Protection against Myocardial Ischemia and Reperfusion: Role of Peroxynitrite in Late Preconditioning and in Postconditioning

Ph.D. Thesis

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Contents

1. List of full papers directly related to the subject of the Thesis:

I. Role of iNOS and peroxynitrite-matrix metalloproteinase-2 signaling in myocardial late preconditioning in rats. Bencsik P, **Kupai K**, Giricz Z, Görbe A, Pipis J, Murlasits Z, Kocsis GF, Varga-Orvos Z, Puskás LG, Csonka C, Csont T, Ferdinandy P.Am J Physiol Heart Circ Physiol. 2010 Aug;299(2):H512-8.

IF.: 3.712

II. Cholesterol diet-induced hyperlipidemia impairs the cardioprotective effect of postconditioning: role of peroxynitrite. **Kupai K**, Csonka C, Fekete V, Odendaal L, van Rooyen J, Marais de W, Csont T, Ferdinandy P.Am J Physiol Heart Circ Physiol. 2009 Nov;297(5):H1729-35.

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III. Matrix metalloproteinase activity assays: Importance of zymography. **Kupai K**, Szucs G, Cseh S, Hajdu I, Csonka C, Csont T, Ferdinandy P. J Pharmacol Toxicol Methods. 2010 Mar-Apr;61(2):205-9.

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2. List of full papers indirectly related to the subject of the Thesis:

I. Matrix metalloproteinase inhibitors: a critical appraisal of design principles and proposed therapeutic utility. Dormán G, Cseh S, Hajdú I, Barna L, Kónya D, **Kupai K**, Kovács L, Ferdinandy P. Drugs. 2010 May 28;70(8):949-64

IF.:4.732

II. Measurement of myocardial infarct size in preclinical studies. Csonka C, **Kupai K,** Kocsis GF, Novák G, Fekete V, Bencsik P, Csont T, Ferdinandy P. J Pharmacol Toxicol Methods. 2010 Mar-Apr;61(2):163-70.

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Cumulative impact factor of papers indirectly related to the thesis: 6.952

3. Abbreviations

1400W	- N-(3-(Aminomethyl)benzyl)acetamidine
Akt	- protein kinase B
AMI	- acute myocardial infarction
AMP	- adenosine monophosphate
ANOVA	- analysis of variance
AP-1	- activator protein 1
ATP	- adenosine-triphosphate
Bax	- Bcl-2–associated X protein
Bcl2	- B-cell lymphoma 2
COX	- Cyclooxygenase
DHGLA	- dihomo-gamma-linolenic acid
DNA	- deoxyribonucleic acid
dNTP	- deoxyribonucleotide triphosphate
ERK	- extracellular-signal-regulated kinases
FeTPPS	- 5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]
GSH	- gluthation
HSP	- Heat Shock Protein
HT-1080	- human fibrosarcoma cell line
I/R	- ishemia/reperfusion
JAK	- Janus kinase
K	- Kelvin
K-ATP	- ATP sensitive potassium channel
MAPK	- mitogen-activated protein kinases
MI	- myocardial infarction
MMPs	- matrix metalloproteinases
mPTP	- mitochondrial permeability transition pore
NFkB	- nuclear factor kappa-light-chain-enhancer of activated B cells
NO	- nitrogen monoxide
NOS	- nitrogen monoxide synthase
O2–•	- superoxide
ONOO-	- peroxynitrite
PARP	- poly-ADP ribose polymerase

PI_3 kinase	- phosphatidylinositol 3-kinases
PKC	- protein kinase C
Post	- postconditioninig
Pre	- preconditioning
PTKs	- protein tyrosine kinase
QRT-PCR	- quantitative real time polymerase chain reaction
RISK	- reperfusion injury salvage kinases
RNA	- ribonucleic acid
ROS	- reactive oxygen species
SERCA	- sarcoplasmic reticulum Ca^{2+}-ATPase
SOD	- superoxide-dismutase
Src PTKs	- Src protein tyrosine kinases
STAT	- signal transducer and activator of transcription
TIMP	- tissue inhibitor of metalloproteinases
VF	- ventricular fibrillation.
XOR	- xanthine oxidoreductase;
X-SH	- SH-group containing molecules
X-SNO	- S-nitroso compounds

4. Epidemiology of ischemic heart diseases

Ischemic heart diseases are currently the leading causes of morbidity and mortality in the industrialized world and is expected to become the leading cause of death worldwide by the year 2020 when it will surpass infectious diseases (Cohen, 2000). About 70% of the people with ischemic heart disease die due to an acute myocardial infarction (AMI) (Murray & Lopez, 1997). According to the American Heart Association, in 2009 the estimated annual incidence of heart attack (myocardial infarction, MI) was 610,000 new events and 325,000 recurrent events. The average age of a person having a first heart attack was 64.5 years old for men and 70.3 years old for women, while the lifetime risk of developing coronary heart disease after age 40 is 49 percent for men and 32 percent for women.

After myocardial infarction, the surviving myocardium undergoes a complex sequence of cardiac remodeling, which may have a beneficial effect on cardiovascular performance in the short-term, but which become detrimental in the long-term and ultimately causes heart failure. In experimental studies, the degree of deleterious remodelling is highly affected by the size of the infarct (Pfeffer & Braunwald, 1990). Also in humans, infarct size is a major determinant of mortality in myocardial infarction. Limitation of infarct size has therefore been an important objective of strategies to improve clinical outcomes.

Furthermore, infarct limitation is an important therapeutic goal since it is also related to other factors such as severity of ventricular arrhythmias, mortality and loss of contractility. Hence, it is of most importance to salvage the myocardium by initiating reperfusion as soon as possible.

5. Pathophysiology of ishemia/reperfusion injury (I/R)

Ischemia can be described as an inadequate flow of blood to a part of the body, caused by constriction or blockage of the blood, narrowing of the coronary arteries during atherosclerosis, or development of coronary spasm. It is well known that myocardial ischemia results in the loss of contractile function and produces myocardial damage as a consequence of cell death from both necrosis and apoptosis (Hearse & Bolli, 1992).

Ischemia induces the accumulation of intracellular sodium, hydrogen, and calcium ions, culminating in tissue acidosis. Under ischemic conditions the decrease in pH stimulates Na^+-H^+ exchange and Na^+-HCO_3^- transporter, which leads to intracellular sodium accumulation and development of intracellular Ca^{2+} overload (Tani & Neely, 1989). Unregulated calcium-influx exerts detrimental effects as it activates a multitude of

intracellular enzymes including proteases and endonucleases important in proapoptotic signaling (Seal & Gewertz, 2005) and it activates plasmamembrane phospholipase A2 which leads to the formation of arachidonic acid metabolites (leukotriene B4, thromboxane A2). This event then increases neutrophil adhesiveness and perpetuate the generation of reactive oxygen radicals via stimulation of neutrophil oxidative burst. The absence of oxygen causes energy depletion (ATP). In addition, cellular ATP-depletion results in mitochondrial dysfunction and initiates the translocation of Bax, a proapoptotic Bcl2 family member protein, from the cytosol to the outer mitochondrial membrane. This causes mitochondrial swelling and induces the efflux of cytochrome c via opening of the permeability transition pore into the cytosol where cytochrome c activates effector caspases and initiates apoptosis. Derangement of the mitochondrial electron transport system promotes the generation of reactive oxygen species (ROS) and development of oxidative stress in the ischemic heart. Under the circumstances of increasing ROS levels and the inability of antioxidant defences, such as glutathione, to protect cellular proteins and lipids against oxidation. ROS react directly with cellular lipids, proteins and DNA leading to cell injury/death and activation of nuclear factor kappa-B (NFkB) (Turer & Hill, 2010). ROS are able to influence extracellular matrix remodelling through the activation of MMPs (Spinale, 2002). There is also evidence supporting the involvement and upregulation of matrix metalloproteinases (MMPs), in particular MMP-2 and MMP-9, in the development of I/R injury in tissues (Fig. 1) (Cheung et al., 2000).

Re-establishment of adequate oxygen and nutrients can limit the size of the ultimate area of infarction. Paradoxically, restoration of normal blood flow to an area of ischemia results in a complex cascade of inflammation and oxidative stress. Increasing evidence points to the fact that reperfusion injury exists and can lead to incremental cell necrosis and apoptosis. At reperfusion, the vascular endothelium upregulates the production of adhesion proteins and releases leukocyte attractants. Attraction and accumulation of leukocytes (primarily neutrophils and monocytes) to these areas triggers multiple mediator cascades leading to cytokine and chemokine release, the generation of ROS and the release of proteolytic enzymes from white blood cells and increased vascular permeability. During ischemia, the hydrolysis of ATP via AMP leads to an accumulation of hypoxanthine. Increased intracellular calcium enhances the conversion of xanthine dehydrogenase to xanthine oxidase which, upon reperfusion and reintroduction of oxygen, may produce superoxide and xanthine from the accumulated hypoxanthine and restored oxygen. This

results in further oxidative stress that can compromise cellular function and survival (Inserte et al., 2009).

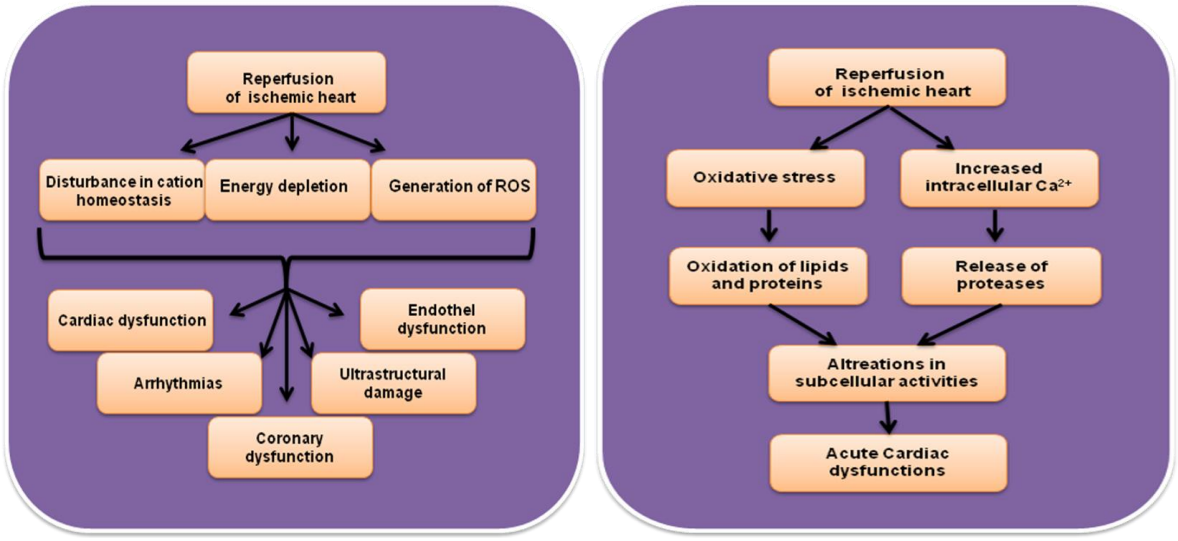

Figure 1.: Schematic diagrams of the acute and chronic detrimental effects of reperfusion in the ischemic myocardium.

6. Endogenous cardioprotection against I/R

Thrombolytic therapy and coronary interventions are the most widely used clinical strategies to reduce infarct size to achieve rapid recanalization of occluded coronary arteries after MI, aimed at reducing infarct size. Pharmacologic approaches such as beta-blockers, nitrates, and mechanical supports such as intraaortic balloon pumping to reduce energy demand and increase coronary flow of the infarcted myocardium are also common practices to minimize infarct size. An alternative approach to minimize infarct size is to stimulate endogenous mechanisms for cardioprotection against I/R injury.

Experimentally the most markedly protective interventions, which are able to limit infarct size in a very significant, consistent, and reproducible manner, are ischemic preconditioning and ischemic postconditioning. Ischemic preconditioning and postconditioning are endogenous protective mechanisms capable of protecting the myocardium from myocardial infarction, stunning, and ventricular arrhythmia induced by I/R injury. The brief conditioning episodes of ischemia and reperfusion can be applied prior to the index ischemic episode (Pre) (Baxter & Ferdinandy, 2001) or at the onset of reperfusion (Post) (Ovize et al., 2010).

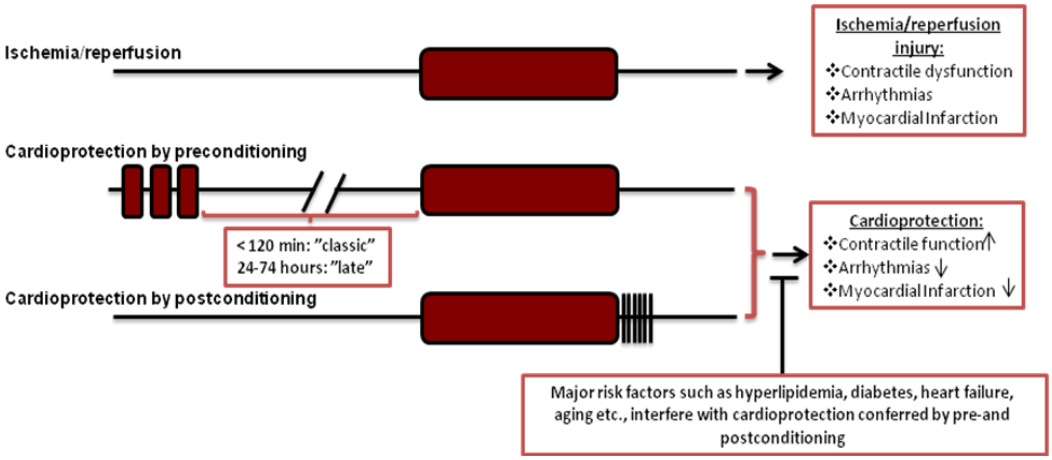

Figure 2.: Shematic representation of ischemia/reperfusion, preconditioning, and postconditioning protocol (Ferdinandy et al., 2007)

6.1 Preconditioning

Pre is the phenomenon whereby brief episodes of ischemia and reperfusion render the heart resistant to ischemic injury and more protected against another similar or greater stress. The phenomenon of Pre was described in 1986 by Murry et al. in a landmark study (Murry et al., 1986). Initially it was believed that ‘‘ischemic Pre’’ could be induced by short cyclic episodes of ischemia and reperfusion, soon it became apparent that a similar phenotype could be elicited by a splendid array of stimuli. For example, a number of pharmacological agents, agonist of adenosine, bradykinin, adrenergic and mascarinic receptors, nitric oxide (NO) donors, phosphodiestersase inhibitors, and various noxious stimuli (endotoxin, cytokine, reactive oxygen species [ROS], etc.) have all been found to generate Pre-like phenotype (Yellon & Downey, 2003;Naderi et al., 2010) and also known as pharmacological preconditioning.

It is now recognized that ischemic Pre consists of two chronologically and pathophysiologically distinct phases: an early phase and a late phase of protection. The early phase occurs immediately after the Pre stimulus and induces robust protection. Unfortunately, early Pre is short-lived (1 to 2 hours), which limits its clinical relevance. In contrast, the late phase of ischemic Pre develops 12 to 24 hours after the initial stimulus and lasts 3 to 4 days, although the magnitude of protection may be somewhat less than in the early phase. Unlike the early phase, the late phase of ischemic Pre protects not only against myocardial infarction but also against reversible postischemic cardiac dysfunction (myocardial stunning) (Fig. 2). Because of its 30-to 50-fold longer duration and the broader protection it provides, considerable interest has been focused on the late phase of Pre and its clinical exploitation.

Since the underlying pathophysiology and mechanisms of these two phases of endogenous cardioprotection appear different, it is important to distinguish the two and discuss them separately.

6.2 Postconditioning

Pre is a powerful cardioprotective intervention with one drawback: it must be applied before ischemia. Therefore, Pre would be useless in the patient presenting to the hospital with AMI since the ischemic process has already been initiated. It was more than 10 years ago when Na et al (Na et al., 1996) described another striking mechanism of endogenous myocardial protection termed ischemic postconditioning. It was found that several very short cycles of occlusion and perfusion immediately at the onset of reperfusion was able to reduce both the incidence of reperfusion-induced ventricular fibrillation and infarct size similarly to that seen with ischemic preconditioning (Fig. 2) (Zhao et al., 2003).

6.3 Mechanism of protection: from receptor to end-effector

The exact mechanism behind the protective effect of ischemic preconditioning and ischemic postconditioning are still not fully understood. Chemical signals trigger the development of late Pre by activating a series of complex signaling events that ultimately result in activation of cardioprotective genes. Among the many signaling pathways implicated, there is now convincing evidence that protein kinase C (PKC), Src protein tyrosine kinases (Src PTKs), mitogen-activated protein kinases (MAPKs), Janus kinase (JAKs), NFkB, and signal transducers and activators of transcription (STATs) play an essential role in the genesis of late Pre (Fig. 3).

Similary to Pre, Post activates signaling pathways, which involve triggers, mediators, and end-effector(s): the contribution of each of these players may vary depending on the experimental model (cell type, species, in vivo, ex vivo and in vitro) and the postconditioning stimulus used (Fig. 3). This observation excluded the critical importance of circulating blood elements or neurologic reflexes, and as in Pre, suggesting that some intracellular biochemical or biophysical modification were leading to protection. Post influences free radical generation and lipid peroxidation, attenuates inflammatory and endothelial cell-cell interactions (Downey & Cohen, 2008), preserves actions of endogenous autacoids such as adenosine, opioids, bradykinin via G-protein-coupled receptor (Kin et al., 2005), stimulates survival kinases such as p42/p44 ERK MAPK, PI_3 kinase-Akt (Tsang et al., 2004), reduces activity of death kinases

including p38, inhibits phosphorylation of inducible transcription factor (i.e., NFkB) (Kin et al., 2008), slows down of tissue pH (Cohen et al., 2008) and activates mitochondrial ATP-sensitive potassium channels and inhibits mitochondrial permeability transition pore opening (mPTP) (Mykytenko et al., 2008). Delay in application of Post, even for a few minutes, eliminates these cellular events, suggesting that these signalling cascades are quickly activated during eary periodes of reperfusion and work as causative mechanisms for protection.

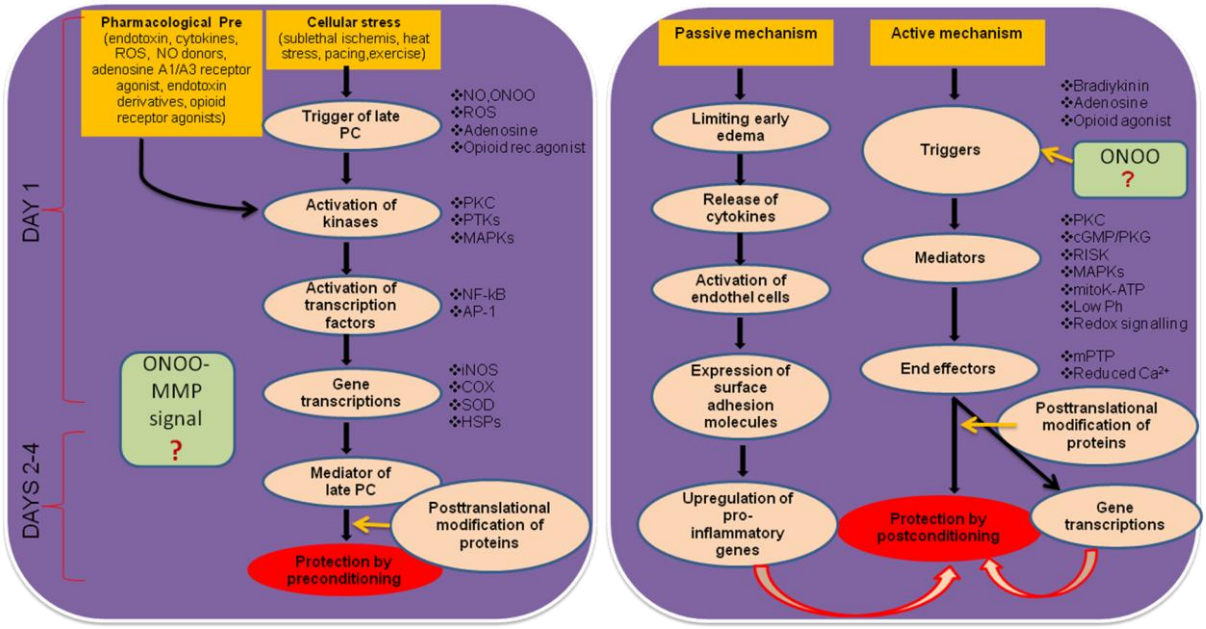

Figure 3.: Possible signalling pathways in late preconditioning and in postconditioning

6.4 Role of peroxynitrite ($ONOO^-$) in myocardial physiology and pathophysiology

$ONOO^-$ is a powerful oxidant species, which can be formed in vivo by the non-enzymatic reaction of nitric oxide (NO) and superoxide ($O2^{-\bullet}$) anion at an extremely rapid rate, limited only by diffusion (Fig. 4). It is widely accepted now that enhanced $ONOO^-$ formation contributes to oxidative and nitrosative stress in a variety of cardiovascular and other pathologies (Pacher et al., 2007). It has been shown that $ONOO^-$ is produced during the acute reperfusion of ischaemic hearts and that drugs which inhibit $ONOO^-$ formation or antagonize its toxicity protect the heart from reperfusion injury (Yasmin et al., 1997). In contrast, increasing evidence suggests that physiological levels of $ONOO^-$ may act as a regulator of several physiological functions (Vinten-Johansen, 2000). However, still very little is known about the physiological roles of endogenous $ONOO^-$ formation, possibly due to the number of technical limitations of detecting low, physiological levels of $ONOO^-$ in biological

systems (Tarpey & Fridovich, 2001). Increasing evidence suggests that $ONOO^-$ may act as a regulator of various cellular functions. Endogenous $ONOO^-$ has been shown to trigger ischaemic stress adaptation of the rat myocardium and to activate stress response pathways (Csonka et al., 2001;Ferdinandy, 2006).

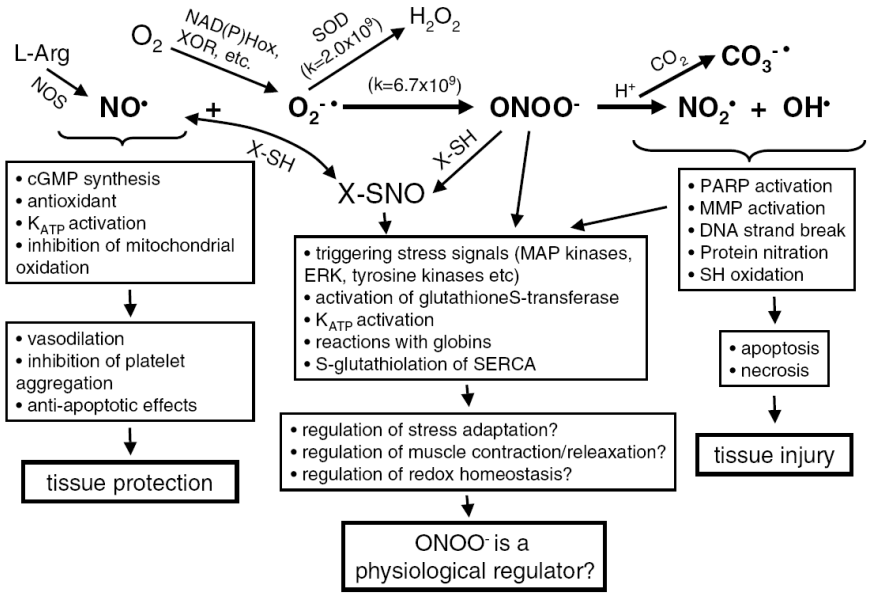

Figure 4.: Cellular mechanisms of the actions of NO, superoxide ($O_2^{-\bullet}$), and ONOO

(MMP, matrix metalloproteinase; NOS, NO synthase; PARP, poly-ADP ribose polymerase; XOR, xanthine oxidoreductase; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; K-ATP, ATP sensitive potassium channel). (Original figure in Ferdinandy, Br J Pharmacol, 2006).

NO is an important cardioprotective molecule via its vasodilator, antioxidant, antiplatelet, and antineutrophil actions and it is essential for normal cellular function. However, excess NO could be detrimental if it combines with $O_2^{-\bullet}$ to form $ONOO^-$ which rapidly decomposes to highly reactive oxidant species leading to tissue injury. There is a critical balance between cellular concentrations of NO, $O_2^{-\bullet}$, and superoxide dismutase (SOD) which physiologically favor NO production but in pathological conditions such as, for example, ischemia and reperfusion result in $ONOO^-$ formation. $ONOO^-$ might be converted to NO donors if it combines with SH-group containing molecules (X-SH) to form S-nitroso compounds (X-SNO) including S-nitrosoglutathione. S-nitrosylation and S-glutathiolation are the proposed mechanisms by which $ONOO^-$ regulates protein functions. Moreover, increasing evidence suggests that physiological levels of $ONOO^-$ act as regulator of several physiological functions.

6.5 Matrix metalloproteinases after myocardial infarction and its clinical significance

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, are implicated in a number of pathological conditions including tumor growth, metastasis, arthritis, periodontal disease. MMPs are also involved in the pathogenesis of cardiovascular disease, including atherosclerosis, restenosis, dilated cardiomyopathy, I/R injury, and myocardial infarction.

Matrix metalloproteinases are inhibited by specific endogenous tissue inhibitors of metalloproteinases (TIMPs), which comprise a family of four protease inhibitors (Dorman et al., 2010;Kupai et al., 2010). Administration of synthetic MMP inhibitors in experimental animal models of these cardiovascular diseases significantly inhibits the progression of atherosclerotic lesion formation, neointima formation, left ventricular remodeling, pump dysfunction, and infarct healing (Johnson et al., 2011;Kim et al., 2000). Moreover, acute treatment with MMP-inhibitors are able to attenuate I/R injury (Cheung et al., 2000) and decrease myocardial infarct size (Giricz et al., 2006) in rats. In addition, MMPs have been shown to target several non-extracellular matrix proteins both inside and outside the cells (Ferdinandy & Schulz, 2003) some of which are important in acute endogenous tissue protective pathways (Ferdinandy et al., 2007;Kandasamy & Schulz, 2009). However, the exact role of MMPs in the development of late Pre is still unknown. The inhibition of MMPs may be a novel pharmacological strategy for the treatment of I/R injury and suggests that inhibition of MMPs may be useful for future therapies in I/R injury.

6.6 Activation of matrix metalloproteinase-2 by $ONOO^-$

MMP-2 has recently emerged as a key enzyme involved in several cardiac conditions associated with enhanced oxidative stress. $ONOO^-$ at cardiotoxic level was reported to activate some full length MMP zymogens, particularly in the presence of glutathione (Viappiani et al., 2009). The release of MMP-2 increases with increasing duration of ischemia and correlates negatively with functional recovery. Lalu et al showed that ischemic preconditioning reduces the ischemia-induced activation and release of MMP-2 into the perfusate of isolated rat hearts (Lalu et al., 2002). In accordance with these findings the MMP inhibitors o-phenanthroline or doxycycline functionally protected the hearts from I/R injury (Cheung et al., 2000;Donato et al., 2010).

Enzymes of this family possess signal peptide, amino-terminal propeptide, catalytic Zn^{2+} binding site, and carboxy terminal domains. Of these, MMP-2, or gelatinase A, is found in nearly all cell types and degrades denatured collagen (gelatin) and intact collagen type IV, a major component of the basement membrane, as well as other extracellular matrix proteins. MMP-9, or gelatinase B, is a cytokine-inducible MMP that is most commonly expressed in leukocytes.

The full-length MMP-2 can be activated in two ways. Proteolytic activation of MMP-2 by proteases occurs by removal of the autoinhibitory propeptide domain resulting in an active truncated MMP-2. The presence of oxidative stress ($ONOO^-$) and cellular glutathione (GSH) causes the S-glutathiolation of the critical cysteine residue in the propeptide domain, disrupting its binding to the catalytic Zn^{2+}-ion, resulting in an active full-length enzyme (Chow et al., 2007).

6.7 Interaction of Cardiovascular Risk Factors with Pre and Post

Ischemic heart disease in humans is a complex disorder caused by or associated with other systemic diseases and conditions. Although, most experimental studies on cardioprotection have been undertaken in juvenile animal models, in which I/R is imposed in the absence of other disease processes and risk factors for cardiovascular diseases. These systemic diseases with aging as a modifying condition, exert multiple biochemical effects on the heart that can potentially affect the development of I/R injury per se and interfere with responses to cardioprotective interventions (Fig. 1). Therefore, the development of rational therapeutic approaches to protect the ischemic heart requires preclinical studies that examine cardioprotection specifically in relation to complicating disease states and risk factors (Ferdinandy et al., 2007). In the present thesis, in part of study 2, we studied cholesterol diet induced hyperlipidemia as a pathological model, in order to test the effectiveness of postconditioning in limiting the infarct size in the presence of underlaying risk factors.

7. Aims

Therefore, the aims of our present thesis were:

***STUDY 1*:**

To investigate the role of NO and $ONOO^-$–MMP signalling in the development of late preconditioning in rats in vivo.

***STUDY 2*:**

A) To study the role of $ONOO^-$ in rat hearts subjected to postconditioning protocol ex vivo.

B) To examine whether experimental hyperlipidemia induced by cholesterol-enriched diet interferes with the cardioprotective effect of postconditioning.

8. Materials and methods

This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

8.1. Experimental Groups-Study 1

Male Wistar rats weighing 300–350 g and housed in a room maintained at 12 h light–dark cycles and a constant temperature of 22±2ºC were used throughout the experiments without any treatment. Animals were preconditioned by 5 consecutive cycles of 4-min ischemia and 4-min reperfusion in vivo. 24 hours after the Pre protocol, 30-min test-ischemia followed by 180-min reperfusion was performed. The control group was subjected to the same procedure without Pre protocol (Fig. 5).

For investigation of the biochemical parameters and gene expression changes during the development of late Pre, additional experimental groups were generated in separate experiments (Fig. 6). A group of animals without any treatment served as 0h control. Four separate groups were preconditioned and 3, 6, 12, or 24 hours after the Pre protocol the hearts were isolated, perfused for 5 min on a Langendorff system to remove blood and frozen in liquid nitrogen. Four separate sham operated control groups undergone the same procedure as the preconditioned groups, but no occlusion was performed with the suture in these groups, then 3, 6, 12, and 24 hours later the hearts were sampled. The frozen hearts were powdered and homogenized in four volumes of ice-cold homogenization buffer (Schulz et al., 1992) with an ultrasonic homogenizer.

Finally, after evaluation of the biochemical parameters in the experimental groups, to verify if late Pre-induced inhibition of MMPs has a causative role in the reduction of infarct size, in separate experiments, 30-min test ischemia followed by 120-min reperfusion was applied after treatment with 1.5 μmol kg^{-1} ilomastat, a non-selective MMP inhibitor, and vehicle, respectively. Ilomastat and its vehicle were administered 5 min before the coronary occlusion and at the 10th and 25th min of ischemia as well as at the 10th min of reperfusion to maintain its plasma level based on the half life of the molecule (Fig. 7).

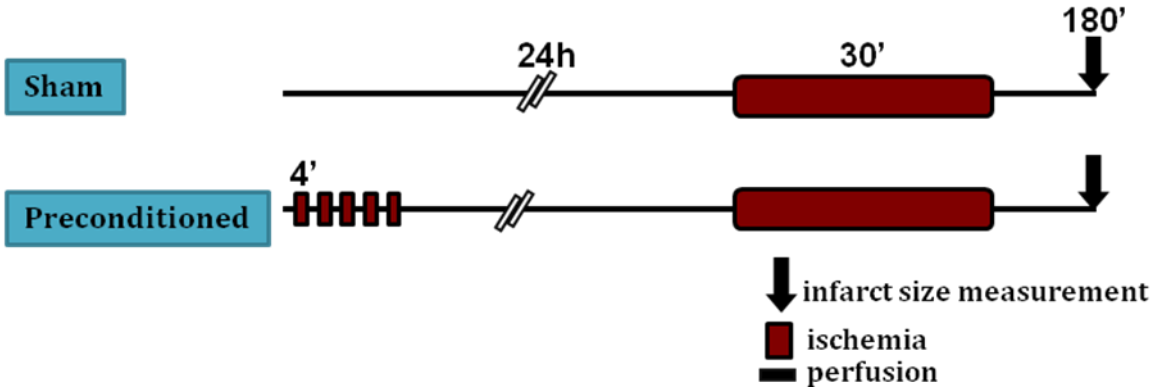

Figure 5.: Infarct size measurement after late Pre

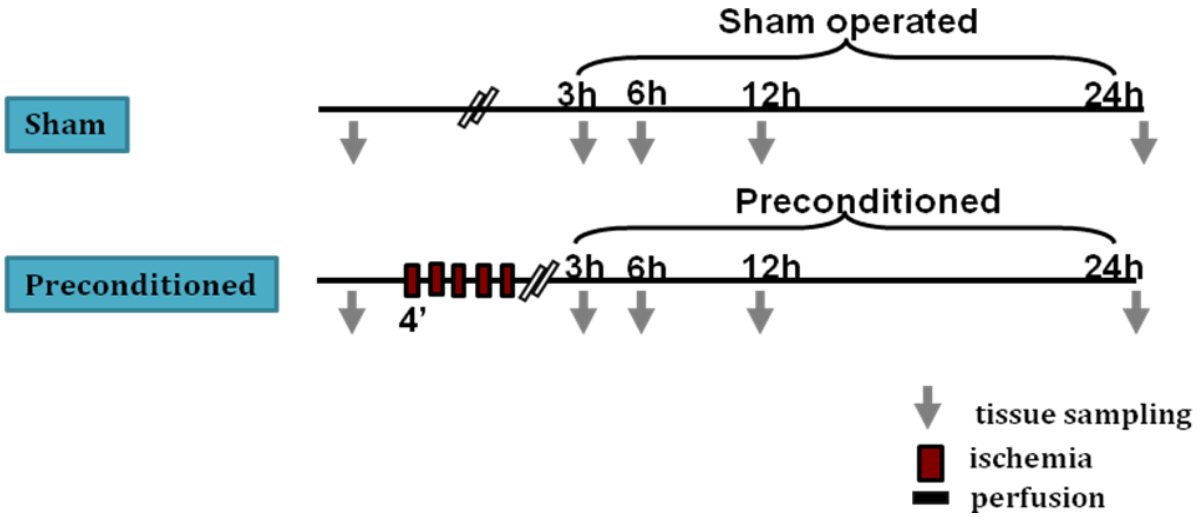

Figure 6.: Tissue sampling for biochemical measurements during the evolution of late Pre

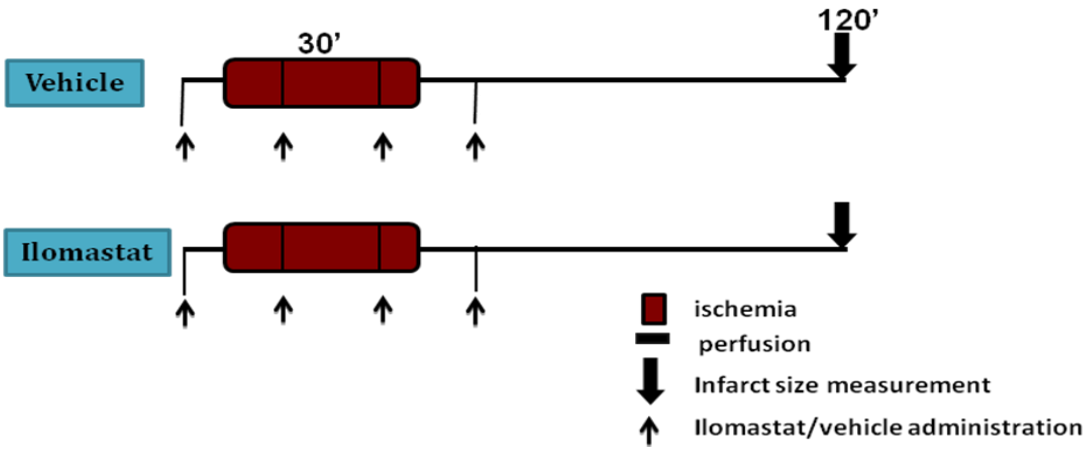

Figure 7.: Infarct size measurements after treatment with the MMP inhibitor ilomastat.

8.1.1 Induction of myocardial infarction

Myocardial infarction was induced as originally described by Selye et al (SELYE et al., 1960). Briefly: male Wistar rats were anaesthetized with 50 mg kg^{-1} pentobarbital sodium (Euthanyl, Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). Animals were intubated and ventilated with a rodent respirator (Harvard Apparatus Inc., Holliston, MA, USA) using room air (tidal volume 2.5 ml, 53±3 breaths/minute). Body temperature was monitored during the experiments and maintained at 37°C by placing the animals on a heating pad. The heart was exposed through a left thoracotomy in the fourth intercostal space. A 5-0 monofil atraumatic suture (Prolene, Ethicon Inc., Somerville, NJ, USA) was passed around the major branch of the left coronary artery, about 2 mm from its origin. A small plastic knob (diameter about 5 mm) was threaded through the ligature and placed in contact with the heart. Both ends of the prolene suture were passed through a small vinyl tube and exteriorized. The heart was replaced in the chest and the occlusion was carried out by a hemostat that pressed the tube and the knob to the myocardium. The Pre protocol was performed by five cycles of 4-min occlusions, each followed by 4-min of reperfusion. Sham operated rats underwent identical procedures, without occlusion of the artery. The chest was closed in layers and all rats were allowed to recover after surgery. Electrocardiogram was monitored throughout the experiments; right place of the ligature was verified by appearance of acute elevation of the ST-segment during the occlusion. To estimate infarct size after coronary occlusion, hearts were isolated and perfused for 5 min in a Langendorff system to wash out the blood, then the coronary artery was re-occluded and the hearts were perfused with 0.1% Evans-blue. After Evans-blue staining, hearts were frozen, cut into slices and incubated in 1% 2,3,5-triphenyl-tetrazolium chloride for 10 minutes at 37ºC. The slices were scanned and the size of the infarcted area related to the area at risk was determined by computer planimetry.

8.1.2 Measurement of cardiac NO and NOS

To measure cardiac NO content directly, ex vivo spin-trapping of NO was applied in separate experiments (Fig. 6.), followed by ESR analysis of left ventricular tissue samples as described (Csont et al., 1998;Onody et al., 2003). In brief, the spin trap N-methyl-D-glucaminedithiocarbamate (MGD, 175 mg kg^{-1}), 50 mg kg^{-1} $FeSO_4$, and 200 mg kg^{-1} sodium citrate were dissolved in 6 ml distilled water (Millipore). After 5 min Langendorff perfusion ventricular samples were minced into small pieces and incubated in MGD-$FeSO_4$-sodium citrate solution for 10 min under inert N_2 gas to avoid oxidation. Then

ventricular samples were dried on blotting paper an placed into quartz ESR tubes, frozen in liquid nitrogen until assayed for ESR spectra of the NO-Fe^{2+}-MGD complex at a temperature of 160 K (Bruker ECS106, Rheinstetten, Germany). In separate experiments, animals were treated with intraperitoneal injection of 1 mg/kg N-(3-(Aminomethyl)benzyl)acetamidine (1400W), a selective iNOS inhibitor, or vehicle saline 23h after Pre and measured cardiac NO content at 24h to estimate the role of iNOS in cardiac NO production in late Pre. To estimate endogenous enzymatic NO production, Ca^{2+}-dependent and Ca^{2+}-independent NOS activities in ventricular homogenates were measured by the conversion of L-[14C]arginine to L-[14C]citrulline as previously described (Ferdinandy & Schulz, 2003). Ventricular homogenates were centrifuged (1,000 g for 10 min) at 4°C and the supernatant was kept on ice for immediate assay of enzyme activities. Samples were incubated for 25 min at 37°C in the presence or absence of EGTA (1 mM) or EGTA plus NG-monomethyl-L-arginine (1 mM) to determine the level of Ca^{2+}-dependent and Ca^{2+}-independent NOS activities, respectively. NOS activities were expressed in pmol min^{-1} mg $protein^{-1}$.

8.1.3 Measurement of cardiac superoxide metabolism

In situ detection of superoxide anion was performed by confocal laser scanning microscopy using a fluorescent dye dihydroethidium (Sigma) as described (Bencsik et al., 2008). Dihydroethidium is freely permeable to cell membranes and emits a red fluorescent signal when oxidized by superoxide. Frozen native heart sections (30 μm) were placed on glass slides covered with PBS buffer (pH 7.4) and collected at 4°C. Then the slides were submerged in 1 μmol/L dihydroethidium (Sigma) in PBS buffer (pH 7.4) and incubated at 37°C for 30 minutes in a dark humidified container. Fluorescence in heart sections was detected by a confocal microscope (Olympus™ FV1000) using a 530 nm long-pass filter for excitation. Images of the hearts that were treated with saline (negative control) were measured first. After the basal settings of the confocal microscope were adjusted, images of the hearts were collected digitally. Eight images were taken randomly of each slides and fluorescence intensity was analysed by ImageJ 129 program. Total activity of SOD was measured by a spectrophotometric assay using a commercially available kit (RanSOD, Randox Laboratories Ltd., Crumlin, UK). Approximately 50 mg ventricular tissue was homogenized in 10x volumes of ice-cold phosphate buffer (0.01 M, pH 7.0). SOD activity in homogenates was determined by the inhibition of formazan dye formation due to superoxide generated by xanthine and xanthine oxidase.

8.1.4 Measurement of cardiac XOR and NADPH oxidase

Activities of xanthine oxidoreductase (XOR; xanthine oxidase and xanthine dehydrogenase) and NADPH oxidase, major sources of superoxide in rat hearts were determined from ventricular homogenates. XOR activity was determined by a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total xanthine oxidoreductase activity) or absence (xanthine oxidase activity) of the electron acceptor methylene blue, as described (Beckman et al., 1989). NADPH oxidase activity was measured by a chemiluminescence assay (Ferdinandy et al., 2000) in Krebs-Henseleit solution containing 0.25 mol/L HEPES (pH 7.4), 5 μmol/L lucigenin, and 30 μL of tissue homogenate in 1 mL of total volume using a Packard-Bell liquid scintillation counter in out-of-coincidence mode. The increase in luminescence signal was monitored for 5 min after adding 100 μmol/L NADPH. Ventricular homogenates were prepared as for the measurement of NOS activity.

8.1.5 Measurement of cardiac $ONOO^-$

To estimate cardiac $ONOO^-$ formation, we measured free 3-nitrotyrosine by enzyme-linked immunosorbent assay (ELISA, Cayman Chemical; Ann Arbor, MI) in myocardial homogenates. Briefly, tissue samples were homogenized by the addition of 4 volumes of homogenization buffer. After centrifugation, the supernatants were incubated overnight with anti-nitrotyrosine rabbit IgG and nitrotyrosine acetylcholinesterase tracer in precoated (mouse anti-rabbit IgG) microplates, followed by development with Ellman's reagent.

8.1.6 Zymographic analysis of MMP activity

To measure myocardial MMP-2 and MMP-9 activities heart ventricular was homogenized in absence of any protease inhibitor. Gelatinolytic activities of MMPs were examined as previously described (Kupai et al., 2010). Briefly, 8% polyacrylamide gels were copolymerized with gelatin (2 mg/ml, type A from porcine skin; Sigma-Aldrich), and 40 μg of protein per lane was loaded. An internal standard (supernatant of phorbol ester-activated HT-1080 cells; American Type Culture Collection, Manassas, VA) was loaded into each gel to normalize activities between gels. Following electrophoresis (150 V, 1.5 h), gels were washed with 2.5% Triton X-100 for 3 x 15 min and incubated for 24 to 48 h at 37°C in incubation buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3, pH 7.4). Gels were then stained with 0.05% Coomassie Brilliant Blue (G-250; Sigma-Aldrich) in a mixture of methanol/acetic acid/water (2.5:1:6.5, v/v) and destained in aqueous 4%

methanol/8% acetic acid (v/v). Gelatinolytic activities were detected as transparent bands against the dark-blue background. Zymograms were digitally scanned, and band intensities were quantified using Quantity One software (Bio-Rad, Hercules, CA) and expressed as a ratio to the internal standard. Band density was expressed as arbitrary units.

8.1.7 Quantitative real-time PCR

Quantitative real-time PCR (QRT-PCR) was performed on a RotorGene 3000 instrument (Corbett Research, Sydney, Australia) with gene-specific primers using SybrGreen protocol to assess expression changes of NOS, MMP and TIMP genes. Two micrograms of total RNA from each sample were reverse transcribed in the presence of random primers in a total volume of 20 μl after dilution with 20 μl of water, 1 μl of the diluted reaction mix was used as template in QRT-PCR. The 20 μl reaction volume contained 0.2 mM of dNTP, 1× PCR reaction buffer (ABGene, Epsom, UK), 6 mM of each primer, 4 mM of $MgCl_2$, 1× SYBR Green I (Molecular Probes, Eugene, OR) at final concentration, and 0.5 U of thermostart Taq DNA polymerase (ABGene). The amplification was carried out with the following cycling parameters: 600 s heat start at 95°C, 45 cycles of denaturation at 95°C for 25 s, annealing at 60°C for 25 s, and fluorescence detection at 72°C for 15 s. Relative expression ratios were normalized to β-actin. Nontemplate control sample was used for each PCR run to check the genomic DNA contaminations of cDNA template. Analysis of results was done using Pfaffl method (Pfaffl, 2001). With the use of this calculation method, differences between the amplification efficiencies of reactions could be corrected.

8.1.8 Statistical analysis

Data were expressed as means ± S.E.M. or S.D. and analyzed with unpaired t-test or One-way ANOVA followed by Tukey's PostC-hoc test as appropriate. Two-way ANOVA has been performed to study the time-course of enzymatic changes in order to determine possible interactions between time-dependence and late Pre. $P \leq 0.05$ was accepted as statistically significant difference compared to control or sham operated control group.

8.2. Experimental Groups-Study 2

8.2.1 Experimental design: diet protocol

Six-week old male Wistar rats were fed normal or 2% cholesterol-enriched rat chow for 12 weeks. Wistar rats were chosen for the study since this species shows a moderate increase in serum cholesterol and triglyceride level due to high-cholesterol diet and no substantial atherosclerosis develops; therefore, the direct myocardial effect of hyperlipidemia, independent from atherosclerosis, can be studied in this model. At the end of the 12-wk diet, serum cholesterol, triglyceride and myocardial free fatty acid levels (FOLCH et al., 1957) were measured from normolipidemic and hyperlipidemic animals to confirm the development of hyperlipidemia.

8.2.2 Infarct size measurements-Postconditioning protocol

At the conclusion of the experimental diet period, animals were anesthetized with diethyl ether and given 500 U/kg heparin. Hearts were isolated and perfused with Krebs-Henseleit buffer (118 NaCl, 4.3 KCl, 2.4 $CaCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 11.1 glucose in mmol/L, gassed with 95% O_2-5% CO_2) with constant pressure according to Langendorff. Regional ischemia was induced by 30 min coronary occlusion followed by 120 min reperfusion. Postconditioning was induced by six consecutive cycles of 10 s coronary occlusion and 10 s reperfusion immediately at the onset of reperfusion (Fig. 8). Electrocardiogram was monitored during the study to measure heart rate and incidence of reperfusion-induced ventricular fibrillation (VF). At the end of the 120 min reperfusion, infarct size was determined by triphenyltetrazolium chloride staining and evaluated by planimetry. Infarct size was expressed as a percentage of area at risk.

To analyze, if $ONOO^-$ plays a role in the cardioprotective mechanism of postconditioning, normolipidemic hearts were subjected to 30 min regional ischemia followed by the 6x10 s cycles postconditioning protocol in separate experiments, in the presence or absence of 5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III] (FeTPPS; 20 mg/L). The dose of 20 mg/L FeTPPS was selected according to our previous studies (Ferdinandy et al., 2000;Onody et al., 2003). Krebs-Henseleit solution contained FeTPPS only during the last 20 s of the 30-min ischemic period and during postconditioning. At the end of the 120-min reperfusion period, infarct size was measured as described above (Fig. 8).

To check if a stronger postconditioning stimulus could postcondition the heart, hearts were isolated from normolipidemic and hyperlipidemic groups and subjected to 30 min coronary occlusion and 120 min reperfusion with or without a postconditioning protocol of 12 cycles of 10 s coronary occlusion and 10 s reperfusion. At the end of the 120-min reperfusion, infarct size was determined as mentioned above (Fig. 9).

8.2.3 Cardiac 3-nitrotyrosine measurement

For biochemical analysis, hearts from control and cholesterol-fed groups were subjected to 30 min coronary occlusion followed by 5 min reperfusion with or without postconditioning, and left ventricular tissue was sampled, homogenized, and used for 3-nitrotyrosine measurements by ELISA as mentioned in 8.1.5 section and by Western Blot analysis (Fig. 8). Heart tissue for Western Blot analysis was homogenized (Schulz et al., 1992) and centrifuged. Protein concentrations of supernatants were measured by the bicinchoninic acid assay. Equal amounts (20 μg) of proteins were separated by 10% SDS-PAGE and transferred to nitrocellulosemembrane (Amersham), then the blot was blocked in Tris buffered saline/Tween 20 supplemented with 5% nonfat dry milk overnight. The primary 3-nitrotyrosine antibody (MAB5404; Chemicon International) was used at the manufacturer-recommended dilution. The membrane was developed with an enhanced chemiluminescence kit (ECL Plus; GE Healthcare), exposed to X-ray film, and scanned. Band density was calculated by integrating the area (pixels x intensity, expressed in arbitrary units). By this method, we detected the degree of nitrosylation of tyrosine side chains of proteins, since low-molecular weight free 3-nitrotyrosine is eliminated in the SDS-PAGE.

8.2.4 Statistical analysis.

Results were expressed as means ± S.E.M. when appropriate. Differences among means were analyzed by using one-way ANOVA followed by Tukey's post hoc test. The incidence of VF was analyzed by Fisher's exact test. Differences were considered significant at $P < 0.05$.

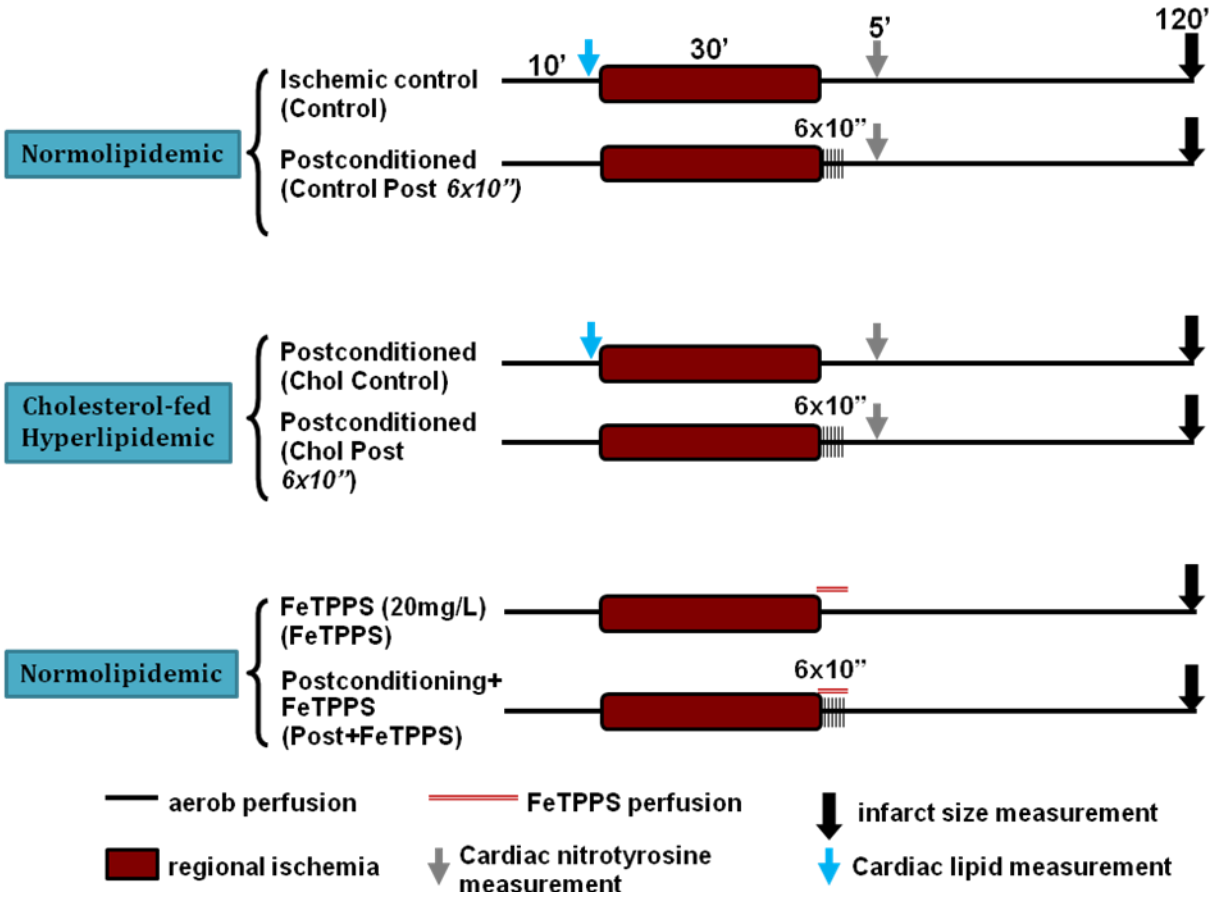

Figure 8.: Experimental design for infarct size measurements and for biochemical measurements

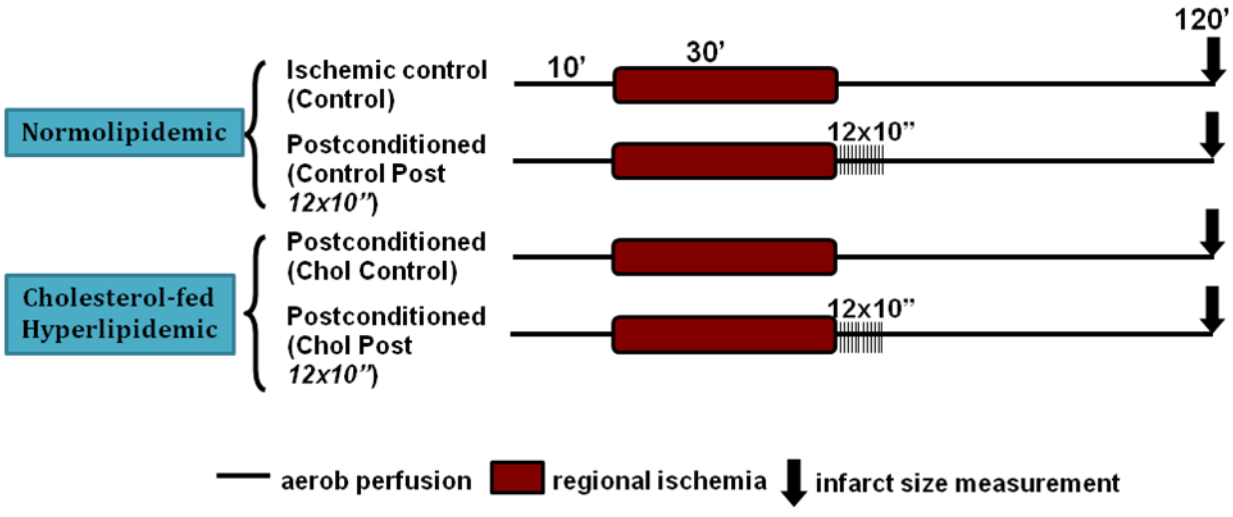

Fig 9: Experimental design for infarct size measurements after 12x10 s cycles postconditioning

9. Results

9.1. Study 1

9.1.1 Infarct size

In control hearts I/R resulted in extensive infarction. When preconditioning was applied before ischemia, infarct size significantly decreased showing the protective effect of preconditioning against acute I/R injury (Fig.10).

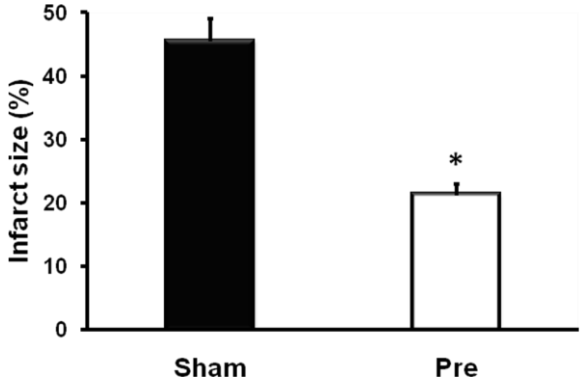

Figure 10. Infarct size in control and preconditioned hearts after 30 min ischemia followed by 120 min reperfusion. Values are mean±S.E.M. (n=8 in each group; *P<0.01 vs. control).

9.1.2 Myocardial NO metabolism

We have determined Ca^{2+}-dependent and Ca^{2+}-independent NOS activities and gene expression of NOS in rat myocardium and measured cardiac NO levels to investigate the role of NO formation during the development of ischemic late Pre. Ca^{2+}-dependent NOS activity and expression levels of endothelial and neuronal NOS remained unchanged (Fig. 11; Table 1.). However, Ca^{2+}-independent NOS activity increased gradually after the Pre protocol and at 12h the elevation reached the level of significance then further increased by 24h. iNOS mRNA content measured at 24h was also increased significantly as compared to the sham operated group, however, gene expression of other NOS isoforms remained unchanged (Fig. 11/; Table 1). Despite the increased iNOS expression and activity, NO signal did not change during the development of late Pre.

To test the contribution of iNOS to cardiac NO generation, cardiac NO after late Pre in the presence of 1400W, a selective iNOS inhibitor was measured in separate experiments. iNOS inhibition did not reduce the amount of cardiac NO in preconditioned hearts (Fig. 12).

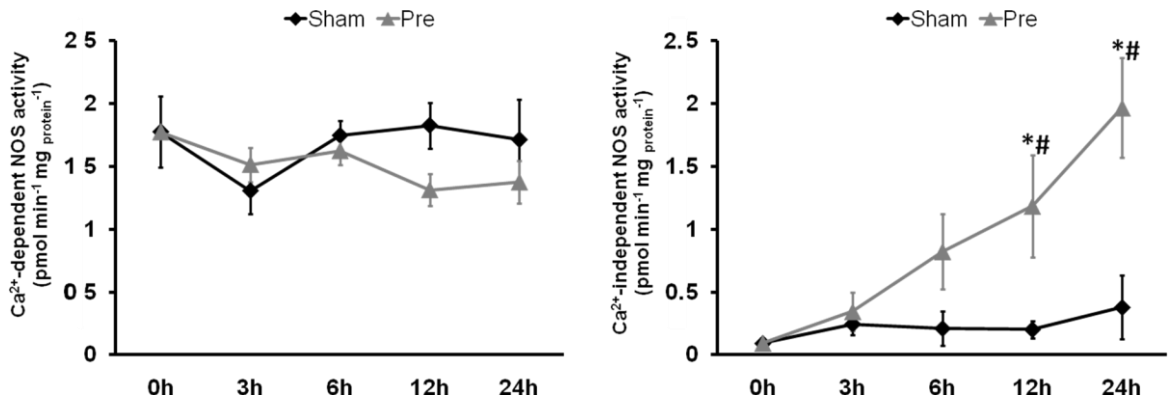

Figure 11. Myocardial activities of Ca^{2+}-dependent NOS and Ca^{2+}-independent NOS. Values are means ± S.E.M. (n=6 in each group, #P < 0.01 vs. sham group; *P <0.01 vs. 0h group).

Gene name	Acc. No	Fold Change in the PC Group Compared With the Sham-Operated Group by Real-Time PCR
Rat neural nitric oxide synthase (Nos 1)	**NM_052799**	**0.85±0.99**
Rat inducible nitric oxide synthase (Nos 2)	**NM_012611**	**1.71±0.91***
Rat endothelial nitric oxide synthase (Nos 3)	**NM_021838**	**0.50±1.01**
Rat matrix metalloproteinase 2	**NM_031054**	**-1.43±0.67#**
Rat matrix metalloproteinase 9	**NM_031055**	**1.34±0.68***
Rat tissue inhibitor of metalloproteinase 1	**NM_053819**	**0.42±1.70**
Rat tissue inhibitor of metalloproteinase 2	**BC084714**	**0.27±0.74**
Rat tissue inhibitor of metalloproteinase 3	**NM_012886**	**-2.08±0.72#**
Rat tissue inhibitor of metalloproteinase 4	**XM_001056766**	**-0.19±0.52**

Table 1. Values are means ± S.E.M. for fold changes in the 24h preconditioned group compared with the 24h sham-operated group (n=4 animals/group). NOS: nitric oxide synthase; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of metalloproteinase. *Significantly upregulated genes; #significantly repressed (downregulated) genes.

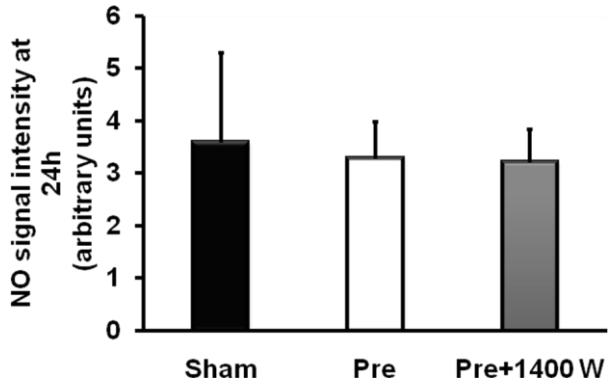

Figure 12. NO content of left ventricular tissue samples in sham operated, in preconditioned groups and in the presence of 1400W measured by electron spin resonance spectroscopy 24h after Pre protocol or sham operation. Values are mean±S.E.M. (n=5-6).

9.1.3 Myocardial superoxide metabolism

Xanthine oxidoreductase (XOR) and NADPH oxidase activities, the major enzymes producing superoxide in the rat heart, were measured to estimate enzymatic superoxide generation during late Pre. We observed a gradual increase of XOR and NADPH oxidase activities in both preconditioned and sham operated groups as compared to the 0h control group, however, there was no difference between Pre and sham operated groups (Fig. 13/A and B). Activity of total SOD, which is responsible for the elimination of superoxide, was measured by a photometric assay to determine the superoxide decomposition during the development of late Pre. A significant decrease of myocardial total SOD activity is shown in the Pre group 24h after the Pre protocol in Fig. 13/C, when compared to 0h control and the 24h sham operated group, respectively. We compared myocardial superoxide content in Pre and sham operated groups and found a significant elevation 24h after the Pre protocol, when measured cardiac superoxide by dihydroethidium staining (Fig. 13/D).

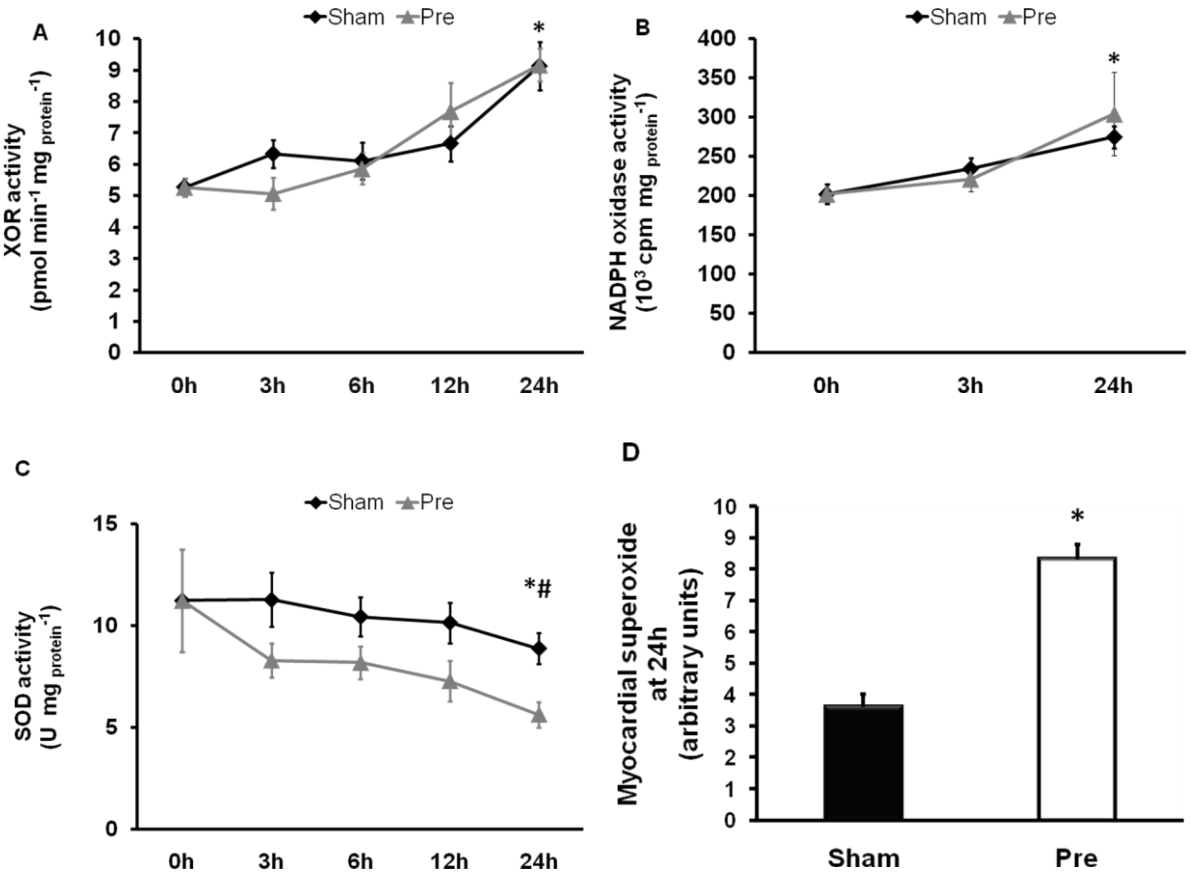

Figure 13. Effect of late Pre on cardiac activities of xanthine oxidoreductase (XOR, Panel A), NADPH oxidase (Panel B) and superoxide dismutase (SOD, Panel C). Values are mean ± S.E.M (n=6 in each group; *P < 0.05 vs. 0h; #P < 0.05 vs. sham 24h group). Panel D shows cardiac superoxide content 24h after the Pre vs. sham operated control. Values are mean ± S.E.M. (n=6 in each group; *P < 0.01 vs. sham group).

9.1.4 Myocardial $ONOO^-$ and activation of MMPs

We determined myocardial nitrotyrosine contents in the Pre and sham operated group 24h after the Pre protocol by ELISA to estimate $ONOO^-$ generation. $ONOO^-$ generation was attenuated significantly when compared to sham operated group 24h after the Pre (Fig. 14).

Zymography was performed to measure the changes in the activity of MMP-9 and MMP-2, characteristic MMPs of the myocardium. Both, MMP-9 (Fig. 15) and MMP-2 (Fig. 15) activities have shown a significant reduction 24h after the Pre protocol compared to the sham operated animals. Ischemic late Pre resulted in a decrease in the gene expression of MMP-2, however, the amount of MMP-9 mRNA was increased. Gene expression of tissue inhibitor of metalloproteinases TIMP1, TIMP2 and TIMP4 were not changed, however,

TIMP-3 mRNA showed a significant decrease in the Pre group, when compared to sham operated group (Table 1.).

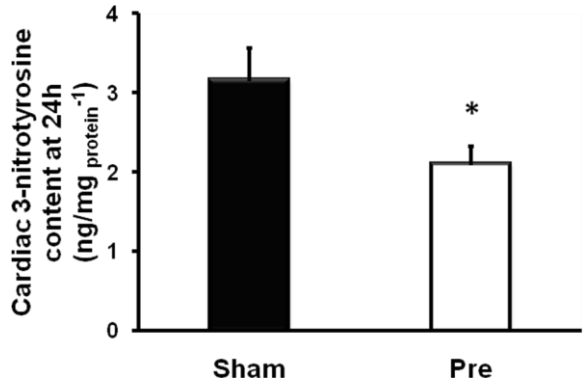

Figure 14. Cardiac nitrotyrosine level (marker for $ONOO^-$ measured by ELISA) in preconditioned and sham operated control 24h after PC. Values are mean ± S.E.M. (n=7-8; *P<0.05 vs. sham group).

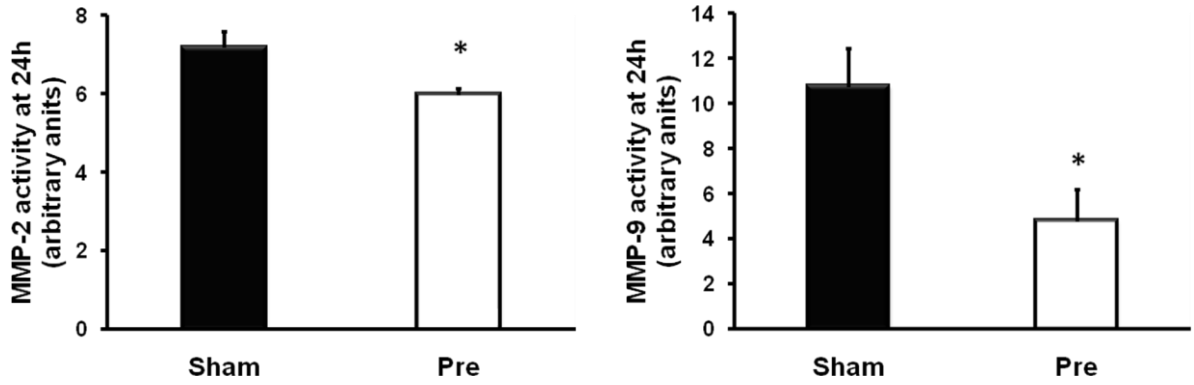

Figure 15. Myocardial activities of MMP-2 and MMP-9 in preconditioned and sham operated controls 24h after Pre protocol as measured by zymography. Values are mean ± S.E.M. (n=4 in each group; *P < 0.05 vs. sham group).

Ilomastat, a non-selective MMP inhibitor, was used to verify if Pre-induced inhibition of MMP activity has a causative role in the reduction of infarct size. Ilomastat (1.5 μmol kg^{-1}) given 5 min before ischemia followed by infusion during ischemia and the first 10 min of reperfusion to maintain plasma concentration, decreased infarct size significantly as compared to the vehicle treated animals (Fig. 16).

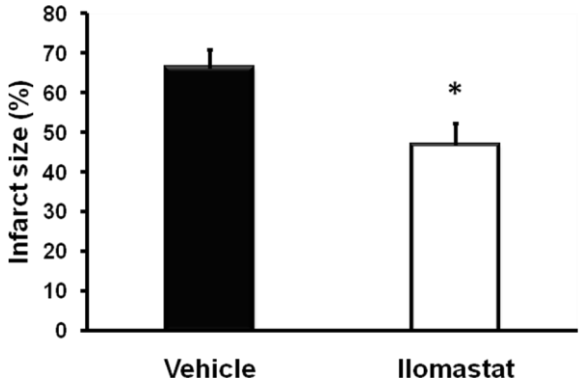

Figure 16. Effect of the non-selective MMP inhibitor ilomastat on infarct size. Values are mean ± S.E.M. (n=7-8; *P< 0.05 vs. vehicle).

9.2. Study 2

9.2.1 Influence of cholesterol-enriched diet on lipid profile

At the end of the 12-wk diet, the body weights of the animals were 400–580 g, and there was no significant difference between groups. In the cholesterol-fed group, serum cholesterol, triglyceride, and tissue dihomo-ɤ-linolenic acid (DHGLA) were increased significantly (Table 2).

	NORMOLIPIDEMIC	HYPERLIPIDEMIC
Serum cholesterol (mmol/L)	**2.57 ± 0.2**	**3.05 ± 0.10***
Serum triglyceride (mmol/L)	**1.47 ± 0.10**	**2.15 ± 0.20***
Myocardial tissue cholesterol (µg/mg)	**0.88 ± 0.02**	**0.93±0.1**
Myocardial free fatty acid content		
DHGLA (µg/g)	**4.13 ± 2.0**	**16.3 ± 4.1***
Palmitic acid (µg/g)	**178 ± 50**	**316 ± 50**
Stearic acid (µg/g)	**162 ± 30**	**206 ± 30**

Table 2. Values are means ±S.E.M. *P <0.05 vs. normolipidemic.

9.2.2 Infarct size measurements in the presence of 6x10 s cycles postconditioning

To examine if experimental hyperlipidemia interferes with the cardioprotective effect of postconditioning, infarct size and the incidence of reperfusion-induced VF were assessed in rat hearts with coronary occlusion in both normolipidemic and hyperlipidemic rats. Postconditioning with 6x10 s cycles significantly decreased infarct size and the incidence of VF in hearts of rats with normolipidemic diet (Fig. 17A and 17B).

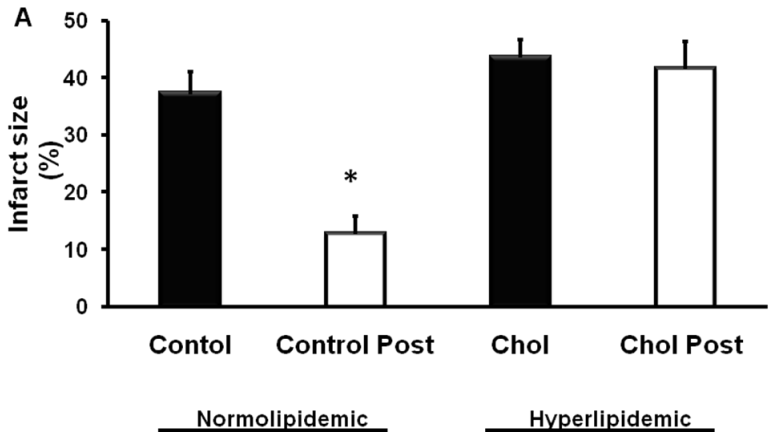

Figure 17/A. Effect of 6x10 s cycles postconditioning on infarct size after 30 min ischemia followed by 120 min reperfusion. Values are mean ± S.E.M. (n=7-10; *P< 0.05 vs. Control).

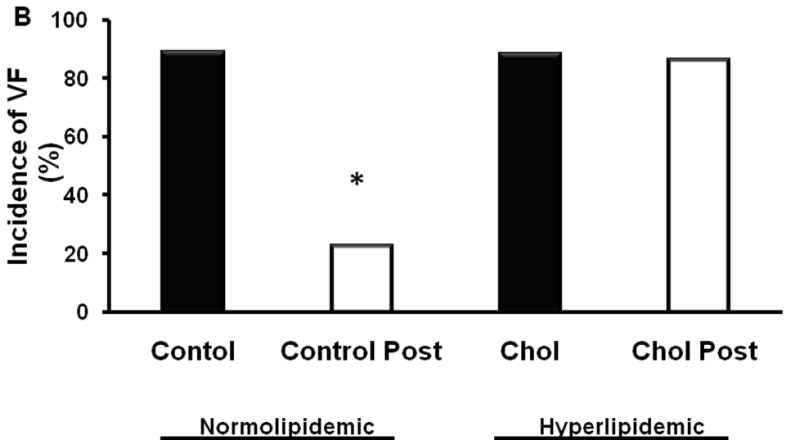

Figure 17/B. Ventricular fibrillation (VF) during the first 30 min of reperfusion in control (Control and Control Post) and cholesterol-fed (Chol and Chol Post) groups. (*P <0.05 vs. control; n=7–9 in each group).

9.2.3 Infarct size measurements in the presence of 12x10 s cycles postconditioning

To test if a more potent postconditioning stimulus could protect the hyperlipidemic heart, in a separate set of experiments, postconditioning with 12x10 s cycles of brief I/R was applied. However, this postconditioning protocol failed to reduce infarct size not only in hyperlipidemic but also in normolipidemic hearts.

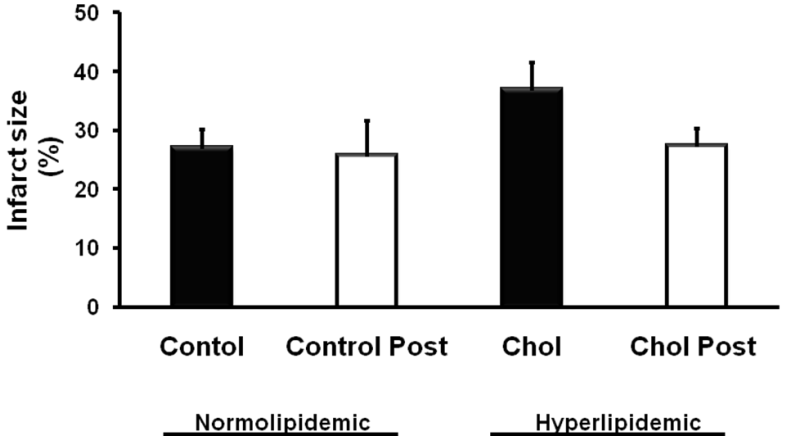

Figure 18. Myocardial injury after 30 min of regional ischemia and 120 min of reperfusion. Infarct size measured at the end of 120 min reperfusion with (Control Post) or without 12x10 s cycles of postconditioning (Control) in control chow-fed rats and cholesterol-fed animals (Chol Post and Chol; n=7–9 rats in each group).

9.2.4 Myocardial $ONOO^-$ content after postconditioning

To investigate the exact role of myocardial nitrosative stress at early reperfusion in normal and cholesterol-fed animals, we measured free and protein-bound 3-nitrotyrosine, a marker for $ONOO^-$ induced nitrosative stress, by ELISA and Western blot, respectively, in left ventricular tissue samples at 5 min of reperfusion after postconditioning (Fig. 8). Postconditioning significantly increased $ONOO^-$-induced nitrotyrosine formation in normolipidemic hearts, but not in hyperlipidemic conditions (Fig. 19).

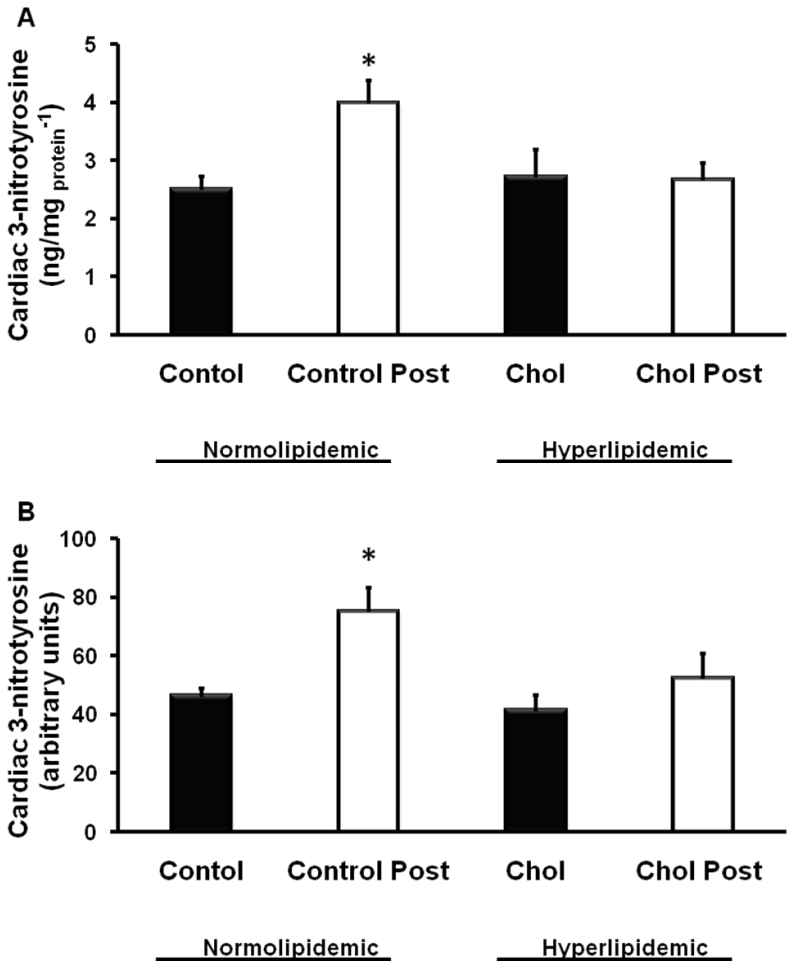

Figure 19. Cardiac 3-nitrotyrosine content, a marker of endogenous $ONOO^-$ induced nitrosative stress. Free 3-nitrotyrosine assayed by enzyme-linked immunosorbent assay (ELISA; *A*) and protein-bound 3-nitrotyrosine by Western blot (*B*) in control (Control and Control Post) and cholesterol-fed (Chol and Chol Post) groups. (*$P<0.05$ vs. control; n=10–13 in each group).

9.2.5 Infarct size in the presence of $ONOO^-$ decomposition catalyst FeTPPS

To test if the early increase in nitrosative stress signal observed in normolipidemic animals is a necessary trigger for the development of cardioprotection by postconditioning, in separate experiments, postconditioning was induced in the presence of a $ONOO^-$ decomposition catalyst FeTPPS in normolipidemic animals. Although FeTPPS did not affect infarct size significantly in the nonpostconditioned group, it abolished the infarct size-limiting effect of postconditioning (Fig. 20).

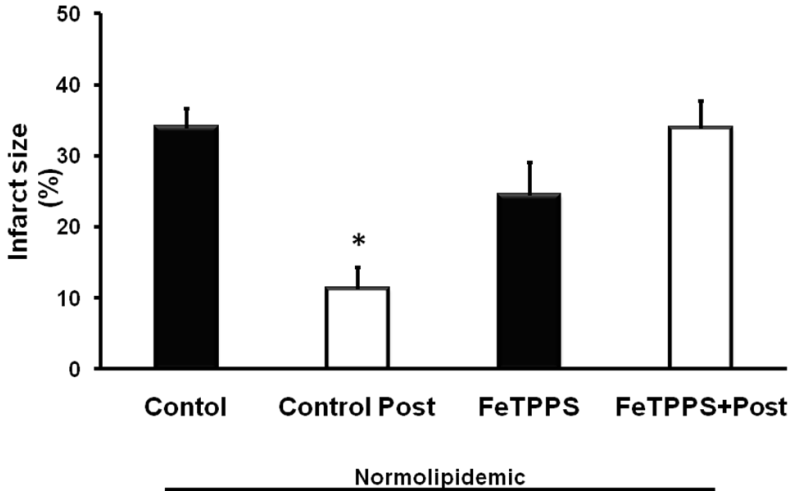

Figure 20. Myocardial injury at the end of 120 min reperfusion with (Control Post) or without 6x10 s cycles postconditioning (Control) in the presence or absence of 20 mg/l FeTPPS, a $ONOO^-$ decomposition catalyst. (*$P<0.05$ vs. control; n=6–9 in each group).

10. Discussion

New findings

Study 1

- Late Pre increased the expression and activity of iNOS but this change did not lead to increased cardiac NO formation
- Late Pre decreased $ONOO^-$ formation, but cardiac superoxide was increased.
- Pharmacological inhibition of MMP activity was able to reduce infarct size in a late Pre model of rats with coronary occlusion in vivo

Study 2

- The infarct size-limiting effect of postconditioning was lost in hearts of hyperlipidemic rats
- Cardiac nitrotyrosine content was increased during early reperfusion after postconditioning, which was not seen in hyperlipidemic hearts
- Postconditioning in the presence of the $ONOO^-$ decomposition catalyst FeTPPS failed to reduce infarct size in normal hearts

10.1 Study 1

10.1.1 The role of iNOS and NO in late preconditioning

We have shown in *Study 1* that late Pre markedly reduced infarct size 24h after the Pre stimulus. We have confirmed that the cardioprotective effect of ischemic late Pre was accompanied by the induction of iNOS as described previously by several studies (Ferdinandy & Schulz, 2003;Bolli et al., 1998). In our present study, surprisingly, iNOS activation did not lead to increased NO level in the myocardium as measured by electron spin resonance spectroscopy 24 h following the Pre stimulus. These findings show that the role of iNOS in late Pre may be not related to increased NO production. This is further supported by the finding that 1400W, a selective iNOS inhibitor, did not decrease cardiac NO content at late Pre in our present study.

10.1.2 The role of $ONOO^-$ and superoxide metabolism in late preconditioning

Many of the physiologic actions of NO are mediated through $ONOO^-$, the reaction product of NO and superoxide (Ferdinandy, 2006). Therefore, we examined myocardial levels of superoxide and $ONOO^-$ as well as the expression and activity of the enzymes involved in superoxide production and its elimination. We have found here a markedly increased superoxide level in the myocardium 24 h after Pre as compared to the non-preconditioned group. Neither XOR activity nor NADPH oxidase activities were changed, however, SOD activity was decreased 24 h after the Pre stimulus as compared to the non-preconditioned controls. These results show that the source of increased myocardial superoxide can be a decreased activity of SOD. Moreover, it is well known that iNOS is able to produce superoxide in certain circumstances e.g. absence of tetrahydrobiopterin and/or L-arginine and altered ratio of reactive oxygen and nitrogen species (Sun et al., 2010). Therefore, since NO production was not increased in the present study, increased iNOS activity could be the source of increased superoxide level during late Pre. We have previously shown that $ONOO^-$ may trigger classic ischemic Pre and subsequent cycles of I/R result in a gradual decrease in $ONOO^-$ formation (Csonka et al., 2001). In *Study 1* we measured cardiac nitrotyrosine levels as a marker of cardiac $ONOO^-$ generation and showed for the first time that late Pre significantly diminishes myocardial $ONOO^-$ formation.

10.1.3 The role of $ONOO^-$-MMP signalling in late preconditioning

It is known that $ONOO^-$ at low concentrations contributes to the beneficial effects of preconditioning on ischemia in isolated rat hearts (Csonka et al., 2001). However, no attempt has been made so far to measure cardiac $ONOO^-$ during late Pre, although $ONOO^-$ was suspected to be a trigger of late Pre in conscious rabbits (Tang et al., 2002). In *Study 1* we measured cardiac nitrotyrosine levels as a marker of cardiac $ONOO^-$ generation and showed that late Pre diminishes myocardial $ONOO^-$ significantly.

It has been described that $ONOO^-$ activates MMPs, which may play a role in a variety of cardiovascular pathologies including I/R injury (Viappiani et al., 2009). Moreover, acute treatment with MMP-inhibitors is able to attenuate I/R injury and decrease myocardial infarct size and acute heart failure induced by pro-inflammatory cytokines in rats (Dorman et al., 2010). To study if late Pre induced MMP inhibition has a causative role in its infarct size reduction, in separate experiments, rats were treated with a non-selective MMP inhibitor ilomastat during coronary occlusion and reperfusion. We have shown that pharmacological

inhibition of MMP also leads to the reduction of infarct size in the rat infarction model *in vivo*. To study if increased TIMPs inhibit MMPs, we measured gene expression of 4 isoforms of TIMPs. As gene expression of TIMP1, TIMP2, and TIMP4 were not changed and that of TIMP3 was decreased, MMP inhibition by altered TIMP levels is unlikely, nevertheless, the role of TIMPs in late Pre cannot be excluded. Therefore, it is plausible to speculate that inhibition of MMP activity is due to a decreased activation of MMPs by Pre-induced decrease in $ONOO^-$. These results show that late Pre-induced inhibition of $ONOO^-$–MMP signalling is involved in the cardioprotective effect of late Pre, and shows that pharmacological inhibition of MMPs is able to reduce infarct size *in vivo*.

10.1.4 Summary of Study 1

Taken together, here we have shown that late Pre decreases cardiac $ONOO^-$ level and MMP activity and that pharmacological inhibition of MMP activity is able to reduce infarct size. This is the first demonstration that late Pre-induced inhibition of $ONOO^-$–MMP signalling is involved in cardioprotection by late Pre and that pharmacological MMP inhibition is able to reduce infarct size *in vivo*. Furthermore, we have shown here that late Pre is associated with increased iNOS and decreased SOD activity, which is accompanied by increased cardiac superoxide levels, but no change in NO levels. One may speculate that iNOS induced superoxide formation also plays a role in the mechanism of late Pre.

10.2 Study 2

10.2.1 Infarct size limiting effect of postconditioning

Numerous systemic diseases such as hyperglycemia, hypercholesterolemia, hypertension, atherosclerosis, heart failure as well as aging have been suggested to modify the pre-conditioning response (Ferdinandy et al., 2007). In *Study 2* we tested the infarct size limiting effect of postconditioning in normal and hyperlipidemic hearts *ex vivo*. We have found that 6x10 s cycles were effective in normal hearts but ineffective in hyperlipidemic rats, in reducing infarct size, so we selected a more potent stimulus involving 12 cycles of 10 sec I/R. However, this potent stimulus was also ineffective in our hyperlipidemic rats. Interestingly, postconditioning was not achieved in normolipidemic hearts either by 12x10 s cycles I/R. These findings confirms that an appropriate postconditioning algoritm is crucial for cardioprotection (Vinten-Johansen et al., 2005). Our present results confirm that of Iliodromitis et al. (Iliodromitis et al., 2006) showing that the infarct size-limiting effect of postconditioning is lost in rabbits with experimental hyperlipidemia and atherosclerosis.

Another study by Zhao et al. (Iliodromitis et al., 2006;Zhao et al., 2007) showed in minipigs that postconditioning under normolipidemic condition can reduce the area of no-reflow and necrosis area, while postconditioning under hypercholesterolemic condition cannot.

10.2.2 Role of $ONOO^-$ in postconditioning

The role of reactive oxygen species (ROS), including $ONOO^-$ in cardioprotection is still not clear. Furthermore, little is known about the balance between the detrimental and protective effects of $ONOO^-$ (Zhao et al., 2007;Iliodromitis et al., 2006;Ferdinandy & Schulz, 2003;Ferdinandy, 2006;Penna et al., 2009).

Our present results show that postconditioning increases formation of cardiac 3-nitrotyrosine, a marker for $ONOO^-$-induced nitrosative stress, at early reperfusion; however, the increased $ONOO^-$ formation was not observed in hyperlipidemic hearts subjected to postconditioning. In hyperlipidemic animals, postconditioning was ineffective and the early increase in $ONOO^-$ after postconditioning was not present, we conclude that the lack of a nitrosative trigger signal may be involved in the loss of postconditioning in hyperlipidemia.

Because $ONOO^-$ has been previously shown to be involved in the trigger mechanism of the cardioprotective effect of preconditioning (Altug et al., 2000;Csonka et al., 2001;Ferdinandy et al., 2007) it is plausible that $ONOO^-$ may also be involved in the triggering mechanism of postconditioning. To test this hypothesis, in normal animals, postconditioning was performed in the presence of a $ONOO^-$ decomposition catalyst FeTPPS at a dose that significantly reduced $ONOO^-$-induced myocardial effects in different models (Ferdinandy et al., 2007;Ferdinandy et al., 2000;Misko et al., 1998). We have found that the infarct size-limiting effect of postconditioning was abolished in the presence of FeTPPS, which shows for the first time that increased nitrosative stress at early reperfusion after postconditioning is necessary to trigger its cardioprotective effect. This is another important example of the physiological regulatory role of mild nitrosative stress (Ferdinandy et al., 2000;Pacher et al., 2007). Moreover, recent studies suggest that some ROS species at low concentrations could protect ischemic hearts (Penna et al., 2009;Penna et al., 2006). ROS scavengers N-acetyl-L-cysteine or mercaptopropionyl glycine given at the beginning of reperfusion abolished postconditioning induced protection (Tsutsumi et al., 2007). Nossuli et al. (Nossuli et al., 1997) showed that, in a feline model of coronary occlusion/reperfusion, intraventricular infusion of authentic $ONOO^-$ (1 μmol/L) 10 min before reperfusion was associated with a reduction in infarct size. Furthermore, it has been shown that $ONOO^-$

regulates mitogen-activated protein kinases, which are also involved in the mechanism of postconditioning (Pesse et al., 2005).

10.2.3 Summary of Study 2

In conclusion, this is the first demonstration that nitrosative stress is involved in the triggering mechanism of postconditioning and that hyperlipidemia leads to the loss of the cardioprotective effect of postconditioning, at least in part via deterioration of the nitrosative trigger. Furthermore, we emphasize the importance of preclinical studies that examine cardioprotective mechanisms specifically in relation to complicating disease states such as hyperlipidemia. This is necessary to maximize the likelihood of identifying rational approaches to therapeutic protection of the ischemic heart in the presence of risk factors.

11. Limitations of the studies

In *Study 1* we measured cardiac NO directly with electron spin resonance spectroscopy. Quantitative measurement of cardiac NO *in vivo* is not possible due to several technical difficulties. In *Study 2*, although FeTPPS is thought to be specific to $ONOO^-$, it cannot be excluded that it reacts with nonperoxynitrite species as well. Because of technical limitations to measure local concentrations and cellular sources of $ONOO^-$, nitrosative stress in the coronary endothelium, endocardial endothelium, cardiac nerves, fibroblasts, and cardiac myocytes could all contribute to changes in nitrotyrosine levels and to the cardioprotective effect of postconditioning. Furthermore, we cannot exclude that nitrotyrosine can be formed by $ONOO^-$-independent pathways as well, for example, via the actions of peroxidases in the presence of nitrite. The stability of the preparation upon long-term perfusion is a general concern in isolated heart preparations. Here we used a Langendorff preparation with no left ventricular balloon to unload the heart from ‘afterload pressure’ and to maintain optimal coronary perfusion throughout the perfusion protocol.

12. Conclusions

In conclusion ONOO plays a dual role, acting as a signalling mediator of cardioprotection during postconditioning, and as a critical determinant of cell death in late preconditioning.

Our results clearly show that late preconditioning reduces infarct size via a mechanism that may involve decreased activity of MMPs due to decreased $ONOO^-$ formation. Late Pre is associated with with increased iNOS and decreased SOD activity which is accompanied by increased cardiac superoxide levels, but no change in NO levels.

Furthermore, we provided evidence that hyperlipidemia blocks the cardioprotective effect of postconditioning. We have found that postconditioning increased cardiac nitrotyrosine concentration. In the presence of FeTPPS postconditioning failed to decrease infarct size. We conclude that $ONOO^-$ formation during postconditioning is involved in the triggering mechanism of cardioprotection induced by postconditioning.

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