increases in the concentrations of platelet cyclic AMP in cells treated with NO/H_2O_2 or peroxide alone (not shown).

YC-1 increased cGMP formation in platelet cytosol at concentrations up to 50 μM. A significant stimulation of activity was noted in the presence of H_2O_2 with 5 μM YC-1, but not at higher concentrations (Fig. 3B). Both NO (or DEA-NO) and YC-1 stimulated the activity of purified sGC strongly, but the presence of H_2O_2 had no influence on the activation by these agents, nor any direct effects of its own (not shown). It appeared that any effects of H_2O_2 on sGC occur when platelets are intact and may not be the only event required for synergism to occur.

The Effects of H_2O_2 on Events Downstream of Soluble Guanylyl Cyclase Activation

The phosphorylation of VASP was investigated using SDS/PAGE and Western blotting. The phosphorylation occurs at three different sites, with phosphorylation of Ser^{157}, the preferred site for PKA, causing a shift in the apparent molecular mass from 46 to 50 kDa (22). In resting platelets probed with an anti-VASP antibody, VASP was found mainly at 46 kDa. Exposure of platelets to prostacyclin for 1 min led to the appearance of a 50-kDa band (Fig. 4). NO (10 nM) alone caused a small shift from 46 or 50 kDa, whereas NO (10 nM) and H_2O_2 (25 μM) significantly increased the 50-kDa band (Fig. 4). However, this increase in the 50-kDa form was also present

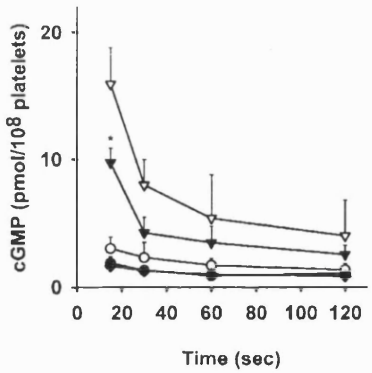

FIG. 2. Platelet cGMP changes induced by NO and hydrogen peroxide in the absence of phosphodiesterase inhibitor (IBMX). Isolated washed platelets were incubated with 0.01 μM NO (●), 0.1 μM NO (▲), and 0.01 μM NO + 25 μM H_2O_2 (○) or 0.1 μM NO + H_2O_2 μM (Δ) or 25 μM H_2O_2 alone (◆) for an interval of 0–120 s, in the absence of IBMX. The reaction was stopped with the addition of 7.5% TCA. cGMP content was measured by ELISA. The results are typical of at least four independent experiments.

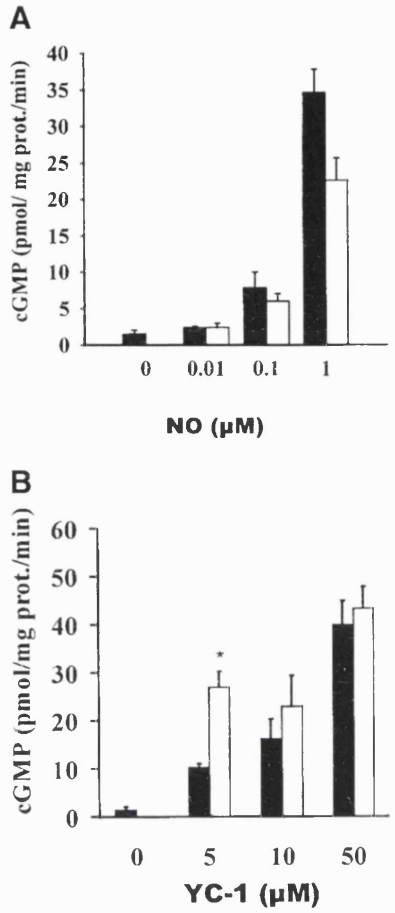

FIG. 3. Interactions of H_2O_2 and NO or YC-1 on cGMP biosynthesis in platelet cytosol and lysates. Cytosolic fractions of human washed platelets (A) were incubated with different concentrations of NO alone (solid bars) or together with 25 μM H_2O_2 (open bars) in the presence of IBMX (200 μM) for 10 min at 37°C with stirring. The reaction was later stopped with the addition of 7.5% TCA. In further experiments (B) YC-1, at a range of concentrations with 25 μM peroxide, was added to cytosol and processed as for A and B. The cGMP contents were determined by ELISA. Values are presented as means ± SE of 3 independent experiments. $^*P < 0.05$.

when platelets were exposed to 25 μM H_2O_2 alone. VASP was immunoprecipitated from platelet proteins with the VASP antibody and reprobed with anti-VASP antibody and anti-phosphoserine antibody to confirm its identity (not shown).

NO, at 10 nM, led to an increase in the serine phosphorylation of 46-kDa and not the 50-kDa VASP protein, but at higher concentrations of NO (100 nM) the 50-kDa band became visible. H_2O_2 at 0.25–25 μM increased the phosphorylation of both bands (Fig. 5), confirming that the peroxide has an effect independent of NO. Mixtures of NO (10 nM) and a range of concentrations of H_2O_2 also enhanced the phosphorylation of both bands (Fig. 5), but only to the same extent (or less at 0.25 μM H_2O_2) as with peroxide alone. The increased phosphorylation persisted for up to 1 h (not shown).

The effects of specific inhibitors for these cA- and cG-kinases were determined, both on inhibition of thrombin-induced platelet aggregation and on VASP phosphorylation by a combination of 10 nM NO and 20 μM H_2O_2. The cyclic GMP-dependent kinase inhibitor KT5823 was found to reverse partially the inhibition (not shown) of platelet aggregation by high concentrations of NO over a range of inhibitor concentrations (100–300 nM), close to its reported K_i (234 nM). KT5823 reversed the inhibition of platelet aggregation by NO/H_2O_2 of platelet aggregation by only 5% (Fig. 6). It had little effect in reducing the phosphorylation of the 46-kDa VASP by the NO/H_2O_2 mixture (Fig. 7), probably because the low dose NO made only a minor contribution to the total phosphorylation. Similar results (not shown) for aggregation and phosphorylation were obtained with a wider range of concentrations of KT5823 (1 nM–1 μM) with an optimum effect between 100 and 500 nM.

In contrast, the cyclic AMP-dependent protein kinase inhibitor (myristoylated PKA inhibitor 14–22 amide) at its 34 nM (reported K_i), reversed the inhibition of aggregation by NO/H_2O_2 by 60% (Fig. 6) and reduced the serine phosphorylation of 50-kDa VASP to a similar extent (Fig. 7). This inhibitor inhibited aggregation and phosphorylation induced by NO/H_2O_2 at concentrations of 1 nM–1 μM (not

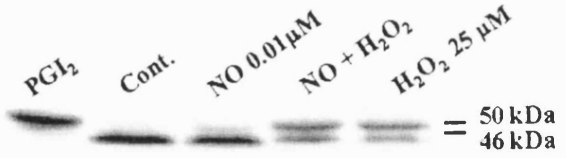

FIG. 4. The effect of NO and hydrogen peroxide on the phosphorylation state of vasodilator-stimulated phosphoprotein (VASP). Washed human platelets were stimulated for 1 min with either 0.01 μM NO or 25 μM H_2O_2 alone or both together. VASP phosphorylation was analyzed using anti-VASP antibodies by immunoblotting a 10% SDS/PA gel. The data are typical of those found in at least four independent experiments.

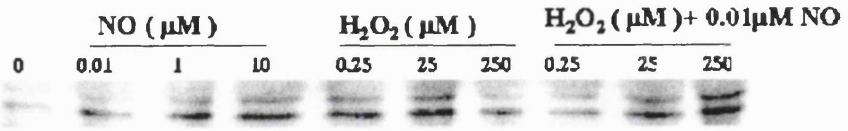

FIG. 5. VASP serine phosphorylation in intact human platelets in the presence of different concentrations of NO and H_2O_2. Washed human platelets were stimulated for 60 s with different concentrations of either NO or H_2O_2 alone or both together at a range of concentrations. VASP phosphorylation was analyzed using monoclonal phosphoserine antibodies following immunoblotting from a 15% SDS/PA gel. The data shown are typical of those in at least five independent experiments.

shown). Neither L-NAME (100 μM) nor ODQ had any marked effect on the phosphorylation of either protein by the combination of NO and H_2O_2 (Fig. 7). Similar observations were obtained with these reagents when serine phosphorylation by H_2O_2 alone was observed (not shown). A combination of the protein kinase inhibitors (34 nM PKA inhibitor and 234 nM KT5823) almost completely blocked serine phosphorylation by the peroxide alone (not shown).

DISCUSSION

H_2O_2, at physiologically relevant concentrations, enhances the effectiveness of NO as a platelet inhibitor (9, 10). In the current work, the possibility that the site of synergism between NO and H_2O_2 was at the level of sGC *alone* was explored. In earlier work, low concentrations of NO and H_2O_2 in the presence of IBMX increased the levels of cGMP in whole platelets compared to NO alone. This study shows that, in the absence of IBMX, H_2O_2 caused only minor and transitory increases in cGMP at all concentrations of NO tested. No increases in cGMP synthesis were observed when NO/H_2O_2 were added to platelet lysates, cytosol, or purified sGC over those seen with NO alone. H_2O_2 has been reported to increase significantly the levels of cGMP in intact endothelial cells (23), aortic smooth muscle (24). However, in all of these studies phosphodiesterase inhibitors were present throughout the experiments. It is possible that H_2O_2 itself inhibits phosphodiesterases and so increases the duration of the cyclic nucleotides in the platelets. If this were the case, an apparent increase in both cGMP and cAMP synthesis in the presence of NO or PGI_2 should have been observed, but this was not the case. These observations are consistent with previous work, which showed little synergism between H_2O_2 and dibutyryl cyclic GMP with respect to platelet inhibition (9).

On the other hand, the synergism between NO and H_2O_2 was almost completely inhibited by the presence of ODQ, the sGC inhibitor (10). The synergy between NO and H_2O_2 appears to require, at least, the presence of low concentrations of cGMP, and small increases in its concentration may be essential to the synergism, even if this is not the complete explanation. H_2O_2 alone does not inhibit the aggregation of washed platelets (9, 10), but it is conceivable that the peroxide may induce a rise in endogenous NO synthesis (25) although this related to iNOS induced by cytokines. This is unlikely to be an important mechanism in this case, since synergism between GSNO and peroxide was only slightly impaired by the presence of L-NAME: H_2O_2 does not increase the stability of NO in solution (10). The extent of synergism between the peroxide and YC-1 was limited to low concentrations of the latter in platelet cytosol, but none was found with purified sGC. Wu *et al.* have reported that H_2O_2 does enhance platelet inhibition by YC-1, but the effect is relatively small at low concentrations of the peroxide (26) as was found in this study. This suggests again that there may be other mechanisms involved that are not simply regulated by cyclic nucleotide concentrations and are unique to NO.

If the mechanism underlying the synergism was not primarily at the level of sGC, the actions of the peroxide on events downstream of cGMP formation may be of importance. The complete series of events mediating NO-dependent inhibition of platelet aggregation has not been fully elucidated. However, cGMP is known to activate cGMP kinase 1 (cGK1), which reduces $[Ca^{2+}]_i$, inhibits platelets, and initiates the phosphorylation of VASP (27), the IP_3 receptor (28), and the thromboxane receptor (29). At

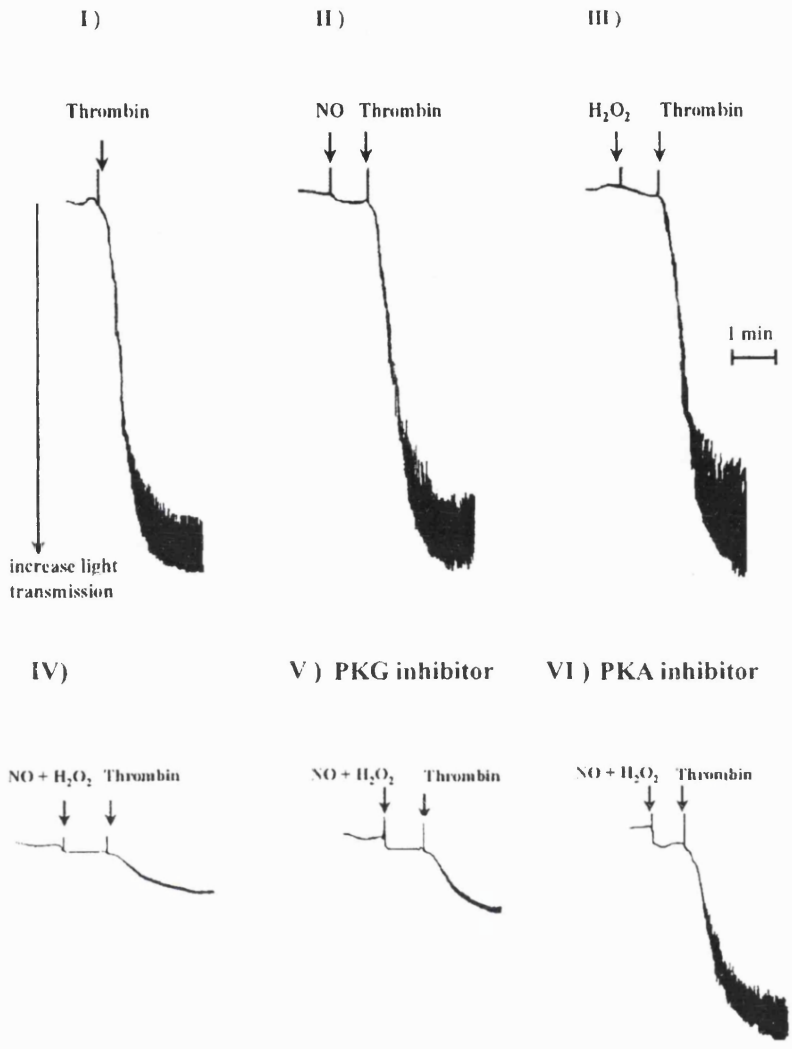

FIG. 6. The influence of inhibitors of protein kinases on inhibition of platelet aggregation induced by NO and hydrogen peroxide. Washed platelets were activated with 0.02 U thrombin/ml and after preexposure to (I) buffer, (II) 10 nM NO, (III) 25 μM H_2O_2, (IV) 10 nM NO + 25 μM H_2O_2, and (V) 10 nM NO + 25 μM H_2O_2 in the presence of myristoylated 14–22 peptide protein kinase A inhibitor 36 nM (30 min prior exposure) and (VI) 10 nM NO + 25 μM H_2O_2 in the presence of KT5823 234 μM and the extent of aggregation measured by conventional aggregometry. The results are typical of three independent experiments.

low concentrations of NO, the phosphorylation of Ser^{239} of VASP occurs, whereas PGI_2 acts primarily via the phosphorylation of Ser^{157}, leading to an apparent change in molecular weight (22). NO at higher concentrations also leads to the phosphorylation of Ser^{157} and a decrease in the exposure of fibrinogen binding sites (22, 30). Indeed, the synergism often observed between NO and PGI_2 may in part involve the enhancement by NO of PGI_2-induced phosphorylation of Ser^{157} on VASP. The importance of VASP in platelet function was indicated by the modification of platelet function in VASP knockout mice (31). In the current studies, it was revealed that H_2O_2 induced phosphorylation of both the 46- and the 50-kDa band in the absence of NO and without a rise in ambient cyclic GMP concentrations. This provides further evidence that the basis of their synergism is more complex than a simple elevation of cGMP levels. When NO and H_2O_2 were added simultaneously, there was again

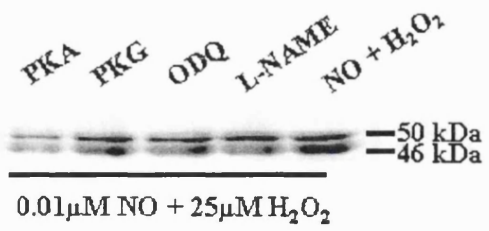

FIG. 7. The influence of inhibitors of protein kinases, NO synthesis, and soluble guanylyl cyclase on VASP serine phosphorylation induced by NO and hydrogen peroxide. Washed human platelets were exposed for 1 min to 0.01 μM NO + 25 μM H_2O_2 (lanes 1–5) after pre-incubation for 30 min with either myristoylated 14–22 peptide protein kinase A inhibitor 36 nM (lane 1), PKG-inhibitor KT5823 234 μM (lane 2), ODQ 10 μM (lane 3) and L-NAME 800 μM (lane 4), or buffer (lane 5). The results are typical of at least three independent experiments.

increased serine phosphorylation at both 46 and 50 kDa, but the effects were little more than additive.

Peroxide alone does not inhibit platelet aggregation but still enhances VASP phosphorylation even at submicromolar concentrations. This suggests that other events are essential to connect VASP phosphorylation with inhibition of platelet activation, events that may be activated by low concentrations of NO. Further work is required to establish this. It was surprising to find that H_2O_2 has no synergism with PGI_2. This may reflect the greater potency of PGI_2 in initiating the phosphorylation of Ser^{157} or that this eicosanoid has further actions required for inhibition, not shared by the peroxide.

Previous studies have shown that H_2O_2, at least at concentrations of 1 mM, induces tyrosine phosphorylation of focal adhesion proteins (32) and that hydroxyl radical, probably produced by the Fenton reaction, was the active mediator of phosphorylation. We have also found that hydroxyl radical scavengers, mannitol and urate, can reduce the synergism between NO and H_2O_2 (9, 10). Thus, it is reasonable to speculate that other radical species may be involved in this phenomenon. Hydrogen peroxide has been reported to activate the $p70^{S6k}$ signaling pathway in mouse epidermal cells (33) by using an enzyme-generating system. Similarly, the enhancement of tyrosine phosphorylation of protein kinase C by hydrogen peroxide was achieved at a concentration of 5 mM (34). Submicromolar concentrations of H_2O_2 did inhibit the inward rectifying potassium current in endothelial cells (35).

The mechanism of action of H_2O_2 is unclear, although many mechanisms have been reported on known signal transduction pathways, mainly at concentrations that stress cells (36). The evidence provided by the inhibitors of the relevant protein kinases suggests that, in the case of both inhibition of aggregation and VASP phosphorylation, cAK may be one pathway for the action of H_2O_2. At low concentrations, the peroxide had no effect on ambient cyclic AMP or cyclic GMP concentrations but nevertheless appears to activate the cAK. H_2O_2 may inhibit phosphatase activity and intensify the phosphorylation of VASP or other proteins. This is worthy of further investigation since H_2O_2 has been reported to have such effects, but usually at much higher, cytotoxic concentrations (37).

VASP is associated with the inhibition of agonist-induced aggregation, but not calcium regulation and degranulation, in NO or PGI_2 treated platelets. The phosphorylation of Ser^{157} is the key to the inhibition of fibrinogen binding (22), and it may be that H_2O_2 enhances the ability of NO to modify VASP, thus enhancing the effectiveness of the latter as a platelet inhibitor. However, although H_2O_2 induces the phosphorylation of Ser^{157} independent of the addition of low doses of NO, this does not preclude the possibility that the observed synergism involves VASP. The peroxide may simply substitute for higher concentrations of NO with respect to VASP phosphorylation, and the low dose of NO may complete the process of inhibition by some other mechanism in which H_2O_2 has no influence.

The precise function of VASP is uncertain; it may act as an adapter protein, linking the extracellular matrix, via integrin associated proteins such as vinculin, to the actin polymerization mechanism of the platelet. Recent work has shown that H_2O_2 increases the nitration of VASP, particularly in the 46-kDa protein, but its importance in the synergism in NO needs further investigation (Sabetkar and Bruckdorfer, unpublished observations).

In summary, hydrogen peroxide, along with other reactive oxygen species, may have the ability to modify the function of VASP at least in platelets, but other events are required (provided by NO) to complete the process of inhibition. This does not preclude the importance of small rises in cyclic GMP in

the presence of hydrogen peroxide which have been proposed in earlier studies.

ACKNOWLEDGMENTS

The authors acknowledge the support of the Wellcome Trust and the British Heart Foundation for this work.

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FEBS 25660 FEBS Letters 511 (2002) 59–64

The role of protein nitration in the inhibition of platelet activation by peroxynitrite

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Received 26 November 2001; accepted 30 November 2001

First published online 19 December 2001

Edited by Barry Halliwell

Abstract Peroxynitrite at low concentrations (3–10 μM) inhibited agonist-induced platelet aggregation by a mechanism not dependent on the formation of cyclic guanosine monophosphate. Platelets recovered completely from peroxynitrite-induced inhibition within 30 min. Peroxynitrite induced nitration of cytosolic proteins, but this diminished to near basal levels within 60 min of exposure to the oxidant. During this period there was a reduction in tyrosine phosphorylation of specific proteins such as syk, but this was not due to direct nitration of these same proteins. The inhibition of phosphorylation was reversible with platelet proteins recovering the ability to be phosphorylated within 15 min of exposure to peroxynitrite. Conversely, peroxynitrite increased phosphorylation of other proteins, but again these events were not directly linked to nitration. Nitration may affect the phosphorylation of tyrosine residues in a number of proteins, but by an indirect route, possibly by acting on proteins upstream in the signalling cascades. We suggest that low concentrations of peroxynitrite reversibly inhibit platelet aggregation by preventing the phosphorylation of key signalling proteins.

Key words: Platelet; Protein nitration; Tyrosine phosphorylation; Cyclic guanosine monophosphate

1. Introduction

Platelet aggregation, induced by specific agonists, is the culmination of several exquisitely integrated signalling pathways, which lead to biochemical and morphological changes in the cell. Excessive platelet activation is limited by the endothelial-derived platelet inhibitors prostacyclin (PGI_2) and nitric oxide (NO) [1]. NO, unlike PGI_2, is able to inhibit both platelet activation and adhesion to the subendothelium. NO, which activates soluble guanylyl cyclase (sGC) leading to the formation of cyclic guanosine monophosphate (cGMP), inhibits platelet activation by reducing the intracellular calcium concentration through the action of cGMP-dependent kinases [2]. The regulation of platelet activity by NO is crucial in preventing excessive platelet aggregation and thrombi.

NO undergoes a diffusion limited reaction with superoxide anion ($O_2^{\bullet-}$) to form peroxynitrous acid (ONOOH), a potent oxidant [3] which has been shown to be released by endothelial cells [4]. This can lead to a reduction in the bioavailability of NO at the endothelial surface, and possibly to a loss of platelet regulation. The influence of peroxynitrite on platelet function has been shown to be concentration-dependent. At high concentrations, in excess of 150 μM, peroxynitrite acts as a platelet agonist by stimulating aggregation [5]. At lower concentrations or in the presence of plasma, peroxynitrite acts as a platelet inhibitor, although much less effectively than NO [5–7]. The mechanism of this inhibition has been proposed to be cGMP-dependent [5,6] since peroxynitrite nitrosates thiol-containing proteins, though inefficiently, to form nitrosothiols [8]. The nitrosothiols then inhibit platelets through the release of NO. However, peroxynitrite also undergoes a range of different reactions at physiological pH, which may affect cell function. Peroxynitrite nitrates phenols such as tyrosine [9], accounting for the presence of nitrated proteins in normal plasma [10]. The presence of nitrated proteins has been used as a marker for peroxynitrite formation in tissues, while the formation of nitrotyrosine intracellularly may have important consequences for cell function. Peroxynitrite-induced nitration of peptides has been shown to reduce their phosphorylation when exposed to specific protein kinases [11,12], suggesting that protein nitration may interfere with protein phosphorylation signalling pathways. Furthermore, we have recently shown that nitrated proteins form spontaneously in platelets undergoing collagen-induced aggregation [13]. The function of these nitrated proteins is unknown.

In the present study, we examined the effects of peroxynitrite on platelet function with the aim of dissecting the relative contributions of the NO-cGMP pathway and protein nitration. Our data indicate that peroxynitrite inhibits platelet aggregation in a cGMP-independent and reversible manner, and that the major action of peroxynitrite is to prevent, reversibly, the agonist-induced phosphorylation of signalling proteins.

2. Materials and methods

Prostacyclin (synthetic sodium salt), thrombin (human), bovine serum albumin (BSA), trichloroacetic acid (TCA) and (3-[3-cholamidopropyl]dimethylammonio)-1-propane-sulphonate (CHAPS) buffer were purchased from Sigma Chemical Co. (Poole, UK), collagen Type I (equine) from Hormonchemie (Munich, Germany) and ODQ from Tocris-Cookson Chemicals (Southampton, UK).

Biotinylated goat anti-rabbit IgG and horseradish peroxidase (HRP)-linked anti-rabbit IgG antibodies and nitrocellulose (pore size 0.45μm) were obtained from Amersham Pharmacia Biotech (Bucks, UK). Avidin–biotin HRP was from DAKO Ltd. (Beaconsfield, UK). Polyclonal anti-nitrotyrosine antibody was produced in

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0014-5793/02/$22.00
PII: S0014-5793(01)03279-3

our laboratory, while anti-phosphotyrosine HRP-linked (PY-20) antibody was from Santa Cruz (Insight Biotechnology Ltd., Middlesex, UK), anti-syk monoclonal antibody and rabbit anti-nitrotyrosine polyclonal antibody were from Upstate Biotechnology (TCS Biologicals, Bucks, UK). Pre-cast NuPAGE 10% and 4–12% gradient gels and NuPAGE sample buffer were purchased from Invitrogen Life Technologies (Paisley, UK). Enhanced chemoluminescence (ECL) reagents were obtained from Perbio (Cheshire, UK). The cGMP ELISA kits were purchased form Cayman Chemicals (USA).

2.1. Platelet preparations

Venous blood was taken with informed consent from healthy volunteers, who denied taking any medication in the previous 14 days, placed into acid–citrate–dextrose anti-coagulant and centrifuged for 20 min at $150\times g$ to yield platelet-rich plasma (PRP). Washed platelets (WPs) were prepared from PRP in the presence of prostacyclin as described elsewhere [14], suspended in buffer (NaCl 137 mM, NaH_2PO_4 4.2 mM, $NaHCO_3$ 11.9 mM, KCl 2.7 mM, pH 7.4) and diluted to a count of 3×10^8 platelets/ml. The platelets were rested for 1 h at room temperature and used within the next 2 h.

To prepare cytosolic and membrane fractions, WPs were sonicated at 4°C (Soniprep, M.S.E., UK) for 2×15 s bursts separated by a 15 s pause, and the cytosol separated from the membrane fractions by centrifugation at $1500\times g$ for 10 min. The pellet, which contained the membrane fraction, was solubilised in 0.1% CHAPS buffer [15].

2.2. Preparation of peroxynitrite

Sodium peroxynitrite was prepared using the acidified nitrite method [16]; controls were prepared by the same procedure, except that the solutions were passed directly into water instead of NaOH, leading to immediate decomposition of the oxidant. NaOH was then added to restore the pH to 10. Residual H_2O_2 was removed by passing the peroxynitrite through a MnO_2 column and checked by absorbance at 220 nm. The concentration of stock peroxynitrite solutions was determined spectrophotometrically (302 nm = 1670 M^{-1} cm^{-1}) [17] using decomposed peroxynitrite as the blank. Working dilutions of both active and decomposed peroxynitrite were prepared in the same manner using 1 mM NaOH.

2.3. Measurement of cGMP concentrations

WPs were pre-incubated with isobutylmethylxanthine (200 μM) for 30 min prior to experimentation. WPs were then incubated with either peroxynitrite or NO and after 1 min the reactions halted by the addition of TCA (5%). The cGMP was extracted with diethylether, with the ether subsequently removed by heating extracted samples to 70°C for 5 min. The cGMP was then analysed using a commercially available ELISA kit.

2.4. Quantification of nitrated proteins

The nitrotyrosine content of proteins in the platelet samples was estimated using a competitive semi-quantitative ELISA developed in this laboratory [10]. The assay used nitrated BSA (NT-BSA) as a standard and a commercially available rabbit anti-human polyclonal anti-nitrotyrosine antibody. The amount of nitrotyrosine present after peroxynitrite treatment of BSA was determined by absorbance at 438 nm (pH 9) using the molar extinction coefficient of 4300 M^{-1} cm^{-1}. The molar ratio of nitrotyrosine to albumin was within 3–6 mol nitrotyrosine/mol protein. The results were extrapolated from a semi-log plot of the standard curve and were expressed as nmol of NT-BSA equivalents/mg of protein. The protein concentrations were established using a modified Lowry method [18].

2.5. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

After various treatments, the reactions were stopped by addition of NuPAGE sample buffer. Proteins were separated by SDS–PAGE on either NuPAGE Bis–Tris 10% or 4–12% gradient gels. The separated proteins were transferred to nitrocellulose membranes using wet transfer techniques. Membranes were blocked with BSA (0.5%), dissolved in PBS containing 1% PVP-10, 1% PEG, 0.2% Tween and 10 mM NaF, for 60 min. Membranes were probed with anti-nitrotyrosine (1:500), anti-phosphotyrosine (1:5000) or anti-syk (1:2500) for 60 min. The membranes were washed several times with PBS/Tween 0.05%, followed by incubation with HRP-linked goat anti-rabbit IgG for 60 min. The protein bands were visualised using ECL reagents.

The in house polyclonal anti-nitrotyrosine antibody was raised against nitrated Keyhole Limpet Hemocyanin and purified using standard affinity purification techniques. Extensive characterisation was performed using nitrated peptides and a variety of tyrosine derivatives. The performance of this antibody in our ELISA was very similar to the commercial polyclonal antibody, but gave clearer resolution on immunoblots. The selectivity of the antibody was checked by treatment of nitrated proteins with the reductant dithionite and by its differential recognition of peptides containing tyrosine, aminotyrosine and nitrotyrosine.

2.6. Statistical analysis

All data are presented as the mean ± S.E.M. of at least three independent experiments, unless otherwise stated. Statistical analysis was performed using Student's unpaired *t*-test.

3. Results

3.1. The influence of peroxynitrite on platelet aggregation

Incubation of WPs with peroxynitrite for 1 min prior to the addition of thrombin (0.02 U/ml) led to the inhibition of platelet aggregation compared to agonist alone (Table 1). The effect of peroxynitrite was concentration-dependent, with the IC_{50} value established at 4.6 ± 0.9 μM. In control experiments, decomposed peroxynitrite failed to inhibit thrombin-induced platelet activation.

In order to ascertain whether peroxynitrite-mediated inhibition of platelet aggregation was NO and cGMP-dependent, the experiments were repeated in the presence of ODQ (10 μM), an inhibitor of sGC [19] and, therefore, of the conventional NO-mediated pathway. The presence of ODQ did not influence the inhibition of thrombin-induced platelet aggregation by peroxynitrite (Table 1a). It was also established that

Table 1
The influence of peroxynitrite on platelet aggregation and cGMP formation

a: Peroxynitrite (μM)	Inhibition of aggregation (%)	
	without ODQ	with ODQ
0.01	4.5 ± 2.5	6.5 ± 6.5
0.1	17.5 ± 6.7	23.4 ± 8.9
1	ND	37.3 ± 7.2
10	67.6 ± 9.3	68.2 ± 17.2
100	98.2 ± 0.2	98.6 ± 1.4
Decomposed (10 μM)	0	0
b: Peroxynitrite (μM)	cGMP (pmol 1×10^8 platelets)	
	without ODQ	with ODQ
0	0.31 ± 0.12	0.09 ± 0.06
0.3	0.34 ± 0.17	0.06 ± 0.02
3	0.62 ± 0.29	0.21 ± 0.13
30	1.18 ± 0.27**	0.34 ± 0.11
Decomposed (10 μM)	0.27 ± 0.09	0.12 ± 0.07

Peroxynitrite (0–100 μM) was incubated with platelets for 1 min before the addition of thrombin (0.02 U/ml) and aggregation measured 3 min later. For cGMP measurement, platelets were pre-incubated with isobutylmethylxanthine (200 μM) for 30 min prior to addition of peroxynitrite. The reaction was halted after a 1 min incubation with peroxynitrite by the addition of ice-cold TCA (5%). The cGMP was extracted using diethyl and measured using a competitive enzyme immunoassay. The results are expressed as pmol cGMP/1×10^8 platelets. In some experiments the platelets were pre-incubated with ODQ (10 μM) for 15 min before the addition of peroxynitrite. Results for both inhibition of aggregation ($n=5$) and cGMP ($n=4$) are expressed as mean ± S.E.M. **$P>0.01$ compared to basal.

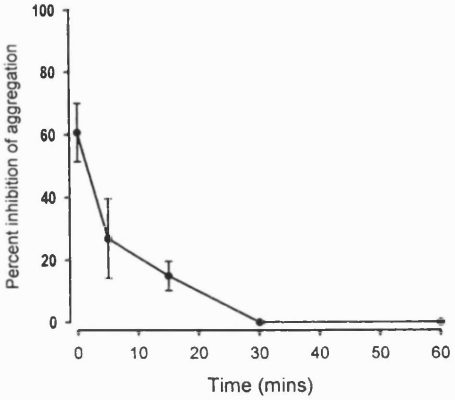

Fig. 1. The influence of peroxynitrite on platelet aggregation as a function of time. Peroxynitrite (5 μM) was added to platelets and incubated for up to 1 h at 37°C without stirring, before the addition of thrombin (0.02 U/ml). Platelet aggregation was measured 3 min after the addition of thrombin. Results are expressed as percent inhibition of aggregation and represent the mean ± S.E.M. of four independent experiments.

the inhibitory action of ODQ on GC activity was not changed by exposure to the peroxynitrite. ODQ was exposed to peroxynitrite (10 μM) prior to addition to platelets and still shown to inhibit the action of NO on platelets (results not shown). These results indicate that peroxynitrite acts primarily through a mechanism independent of cGMP.

In a second series of experiments, we investigated whether the effects of peroxynitrite on platelets were reversible. Platelets were incubated with peroxynitrite for up to 1 h before stimulation with thrombin (0.02 U/ml). Incubation of peroxynitrite (5 μM) for 1 min resulted in 60.7 ± 9.3% inhibition of thrombin-stimulated aggregation. After a 15 min incubation, the level of inhibition had declined to 14.9 ± 4.7% and at 30 min, no inhibition was evident (Fig. 1). These data suggest that the actions of peroxynitrite are transient and non-toxic, since it does not permanently impair the ability of platelets to respond to thrombin. Similar data were obtained using collagen as an agonist (data not shown).

3.2. cGMP synthesis in response to peroxynitrite and NO

The results of the aggregation experiments suggested that the effects of peroxynitrite were independent of both NO and cGMP. To confirm this, the ability of peroxynitrite to induce cGMP formation was assessed, and compared with that of authentic NO solutions. When this oxidant was added to platelets as a bolus and incubated at 37°C for 1 min, peroxynitrite caused a concentration-dependent increase in the formation of cGMP from the basal level. However, this increase was only significant at the highest concentration of peroxynitrite used (30 μM) which elevated cGMP from a basal level of 0.31 ± 0.12 to 1.18 ± 0.27 pmol/1 × 10^8 platelets ($P < 0.01$) (Table 1); 0.3 and 3 μM peroxynitrite had no significant effect on basal levels. The presence of ODQ (10 μM) prevented the rise of cGMP above basal levels with all concentrations of peroxynitrite (Table 1b). The rates of cGMP synthesis were much lower than those measured for NO (1 μM NO produces 4.1 ± 0.6 pmol cGMP/10^8 platelets after 1 min). The inhibition of platelet aggregation by peroxynitrite at 3–5 μM (the approximate IC_{50}) does not appear to be due to increases in cGMP formation.

3.3. Peroxynitrite-induced nitration of platelet proteins

Measurement of nitrotyrosine levels using a semi-quantitative ELISA demonstrated the presence of low amounts of nitrated proteins in both cytosolic and membrane fractions of unstimulated platelets. The subsequent addition of peroxynitrite (3 μM) led to a significant increase in nitrated platelet proteins, but only of cytosolic proteins. In the cytosolic fraction, peroxynitrite increased nitrotyrosine levels from a basal level of 0.3 ± 0.1 to 6.4 ± 2.8 nmol NT-BSA equivalents/mg protein ($P < 0.01$), whereas in the membrane fraction the increase in nitration was not significant.

The inhibition of platelet aggregation by peroxynitrite (Fig. 1) was reversible, and therefore if protein nitration was a regulator of platelet function, this too would be reversible. After a 1 min incubation, peroxynitrite (3 μM) formed 6.4 ± 2.8 NT-BSA equivalents/mg protein in the cytosolic fraction. The amount of nitrotyrosine fell, by 60 min, to 3.1 ± 0.9 NT-BSA equivalents/mg protein ($P > 0.05$ compared to levels after 1 min) and, after 90 min, the concentration was only 1.2 ± 0.4 NT-BSA equivalents/mg protein ($P < 0.01$; Fig. 2). We also observed considerable variations between individual platelet preparations in both the initial levels of nitration and in the speed of the loss of nitration. In these experiments, the low levels of nitrotyrosine in the membrane fraction did not increase and remained constant over 90 min.

3.4. Influence of peroxynitrite on thrombin-stimulated protein phosphorylation

Previously, it had been shown that peroxynitrite (at high concentrations above 150 μM) caused both nitration and phosphorylation of proteins in platelets [20]. WPs were incubated with 10 μM peroxynitrite, for time intervals up to 60 min and the extent of protein nitration was determined by Western blotting. The incubation of platelets with a range of peroxynitrite concentrations resulted in increased nitration of many different protein bands (Fig. 3A), whereas thrombin

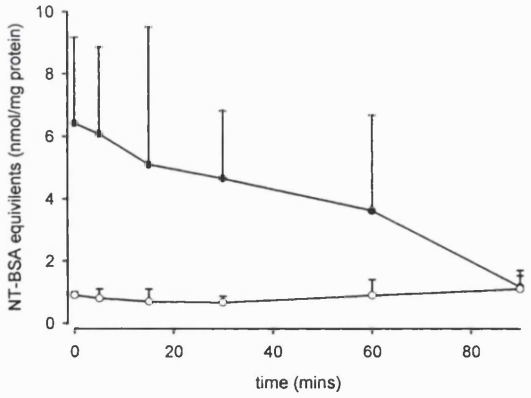

Fig. 2. The influence of time on peroxynitrite-induced nitration of platelet proteins. Peroxynitrite (3 μM) was incubated with WPs for up to 90 min at 37°C without stirring. Aliquots were taken at various time points before being cooled and sonicated. The platelets were subsequently centrifuged at 1500 × *g* for 5 min to separate the membrane (○) and cytosolic fractions (●). The membrane fraction was solubilised using CHAPS buffer (0.1%). Nitrotyrosine was then measured in the membrane and cytosolic samples using a semi-quantitative ELISA. Results are expressed as nmol NT-BSA equivalents/mg protein and represent the mean ± S.E.M. of five independent experiments.

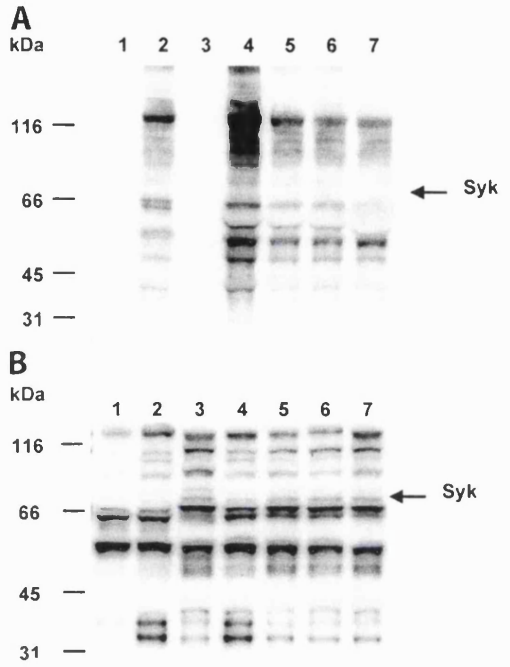

Fig. 3. A: The effect of peroxynitrite on the nitration of platelet proteins. WPs were treated with peroxynitrite (10 μM) for up to 60 min before the addition of thrombin (0.02 U/ml), and then incubated for 1 min with stirring at 37°C. Platelets were lysed with NuPAGE sample buffer and subjected to SDS–PAGE using a NuPAGE 4–12% gradient gel. The separated proteins were transferred onto nitrocellulose membrane and then probed with anti-nitrotyrosine antibody (1:500). The antigen was visualised using the ECL system. Lane 1: basal, 2: peroxynitrite 10 μM (1 min), 3: thrombin 0.02 U/ml, 4: peroxynitrite (1 min)+thrombin 0.02 U/ml, 5: peroxynitrite (15 min)+thrombin 0.02 U/ml, 6: peroxynitrite (30 min)+thrombin 0.02 U/ml, 7: peroxynitrite (60 min)+thrombin 0.02 U/ml. Each well contained 20 μg platelet protein. The blot is a representative of four separate experiments. B: The influence of peroxynitrite on the phosphorylation of platelet proteins. WPs were treated with peroxynitrite (10 μM) for up to 60 min before the addition of thrombin (0.02 U/ml), and then incubated for 1 min with stirring at 37°C. Platelets were lysed with NuPAGE sample buffer and subjected to SDS–PAGE using a NuPAGE 4–12% gradient gel. The separated proteins were transferred onto nitrocellulose membrane and then probed with anti-phosphotyrosine antibody (1:5000). The antigen was visualised using the ECL system. Lane 1: basal, 2: peroxynitrite 10 μM (1 min), 3: thrombin 0.02 U/ml, 4: peroxynitrite (1 min)+thrombin 0.02 U/ml, 5: peroxynitrite (15 min)+thrombin 0.02 U/ml, 6: peroxynitrite (30 min)+thrombin 0.02 U/ml, 7: peroxynitrite (60 min)+thrombin 0.02 U/ml. Each well contained 20 μg platelet protein. The blot is a representative of four separate experiments.

alone produced little nitration. Interestingly, the combination of peroxynitrite, followed by thrombin 1 min later, appeared to enhance the nitration; again the extent of nitration of most of the protein bands decreased markedly after 60 min, even after addition of thrombin at each interval.

To assess the influence on platelet-signalling proteins, WPs were incubated with peroxynitrite for time intervals up to 60 min before stimulation with thrombin (0.02 U/ml), and protein phosphorylation assessed by Western blotting. Thrombin stimulation of platelets increased phosphorylation over a broad range of protein sizes compared to unstimulated cells (Fig. 3B). Incubation of platelets with peroxynitrite (10 μM) for 1 min prior to thrombin stimulation led to a decrease in phosphorylation of proteins with apparent molecular weights of 72, 94 and 112 kDa. However, this inhibitory effect of peroxynitrite on the phosphorylation of some proteins was reversible. The thrombin-induced phosphorylation of the 72 kDa protein, subsequently identified as syk (results not shown), was strongly diminished by a 1 min pre-incubation of the platelets with peroxynitrite. However, if the cells were left to recover for 15 min after peroxynitrite, thrombin-stimulated phosphorylation returned to control levels. Similarly, phosphorylation of the 112 kDa began to recover within 15 min and was fully phosphorylated after 60 min, while the thrombin-stimulated phosphorylation of the 94 kDa was still only partial 60 min after peroxynitrite treatment. These data suggest that peroxynitrite at low concentrations inhibits protein phosphorylation of specific proteins, and that the mechanism is reversible in some cases. Immunoblotting experiments did not reveal any nitrated proteins with the same molecular weight as syk, indicating that syk may not be nitrated by peroxynitrite or thrombin (Fig. 3A).

In contrast, peroxynitrite was able to increase the tyrosine phosphorylation of certain proteins. After 1 min of peroxynitrite treatment, increased levels of protein phosphorylation were observed in the 35 and 38 kDa proteins in the presence or absence of thrombin, and to a greater extent than with thrombin alone (Fig. 3B). However, these effects were transient, since they returned to levels seen with thrombin alone 15 min after addition of peroxynitrite. As shown many times before, the src group of proteins was phosphorylated even in the resting platelets and this was not changed by addition of peroxynitrite. The level of phosphorylation of a 64 kDa protein was reduced by thrombin; the significance of this is unknown.

4. Discussion

The influence of peroxynitrite on platelet function has been studied previously, but the results have been contradictory. In earlier studies, peroxynitrite at concentrations above 150 μM was shown to stimulate platelet aggregation in plasma-free platelet preparations [5,6], while only in the presence of plasma did it act as a platelet inhibitor [5,6]. The inhibitory effect of peroxynitrite was attributed to the formation of nitrosothiols, which act as NO donors and therefore inhibit platelets via the stimulation of sGC. Indeed, in the presence of glutathione, peroxynitrite does activate sGC [21]. We tested the role of sGC in peroxynitrite-mediated inhibition using much lower concentrations of the oxidant. At 1–10 μM, peroxynitrite was a potent inhibitor of platelets, but only a modest activator of sGC compared to authentic NO (1 μM). Furthermore, peroxynitrite still inhibited platelets that had been exposed to the sGC inhibitor ODQ, which was shown to be active even after addition of low concentrations of peroxynitrite. Therefore, the data indicate that the inhibition of platelets by peroxynitrite occurs by a process in which cGMP plays only a minor part. This is reminiscent of our recent work in which a synergistic inhibition of very low concentrations of NO and hydrogen peroxide did not appear to require elevated concentrations of cGMP [22]. However, in this case, a basal concentration of cGMP was required because the inhibition was blocked by ODQ [22].

The inhibition of platelets by peroxynitrite was fully reversible, indicating that this was not simply a destructive effect of

the oxidant on platelet proteins and lipids. The recovery occurred within 30 min and was 70% recovered after 15 min after addition of 5 μM peroxynitrite (Fig. 1). An alternative explanation of a role for cGMP is that the inhibition of platelets is due to the nitration of platelet proteins by the formation of nitrotyrosine. Nitration did occur, even at low concentrations of peroxynitrite and the extent of nitration increased significantly at concentrations immediately above the IC_{50}, as detected by the ELISA. This increase occurred primarily in the soluble fraction, suggesting that the nitrating species passed through the membrane into the cytosol; this has been reported in red blood cells [23]. The extent of the cytosolic nitration also diminished after a period of 1 h, which was slightly longer than the recovery of the platelets in terms of their response to thrombin. There was a significant variation in the rate of loss of nitrated proteins in platelets from individual donors. Nitrotyrosine is stable and its direct reversion to tyrosine seems unlikely, although an intermediate non-enzymatic reduction to amines in tissues has been proposed [24]. The alternative would be proteolysis of the modified proteins or further modification of the nitro group. Certainly, a recent study has demonstrated that protein nitration enhances the susceptibility to proteolytic cleavage by proteasomes [25]. However, there appear to be no reports of proteasomal activity in platelets although they do contain lysosomes. Further work is required to establish the mechanism of nitrotyrosine disappearance.

The stimulation of platelets results in the activation of several key tyrosine kinases and phosphatases, leading to the phosphorylation of several key cellular proteins [26]. The events occur very early in the activation response and are critical to further cellular activation and aggregation. Therefore, it is possible that peroxynitrite inhibited platelet aggregation through the nitration of key signalling proteins which are normally phosphorylated during activation. We have shown that pre-treatment of platelets with peroxynitrite inhibited thrombin-induced phosphorylation of several proteins, but not of others. These results may indicate that peroxynitrite inhibits platelet activation through the temporary impairment of tyrosine phosphorylation of specific signalling proteins. Implicit in this mechanism is the question of whether the reversibility of platelet inhibition and protein nitration, following exposure to peroxynitrite, is associated with a recovery of platelet responsiveness to thrombin-induced tyrosine phosphorylation of specific proteins. Thrombin-induced phosphorylation of several proteins was lost, including syk, after a 1 min pre-incubation with peroxynitrite. However, the phosphorylation returned within 15–30 min of exposure to the oxidant; this correlated temporally very well with the recovery of aggregation response. The rate of recovery for phosphorylation was different for individual proteins, and not all proteins fully recovered. This is new evidence that the effect of peroxynitrite on tyrosine phosphorylation is reversible. In other studies, the recovery may be blocked due to damage caused by excessive nitration and oxidation of sulphydryl groups by the use of peroxynitrite at concentrations of up to 1 mM peroxynitrite. In one study in platelets, accumulative concentrations up to 800 μM peroxynitrite were used and no reversal of the high levels of nitration were observed over 60 min [20].

An obvious mechanism for this inhibition may be that nitration of the same tyrosine residue blocks its phosphorylation. However, no correlation existed between the inhibition of tyrosine phosphorylation and the increase in nitration, and subsequent changes occurring during the hour period. We have been unable to demonstrate that proteins such as syk are nitrated by peroxynitrite and, therefore, the reduction in phosphorylation is likely to be due to the inhibition of another protein kinase upstream from syk. In neuroblastoma cells, the inhibition of tyrosine phosphorylation is associated with compromised phosphoinositide signalling [27], an event crucial for platelet function. The loss of nitro groups from specific proteins, in a short time frame, may be a key event in restoring platelet sensitivity to agonists. Non-specific nitration of proteins may not be important, except when exposed to excess peroxynitrite. Therefore, partial loss of nitrated proteins, or selective loss of nitro groups may be sufficient to reverse the inhibition of platelet function.

Peroxynitrite enhanced the phosphorylation of proteins to a greater extent than did thrombin, in particular those at 35 and 38 kDa; the latter co-migrates with p38 MAP kinase, which his known to be activated by peroxynitrite [28]. Conversely, some proteins are phosphorylated only after addition of thrombin and not by peroxynitrite (e.g. 68 kDa protein). The peroxynitrite-induced protein phosphorylation was transient and diminished after 15 min. There is no evidence that they are directly related to protein nitration and again may be due to activation of kinase upstream of these signalling proteins. There are several mechanisms by which peroxynitrite has been proposed to increase tyrosine phosphorylation. These include the inhibition of specific phosphatases [29], direct activation of proteins kinases [30] or via the activation of specific tyrosine kinase receptors [31]. The precise molecular relationship between protein nitration and phosphorylation in platelets remains to be determined, but it is clearly indirect.

Acknowledgements: The authors wish to thank The Wellcome Trust for their support of this work.

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Free Radical Biology & Medicine, Vol. 33, No. 6, pp. 728–736, 2002

0891-5849/02/$–see front matter

ELSEVIER

PII S0891-5849(02)00890-0

Serial Review: Reactive Nitrogen Species, Tyrosine Nitration and Cell Signaling
Guest Editor: Harry Ischiropoulos

THE NITRATION OF PROTEINS IN PLATELETS: SIGNIFICANCE IN PLATELET FUNCTION

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(*Received* 23 *April* 2002; *Accepted* 23 *April* 2002)

Abstract—Exogenous peroxynitrite has been shown to inhibit or activate platelets according to the concentration added and, at the same time, nitrate platelet proteins. Here, recent evidence is discussed which indicates that nitration of proteins may also occur during normal platelet activation by collagen, by mechanical stimulation during isolation and by exposure to low levels of hydrogen peroxide. Furthermore, this nitration appears to be transient. The implications of these findings are discussed in terms of platelet biology and cell signaling processes. © 2002 Elsevier Science Inc.

Keywords—Free radicals, Peroxynitrite, Nitric oxide, Tyrosine nitration, Hydrogen peroxide, Vasodilator sensitive phosphoprotein

INTRODUCTION

One of the earliest discoveries among the many properties of nitric oxide is its ability to inhibit the activation of platelets by physiological agonists such as thrombin and collagen [1]. It was clear that this occurred by a conventional mechanism mediated via cyclic GMP [2] and that NO would act synergistically with prostacyclin [3], another potent platelet inhibitor which increased intracellular cyclic AMP concentrations. The origin of the NO in vivo was originally assumed to be the endothelium, which releases both NO and prostacyclin, but it was demonstrated that platelets biosynthesized NO during their activation by a NOS III type of enzyme [4]. Activation by collagen produced the greatest release of NO. The role of this platelet-derived NO may be to limit the activation process and thrombus formation and to augment the action of endothelial NO [5].

REACTIVE OXYGEN SPECIES AND PLATELET ACTIVITY

It has been established that platelets also produce reactive oxygen species, superoxide anions and hydrogen peroxide during the activation [6]. The effects of hydrogen peroxide have been a matter for speculation for some time, some authors showing enhancement of the effects of platelet activators [7] while others show inhibition [8,9]. We have resolved this by showing that preincubation with hydrogen peroxide will enhance inhibition of platelets by NO and therefore diminish activation by agonists [10,11]. However, en-

This article is part of a series of reviews on "Reactive Nitrogen Species, Tyrosine Nitration and Cell Signaling." The full list of papers may be found on the homepage of the journal.

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hancement of activation may occur if the peroxide is added after the agonists [12]. Therefore, hydrogen peroxide acts synergistically with NO on platelet inhibition. This complements the other role for H_2O_2 in the arterial wall as an agent that promotes vasodilatation. It has recently been proposed that an endothelium-dependent hyperpolarizing factor may actually be H_2O_2 and is biosynthesized by eNOS, at least in mice [13].

PEROXYNITRITE AND PLATELET FUNCTION

The availability of both superoxide anions and NO raises the possibility that peroxynitrite may be formed endogenously in platelets. Furthermore, this oxidant may also be released from the endothelium by agonist-stimulated process [14]. Earlier work had demonstrated that, at high concentrations (> 150 μM), peroxynitrite may activate isolated platelets, and at lower concentrations was inhibitory [15,16]. In the presence of plasma, only the inhibitory actions were observed [15]. Indeed, we have shown that, against low doses of collagen or thrombin when delivered as a bolus, peroxynitrite has an IC_{50} of approximately 5 μM [10], significantly higher than that of NO (IC_{50} approximately 100 nM). Values in this range for peroxynitrite-induced inhibition have also been shown during activation of platelets by arachidonic acid [17]. However, taking into account the losses in peroxynitrite during dilution at pH 7, the actual efficacy of these two compounds, as inhibitors, will be less disparate.

The inhibition of platelets by peroxynitrite may be attributed to an increase in the concentration of cyclic GMP, which results from the formation of nitrosothiols, in the presence of glutathione [18]; glutathione is present in large amounts in platelet cytosol. This appears not to be the main mechanism, as we have recently shown that ODQ, a specific inhibitor of guanylyl cyclase, reverses this inhibition by peroxynitrite only to a limited extent [19], implying that other inhibitory mechanisms are at work. This is reminiscent of our recent work in which a synergistic inhibition of very low concentrations of NO and hydrogen peroxide did not appear to require elevated concentrations of cyclic GMP [20].

Another important point is that the inhibitory effects of peroxynitrite, in common with that of NO, is a reversible process. The responses of platelets to agonists are fully reversible and therefore at these concentrations peroxynitrite appears not to be cytotoxic [19].

THE NITRATION OF PROTEINS BY PEROXYNITRITE

As with other cell types, the addition of peroxynitrite leads to the nitration of platelet protein [21]. Nitration of proteins, as measured by an ELISA for nitrotyrosine, occurred in both cell membranes and the cytosol at concentrations of peroxynitrite that inhibit platelets [22]. The easy access of peroxynitrite to intracellular sites was demonstrated using the membrane-permeable dye DCFH and the transport may occur via the HCO_3^-/Cl^- transporter [17]. Using much higher concentrations of peroxynitrite (150 μM), high levels of protein nitration were observed [21]. Peroxynitrite then showed some inhibition of platelet activation, but it was less effective than at lower concentrations. Under these conditions, these authors found a persistent, irreversible nitration of the proteins. We have found that, at lower concentrations of peroxynitrite, nitration, particularly in the proteins of the cytosolic fraction, is also reversible. The rate of disappearance of the nitrated protein does vary between different platelet preparations and possibly between different donors [20]. The apparent reversal of nitration, detected by the polyclonal antibody, was complete within a space of 2 h in cytosolic proteins, but the nitration of proteins in the membrane fraction appeared to be much lower and does not change significantly with time.

SPONTANEOUS NITRATION OF PROTEINS IN PLATELETS

Exposure to extracellular sources of peroxynitrite may be important in the regulation of platelet function, but there may also be a role for protein nitration caused by the endogenous processes within the platelet. Activation of isolated platelets by collagen, but only to a lesser extent by thrombin, led to a significant increase in the basal levels of nitration in platelet proteins: this process could be significantly inhibited, by the presence of high concentrations of the NOS inhibitor L-NAME [22]. The extent of this nitration was significantly less (approximately 15–20-fold) than that induced by concentrations of peroxynitrite sufficient to induce inhibition.

Several proteins appear to be endogenously nitrated, one of which was identified as being the vasodilator-sensitive phosphoprotein (VASP), a member of the enabled protein family found in Drosophila, and which is present in platelets, endothelium, and smooth muscle cells. Its normal role is to permit the assembly of a number of proteins, including actin, facilitating the formation of focal adhesions and the polymerization of actin [23]. This protein is a target protein both for NO and for prostacyclin, since its activity is inhibited by phosphorylation of specific serine/threonine residues through cAMP- and cGMP-dependent protein kinases (Fig. 1), preventing the polymerization of actin and the shape change characteristic of platelet activation [24]. The phosphorylation of the serine^{157} residue of VASP

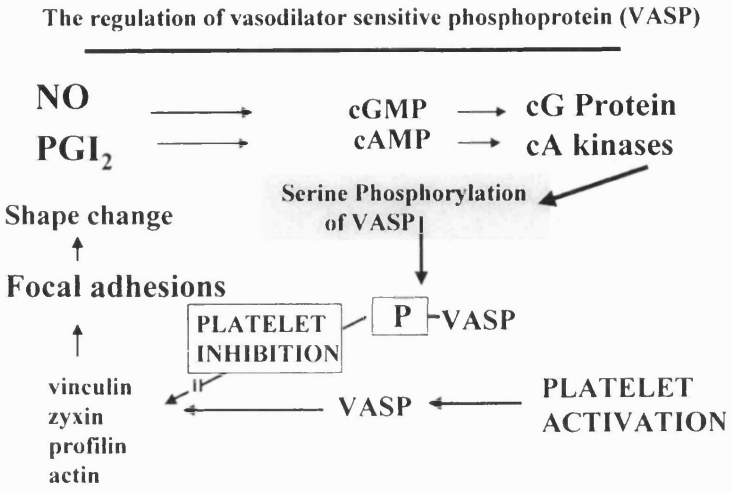

Fig. 1. The role of vasodilator stimulated phosphoprotein (VASP) in the activation platelets and in their inhibition by NO and prostacyclin.

gives rise to an apparent shift in molecular weight from 46 to 50 kDa, which assists in the identification of the protein; this occurs particularly in the presence of prostacyclin (via the cAMP-dependent kinase). Both forms of VASP appear to be capable of being nitrated, and nitration of VASP following activation by collagen was inhibited by the presence of L-NAME, but not ODQ [22].

It is important at this point to mention the methods for the preparation of platelets (Fig. 2). Platelets are sensitive cells and can be readily activated during the process of isolation. The gentlest way of isolating the platelet from plasma is by addition of low concentrations of prostacyclin and a double centrifugation after resuspension in buffer to remove traces of plasma [25]. The platelets are then rested for 90–120 min to effect full recovery of platelet activity following the return to nor-

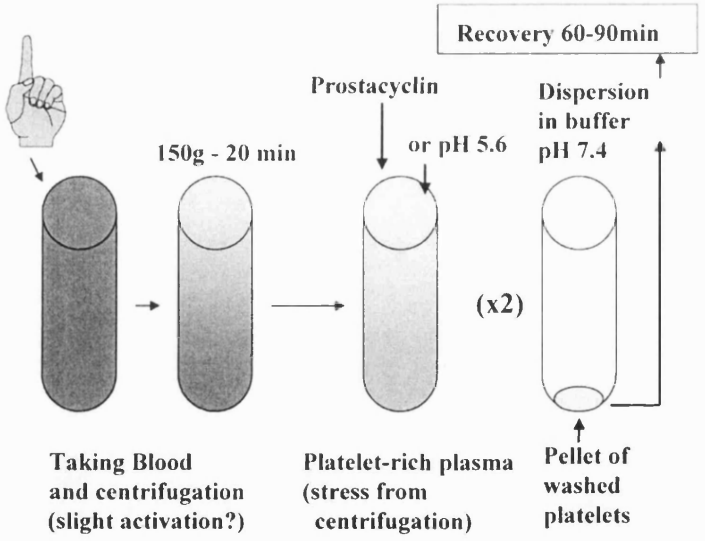

Fig. 2. Procedures for the isolation of platelets from plasma using either prostacyclin or low pH to minimize platelet activation. Blood was collected into acid/citrate/dextrose anticoagulant from human volunteers with informed consent and with permission of the Royal Free Hospital ethical committee. The blood was centrifuged slowly at 200 × *g* for 15 min and the platelet-rich plasma separated from the red cells and buffy coat. The platelets were isolated from the blood by one of two methods (addition of 50 nM prostacyclin or by lowering pH to 6.5 with citrate) with two washes in Tyrode's buffer pH 7.4 to remove the plasma. The platelets are then rested for up to 90 min before use.

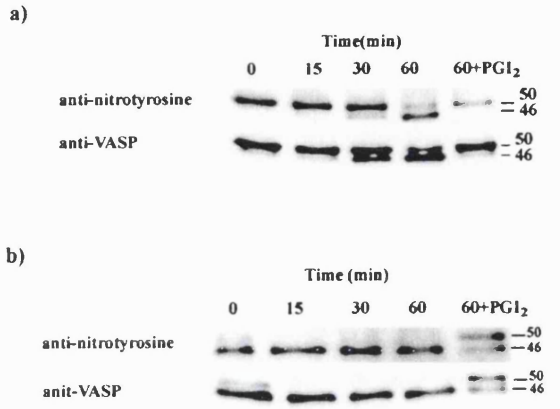

Fig. 3. Nitration of VASP in freshly isolated human blood platelets. Washed platelets were prepared (see Fig. 3a) in the presence of prostacyclin and (Fig. 3b) by lowering the pH to pH 6.5. Platelets were resuspended in modified Tyrode's buffer pH 7.4 and incubated at room temperature for 1–60 min, after which 50 nM PGI_2 was added and left for a further 15 min. The proteins were separated on 10% SDS/PAGE gels and transblotted on to nitrocellulose. The membranes were probed with antinitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, USA) and anti-VASP antiserum (Alexis Corp., Nottingham, UK). Anti-rabbit IgG peroxidase labeled and avidin-horseradish peroxidase conjugate from Amersham-Pharmacia (Buckinghamshire, UK) were used for detection of the bands.

mal of cyclic AMP concentrations. Alternatively, the pH of the PRP may be lowered, which also inhibits platelet activation during centrifugation [26]. Low levels of nitration in VASP, or indeed in any other proteins, were found. However, immediately after the isolation of platelets, there was significant nitration of proteins, without addition of agonists, which disappeared over the following 60–90 min (Fig. 3). The nitration could not be attributed to prostacyclin because when it was added to platelets that had rested 60 min, there was no increase in protein nitration. However, prostacyclin did induce a shift of VASP to the 50 kDa position, indicating that the VASP could still be phosphorylated by the cAMP-dependent protein kinase. Similar data were found when the low pH method was used (Fig. 3). These data were obtained using 10% SDS/PAGE gels where VASP appears as one of the prominent nitrated proteins. Because of differences in blotting conditions and other factors, VASP is less prominent on NOVEX gradient gels, where other proteins appear to be more strongly nitrated (Fig. 4). Using specific antibodies we have shown the p^{38} MAP kinase, the src kinase protein *lyn*, paxillin are tentatively identified and, like VASP, lose their nitration with time. However, others such as profilin and another unidentified protein at 33 kDa do not change significantly in terms of the extent of nitration.

These findings suggest that either nitration is a constant feature of platelet proteins in the turbulent circulation or that it is due to the handling of the platelets during isolation. It also means that a second wave of nitration could be induced by addition of collagen after the initial nitration had subsided. To address the question of whether the initial nitration was endogenous to platelets

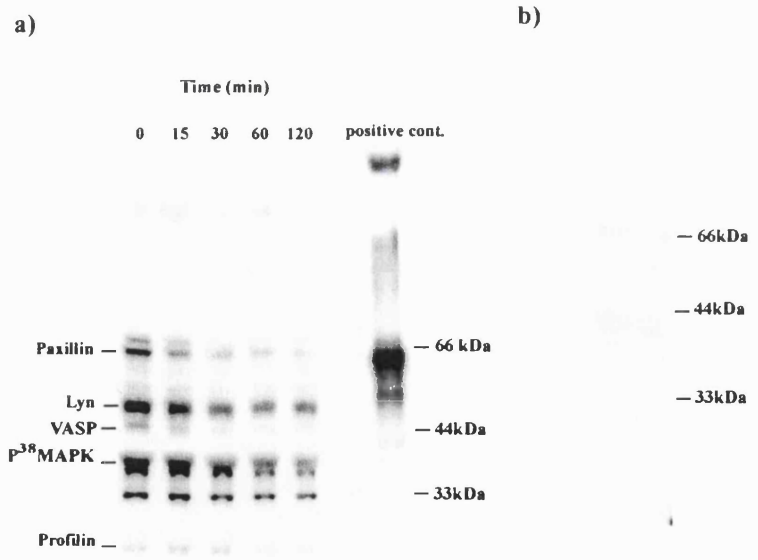

Fig. 4. The nitration of platelet proteins prepared following the isolation of platelets. Washed human platelets were prepared with addition of 50 nM PGI_2 and resuspended in Tyrode's buffer pH 7.4. After resuspension, platelets were incubated at 20°C for 1–60 min. Platelet proteins were separated on NOVEX precast gradient gels and blotted into nitro-cellulose membranes. The blots were probed with antinitrotyrosine antibody-RF1256 prepared in this laboratory (a) or in the presence only of the secondary antibody (b).

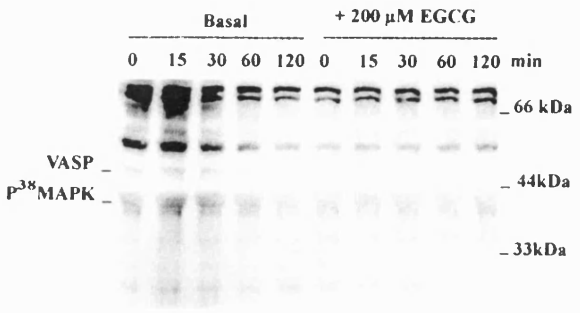

Fig. 5. The effect of −(−) epigallocatechin-gallate (EGCG) on the inhibition of protein tyrosine nitration during isolation of platelets. The EGCG (200 μM) was added immediately into blood together with anticoagulant during collection from the volunteer. Platelets were isolated as described in Fig. 2 with prostacyclin. The platelet proteins were run on 4–12% NOVEX precast gels and the Western blot was incubated with antinitrotyrosine antibody for detection of nitrated proteins.

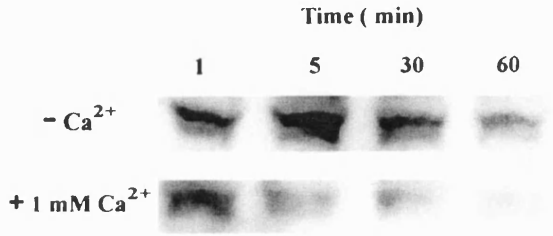

Fig. 6. Denitration of VASP in the presence of Ca^{2+}. Washed human platelets were prepared with addition of PGI_2 and resuspended in Tyrode's buffer pH 7.4. The platelet suspension was then incubated at 20°C for 1–60 min in presence and absence of 1 mM of Ca^{2+}. Platelet proteins were separated on 4–12% NOVEX precast gradient gels and blotted on to nitrocellulose membranes. The blots were probed with antinitrotyrosine antibody.

or caused by the isolation process, measures were taken to reduce the chance of platelet activation even further. Donors took 300 mg aspirin 24 h prior to the blood sampling, which was taken directly into a cocktail of creatine/creatine kinase to reduce the activating effects of the release of ADP from platelets or erythrocytes. No change in the extent of nitration was observed. This suggests that the process of nitration is not influenced by the cyclo-oxygenase pathway and may occur in the circulation.

−(−) Epigallocatechin gallate is a polyphenol, which has been shown to be active against peroxynitrite [27] but only against nitration and not oxidation of thiols [28]. When the polyphenol was added to freshly isolated blood and the platelets then isolated, there was a significant reduction in the nitration of a wide spectrum of the platelet proteins, including VASP, but some of the higher molecular weight proteins were still nitrated (Fig. 5). Ascorbate (100 μM) has no effect on nitration.

It was noted that addition of calcium to freshly isolated platelets accelerated the decrease in the extent of nitration (Fig. 6). The simultaneous addition of a cocktail of protease and proteosome inhibitors had no effect on the rate of disappearance, suggesting that the loss of nitration was not simply due to normal proteolysis (not shown). Therefore, it is possible that, even during the process of isolation, losses in nitrated proteins may be occurring, which are accelerated by the presence of intraplatelet calcium and that epigallocatechin gallate prevents further nitration occurring. These results may have implications for the presence of nitrated proteins in other cell types. The nitration of these proteins may be attributed to stress effects in cultured cells. Alternatively, it may be argued that low levels of nitration may be of importance in cell signaling processes.

The key message here is that platelet proteins may be nitrated endogenously, either while present in the circulation or during isolation, and then this may be lost rapidly in the presence of calcium. Addition of collagen initiates a second wave of endogenous nitration, indicating that this is a reversible process that can be repeated with a second stimulus.

MECHANISMS BY WHICH NITRATION OF PROTEINS MAY OCCUR IN PLATELETS

The fact that protein nitration occurs during cell activation by collagen suggests that the process may be linked to the associated cell signaling processes. This may be due to an increase in NO production following rises in intracellular calcium or the production of superoxide anions by either mitochondrial oxidation or from NADPH oxidase. Certainly L-NAME is inhibitory, indicating a requirement for endogenous NO, but nitration was not enhanced by the addition of 100 μM S-nitrosoglutathione (not shown). Surprisingly we found that hydrogen peroxide could increase protein nitration in platelets, although there have been other reports associating increased hydrogen peroxide concentrations with nitration [29–31], but usually in isolated systems with pure proteins or amino acids and sources of NO and specific enzymes. However, addition of hydrogen peroxide led to nitration of F-actin in Caco-2 cells [32].

We have recently shown that at low concentrations, hydrogen peroxide (from 250 nM to 25μM) increased the nitration of VASP in resting isolated platelets [33]. This process was also reversible and inhibited by L-NAME and epigallocatechin gallate. The mechanism underlying the nitration of proteins by hydrogen peroxide is somewhat enigmatic since hydrogen peroxide alone does not activate platelets at these low concentrations. However, we have shown a strong synergism between NO and H_2O_2 in the inhibition of platelet activation by collagen or thrombin. Again H_2O_2 does not have an inde-

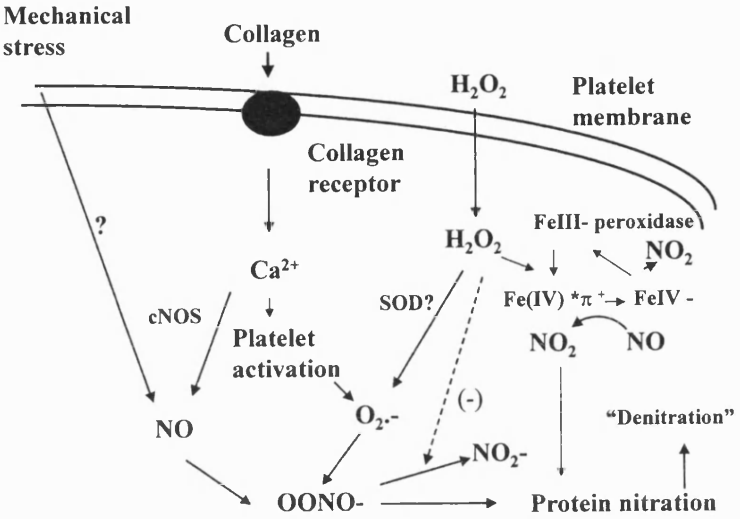

Fig. 7. Possible mechanisms leading to the nitration of proteins in platelets.

pendent inhibitory activity. This appears to be related to the direct effects of both of these inhibitors on VASP phosphorylation as we have demonstrated recently [22].

One explanation of this effect of H_2O_2 is that it may stimulate the reverse action of superoxide dismutase to provide superoxide anion [34,35]. However, hydrogen peroxide also stimulates the formation of higher oxidation states of heme-containing proteins. It was proposed by Abu-Soud and Hazen [36] that, in the presence of ferryl radicals, $NO_2^{\bullet}$ may be generated in the presence of NO and this may nitrate tyrosine residues on platelet proteins. Also, Alvarez and Radi [37] have shown that H_2O_2 was able to inhibit the decay of peroxynitrite, which would enhance the effect of the endogenously produced oxidant.

Alternative mechanisms may be considered. The nitration of tyrosyl radicals has been proposed for prostaglandin H synthase [38], which may be enhanced by the presence of H_2O_2, although this may not lead to the nitration of other proteins. The consumption of NO by lipoxygenases has also been demonstrated [39]. This process may result in the formation of peroxynitrite if 12-lipoxygenase, (for example in platelets), is activated by peroxides [40]. Increased availability of exogenous NO also led to enhanced nitration of proteins in rabbit aortic rings that could be reduced in the presence of superoxide scavengers, indicating that availability of NO was an important determinant of nitration [41].

In summary, the nitration of proteins seems to occur as the result of a range of stimuli, which are not mutually exclusive, but may arise through different mechanisms, which may or may not directly involve nitration by peroxynitrite (Fig. 7).

THE NITRATION OF TYROSINE AND TYROSINE PHOSPHORYLATION

There is a possibility that nitration of tyrosine may directly inhibit the phosphorylation of proteins. This was examined in endothelial cells where exposure to peroxynitrite led to a reduction in the tyrosine phosphorylation of a number of proteins with a concomitant increase in nitration of some of the protein bands [42]. Early work in platelets showed that the addition of peroxynitrite to platelets, at relatively high concentrations, brought about the nitration of proteins and a rapid increase in the phosphorylation of tyrosine residues [21]: the phosphorylation decreased again after 5 min. Proteins with a range of molecular weights were nitrated from 50–208 kDa, most of them from the cytosol. However, if the platelets were activated by low doses of thrombin, the amount of phosphorylation was decreased, but if high doses of thrombin were used, then the phosphorylation was increased by peroxynitrite. There was no direct evidence that phosphorylation and nitration occurred on the same proteins. Indeed, in recent studies [19], tyrosine phosphorylation of certain proteins, e.g., syk, was decreased by low doses of peroxynitrite, without the nitration of this protein. In the same study, there was some increase in the phosphorylation of other proteins without a concomitant increase in nitration. The evidence for a direct relationship between nitration and tyrosine phosphorylation in a competitive sense is, as yet, not strong. However, there may be an explanation of the effects of peroxynitrite on tyrosine phosphorylation because, in an isolated system, protein tyrosine phosphatases were shown to be exquisitely sensitive to this oxidant, which

would reduce the turnover of tyrosine phosphates [43]. Peroxynitrite was much more effective as an inhibitor of these phosphatases than hydrogen peroxide or NO donors. Direct nitration of substrates for tyrosine kinases clearly can inhibit phosphorylation when pure tyrosine kinases were used [42]. In lymphocytes, treatment of the cdc2(6–20)NH_2 peptide prevented its phosphorylation by lymphocyte-specific tyrosine kinase [44].

THE REVERSIBILITY OF NITRATION

The concept that the nitration of protein tyrosine residues may not be permanent has been shown by incubation of nitrated albumin with red cell lysates or plasma, which both had activities that led to the loss of the nitro group. This may not have simply been due to proteolysis [42]. Murad and coworkers [45] found that homogenates of rat tissues decrease the extent of nitration over time when exogenous nitrated albumin is used as substrate. They have provided further evidence that the loss of the stable nitro group may be by way of reduction of hydroxylamines [46]. The existence of mammalian nitroreductases is well established for nonpeptide drugs [47], but to date evidence for the existence of such enzymes for protein denitration has not been established. In the brain, these putative "nitratases" also appear to be very active [48] and were also stimulated by calcium. These authors proposed a direct removal of the nitro group. In platelets, there is clear evidence of a decrease in the endogenous nitration in specific proteins over time, although we have found in recent studies that not all proteins are so variable. With VASP there is a clear decrease in nitration, which can be enhanced by the addition of calcium (see above). This may be due to calcium-activated proteases, but we have been unable to block this disappearance by the addition of protease inhibitors. Nevertheless, more direct proof is required to demonstrate the origin of this enzymatic activity.

THE BIOLOGICAL SIGNIFICANCE OF NITRATION IN PLATELETS

It is clear that nitration of proteins may occur during normal physiological processes and this has been clearly demonstrated to be the case in platelets. In other cell types there are examples of spontaneous nitration of proteins; e.g., NH_2 terminal cJun kinase and prostacyclin synthase in endothelial cells during activation of the cells [49,50]. Furthermore, peroxynitrite appears to activate the PI_3 kinase/Akt pathway [51]. The extent of the nitration is clearly less than that seen following exposure of cells to a bolus of peroxynitrite. The question is whether these modifications are of biological significance or just an epiphenomenon. It is clear that excessive nitration may cause extensive protein damage, as may occur in certain disease states. However, specific targeting of tyrosine or other residues on proteins may be the basis for a signaling system comparable to that of tyrosine phosphorylation. In platelets, some of the proteins exhibit tyrosine phosphorylation even at rest (*src* family) and resting platelets do have some nitrated proteins. Of course, for this to be subject to regulation, it would be preferable that nitration is under enzymatic control. It has now clear that certain enzymes, such as superoxide dismutase, myeloperoxidase, and possibly xanthine oxidase or nitric oxide synthase, enhance nitration either from peroxynitrite or other nitrogenous substrates, but these may not be directly involved in the nitration processes described above. In a recent publication, it was shown the nitrotyrosine could replace phosphotyrosine in SH2-specific peptides that bind to the SH2 domain of the *src* family tyrosine kinase, *lyn* [52], a kinase that is also important in platelet activation [53]. Indeed, we have preliminary evidence that *lyn* itself may be nitrated in fresh platelets (Fig. 4). Clearly, a great deal of work needs to be done in the isolation of individual signaling proteins and the identification of changes in nitration or other modification which can be linked with specific cellular events. A proteomic approach offers the best way forward for this, as illustrated by the work of Aulak et al. [54].

It is always tempting to assume that nitration of proteins has many parallels with the signaling mechanisms afforded by tyrosine phosphorylation, but the evidence for this is still scant. Even though nitration of proteins occurs during activation of platelets, the process appears to involve radicals (nitric oxide and hydrogen peroxide) and indeed peroxynitrite, all of which are inhibitory to platelet activation. From this, it appears that nitration is more likely to have a role in limiting the activity of platelet activation mechanisms. Nitration may occur in the circulation as a result of shear stress, which is also know to activate phospholipase C and fibrinogen binding.

It is, therefore, possible that nitration is a protective mechanism that is reversible when the cells are no longer stressed. This may prevent unwanted activation from occurring. In pathological states, overproduction of peroxynitrite may lead to excessive nitration and denaturation, so that the nitro groups are less amenable to enzymatic reduction or the proteins are less readily cleaved by proteolytic enzymes, although there is evidence that nitration may actually increase proteolysis [42,55]. The least appealing proposition is that nitration is if no importance in normal physiology or in pathology. The search for a clear role continues. A recent publication showing that protein nitration due to production of

NO and superoxide anion is closely associated with the capacitation of spermatozoa [56] encourages the idea that there may be much to discover.

CONCLUSION

There is now clear evidence that proteins can become nitrated spontaneously in platelets, without the addition of exogenous peroxynitrite. Furthermore, this process appears to be reversible, although the nature of the products of "denitration" cannot be ascertained readily using antibody technology. The rate of denitration seems to be determined by cellular regulators such as the concentration of calcium. Nitration of proteins seems to be stimulated by normal physiological processes, such as the activation of platelets by collagen as well as the presence of low concentrations of hydrogen peroxide. It appears that mechanical stress on the platelets, during the isolation of the cells from blood, is sufficient to induce a transitory nitration. The biological significance of protein nitration is still not clear, although a nitrating species such as peroxynitrite does seem to influence the phosphorylation of tyrosine in important signaling proteins. These effects may be indirect and not due to direct competition on specific tyrosine residues for nitration or phosphorylation. There is much to be done to ascertain whether nitration of proteins is an important cellular process or just an inconsequential epiphenomenon. The application of proteomic techniques will be critical to the outcome of this quest.

Acknowledgement — This research was supported by The Wellcome Trust and The British Heart Foundation.

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