Nitroglycerin limits infarct size through S-nitrosation of Cyclophilin D: A novel mechanism for an old drug

Sofia-Iris Bibli[1,2*], Andreas Papapetropoulos[1*], Efstathios K. Iliodromitis[3], Andreas Daiber[4,5], Voahanginirina Randriamboavonjy[2], Sebastian Steven[4,6], Peter Brouckaert[7,8], Athanasia Chatzianastasiou[1], Kyriakos E. Kypreos[9], Derek J Hausenloy[10-16], Ingrid Fleming[2], Ioanna Andreadou[1*]

[1]Laboratoty of Pharmacology, Faculty of Pharmacy, National and Kapodistrian University of Athens, Greece;

[2] Institute for Vascular Signaling, Goethe University, Frankfurt, Germany

[3]Faculty of Medicine, Second Department of Cardiology, Attikon University Hospital, National and Kapodistrian University of Athens, Athens, Greece

[4] University Medical Center of Mainz, Center for Cardiology, Cardiology I, Molecular Cardiology, Mainz, Germany;

[5]German Center for Cardiovascular Research (DZHK), Partner Site Rhine-Main, Mainz, Germany;

[6]University Medical Center of Mainz, Center for Thrombosis and Hemostasis (CTH), Mainz, Germany;

[7]Ghent University, Department of Biomedical Molecular Biology, Ghent, Belgium; [8]Department of Molecular Biomedical Research, VIB, Ghent, Belgium;

[9]Department of Pharmacology, University of Patras Medical School, Patras, Greece;

[10]Cardiovascular & Metabolic Disorders Program, Duke-National University of Singapore Medical School, Singapore

[12]National Heart Research Institute Singapore, National Heart Centre, Singapore

[13]Yong Loo Lin School of Medicine, National University Singapore, Singapore

[14]The Hatter Cardiovascular Institute, University College London, London, UK

[15]The National Institute of Health Research University College London Hospitals Biomedical Research Centre, Research & Development, London, UK

[16]*Department of Cardiology, Barts Heart Centre, St Bartholomew's Hospital, London, UK*

Address for correspondence:

Ioanna Andreadou, Ph.D, Faculty of Pharmacy, Panepistimiopolis, Zografou, Athens 15771, GREECE; tel:+30 210 7274827; fax:+30 210 7274747; e-mail: jandread@pharm.uoa.gr

Sofia Iris Bibli, PhD. Institute for Vascular Signalling, Centre for Molecular Medicine, Goethe University. Theodor Stern Kai 7, 60596 Frankfurt am Main, Germany. Tel: +49 69 6301 6973 Email: bibli@vrc.uni-frankfurt.de

Andreas Papapetropoulos, Ph.D, Faculty of Pharmacy, Panepistimiopolis, Zografou, Athens 15771, GREECE; tel:+30 210 7274827; fax:+30 210 7274747; e-mail: apapapet@pharm.uoa.gr

Word count

Abstract

Aims: Nitroglycerin (NTG) given prior to an ischemic insult exerts cardioprotective effects. However, whether administration of an acute low dose of NTG in a clinically relevant manner following an ischemic episode limits infarct size, has not yet been explored. *Methods and Results:* Adult mice were subjected to acute myocardial infarction *in vivo* and then treated with vehicle or *low dose* NTG prior to reperfusion. This treatment regimen minimized myocardial infarct size without affecting hemodynamic parameters, but the protective effect was absent in mice rendered tolerant to the drug. Mechanistically, NTG was shown to nitrosate and inhibit cyclophilin D (CypD), and NTG administration failed to limit infarct size in CypD knockout mice. Additional studies revealed lack of the NTG protective effect following genetic (knockout mice) or pharmacological inhibition (L-NAME treatment) of the endothelial nitric oxide synthase (eNOS). The protective effect of NTG was attributed to preservation of the eNOS dimer. Moreover, NTG retained its cardioprotective effects in a model of endothelial dysfunction (ApoE knockout) by preserving CypD nitrosation. Human ischemic heart biopsies were found to express reduced eNOS activity and exhibited reduced CypD nitrosation.

Conclusions: Low dose NTG given at reperfusion reduces myocardial infarct size by preserving eNOS function, and the subsequent eNOS-dependent S-nitrosation of cyclophilin D, to inhibit cardiomyocyte necrosis. This novel pharmacological action of NTG warrants confirmation in patients.

Key words: Nitroglycerin; cardioprotection; eNOS; CypD nitrosation

Introduction

Prevention of myocardial ischemia-reperfusion injury is the focus of considerable attention and the target of therapeutic interventions.[1] Pharmacological agents given during the ischemic insult to minimize infarct size appear as the preferable approach, to protect against reperfusion injury.[2] In the complex procedure of rescuing the infarcted heart muscle, nitric oxide (·NO) has been proposed as a promising candidate.[3] Sublingual, intravenous, and oral nitrate preparations are used in the management of acute coronary syndromes. Most of the published data come from patients with myocardial infarction (MI), but the conclusions apply to patients with unstable angina. Some organic nitrates effectively improve the function of the post-infarcted heart due to their haemodynamic effects and also reduce final infarct size.[4] Among them, glyceryl trinitrate (commonly known as nitroglycerin, NTG), is a potent vasodilator that has been used in clinical practice for over a century.[5,6] Mechanistically, nitrates such as NTG, produce their effects in vascular smooth muscle cells by stimulating guanylate cyclase to produce cyclic guanosine monophosphate (cGMP), which in turn causes smooth muscle relaxation by decreasing myosin light chain kinase phosphorylation.[7] However, NTG has also been shown to have cGMP-independent effects.[8] The main limitation of nitroglycerin therapy is the fact that continuous infusion of the drug leads to the rapid development of nitrate tolerance that occurs in most patients within 24 hours.[9] To date, limited data exist concerning the cardioprotective effects of NTG when it is administered in a clinically meaningful manner, i.e. acutely during sustained ischemia and prior to reperfusion. Our group has characterized the beneficial effects of acute NTG treatment given before the ischemic insult.[10] In addition, the effect of other nitrovasodilators and NO donors, as cardioprotective strategies, have only been sporadically and superficially assessed. Conflicting findings have been reported concerning NTG's beneficial effects on the infarcted heart when administered during myocardial infarction[11-13], and the underlying mechanism of its action is incompletely understood.

Given that NTG i) exerts cardioprotective effects following a low dose acute administration, ii) has been shown to behave as an ·NO donor, and iii) ·NO donors intracellularly can act as nucleophilic precursors to modify cysteine residues, we hypothesized that low dose NTG administered therapeutically i.v., i.e. at the end of

ischemia and during reperfusion in *in vivo* models of myocardial infarction, could limit infarct size through S-nitrosation.

Methods

2.1 Animals

In the present study C57BL/6, eNOS$^{-/-}$, CypD$^{-/-}$ and ApoE$^{-/-}$ male mice 12-16 weeks old were used.

2.2 Ethical statement

Animals received proper care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health. Animals received the usual laboratory diet and all studies were approved by the animal research ethics committees in Athens and Mainz (787/13-2-2014, 23 177-07/G 10-1-051 E3 for CypD$^{-/-}$ mice, Landesuntersuchungsamt Koblenz, Germany)

2.3 Surgical Procedures

2.3.1 Murine in Vivo Model of Ischemia-Reperfusion Injury

16 week old male C57BL/6 wild type, eNOS$^{-/-}$, CypD$^{-/-}$ and ApoE$^{-/-}$mice were anesthetized by intraperitoneal injection with a combination of ketamine, xylazine and atropine (0.01 mL/g, final concentrations of ketamine, xylazine and atropine 10 mg/mL, 2 mg/mL, 0.06 mg/kg respectively). The depth of anaesthesia was monitored at a regular basis by eye reflex and haemodynamics. A tracheotomy was performed for artificial respiration at 120-150 breaths/min and PEEP 2.0 (0.2 mL tidal volume) (Flexivent rodent ventilator, Scireq, Montreal, Ontario, Canada). For groups in which blood pressure is provided, following anesthesia the left carotid artery was catheterized by the use of a 29G catheter. Mean arterial blood pressure was monitored through a fluid filled transducer connected to a BIO AMP amplifier and indicated electrocardiogram recording was performed by a Lead I ECG recording with PowerLab 4.0 (ADInstruments, UK). Recordings were analyzed by LabChart 7.0 software. A thoracotomy was then performed between the fourth and fifth ribs and the pericardium carefully retracted to visualize the left anterior descending artery (LAD), which was ligated using an 8-0 prolene monofilament polypropylene suture placed 1

mm below the tip of the left ventricle. The heart was allowed to stabilize for 15 minutes prior to ligation to induce ischemia. After the ischemic period, the ligature was released allowed reperfusion of myocardium. Throughout experiments, body temperature was maintained at 37^{o}C $\pm$ 0.5^{o}C. After reperfusion hearts were rapidly excised from mice and directly cannulated and washed with 2.5 ml saline-heparin 1% for blood removal. 5 ml of 1% triphenyltetrazolium chloride (TTC) phosphate buffer 37^{0} C were infused via the cannula into the coronary circulation followed by incubation of the myocardium for 5 minutes in the same buffer; 2.5 ml of 1% Evans Blue, diluted in distilled water was then infused into the heart. Hearts were kept in -20^{0}C for 24h and then sliced in 1mm sections parallel to the atrio-ventricular groove, and then fixed in 4% formaldehyde overnight. Slices were then compressed between glass plates 1 mm apart and photographed with Cannon Powershot A620 Digital Camera through a Zeiss 459300 microscope and measured with the Scion Image program. The areas of myocardial tissue at risk and infracted were automatically transformed into volumes (considering the height of each slice stable to 1mm). Infarct and risk area volumes were expressed in cm^{3} and the percent of infarct to risk area ratio (%I/R) is calculated.[14]

2.4 Experimental protocols

I. C57BL/6 cohort

C57BL/6 male mice 12-16 weeks old were subjected to 30 minutes regional ischemia of the myocardium followed by 2 hours of reperfusion with the following interventions. Vehicle A group (n=6): Vehicle NaCl 0.9% administered 3 times per day for 3.5 days before the ischemia/reperfusion induction. Mice were also treated with NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml $\cdot$ h^{-1} .

NTG A group (n=6): Vehicle NaCl 0.9% administered 3 times per day for 3.5 days before the ischemia/reperfusion induction. NTG was administered at a dose of 24 μg $\cdot$ kg^{-1} $\cdot$ min^{-1} in NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml $\cdot$ h^{-1}.[15]

NTG tolerance group (n=7): NTG in NaCl 0.9% at a dose of 20 mg/kg administered 3 times per day for 3.5 days to induce nitrate tolerance[16] before the

ischemia/reperfusion induction. Mice were also treated with NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml · h^{-1}.

Vehicle B group (n=9/in total): Mice were treated with NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml · h^{-1}.

NTG B group (n=9): Administration of NTG at a dose of 24μg · kg^{-1} · min^{-1} in NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml · h^{-1}.[15]

The above groups of animals were used for infarct size determination.

II. eNOS$^{-/-}$ study arm

Twelve eNOS$^{-/-}$ male mice 13-16 weeks old were subjected to 30 minutes regional ischemia of the myocardium followed by 2 hours of reperfusion with the following interventions.

eNOS$^{-/-}$ group (n=6): Vehicle NaCl 0.9% administered at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml · h^{-1}.

eNOS$^{-/-}$+ NTG B group (n=6): Administration of NTG at a dose of 24μg · kg^{-1} · min^{-1} in NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml · h^{-1}.

III. CypD$^{-/-}$ study arm

Sixteen CypD$^{-/-}$ male mice 12-14 weeks old were subjected to 30 minutes regional ischemia of the myocardium followed by 2 hours of reperfusion with the following interventions.

CypD$^{-/-}$ group (n=8): Vehicle NaCl 0.9% administered at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml · h^{-1}.

CypD$^{-/-}$+ NTG B group (n=8): Administration of NTG at a dose of 24μg · kg^{-1} · min^{-1} in NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml · h^{-1}.

eNOS$^{-/-}$ and CypD$^{-/-}$ animals have the same C57BL6/J background. To avoid extensive use of Vehicle B treated animals, we performed initially the experiments on

the eNOS$^{-/-}$ animals and the appropriate controls (n=6) and subsequently on the CypD$^{-/-}$ in which we added (n=3) additional controls. As no differences were detected among the Vehicle B treated animals in the 2 different cohorts we summarized the observed values. Similar for the WT treated with NTG B groups.

IV. ApoE$^{-/-}$ mice

Fifteen ApoE$^{-/-}$ male mice, C57BL6/J background, 13-16 weeks old were subjected to 30 minutes regional ischemia of the myocardium followed by 2 hours of reperfusion with the following interventions.

ApoE$^{-/-}$ group (n=7): Vehicle NaCl 0.9% administered at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml $\cdot$ h^{-1}.

ApoE$^{-/-}$ +NTG group (n=8): Administration of NTG at a dose of 24 μg $\cdot$ kg^{-1} $\cdot$ min^{-1} in NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml $\cdot$ h^{-1}.

V. Molecular Analysis Cohorts

Additional animals (5 per group) from groups Vehicle B, NTG B in presence and absence of N$^{\omega}$nitro-L-arginine methyl ester (L-NAME) (Cayman Chemicals, Lab Supplies P. Galanis & Co., Athens, Greece) (10 mg/kg IV in the 20th minute of ischemia), eNOS$^{-/-}$, CypD$^{-/-}$ and ApoE$^{-/-}$ treated with Vehicle B or NTG B were subjected to 30 minutes regional ischemia followed by 10 minutes of reperfusion. In the 10th minute of reperfusion tissue samples from the ischemic or non ischemic part of the myocardium was emerged in liquid nitrogen for nitrite assessment and S-nitrosation studies.

2.5 Western blot analysis

Tissue samples from the ischemic myocardium were pulverized in liquid nitrogen and dry ice. The sample powder was moisturized with lysis solution (1% Triton X100, 20 mmol/L Tris pH 7.4–7.6, 150 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L ethylene glycol tetraacetic acid, 1 mmol/L glycerolphosphatase, 1% sodium dodecyl sulfate [SDS], 100 mmol/L phenylmethanesulfonyl fluoride, and 0.1% protease phosphatase inhibitor cocktail) and homogenized. After centrifugation at 11.000 RPM for 15 minutes at 4°C, supernatants were collected. The supernatant was mixed with Dave buffer (4% Sodium dodecyl sulfate-SDS, 10% dithioltrietol-DTT,

20% glycerol, 0.004% bromophenyl blue, and 0.125 mol/L Tris/HCl). The samples were heated at 100°C for 10 minutes and stored at −80°C. The protein concentration was determined based on the Bradford dye-binding procedure. An equal amount of protein was loaded in each well and then separated by SDS-polyacrylamide gel electrophoresis 7.5% to 11% and transferred onto a polyvinylidene difluoride membrane (PVDF). After blocking with 5% nonfat dry milk, membranes were incubated overnight at 4°C with the following primary antibodies: phospho-eNOS (Ser1177), eNOS, CypD and β-tubulin. All antibodies were purchased from Cell Signaling Technology, Beverly, Massachusetts while CypD was purchased from Abcam. Membranes were then incubated with secondary antibodies for 1 to 2 hours at room temperature (Biorad goat anti-mouse and goat anti-rabbit horseradish peroxidase) and developed using the GE Healthcare ECL Western Blotting Detection Reagents (Thermo Scientific Technologies, Thermo Fisher Scientific Inc.,Waltham). Relative densitometry was determined using a computerized software package (NIH Image, Image J 1.44P, National Institutes of Health), and the values for phosphorylated eNOS, were normalized to the values for total eNOS.[14] For the assessment of monomer to dimer eNOS ratio, samples were prepared as described without the addition of the reducing agent DTT and the detergent SDS, while naïve acrylamide gels were used for western blot.

2.6 Nitrite measurement

Tissues from Vehicle B and NTG B groups with or without the addition of L-NAME or human material were homogenized in phosphate buffer saline-PBS, in the presence of 0.5% Triton-X100 in pH 7.4 and centrifuged at 10000g for 10 minutes. Supernatants were ultrafiltered using a 10 kDa molecular weight cut-off filter using a commercially available microfuge ultrafiltration device (Millipore). NOx levels were evaluated in the filtrate by Nitrite Colorimetric Assay Kit (#780001, Cayman Chemicals) according to manufacturer’s protocol.

2.7 Biotin switch assay

All chemicals were purchased from Sigma. Iodoacetyl-PEG2-biotin and streptavidin were purchased from Thermo Scientific.

S-nitrosation was detected using a modified biotin switch assay. In brief, samples were precipitated with 20% trichloroacetic acid (TCA) and stored at -80ºC. Precipitates were washed with 10% and then 5% TCA and then centrifuged (16000g, 30 minutes, 4°C) before being suspended in HENs buffer (250 mmol/L HEPES-NaOH, 1 mmol/L EDTA, 0.1 mmol/L neocuproine, 100 µmol/L deferoxamine, 2,5% SDS) containing 20 mmol/L methanethiosulfonate (MMTS) to block free thiols and protease and phosphatase inhibitors. Acetone precipitation was performed and pellets were re-suspended in pellets re-suspended in 200 µL lysis buffer (6 mol/L urea, 100 mmol/L NaCl, 2 % SDS, 5 mmol/L EDTA, 200 mmol/L Tris pH 8.2) supplemented with 20 mmol/L L-ascorbate for 10-15 minutes at room temperature (RT). Acetone precipitation was performed and pellets were re-suspended in 200 µL lysis buffer (6 mol/L urea, 100 mmol/L NaCl, 2 % SDS, 5 mmol/L EDTA, 200 mmol/L Tris pH 8.2). Equal amount of protein (500µg per sample) was divided in two aliquots and supplemented with 50 mmol/L iodoacetyl-PEG2-biotin. In one of them 1mM dithioltreitol (DTT) was added and samples were incubated at RT for 10 minutes. Subsequently samples were sonicated and incubated for 2 hours at RT in the dark. Lysates were precipitated with acetone and protein pellets were re-suspended in 50 µl Tris/HCl (50 mmol/L, pH 8.5) containing guanidinium chloride (GdmCl 6 mmol/L), and incubated at 95°C for 5 minutes. Biotin was then immunoprecipitated overnight (4ºC) using a high capacity streptavidin resin. Elution was performed by addition of 3% SDS, 1% β-mercaptoethanol, 8 mol/L urea and 0.005% bromophenol blue in H_2O for 15 minutes at room temperature followed by 15 minutes at 95ºC. Targets were detected following SDS-PAGE by Western blotting.[17]

2.8 cGMP measurement

Cyclic nucleotides were extracted by HCl and measured using a commercially available EIA kit (Enzo Life Sciences, P. Zafiropoulos S.A., Greece) following the manufacturer's instructions.[14]

2.9 Vascular reactivity studies

Aorta from ApoE and wild type mice in C57BL6/J background were cleaned of fat and connective tissue, and cut into 2 mm long segments. The presence of a functional endothelium was assessed in all preparations by the ability of acetylcholine (1 µmol/L) to induce more than 60% relaxation of vessels pre-contracted with

phenylephrine (1 µmol/L) and only arteries with an intact endothelium were used for further studies. A concentration-relaxation curve to acetylcholine was generated using arteries pre-contracted to 80% of their maximal response to phenylephrine in the presence of the cyclooxygenase inhibitor diclofenac (10 µmol/L).

2.10 Human Cohort

Samples from the right ventricle of healthy donors collected post-mortem after accidents or from patients undergone unsuccessful coronary artery GABG after acute myocardial infarction were used. Patients' characteristics are presented in Table 1. Samples were aged matched and subjects were not in treatment with nitrites.

2.11 Statistical analysis

All results are presented as mean ± standard error mean. One-way Analysis of Variance model (ANOVA) with Kruskal Wallis correction or Two-way Analysis of Variance model with Bonferonni post-hoc analysis were used as described in the figure legends. For the human cohort Student's t test with Mann Whitney was used. Analyses were performed using a Stata 13.1 statistical software package (StataCorp, TX, USA). A calculated p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1 Acute low dose NTG administration limits myocardial infarct size *in vivo*

Acute administration of NTG at a dose that did not affect hemodynamic parameters (Table 2) when given at the end of ischemia to naïve mice, markedly reduced infarct size (22.9±1.6% vs 48.1±1.7% for vehicle-treated mice, $p<0.05$) (Figure1A). Continuous delivery of NTG is known to induce nitrate tolerance in both the vasculature and in cardiomyocytes.[18] In order to determine if NTG tolerance interferes with the cardioprotective effects exerted by acute NTG, we used a well-described NTG tolerance protocol.[16] We observed that the NTG tolerant group exhibited a myocardial infarct size similar to that of the Vehicle group (46.1±3.3% and 48.1±1.7%, respectively). None of the treatments affected the area at risk (Figure 1B). Since signaling in the rabbit heart more closely resembles that of the human heart,[14] experiments were also performed in rabbits and comparable results were obtained (Supplementary Figure 1, Supplementary Results section).

3.2 NTG induced cardioprotection is cyclophilin-D (CypD) mediated

Acute NTG administration did not increase cGMP levels in mice (Figure 2A); a finding that is in agreement with previous reports of cGMP-independent effects of NTG.[8,19-20] Having ruled out that the effects of acute NTG are mediated by cGMP/PKG pathways (Supplementary Figure 1), we evaluated the contribution of mitochondrial permeability transition pore (mPTP) opening to the action of NTG. To this end, we used mice lacking cyclophilin D (CypD), a major regulator of mPTP opening. No significant differences were detected in the areas at risk between vehicle and NTG-treated wild-type and $CypD^{-/-}$ mice (Figure 2B). However, in agreement with previous findings,[21] the infarct size in $CypD^{-/-}$ mice was lower than that of the wild type animals (19.2 ± 1.9% vs 51.9± 2.4%, $p<0.05$). Administration of NTG to $CypD^{-/-}$ mice had no additional effect on infarct size (18.6 ± 2.9% in $CypD^{(-/-)}$ versus 23.3 ± 2.7% in wild type) suggesting that the protective effect of NTG administration is mediated via mPTP inhibition (Figure 2C).

To identify the mechanism through which CypD contributes to the cardioprotection afforded by NTG, we used the biotin switch assay to determine whether NTG elicited the S-nitrosation of CypD. Using an online software tool for the prediction of cysteine

modifications (http://clavius.bc.edu/~clotelab/DiANNA) multiple nitrosation sites on CypD were identified. Indeed, S-nitrosated CypD was detected in non-ischemic hearts and the signal was diminished by ischemia (Figure 2D). CypD S-nitrosation levels were, however, restored by the acute application of NTG given at the end of the ischemic period. Surprisingly, the ability of NTG to enhance CypD S-nitrosation was abolished by NOS inhibition using L-NAME (Figure 2E), suggesting that NTG acts in an eNOS-dependent manner. The S-nitrosation of other proteins such as SERCA,[22] is known to regulate myocardial function, however, NTG did not enhance SERCA nitrosation (data not shown).

3.3 Genetic evidence for eNOS dependent NTG protection

To determine whether the NTG- and eNOS-dependent changes in CypD S-nitrosation were functionally important, infarct sizes were compared in wild type and $eNOS^{-/-}$ mice treated with NTG during the ischemic insult. No significant differences were detected in the areas at risk among the studied groups (Figure 3A). The infarct size in vehicle-treated wild-type mice was similar to that of $eNOS^{-/-}$ animals (51.9 ± 2.4% vs 51.4± 3.5%, p=NS). However, the infarct size limiting effects of NTG were evident only in the wild type mice (23.3 ± 2.7% *vs* 46.4 ± 2.9% $p<0.05$ for $eNOS^{(-/-)}$ and wild-type, respectively) (Figure 3B). Similarly, in rabbits the effects of NTG were also eNOS mediated as the cardioprotective effects of NTG were inhibited by L-NAME administration (Supplementary Figure 1). The pharmacological inhibition of nNOS or iNOS failed to reduce the effect of NTG. To further confirm that NTG-stimulated CypD S-nitrosation was eNOS mediated, biotin switch assays were performed using tissues from $eNOS^{-/-}$ animals treated with vehicle or low dose NTG. In eNOS-deficient mice the administration of NTG to the ischemic heart was not able to increase CypD S-nitrosation (Figure 3C).

3.4 Nitroglycerin preserves eNOS dimerization and increases nitrite in the ischemic myocardium

To study the mechanism by which NTG affects eNOS to promote cardioprotection, eNOS phosphorylation on the activator Ser1176 site was evaluated. In these experiments we found no evidence for enhanced eNOS Ser1176 phosphorylation (Fig. 4A). However, we observed that NTG did increase the eNOS dimer to monomer ratio (Figure 4B). In line with the notion that NTG preserves eNOS active dimeric

form, NO metabolites in the heart increased in response to acute NTG treatment in a L-NAME-dependent manner (Figure 4C).

3.5 The beneficial effects of NTG are preserved in a model of endothelial dysfunction

To assess the effect of NTG in a model of endothelial dysfunction that more closely mimics the clinical state of patients suffering AMI, we used ApoE$^{-/-}$ mice. The ApoE$^{-/-}$ mice studies demonstrated an impaired relaxation to acetylcholine compared to wild-type mice, indicating the presence of endothelial dysfunction (Figure 5A). Subsequently, the effects of acute NTG treatment on myocardial infarct size and CypD S-nitrosation in the ischemic heart were evaluated. There was no observed difference in the area at risk (Figure 5B), and infarct sizes were similar in ApoE$^{-/-}$ and wild type mice (49.7 ± 4.4% vs 51.9± 2.4.%, p=NS). The administration of NTG, however, protected both ApoE$^{-/-}$ and wild-type mice to a similar extent (20.0 ± 3.6% vs 23.3± 2.7% respectively, p=NS) (Figure 5C). While CypD S-nitrosation was lower in vehicle-treated ApoE$^{-/-}$ versus wild-type mice, NTG administration increased CypD S-nitrosation to similar levels in the two mouse lines (Figure 5D).

3.6 Evidence for reduced NO bioavailability and CypD S-nitrosation in human post-infarction myocardium

To add translational value to our findings we determined markers of eNOS activity and CypD S-nitrosation in human samples. Our cohort consisted of material collected post-mortem from right ventricles of healthy individuals or right ventricles from patients who underwent unsuccessful GABG surgery. There was a clear reduction in CypD S-nitrosation in the ischemic/reperfused myocardium compared to the non-infarcted myocardium (Figure 6A). These observations were coupled with a low endogenous eNOS activity as mirrored by the intracardial L-arginine, L-citrulline (Figure 6B) and NOx levels (Figure 6C).

Discussion

The present study provides evidence that an acute, non-hypotensive dose of NTG administered in a clinical relevant manner following an ischemic episode is cardioprotective. Mechanistically, the actions of NTG, encompasses the dominant role of eNOS, and inhibition of mPTP opening by S-nitrosation of CypD. If confirmed in humans, these data would provide the basis for administration of a well-titrated nitroglycerin dose as front-line therapy in all patients with acute coronary syndromes, independently of their hemodynamic status, as NTG possesses an additional benefit, i.e. cardioprotection.

During the first 10 minutes of reperfusion following ischemia, reperfusion injury is triggered by endothelial dysfunction, which is results from a decrease in endothelial derived nitric oxide (·NO) release and increased oxidative stress.[23] Recently we were able to identify that eNOS inhibition during early reperfusion results in elevated myocardial infarct size in mice.[24] Re-activation[24] or preservation of eNOS function[25] during early reperfusion serves as promising therapeutic cardioprotective strategy.[26] Endogenously generated and exogenously applied ·NO exerts its beneficial effects either through cGMP/PKG-dependent pathways or by directly targeting the mitochondria.[27,28] The former pathways have been shown to involve, among others, K_{ATP} channels, the sodium–proton exchanger and protein kinase Cε leading to inhibition of mPTP opening,[3,29] while the latter relies on S-nitrosation of complex I to reduce ROS production.[30] In the present study we report a novel S-nitrosation target which is regulated by endothelial dysfunction during early reperfusion and results in elevated cardiomyocyte death. We observed that compared to the non-ischemic hearts, the ischemic ones developed reduced S-nitrosation of the mitochondrial protein Cyclophilin D (CypD). CypD which increases mPTP opening, undergoes S-nitrosation on cysteine 203 leading to reduced mPTP opening in fibroblasts from wild type mice but not in $CypD^{-/-}$ fibroblasts.[31] Since one of the criteria of identifying a functionally important therapeutic target for clinical translation is to validate the target in human myocardium,[32] we sought to evaluate if CypD was indeed de-nitrosated in the human ischemic left ventricle. Interestingly, in human ischemic necropsy samples a reduced activity of eNOS was observed as defined by the increased substrate levels and the reduced production of NOx. We were able to identify that under these

conditions of insufficient ·NO bioavailability, endogenous S-nitrosation of CypD was also reduced. Taken together, our observations suggest that S-nitrosation of CypD might serve as a novel therapeutic target for infarct size limitation. It should also be pointed out that results from clinical studies with cyclosporine (CsA), a CypD inhibitor, have been disappointing probably due to patient selection and the dose of CsA used. mPTP inhibition by more potent and selective agents is needed to investigate whether mPTP is an effective therapeutic strategy in reperfused STEMI patients.[32]

·NO donors are among the most promising candidates that affect CypD S-nitrosation in early reperfusion and prevent mPTP opening. Interestingly, among 20 *in vivo* animal studies published so far that evaluate different ·NO donors as cardioprotective agents, only three have investigated the role of the well established and widely used nitrate donor- NTG in myocardial infarction.[33] Additionally, controversial findings regarding the reduction of myocardial necrosis after the administration of NTG prior to reperfusion have been reported.[11-13] In our study, NTG given in a manner that recapitulates the clinical setting, i.e. at the end of the ischemia and during reperfusion, at a dose which does not affect the hemodynamic parameters[34] reduced infarct size in mice and rabbits. The divergent results between previous studies and our work may be due to i) the observed hemodynamic instability[12], which suggests a progressive decline of left ventricular (LV) function and ii) the dosing protocol[13], in which a constant rate of 50 ml·h^{-1} for 65 min was used. In our study the dose administered was 2 μg kg^{-1} min^{-1} at a constant rate of 1 ml·h^{-1} for 65 min starting at 10 min prior to the onset of reperfusion. Guidelines indicate that optimal administration volume must be <10% of the circulating blood volume of the animal. Considering that the plasma volume of rabbit is approximately 55.3 $\pm$ 5.3 ml· kg^{-1},[35] this leads to the conclusion that volumes greater than 8 ml h^{-1} are not well tolerated and cause serious volume expansion.[36]

A greater ·NO bioavailability has been associated with increased plasma NOx levels,[12] and since it has been proposed that low dose of NTG is converted to nitrite,[37] we determined nitrite levels. We observed that NTG was able to produce increased levels of nitrite compared to the control group that was L-NAME sensitive, indicating increased ·NO production. This is in agreement with results from a very recent study indicating that plasma nitrite levels were confirmed to be 6 ± 2-fold greater in mice treated with a NTG patch than in mice with the control tape.[38] We

next correlated the elevated ·NO production after NTG and the observed S-nitrosation of CypD with functional outcomes. Indeed, administration of NTG in CypD$^{-/-}$ mice did not enhance the cardioprotection already observed in these hearts, indicating that it acts in a CypD dependent manner. We should mention that other pharmacological agents have shown protection against ischemia/reperfusion-induced myocardial injury in CypD$^{-/-}$ hearts, suggesting that additional cardioprotective signaling pathways exist.[21,39] This does not seem to be the case for the cardioprotective activity of NTG, as it is exclusively mediated through CypD.

Exogenous applied ·NO had diverse and concentration-dependent effects on mPTP opening. The high concentrations of ·NO opened mPTP and these effects were related to both disulfide bonds and $ONOO^-$ formation. In contrast, physiological concentrations of ·NO inhibited mPTP opening by modifying thiol residues possibly through S-nitrosation.[40] To test the role of endogenously produced NO in the effect of NTG on S-nitrosation of CypD, we repeated the experiments in the presence of the eNOS inhibitor, L-NAME. Surprisingly, the observed post-translation modification was abolished suggesting, that the observed effects were eNOS mediated. No studies have been performed so far to delineate the mechanistic basis of NTG's cardioprotective effects; most of the studies have focused on the molecular mechanism which mediates the vasodilatory effects of NTG. Although NTG is considered as an endothelium-independent vasodilator, eNOS was reported to play a role in the vasodilatory signaling of NTG under some conditions.[41] Moreover, eNOS is critically involved in the amplification of the vasodilator effects elicited by *low dose* NTG.[42] Furthermore, other studies showed an endothelium dependence of NTG effects in animals and in patients.[43] However, these findings remain contradictory since the majority of literature shows improved NTG-dependent relaxation in endothelium-denuded or vessels of eNOS$^{-/-}$.[44] Thus, although the role of eNOS in NTG vasorelaxation has been investigated, there are no studies on the contribution of eNOS in cardioprotection. To further test the requirement of eNOS for NTG-induced cardioprotection, we measured whether NTG reduces infarct size in eNOS$^{-/-}$ mice. We observed that in contrast to the wild type animals, in which NTG indeed reduced infarct size, eNOS$^{-/-}$ animals were not protected. In addition, S-nitrosation of CypD in eNOS$^{-/-}$ animals was not detectable independent of NTG administration. These data indicate that CypD nitrosation is derived from endogenous NO.

To further address the mechanism through NTG affects eNOS to promote cardioprotection, we examined the possibility that NTG affects eNOS phosphorylation on the activator Ser1176 site. Although NTG administration was not able to increase phosphorylation of eNOS, interestingly, we observed preservation of eNOS dimer upon NTG treatment. Although, the mechanisms by which NTG administration preserves eNOS dimer are not yet clear, our data suggest that exogenous administered NTG during ischemia results in a preservation of endothelial nitric oxide synthase function which leads to increase ·NO bioavailability and further inhibition of CypD via S-nitrosation.

There is a need for improved design of animal studies to reflect more accurately the comorbidities and other confounding factors seen in clinical AMI.[33] Therefore, in order to further investigate the role of NTG in conditions of endothelial dysfunction, we administered NTG in ApoE$^{-/-}$ mice.[45] We confirmed that ApoE$^{-/-}$ mice without the addition of any atherogenic diet developed impaired aortic endothelial function in response to achetylcholine. In addition, we observed that ApoE$^{-/-}$animals were still protected upon NTG administration. Interestingly, NTG preserved CypD S-nitrosation in the ischemic ventricles of the tested animals. This finding leads us to propose that NTG could be a potent cardioprotective agent even in the presence of endothelial dysfunction.

Nitroglycerin was widely used in the past decades as one of the main drugs for coronary artery disease treatment. NTG is capable of promoting coronary vasodilation thereby increasing coronary blood flow and its effects are limited to large arteries while the smaller ones are nitrate resistant. Reports of clinical trials that investigated the role of NO donors as adjuncts to reperfusion in acute myocardial infarction have not found evidence for infarct size reduction after nitrate or nitrite infusion.[33] In contrast, NTG can have cardioprotective effects when given 24 h before coronary angioplasty as defined by improved ST-segment shifts, regional wall motion abnormalities, and chest pain scores following balloon inflation compared with patients who received saline.[46] On a multinational metanalysis level, patients with acute myocardial infarction under chronic nitrate treatment developed reduced levels of cardiac AMI associated biomarkers.[47] The results of the above mentioned study were supported by a very recent study indicating that long-term nitrate treatment is

cardioprotective.[48] Interestingly in the above study is mentioned that while the mechanism behind NTG-induced protection remains to be fully elucidated, it is likely that the cardioprotective effect of NTG involves ·NO and we should mention that herein we presented the mechanism by which NTG exerts its cardioprotective effects. Additionally, in a recent study, intracoronary but not IV infusion of nitrites reduced infarct size in STEMI patients with completely occluded arteries at admission. [49,50]

Very recently an experimental study showed that a NTG patch (designed to deliver 5 mg/day, so the 1/8 patch that was used is expected to deliver 0.026 mg/h) reduced myocardial infarct size when applied after the initiation of ischemia and 15 min prior to reperfusion in a mouse model of I/R similar to our protocol described herein.[38] Based on all the above, our study yields useful novel information that can be exploited to design future clinical studies where NTG can be safely used resulting in beneficial effects for patients.

Herein we demonstrated that low dose NTG reduces myocardial infarct size when administered during the ischemic insult both in normal animals and animals exhibiting endothelial dysfunction. Our findings in two *in vivo* different animal species suggest that apart from the known hemodynamic consequences, NTG protects the heart through an eNOS-dependent pathway, which preserves mitochondrial integrity by inhibition of CypD through S-nitrosation. The present data indicate an entirely novel target of cardioprotection and a novel benefit of NTG administration for patients suffering from acute coronary syndromes. Appropriate use of an old drug may prove to be a useful novel tool in the armamentarium against reperfusion injury.

Acknowledgments

This work has been co-financed by the COST Action BM1005 (ENOG:European network on gasotransmitters), by the COST Action BM1203 (EU-ROS) and by the EU-CARDIOPROTECTION COST-ACTION (CA16225). This work was also supported by the European Society of Cardiology (Basic Research Fellowship 2016 and Research Grant R-2016-054 to S.-I.B.), the Bodossaki Foundation (Mozamvinos Post-Doctoral Fellowship 2017, to S.-I.B.), the Schering Stiftung (Young Investigator Fund for Innovative Research 2017, to S.-I.B), and the Deutsche Forschungsgemeinschaft (SFB 815/A16 and SFB 834/B13 to I.F. and Exzellenzcluster 147 "Cardio-Pulmonary Systems"). DJH was supported by the

British Heart Foundation (FS/10/039/28270), the National Institute for Health Research University College London Hospitals Biomedical Research Centre, Duke-National University Singapore Medical School, Singapore Ministry of Health's National Medical Research Council under its Clinician Scientist-Senior Investigator scheme (NMRC/CSA-SI/0011/2017) and Collaborative Centre Grant scheme (NMRC/CGAug16C006), and the Singapore Ministry of Education Academic Research Fund Tier 2 (MOE2016-T2-2-021).

Conflict of interest

On behalf of all authors, the corresponding authors state that there is no conflict of interest.

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Table 1. Clinical data from the studied tissues.

Characteristics	***Non infarcted***	***STEMI***
Demographic data		
No	6	6
Mean age (range)	54.3 (40–68)	55.6 (42–64)
Male /female	6	6
Smokers	6	6
Clinical data		
Hypertension	0	0
Diabetes	0	0
Hyperlipidemia	0	6
Coronary disease	0	6
Myocardial Infarction	0	6
Valve insufficiency	0	0
Renal disease	0	0
Heart failure	0	0
Lung Cancer (SCLC)	6	0
Medication		
Statins	0	6
ACE inhibitors	0	0
b-blockers	0	0
cisplatin and etoposide	6	0

Table 2:Haemodynamic variables of the murine in vivo ischemia reperfusion model

Study group	HW	Baseline		20 min Ischemia		NTG infusion		180 min Reperfusion		1st minute	180th minute
		HR	MAP	HR	MAP	HR	MAP	HR	MAP	HR	MAP
WT+Vehicle	152±05	384±3	97.1±1.9	378±2	95.2±1.9	377±2	96.0±1.6	373±2	99.6±1.3	370±2	93.4±1.2
WT+NTG	151±05	390±4	98.6±4.8	385±4	99.8±2.9	385±4	99.8±3.0	380±3	100.6±3.3	374±2	105.8±0.5

HW: Heart weight in mg, HR: Mean heart rate in beats/min, MAP: Mean arterial blood pressure in mmHg.

Figures Legends

Figure 1. Acute NTG administration reduces myocardial infarct size in mice. A. Infarcted area to area at risk ratio presented as %. **B.** Area at risk to while myocardial area. n=6-7 per group, One Way Anova, Kruskal Wallis. (**p<0.01)

Figure 2. NTG confers cardioprotection by increased S-nitrosation of CypD. A. Acute NTG administration did not increase cGMP levels in mice. **B.** Area at risk to while myocardial area. **C.** Infarcted area to area at risk ratio presented as %. **D-E.** Representative western blot and relative densitometric analysis of S-nitrosated CypD (upper- determined by biotin switch assay) and total CypD of the Vehicle and NTG treated animals in the (D) non ischemic and ischemic heart or (E) ischemic biopsies from Vehicle or L-NAME treated animals. n=5-9 per group, Two Way Anova, Bonferonni. (*p<0.05, **p<0.001, ***p<0.0001)

Figure 3. Ablation of eNOS abrogates the NTG infarct limiting effects. A. Area at risk to whole myocardial area. **B.** Infarcted area to area at risk ratio presented as %. **C.** Representative western blot and relative densitometric analysis of S-nitrosated CypD (upper- determined by biotin switch assay) and total CypD of the Vehicle and NTG treated animals in the ischemic heart of WT and $eNOS^{-/-}$ mice. n=5-9 per group, Two Way Anova, Bonferonni. (*p<0.05, **p<0.001, ***p<0.0001)

Figure 4. NTG stabilizes eNOS dimer. **A.** Representative western blot and densitometric analysis of phospho eNOS S1176 normalized to total eNOS of ischemic heart of the indicated study groups (p=NS among groups). **B.** Representative western blot and densitometric analysis of eNOS dimer to monomer ratio in the ischemic left heart of vehicle or NTG treated animals(*p<0.05, ***p<0.001). **C.** Nitrite levels in nM in the ischemic hearts of the indicated studied groups. n=5-6 per group, One Way Anova, Kruskal Wallis (A), Two Way Anova, Bonferonni (B,C). (*p<0.05, ***p<0.0001)

Figure 5. NTG preserves its infarct limiting effects in ApoE$^{-/-}$ animals. A. Impaired vasorelaxation to achetylcholine compared to WT mice was observed in ApoE$^{-/-}$ mice. Aortic relaxation from WT and ApoE$^{-/-}$ mice in response to increasing concentrations of achetycholine, (n=9 per group). **B.** Area at risk to while myocardial area. **C.** Infarcted area to area at risk ratio presented as %. **D.** Representative western blot and relative densitometric analysis of S-nitrosated CypD (upper- determined by biotin switch assay) and total CypD of the Vehicle and NTG treated animals in the ischemic heart of WT and ApoE$^{-/-}$ mice. n=5-9 per group, Two Way Anova, Bonferonni. (*p<0.05, **p<0.001, ***p<0.0001)

Figure 6. Ischemia/Reperfusion injury reduces endogenous NO levels and CypD nitrosation in human right ventricles. A. Representative western blot and relative densitometric analysis of S-nitrosated CypD (upper- determined by biotin switch assay) and total CypD of the non ischemic versus the ischemic human right ventricles. **B.** Intracardial L-arginine and L-citrulline levels in the same samples as in panel A. **C.** Intracardial NOx levels in the same samples as in panel A. n=6 per group. Student's t test, Mann Whitney (**p<0.001).