CARDIAC STRESS AND STRESS ADAPTATION: ROLES OF PEROXYNITRITE AND CHANGES IN GENE EXPRESSION

Ph.D. Thesis

Annamária Ónody, MSc

Cardiovascular Research Group
Department of Biochemistry
University of Szeged
www.cardiovasc.com

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Contents:

1. Abbreviations

$ONOO^-$	-	Peroxynitrite
FeTPPS	-	5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]
NO	-	Nitric oxide
$O_2^{\cdot-}$	-	Superoxide
cGMP	-	Cyclic guanosine-monophosphate
NOS	-	Nitric oxide synthase
XOR	-	Xanthine oxidoreductase
SOD	-	Superoxide dismutase
GSH	-	Glutathione
LDH	-	Lactate dehydrogenase
LVEDP	-	Left ventricular end-diastolic pressure
MGD	-	N-methyl-D-glucamine-dithiocarbamate
L-NMMA	-	N^G-monomethyl-L-arginine
MnTBAP	-	[5,10,15,20-tetrakis(4-carboxyphenyl)-porphyrinato]manganese(III) chloride
SSC	-	Saline sodium citrate
SDS	-	Sodium dodecyl sulfate
BSA	-	Bovine serum albumin
PPARγ	-	Peroxisome proliferator activator receptor
DMSO	-	Dimethylsulfoxide

2. List of publications

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

I. Csonka C, Csont T, **Ónody A**, Ferdinandy P. Preconditioning decreases ischemia/reperfusion-induced peroxynitrite formation. *Biochem Biophys Res Commun.* 285(5):1217-1219 (2001). [IF: 2.935]

II. **Ónody A**, Zvara A, Hackler L Jr, Vigh L, Ferdinandy P, Puskas LG. Effect of classic preconditioning on the gene expression pattern of rat hearts: a DNA microarray study. *FEBS Lett.* 536(1-3):35-40 (2003). [IF: 3.912]

III. **Ónody A**, Csonka C, Giricz Z, Ferdinandy P. Hyperlipidemia induced by a cholesterol-rich diet leads to enhanced peroxynitrite formation in rat hearts. *Cardiovasc Res.* 58(3):663-70 (2003). [IF: 4.692]

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

IV. Giricz Z, Csonka C, **Ónody A**, Csont T, Ferdinandy P. Role of cholesterol-enriched diet and the mevalonate pathway in cardiac nitric oxide synthesis. *Basic Res Cardiol.* 98(5):304-10 (2003). [IF:1.994]

V. Csonka C, **Ónody A**, Csont T, Ferdinandy P. Defibrillatory action of glibenclamide is independent from ATP-sensitive K^+-channels and free radicals. *J Cardiovasc Pharmacol.* 41(6):916-922 (2003). [IF:1.602]

VI. D'Souza SP, Yellon DM, Martin C, Schulz R, Heusch G, **Ónody A**, Ferdinandy P, Baxter GF. B-type natriuretic peptide limits infarct size in rat isolated hearts via K^+-ATP channel opening. *Am J Physiol Heart Circ Physiol.* 284(5):H1592-H1600 (2003). [IF:3.369]

VII. Csont T, Csonka C, **Ónody A**, Görbe A, Dux L, Schulz R, Baxter GF, Ferdinandy P. Nitrate tolerance does not increase production of peroxynitrite in the heart *Am J Physiol Heart Circ Physiol.* 283(1):H69-H76 (2002). [IF:3.369]

3. Summary

Ischemic preconditioning is a well-described adaptive response in which brief exposure to ischemia markedly enhances the ability of the heart to withstand a subsequent ischemic injury. The exact molecular mechanisms of ischemic preconditioning are very complex, and still a question of debate. Among several other mediators, nitric oxide and superoxide have been suggested to be as key triggers and mediators of preconditioning. However, classical preconditioning may be a 'healthy-heart phenomenon', since several risk factors i.e. hyperlipidemia lead to the loss of preconditioning due to the impairment of cardiac nitric oxide synthesis. Therefore, the present study was devoted to examining the role of peroxynitrite ($ONOO^-$), the reaction product of nitric oxide and superoxide, in the mechanism of ischemic preconditioning and in hyperlipidemia. In addition, we attempted to identify genes and proteins that are possibly involved in the molecular background of preconditioning.

First, we studied the effect of preconditioning and subsequent ischemia/reperfusion on myocardial $ONOO^-$ formation in isolated rat hearts. Hearts were subjected to either a test ischemia/reperfusion protocol or a preconditioning protocol followed by a test ischemia/reperfusion. Generation of free 3-nitrotyrosine, as a marker for endogenous cardiac $ONOO^-$ formation, was measured during preconditioning induced by three brief cycles of ischemia/reperfusion and also during subsequent test ischemia/reperfusion in isolated working heart. Preconditioning significantly decreased test ischemia/reperfusion-induced 3-nitrotyrosine formation and improved postischemic cardiac performance when compared to non-preconditioned hearts. During preconditioning, however, the first period of short ischemia/reperfusion markedly increased 3-nitrotyrosine formation, which was reduced after subsequent cycles of brief ischemia/reperfusion. These results show that classic preconditioning inhibits ischemia/reperfusion-induced endogenous formation of $ONOO^-$ in rat hearts and that subsequent periods of ischemia/reperfusion result in a gradual attenuation of reperfusion-induced $ONOO^-$ generation. This mechanism might be involved in ischemic adaptation of the heart.

In the hope of identifying new cellular pathways involved in cardiac ischemia and ischemic adaptation, we monitored global gene expression changes by DNA microarray analysis

of 3200 rat specific genes, and by real-time quantitative PCR in rat hearts. Genes with altered expression due to ischemia and/or preconditioning included metabolic enzymes, regulatory proteins and others controlling protein degradation, stress responses, apoptosis, and several ones with unknown cellular functions. Some genes were observed to change specifically in response to preconditioning: the genes of oligoadenylate synthase, chaperonin subunit epsilon, a cGMP phosphodiesterase (PDE9A1), a secretory carrier membrane protein, an amino acid transporter, and protease 28 subunit.

In separate studies, we studied the direct effect of hyperlipidemia on $ONOO^-$ formation in myocardium. Rats were fed cholesterol-enriched diet or normal diet for 8 weeks, and the hearts were perfused in isolated working mode. Cholesterol-enriched diet resulted in a decrease of cardiac nitric oxide level, an increase of cardiac formation of superoxide and their reaction product $ONOO^-$. Dityrosine in the perfusate, a marker of cardiac $ONOO^-$ formation, and plasma 3-nitrotyrosine, a marker for systemic $ONOO^-$ formation, were both elevated in hyperlipidemic rats. In cholesterol-fed rats, left ventricular end-diastolic pressure (LVEDP) was significantly elevated as compared to controls. Administration of FeTPPS, a $ONOO^-$ decomposition catalyst, normalized LVEDP in the cholesterol-fed group.

Our results show that (i) $ONOO^-$ formation during ischemia/reperfusion might act as a trigger for cardiac preconditioning, however, preconditioning in turn decreases formation of $ONOO^-$ upon subsequent cycle(s) of ischemia/reperfusion; (ii) there are a number of genes the expression of which is significantly altered due to ischemia and/or preconditioning in the heart; (iii) sustained exposure to dietary cholesterol leads to an increase in cardiac $ONOO^-$ formation which can be associated with the deterioration of cardiac function and that of the endogenous adaptive mechanisms.

4. Introduction

4.1. Myocardial ischemia/reperfusion

Ischemic heart disease, a major cause of mortality in industrialized societies, is characterized by insufficient blood supply to regions of the myocardium which leads to tissue necrosis (infarction). Ischemic heart disease may develop as a consequence of hypertension, atherosclerosis, hyperlipidemia, and diabetes. Although reperfusion of ischemic myocardium is the definitive treatment to attenuate myocardial injury, reperfusion itself causes additional tissue damage mediated by an inflammatory-like response and other factors (calcium overload), which may lead to further complications such as diminished cardiac contractile function and arrhythmias (see for review: Braunwald, 1985). Therefore, development of cardioprotective agents to improve myocardial function, to decrease the incidence of arrhythmias, to lessen the necrotic tissue mass, and to delay the onset of necrosis during ischemia/reperfusion is of great clinical importance. Previous attempts to attenuate the consequences of ischemia/reperfusion injury with pharmacological tools have been largely unsuccessful. However, the heart was found to be able to adapt to ischemic stress (Murry *et al.,* 1986).

4.2. Myocardial adaptation to ischemia: preconditioning

Preconditioning confers a remarkable cardioprotection in a variety of species including humans (see for review: Przyklenk and Kloner, 1998). Preconditioning can be elicited by different sublethal stress signals, such as brief periods of ischemia, hypoxia, rapid electrical pacing, heat stress, or administration of bacterial endotoxin, etc. Ischemic preconditioning is a well-described adaptive response in which brief exposure to ischemia markedly enhances the ability of the heart to withstand a subsequent ischemic injury (see for review: Przyklenk and Kloner, 1998), although the cardioprotective effectiveness of ischemic preconditioning might be attenuated in the heart during aging and some disease states such as hyperlipidemia and diabetes (see for review: Ferdinandy *et al.,* 1998).

The cardioprotective effect of preconditioning shows two distinct phases. The early phase

("classic preconditioning") is manifested within min after the preconditioning stimulus and has a duration of less than 3 h. The late phase (second window of protection) is characterized by a slower onset (20 h) and a duration of up to 72 h. Both phases of preconditioning involve reduction of necrotic tissue mass (infarct size), improvement of cardiac performance and reduction of arrhythmias following ischemia/reperfusion (see for review: Przyklenk and Kloner, 1998; Baxter and Ferdinandy, 2001; Ferdinandy *et al.*, 1998).

Among several other mediators, NO, oxygen free radicals, and antioxidant enzymes have been suggested to be, and also refuted as key triggers and mediators of preconditioning. NO has been also suggested to play a role in preconditioning, such as adenosine, bradykinin, and opioids (see for review: Bolli, 2001). There is still a considerable debate regarding the exact cellular mechanism of ischemic preconditioning (see for review: Schulz *et al.*, 2001). Further discrepancies are generally attributed to species differences, different stimuli to induce preconditioning, and different study end-points, i.e. myocardial function, arrhythmias, or infarct size. Understanding the cellular pathways involved in the ischemic adaptation of the myocardium may lead to the development of "preconditioning mimetic" drugs for patients suffering from ischemic heart disease.

4.3. Peroxynitrite

In 1990, Beckman *et al.* described the generation of potentially toxic nitric oxide (NO) metabolite, peroxynitrite ($ONOO^-$), formed in the diffusion-limited biradical reaction between nitric oxide (NO) and superoxide ($O_2^{\cdot-}$). This led to an explosion of research into both the chemical and physiologic actions of $ONOO^-$, and the relationship of $ONOO^-$ to the well documented biological effects of both NO and $O_2^{\cdot-}$ (Lopez *et al.*, 1997; Ma *et al.*, 1997; Liu *et al.*, 1997; Xia *et al.*, 1996). With the increased interest in using NO therapy to treat a number of human diseases, the understanding of the physiological properties unique to $ONOO^-$ as an important NO metabolite is of crucial importance.

4.4. Production of $ONOO^-$

NO is necessary for normal cardiac physiology. NO plays an important physiological role in the regulation of cardiac function by initiating coronary vasodilation, inhibiting platelet and neutrophil actions, antioxidant effects, modulation of cardiac muscle contractile function, and by inhibiting oxygen consumption (Xie and Wolin, 1996). In physiological conditions, NO is generated by isoforms of NO synthase (NOS) which catalyzes the conversion of L-arginine to NO and L-citrulline (see for review: Moncada *et al.*, 1991). NOS belongs to a family of P-450 cytochrome oxidoreductase enzymes (see for review: de Belder and Randomski, 1994). In the heart, NO is generated by Ca^{2+}-dependent NO synthases in cardiac myocytes, vascular and endocardial endothelium (NOS III, endothelial NOS (Moncada *et al.*, 1997) as well as in specific cardiac neurons (NOS I, neuronal NOS). Under pathological conditions (e.g. during inflammation), temporary high level of NO is produced after the expression of an Ca^{2+}-independent, inducible NO synthase (NOS II, inducible NOS).

The regulation of basal NO release is not precisely known, pulsative flow and shear stress may provide the stimulus for NO synthase activation (de Belder and Radomski, 1994). Myocardial ischemia leads to increased activity of Ca^{2+}-dependent NOS (Depre *et al.*, 1997) and to accumulation of NO which might contribute to ischemia/reperfusion injury (Zweier *et al.*, 1995; Schulz and Wambolt, 1995; Yasmin *et al.*, 1997).

However, it is known that NO can also contribute to cardiac pathologies when formed in excess quantities or when synthetized in the presence of other oxidants (see for reviews: Christopherson and Bredt, 1997; Fleming and Busse, 1999). Nevertheless, many of the toxic actions of NO are not directly due to NO itself, but are mediated via production of $ONOO^-$, the reaction product of NO with $O_2^{\cdot-}$ (Fig.1) (Beckman and Koppenol, 1996; Rubbo *et al.*, 1996).

Possible sources of $O_2^{\cdot-}$ in the heart include NAD(P)H oxidoreductases (Mohazzab *et al.*, 1997), xanthine oxidoreductases (Hille and Nishino, 1995), mitochondrial electron transport chain activity, respiratory burst of activated neutrophils, arachidonic acid metabolism, and autooxidation of certain tissue metabolites.

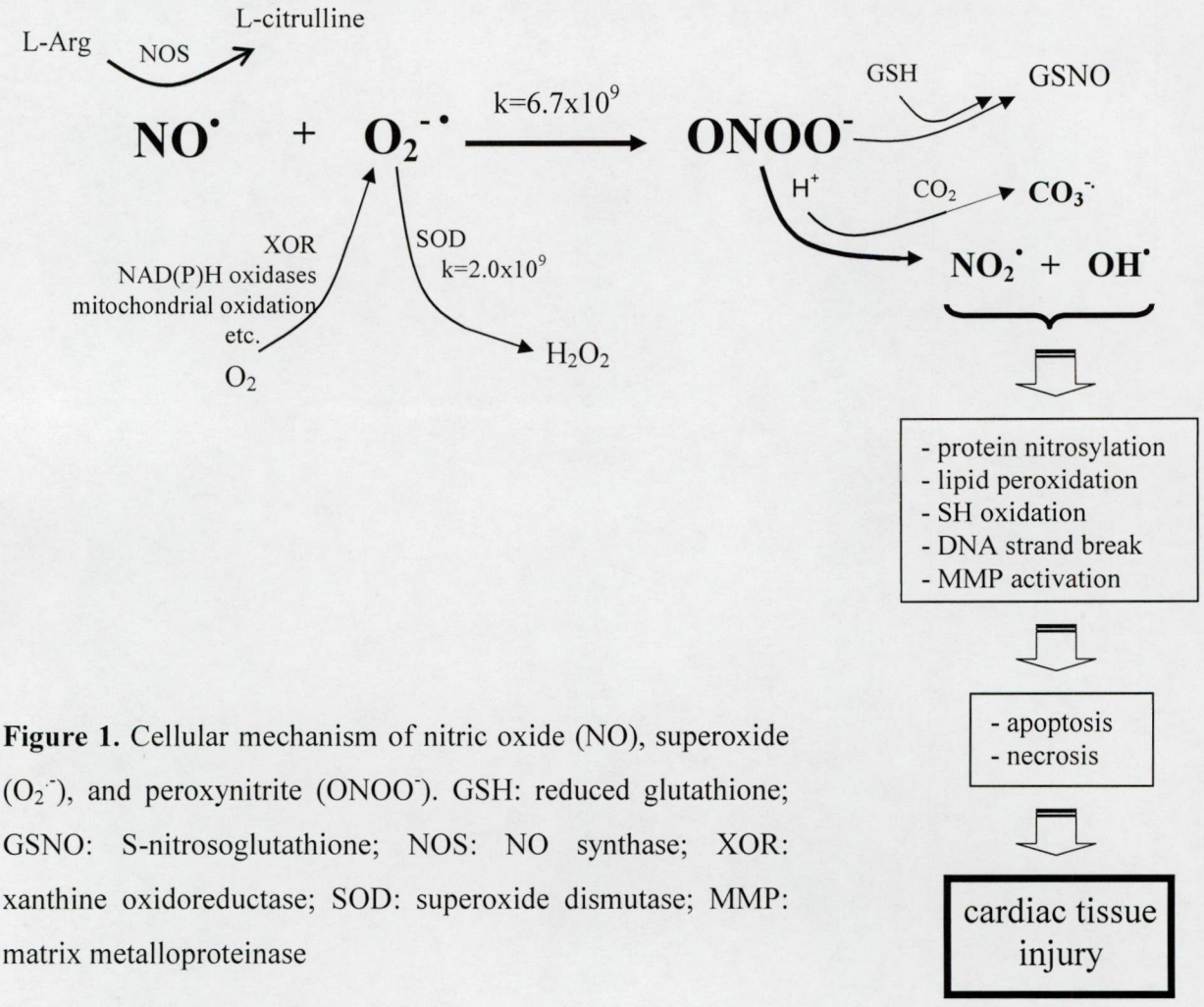


Figure 1. Cellular mechanism of nitric oxide (NO), superoxide (O_2^{-}), and peroxynitrite ($ONOO^-$). GSH: reduced glutathione; GSNO: S-nitrosoglutathione; NOS: NO synthase; XOR: xanthine oxidoreductase; SOD: superoxide dismutase; MMP: matrix metalloproteinase

NO and O_2^{-} combine at a reaction rate which is only limited by diffusion to form $ONOO^-$. $ONOO^-$ at pH<8 is protonated to form the unstable intermediate, peroxynitrous acid, which spontaneously decomposes to yield highly reactive oxidant species such as nitrogen dioxide radical ($NO_2^{\cdot}$) and hydroxyl radical (Fig.1). Understanding the balance between local concentrations of NO, O_2^{-}, and SOD is critical in understanding NO biology and its potential toxicity in the form of $ONOO^-$ (Beckman and Koppenol, 1996; Rubbo *et al.*,1996). One must consider the competition between NO and SOD for O_2^{-}. Under normal physiological conditions in vascular endothelium, [NO] is ~10 nM, [SOD] is ~1 μM. Therefore, the reaction rate of O_2^{-} to form $ONOO^-$ is only a fraction of the dismutation rate of O_2^{-} by SOD, and as a result, very little $ONOO^-$ is formed. However, at maximal vascular rates of NO production (i.e. which may occur

during acute reperfusion of ischemic tissue or during inducible NOS expression), [NO] is 1 μM. Therefore, the formation of $ONOO^-$ will predominate over the dismutation of $O_2^{\cdot-}$.

The antioxidants of the heart are crucial for maintenance of normal cardiac mechanical function. Unchecked, the highly oxidative heart muscle can potentially be subjected to its own basal production of $O_2^{\cdot-}$ (Boveris and Chance, 1973), and NO (Hare and Colucci, 1995). Mitochondrial MnSOD, cytosolic Cu-Zn SOD, extracellular Cu-Zn SOD, glutathione (GSH), uric acid, and catalase are the most important endogenous antioxidants opposing oxidative stress. A variety of myocardial insults reduce GSH level, whereas its oxidized form is increased (Janssen *et al.*, 1993). Hearts with enhanced endogenous GSH levels are less susceptible to ischemia/reperfusion injury (Kirschenbaum and Singal, 1993) and micromolar concentrations of GSH added to the perfusate protect isolated hearts from stunning injury (Cheung *et al.*, 2000).

4.5. Cellular targets of $ONOO^-$

The possible downstream targets of $ONOO^-$ which mediate its toxicity are several (Fig.1). Its highly reactive decomposition products at physiological or acidic pH can attack proteins (oxidation of sulfhydryls, nitration of tyrosine residues), lipids (formation of lipid peroxides), and DNA (strand breakage). These result in the depletion of antioxidants such as glutathione and most often in the inhibition of several enzymes including superoxide dismutase, glutathione peroxidase, aconitase and other enzymes of the mitochondrial respiratory chain, creatine kinase, Ca^{2+}-ATPase, Na^+-K^+-ATPase, prostacyclin synthase, α-antiproteinase and many others, just to name a few (see for reviews: Beckman and Koppenol, 1996; Ronson *et al.*, 1999; Rubbo *et al.*, 1996; Szabó, 1996a). As a result of DNA strand breakage, activation of the NAD^+ consuming DNA repair enzyme poly-ADP ribose synthase contributes further to the depletion of cellular energy stores (Szabó *et al.*, 1996b). $ONOO^-$ has been also shown to cause irreversible inhibition of the mitochondrial respiratory chain (Brown, 2001; Xie and Wolin, 1996) and to trigger apoptosis of cardiac myocytes (Arstall *et al.*, 1999; Beckman, 1999).

4.6. Pathophysiology of $ONOO^-$ in myocardial ischemia/reperfusion

Yasmin *et al.*, (1997) showed that $ONOO^-$ is produced during the acute reperfusion of ischemic hearts and that drugs which inhibit $ONOO^-$ formation or antagonize its toxicity protect

the heart from this injury. There is a novel, simple and effective methodology to detect $ONOO^-$ based on the reaction of $ONOO^-$ with tyrosine to form dityrosine (Ferdinandy and Schulz, 2001a). Rapid generation of $ONOO^-$ during reperfusion of the ischemic heart has also been detected using luminol chemiluminescence in the perfusate and anti-nitrotyrosine labelling of myocardial proteins (Wang and Zweier, 1996). Yasmin *et al.,* (1997) showed that low concentrations of the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA), or a cell permeable SOD mimetic, MnTBAP protected the hearts from ischemia/reperfusion injury. The beneficial effect of L-NMMA fell within a narrow range of concentrations and was lost at higher concentrations which further reduced coronary flow. Their data also showed that a NO donor, at subvasodilatory concentration, protected hearts from endogenous $ONOO^-$-mediated injury. Their study provided the first mechanistic evidence of how either NO donors or NOS inhibitors reduce ischemia/reperfusion injury.

4.7. Hyperlipidemia

High-cholesterol diet is regarded as an important factor in the development of cardiac diseases since it leads to development of hyperlipidemia, atherosclerosis, and ischemic heart disease. The heart of hyperlipidemic/atherosclerotic patients adapts poorly to oxidative or other kinds of stress, suggesting that the endogenous adaptive mechanisms are impaired (Roberts, 1995). The focus of research so far has been mainly on the coronary effects of cholesterol, i.e. coronary sclerosis, and the possible direct effect of hypercholesterolemia on the heart was neglected. Very few studies looked at the cellular effects of cholesterol-enriched diet on the myocardium, however, intracellular lipid accumulation in cardiomyocytes and several alterations in the structural and functional properties of the myocardium have been observed (Hexeberg *et al.,* 1993; Haukur and Leeson, 1975).

Increasing evidence accumulated in recent years showing that high-cholesterol diet impairs NO-cGMP signaling in both endothelial and non-endothelial cells (Ferdinandy *et al.,* 1997; Lefer and Ma, 1993; Simonet *et al.,* 1993; Deliconstantinos *et al.,* 1995). Atherosclerosis is a well known "NO deficient state" in the vasculature which leads to sustained arterial hypertension (see for review: Dusting, 1995) and to reduced cardiovascular tolerance to stress (see for review: Ferdinandy *et al.,* 1998). Ferdinandy *et al.* (1997) have shown that cardiac NO level is

significantly decreased in hearts of cholesterol-fed rats, however, the mechanism of reduced NO level in the heart is not known. Reduced vascular NO release in hyperlipidemia has been also shown as a consequence of increased formation of $O_2^{\cdot-}$, which then reacts with NO to form $ONOO^-$ (White *et al.*, 1994; Szilvássy *et al.*, 2001). Although it is not known if hyperlipidemia leads to increased formation of reactive oxygen species in the heart, it is plausible to speculate that this mechanism is involved in the enhanced breakdown of NO in the myocardium in hyperlipidemia.

5. Aims

Since it is not known (i) what the possible role of $ONOO^-$ in preconditioning is, (ii) what kinds of genes and proteins are possibly involved in the molecular background of preconditioning, and (iii) what the mechanism is, by which cardiac preconditioning is abolished in hyperlipidemia, therefore, the aims of our present studies were:

study 1.: to examine the effect of preconditioning on $ONOO^-$ formation in the myocardium during ischemia/reperfusion, and to identify new cellular pathways involved in cardiac ischemia and ischemic preconditioning.

study 2.: to identify the mechanism by which cardiac NO content is decreased due to experimental hyperlipidemia.

6. Methods

The 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) and was approved by the ethics committee of the University of Szeged.

6.1. Animals, pretreatments

6.1.1. Study 1.

Male Wistar rats (300-350g) (housed in a room maintained at 12 h light-dark cycles and at a constant temperature of 22±2 °C) were used without any pretreatment.

6.1.2. Study 2.

Male Wistar rats (18 weeks old) (housed in a room maintained at 12 h light-dark cycles and at a constant temperature of 22±2°C) were fed laboratory chow enriched with 2% cholesterol or standard chow for 8 weeks. At the end of the 8-week diet period, body weight of the animals were 350-400 g, and there were no significant differences between groups; plasma cholesterol and trigliceride level increased by 20% and 300%, respectively, which was consistent with our previous findings (Csont *et al.*, 2002).

Separate groups of normal and hyperlipidemic rats were injected twice intraperitoneally with 2x20 μmol/kg FeTPPS (5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]), a $ONOO^-$ decomposition catalyst, 24 hours and one hour before the isolation of the hearts to allow sufficient time for repair mechanisms and *de novo* protein synthesis to recover $ONOO^-$-induced cellular injury.

6.2. Isolated heart preparation, measurement of cardiac functions

The preparation of isolated rat hearts was essentially the same in both series of studies. Rats were anesthetized with diethyl ether. After intravenous administration of heparin (500 U/kg) the chest was opened, the heart was rapidly excised and perfused in Langendorff mode or in a

"working" mode according to Neely *et al.* (1967) modified by Tósaki and Hellegouarch (1994). The perfusion was carried out with an oxygenated, normothermic Krebs-Henseleit buffer containing (in mM) NaCl 118.4, KCl 4.1, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.17, $MgCl_2$ 1.46 and glucose 11.1; gassed with 95% O_2 and 5% CO_2 and supplemented with 0.3 M L-tyrosine. Preload (1.7 kPa) and afterload (9.8 kPa) were kept constant throughout the experiments. To prevent the myocardium from drying out, a thermostated glassware (in which the heart was suspended) was sealed.

Cardiac mechanical functional and hemodynamic parameters including heart rate (HR), coronary flow (CF), aortic flow (AF), left ventricular developed pressure (LVDP) and its first derivatives ($+dP/dt_{max}$, $-dP/dt_{max}$), and left ventricular end-diastolic pressure (LVEDP) were monitored as described (Ferdinandy *et al.*, 2000; Csonka *et al.*, 1999). Plasma, coronary effluent, and myocardial tissue were sampled and frozen in liquid nitrogen for further biochemical measurements.

6.3. Experimental design

6.3.1. Study 1A.

A non-preconditioning and a preconditioning protocol were applied before induction of test ischemia/reperfusion as described in detail (Csonka *et al.*, 1999). After 10 min of aerobic perfusion as working hearts, preconditioning was induced by three intermittent cycles of 4 min and 45 sec no-flow ischemia, separated by 30 sec Langendorff perfusion followed by 4 min and 45 sec aerobic working perfusion. The 30 sec Langendorff perfusion allowed for the spontaneous restoration of sinus rhythm before switching to working mode between no-flow periods. Non-preconditioned control and preconditioned hearts were then subjected to 30 min global no-flow ischemia followed by 15 min reperfusion.

6.3.2. Study 1B.

To assess the effect of ischemia/reperfusion injury and of preconditioning on the gene expression pattern, three different perfusion protocols were applied (n=5-8 in each group). After 10 min equilibration, hearts were subjected to either a preconditioning or a non-preconditioning

protocol (as described above) followed by 30 min global no-flow ischemia and by 120 min reperfusion. A time-matched control group was aerobically prefused for 190 min.

To profile gene expression patterns associated with ischemia/reperfusion, we have used cDNA microarrays of 3200 rat genes to monitor transcript levels in rat hearts. In order to confirm the differential expression of genes revealed by microarray analysis of rat hearts after ischemia with and without preconditioning, several genes were analyzed by real-time quantitative fluorescent RT-PCR.

6.3.3. Study 2.

In all groups from this study after a 10 min normoxic, normothermic perfusion cardiac mechanical functional and hemodynamic parameters were monitored.

6.4. Measurement of cardiac NO and $O_2^{\cdot-}$ content, and LDH release

NO content of ventricular tissue was measured using electron spin resonance spectroscopy after loading the heart with the NO-specific spin trap Fe^{2+}-N-methyl-D-glucosamine-dithiocarbamate (MGD) as described (Ferdinandy *et al.*, 2000; Csonka *et al.*, 1999). The spin-trap for NO was prepared freshly before each experiment. MGD (175 mg) and $FeSO_4$ (50 mg) dissolved in distilled water (pH 7.4, volume 6 ml) was infused into the aortic cannula under Langendorff perfusion (constant pressure at 9.8 kPa) for 5 min at a rate of 1 ml/min in order to measure basal myocardial NO content. Tissue samples from the apex of the heart (approximately 150 mg) were collected at the end of the infusion of $Fe^{2+}(MGD)_2$ and placed into quartz ESR tubes and frozen in liquid nitrogen. Electron spin resonance spectra of NO-Fe^{2+}-$(MGD)_2$ adducts were recorded with a Bruker ECS106 spectrometer (Rheinstetten, Germany; ESR parameters: X band, 100 kHz modulation frequency, 160 K temperature, 10 mW microwave power, 2.85 G modulation amplitude, 3356 G central field) and analyzed for NO signal intensity as described (Csonka *et al.*, 1999).

Superoxide production in freshly minced ventricles was assessed by lucigenin-enhanced chemiluminescence (Ferdinandy *et al.*, 2000). Approximately 100 mg of the apex of the heart was placed in 1 ml air-equilibrated Krebs-Henseleit solution containing 10 mM HEPES-NaOH (pH 7.4) and 5 μM lucigenin. Chemiluminescence was measured at room temperature in a liquid scintillation

counter using a single active photomultiplier positioned in 'out-of-coincidence' mode in the presence or absence of the $O_2^{\cdot-}$ scavenger nitro blue tetrazolium (NBT, 200 μM). NBT-inhibitable chemiluminescence was considered an index of myocardial $O_2^{\cdot-}$ generation. It should be noted that NBT, like other $O_2^{\cdot-}$ scavengers, is not entirely specific for $O_2^{\cdot-}$.

Cardiac lactate dehydrogenase (LDH) release was estimated from coronary effluent collected for 5 min. LDH release was assayed by spectrofotometer by using Sigma kits and was expressed as mU per min per g (Csonka *et al.*, 1999).

6.5. Determination of $ONOO^-$ formation

We measured both dityrosine by spectrofluorometry and free 3-nitrotyrosine by ELISA (Cayman Chemical, Ann Arbor, MI) in the perfusate as markers of cardiac $ONOO^-$ formation (Ferdinandy *et al.*, 2000). $ONOO^-$ promotes nitration of phenolic compounds such as tyrosine, the nitration of which leads to the formation of stable products, dityrosine and 3-nitrotyrosine (Fig.2). Therefore, to measure cardiac $ONOO^-$ generation, Krebs-Henseleit buffer was supplemented with 0.3 mM L-tyrosine, and dityrosine and free 3-nitrotyrosine formation was detected in the coronary effluent as described (Ferdinandy *et al.*, 2000; Yasmin *et al.*, 1997). Tyrosine at 0.3 mM does not affect cardiac mechanical function (Yasmin *et al.*, 1997). Samples of coronary effluent during Langendorff perfusion periods were collected for 30 sec and stored in -80 °C until assayed for 3-nitrotyrosine. Dityrosine and 3-nitrotyrosine formation was normalized to coronary flow and to wet weight of the hearts and expressed as pmoles per min per g protein.

We also measured plasma 3-nitrotyrosine as a marker of systemic $ONOO^-$ generation as described (Szilvássy *et al.*, 2001). Plasma $ONOO^-$ concentration was expressed as nmoles per liter.

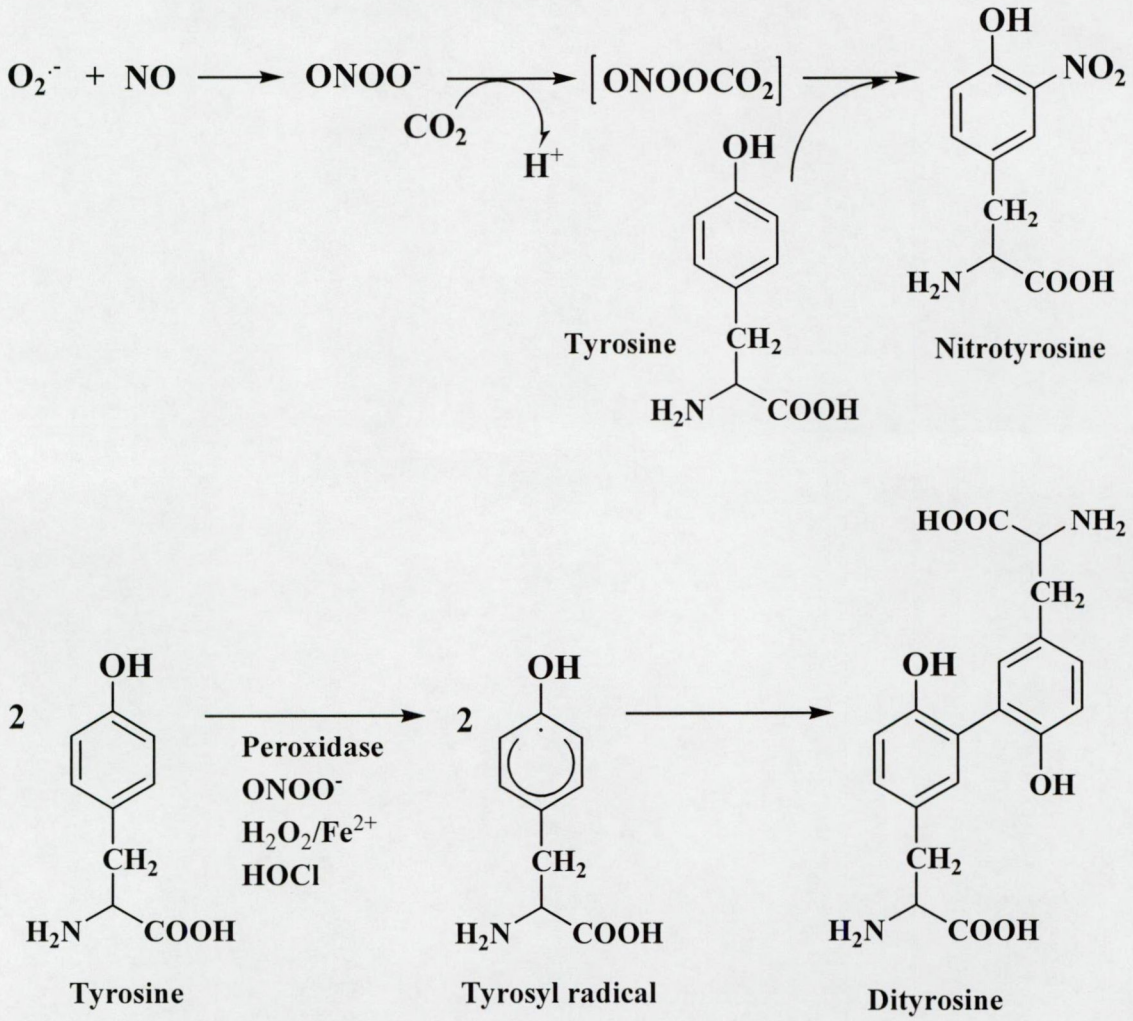


Figure 2. Formation of the markers of peroxynitrite ($ONOO^-$). $ONOO^-$ promotes nitration of phenolic compounds such as tyrosine, the nitration of which leads to the formation of stable products, dityrosine and 3-nitrotyrosine.

6.6. Methods for gene expression measurement

6.6.1. RNA preparation

Total RNA was purified from each group (25-25 mg tissue from each heart) with NucleoSpin RNA purification kit (Macherey-Nagel, Dürren, Germany) according to the manufacturer's instructions. The quantities and qualities of RNA from each sample was assessed

by gel electrophoresis as well as spectrophotometry (NanoDrop spectrophotometer, NanoDrop, USA). Two RNA pools were prepared from each group (n=3, randomly selected from each group) and used in replica experiments. Total RNA was used for microarray analysis as well as for reverse transcription quantitative PCR.

6.6.2. Microarrays and probes

Construction and use of microarrays were performed as described (Puskás *et al.*, 2002a; Kitajka *et al.*, 2002). Briefly, 3200 amplified cDNA inserts from different rat cDNA libraries were amplified with vector-specific primers, analyzed with agarose gel-electrophoresis and purified with Millipore PCR purification plates. Purified PCR products were reconstituted in 50% DMSO/water and arrayed in duplicate on FMB cDNA slides (Full Moon Biosystems, Sunnyvale, CA) using a MicroGrid Total Array System spotter (BioRobotic's, Cambridge, UK) with 16 pins in a 4x4 grid format. After printing, DNA was UV crosslinked to the slides with 700 mJ energy (Stratalinker, Stratagene). Microarray probes were generated by a modified version of a linear amplification technique described before (Puskás *et al.*, 2002b). Briefly, 2 μg total RNA from each pooled sample was amplified. Three μg of amplified RNA was labeled with both Cy5 and Cy3 fluorescent dyes (dye-swap experiments) during reverse transcription (RT) with RNAse H (-) point mutant M-MLV reverse transcriptase (Fermentas, Vilnius, Lithuania) and random nonamers. After RT, RNA was alkali hydrolyzed and labeled cDNA was purified with NucleoSpin™ PCR purification kit (Macherey-Nagel) according to the manufacturer's instructions. Probes generated from the control and treated bone samples were mixed, reconstituted in 16 μl hybridization buffer (50% formamide, 5x SSC, 0.1% SDS, 100 mg/ml salmon sperm DNA) and applied onto the array after denaturation by heating for 1 min at 90 °C. Prior to hybridization, the slides were blocked in 1x SSC, 0.2% SDS, 1% BSA for 30 min at 45 °C, rinsed in water and dried. The slide was covered by a 22 mm x 22 mm coverslip, and sealed with DPX Mountant (Fluka, Buchs, Switzerland) in order to prevent evaporation. Slides were incubated at 42 °C for 20 hours in a humid hybridization chamber. After hybridization the mountant was removed and the arrays were washed by submersion and agitation for 10 min in 1x SSC with 0.1% SDS, for 10 min in 0.1x SSC with 0.1% SDS and for 10 min in 0.1x SSC at room temperature, then rinsed briefly in water and dried.

6.6.3. Scanning and data analysis for gene expression studies

Each array was scanned under a green laser (543 nm for Cy3 labeling) or a red laser (633 nm for Cy5 labeling) using a ScanArray Lite (GSI Lumonics, Billerica, MA) scanning confocal fluorescent scanner with 10 μm resolution (Laser power: 85% for Cy5 and 90% for Cy3, Gain: 75% for Cy5 and 70% for Cy3). Scanned output files were analyzed using the software program SCANALYZE2 (http://www.microarrays.org/software.html). Each spot was defined by manual positioning of a grid of circles over the image. The average pixel intensity and the local background of each spot were determined. A measure, i.e. "expression ratio" (MRAT, denotes the median of the set of background-corrected single pixel intensity ratios of the two channels within the spot) was determined (Eisen *et al.,* 1998). This average expression ratio for all genes on the array was normalized to 1.0. For background corrections those data were calculated as negatives where the average intensity of the spot was smaller than two times of the average background of the same area. Each experiment was performed twice using both fluorescent dyes for labeling control and sample to reduce the number of false positive or false negative ratios deriving from possible uneven incorporation of fluorescent dyes during labeling, or from other experimental variables introduced by hybridization, washing conditions or array features. Therefore, from each RNA pool two probes were generated: a Cy5-labeled and a Cy3-labeled one in order to perform replicate "color-flip" experiments suggested by other authors (Lee *et al.,* 2000; Stuart *et al.,* 2001). Replica spots (on the same array) and replica experiments (two different arrays) resulted in four data points for every gene. Those spots were excluded from further analysis, where ratios of the replica spots have more than 2-fold differences. The same restriction was applied for the average ratios of the replica experiments.

6.6.4. Real-time quantitative PCR

The confirmatory real-time quantitative reverse transcription-PCR (QRT-PCR) was performed on a RotorGene 2000 instrument (Corbett Research, Sydney, Australia) with gene-specific primers and SybrGreen protocol to confirm the gene expression changes observed by DNA microarrays as described. In brief, 20 μg of total RNA from each pool was reverse transcribed in the presence of poly(dT) sequences in total volume of 20 μl. After dilution of the mix with 80 μl of water, 2 μl of this mix was used as template in the QRT-PCR. Reactions were

performed in a total volume of 20 µl (8 pmol/each forward and reverse primer, 1x BioRad SYBRGreen buffer, BioRad, Hungary) with the following protocol: 10 min denaturation at 95 °C, and 45 cycles of 25 sec denaturation at 95 °C, 25 sec annealing at 59 °C and 25 sec extension at 72 °C. Fluorescent signals were gathered after each extension step at 72 °C. Curves were analyzed by the RotorGene software using dynamic tube and slope correction methods with ignoring data from cycles close to baseline. Relative expression ratios were normalized to β-actin and calculated with the Pfaffl method (Pfaffl, 2001). In this study we used the PCR primers of the following gene products: β-actin, chaperonin subunit ε, natriuretic peptide precursor type B, anion exchange protein 2, metallothionein-II, peroxisome proliferator activator receptor γ (PPARγ), betaine-homocysteine methyltransferase, cysteine proteinase inhibitor. All the PCRs were performed four times in separate runs.

6.7. Statistics

Data were expressed as mean±SEM and analyzed with unpaired t-test or Fischer's Exact test or one way analysis of variance (ANOVA) followed by Tukey's test as appropriate. $P<0.05$ was accepted as a statistically significant difference. (The statistical analysis of gene expression is described in *chapter 6.6.3.*)

7. Results

7.1. Study 1A.

7.1.1. Cardiac function and LDH release

In non-preconditioned control hearts, test ischemia/reperfusion resulted in a marked decrease in aortic flow (AF) and a considerable increase in left ventricular end-diastolic pressure (LVEDP) and LDH release (Table 1). When preconditioning was applied before test ischemia, postischemic AF increased and LVEDP and LDH release decreased showing the protective effect of classic preconditioning against acute ischemia/reperfusion injury.

Table 1. Aortic flow (AF), left ventricular end-diastolic pressure (LVEDP), and lactate dehydrogenase (LDH) release in non-preconditioned and preconditioned hearts before and after test ischemia/reperfusion

	AF mL/min	LVEDP (kPa)	LDH (mU/min/g)
	Before test ischemia-reperfusion		
Nonpreconditioned	45.9±1.2	0.54±0.05	n.d.
Preconditioned	43.6±2.1	0.52±0.05	n.d.
	After ischemia-reperfusion		
Nonpreconditioned	16.4±1.1	1.76±0.05	163.0±11.6
Preconditioned	25.1±1.6*	1.34±0.08*	63.9±12.3*

Values are means ± SEM (n=7 in each group); n.d., nondetectable; *P<0.05 corresponding nonprecontitioned group.

7.1.2. Cardiac $ONOO^-$

In the non-preconditioned control group, test ischemia/reperfusion markedly increased cardiac 3-nitrotyrosine formation (Fig.3). Preceding preconditioning with three brief periods of no-flow ischemia significantly decreased test ischemia/reperfusion-induced 3-nitrotyrosine formation. In the preconditioned group, however, the first cycle of no-flow ischemia followed by

reperfusion markedly increased 3-nitrotyrosine formation, which was attenuated after the third cycle of no-flow ischemia.

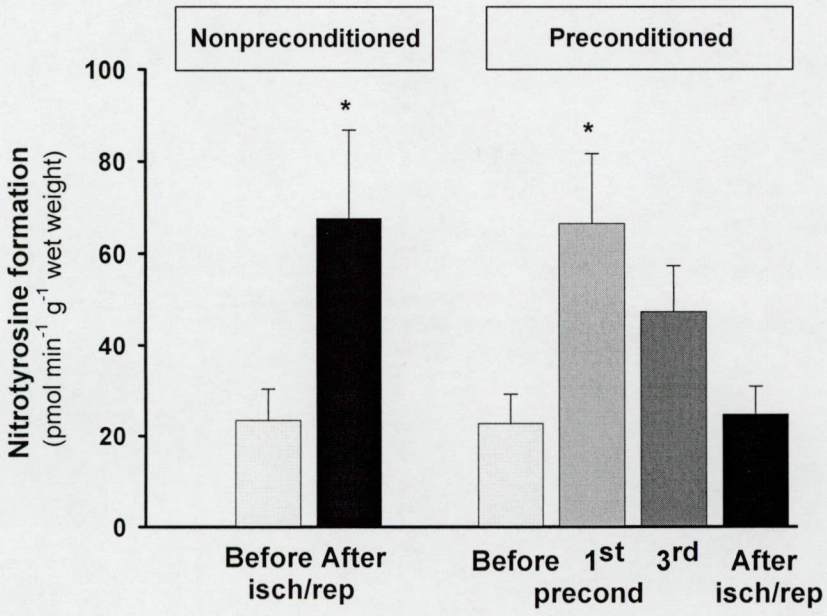


Figure 3. Effect of preconditioning and test ischemia/reperfusion on cardiac formation of free 3-nitrotyrosine, a marker for endogenous peroxynitrite formation. Precond: preconditioning, isch/rep: ischemia/reperfusion; *P<0.05 vs. corresponding "before isch/rep" or "before precond" values. n=7 in each group.

7.2. Study 1B.

7.2.1. Gene expression alteration after ischemia/reperfusion

Relative gene expression changes in response to ischemia/reperfusion were determined using the expression profiles of time-matched control hearts as baseline. Changes of 3200 genes were followed by rat-specific cDNA microarrays. In response to ischemia/reperfusion, out of 3200 genes 1468 showed significant intensity (see Materials and Methods for statistical calculations) and 1.6% showed altered expression: 28 genes exhibited significant up-regulation and 20 were down-regulated.

Just to mention a few, we found several genes with altered expression due to myocardial ischemia, up-regulated: atrial natriuretic factor, procollagen, tubulin, ubiquinone oxidoreductase,

heat-shock proteins, isocitrate dehydrogenase gene; down-regulated: glycine-N-acyltransferase, and several metabolic enzymes gene (Table 2).

Table 2. Several genes were shown with altered expression in response to ischemia/reperfusion when compared to nonischemic hearts. All experiments were done in duplicates and data were calculated from four intensity ratios.

Functional clusters	Gene product	Acc. No.	Ratio	SD
Bioactive peptides	Atrial natriuretic factor	M27498	2.68	0.45
Cytoskeleton, extracellular matrix proteins	Procollagen, type III, α 1	W89883	2.64	0.38
	α-tubulin	NM_022298	2.66	0.98
Energy metabolism	NADH-ubiquinone oxidoreductase B15	NM_012985	2.28	0.02
Heat shock proteins	Chaperonin subunit ε	AA956164	1.87	0.11
	Wagneri gene for 105-kDa heat shock protein	AW544862	2.47	0.24
	Heat shock protein, 86 kDa 1	AJ428213	3.5	0.5
Metabolic enzymes	Glycine-N-acyltransferase	AA237628	0.57	0.03
	Isocitrate dehydrogenase 3 (NAD^+) α	NM_053638	1.87	0.38
	Formiminotransferase cyclodeaminase	NM_053567	0.5	0.05
	Aconitase 1	AA875134	0.58	0.1
Ubiquitin system	Non-Canonical Ubiquitin Conjugating Enzyme 1	AA250689	0.6	0.01
	Rattus norvegicus ubiquitin-like protein	AW545652	2.45	0.39

7.2.2. Gene expression alteration due to preconditioning

To study the effects of preceding preconditioning on ischemia/reperfusion-induced gene-expression pattern, we have used cDNA microarrays to monitor alteration in gene expression of ischemic rat hearts with and without preconditioning. Out of 3200 rat genes 1450 had significant intensity values, but only 1% of them showed altered expression: 15 clones were overexpressed and 16 repressed. Some of these genes are shown in Table 3.

The expression of some genes changed considerably upon preconditioning followed by test ischemia/reperfusion when compared to ischemia/reperfusion alone: cGMP phosphodiesterase and a chaperonin gene were dramatically overexpressed, peroxisome proliferator activator receptor γ, betaine-homocysteine methyltransferase, myosin light chain kinase genes, and protease 28 subunit gene were dramatically repressed.

Table 3. Several genes were shown with altered expression due to preconditioning followed by ischemia/reperfusion when compared to ischemic/reperfused hearts without preconditioning (Prec/Isc) and several genes with altered expression due to ischemia/reperfusion when compared to nonischemic controls (Isc/Norm), respectively. All experiments were done in duplicates and data were calculated from four intensity ratios. *Isc* denotes for ischemia/reperfusion, *Norm* for nonischemic controls, and *Prec* for preconditioning.

Functional cluster	Gene product	Acc. No.	Ratio 1. (Isc/Norm)	SD (1)	Ratio 2. (Prec/Isc)	SD (2)
Bioactive peptides	Natriuretic peptide precursor type B	NM_031545	0.73	0.06	1.76	0.21
Cytoskeleton, extracellular matrix proteins	Class I β-tubulin	AB011679	0.65	0.18	0.66	0.01
Heat shock proteins	Chaperonin subunit 5, ε	AA955792	1.87	0.11	2.18	0.21
Metabolic enzymes	(2'-5')oligoadenylate synthase 1	Z18877	1.17	0.15	1.75	0.14
	cGMP phosphodiesterase (PDE9A1)	AA273765	1.51	0.26	3.29	0.89
	Peroxisome proliferator activator receptor γ	NM_013124	0.95	0.23	0.50	0.14
	Protease (macropain) 28 subunit, α	NM_017278	1.1	0.31	0.56	0.13
	Betaine-homocysteine methyltransferase	NM_030850	1.36	0.21	0.57	0.05
Metal binding proteins	Metallothionein II	H32024	1.08	0.16	1.69	0.08
Others	18S, 5.8S, and 28S ribosomal RNAs	V01270	2.8	0.29	1.85	0.14
	Coagulation factor VII	AA271041	1.29	0.2	1.99	0.21
	Cysteine proteinase inhibitor	M92418	0.98	0.09	0.63	0.11
Receptors, ion channels, membrane proteins	Secretory carrier membrane protein (SCAMP3)	AF005036	1.78	0.27	1.59	0.05
	Neutral and basic amino acid transporter	U10110	1.78	0.38	1.64	0.14
	Anion exchanger 2	NM_017048	1.76	0.29	1.84	0.28
Regulatory proteins, kinases, phosphatases	Myosin light chain kinase	AW142114	1.02	0.24	0.59	0.14

7.2.3. Real-time quantitative PCR

In order to confirm the differential expression of genes revealed by microarray analysis of rat hearts after ischemia with and without preconditioning, several genes were analyzed by real-time quantitative fluorescent QRT-PCR. We have selected seven genes the expression of which has been significantly altered in preconditioned hearts for real-time RT-PCR analysis (Fig.4). The

differential expression of these genes revealed an almost perfect concordance with the microarray data. Genes encoding chaperonin subunit ε, anion exchange protein 2 and metallothionein-II had very significant rise in transcription rate, while natriuretic peptide precursor type B gene showed a less pronounced induction. Genes encoding PPARγ and betaine-homocysteine methyltransferase showed repression, although cysteine proteinase inhibitor gene exhibited moderate repression at the mRNA level.

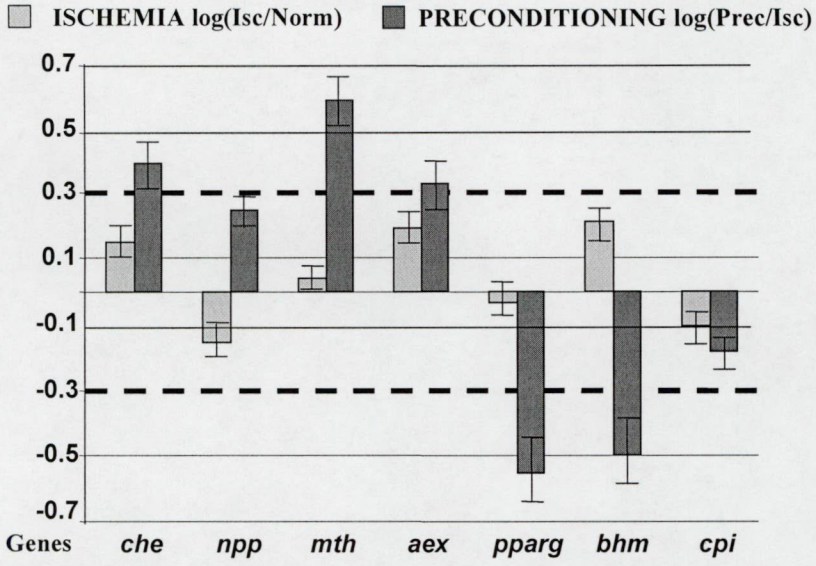


Figure 4. Quantitative determination of transcript levels by real-time PCR. Changes in transcript levels in rat hearts during ischemia (compared to nonischemic controls; light box), and ischemia with preconditioning (compared to ischemic/reperfused hearts; dark box) were confirmed by triplicate real-time PCR. β-actin was used as a control. The expression of the following genes were determined: chaperonin subunit ε: *che*; natriuretic peptide precursor type B: *npp*; anion exchange protein 2: *aex*; metallothionein-II: *mth*; peroxisome proliferator activator receptor γ: *pparg*; betaine-homocysteine methyltransferase: *bmh*; cysteine proteinase inhibitor: *cpi*. Dashed lines indicate the interval –1.8 to 1.8-fold regulation (corresponding to $\log_2$=0.85) in which changes in expression were considered not significant.

7.3. Study 2.

7.3.1. Cardiac NO content and $O_2^{\cdot-}$ production

Myocardial NO content was significantly decreased in the hyperlipidemic group as measured by electron spin resonance spectroscopy after *ex vivo* spin trapping of NO in isolated hearts (Fig.5). To test if cholesterol-enriched diet increases cardiac $O_2^{\cdot-}$ generation, we performed lucigenin-enhanced chemiluminescence assay in cardiac tissue. Cardiac $O_2^{\cdot-}$ generation was significantly increased due to high-cholesterol diet as compared to controls.

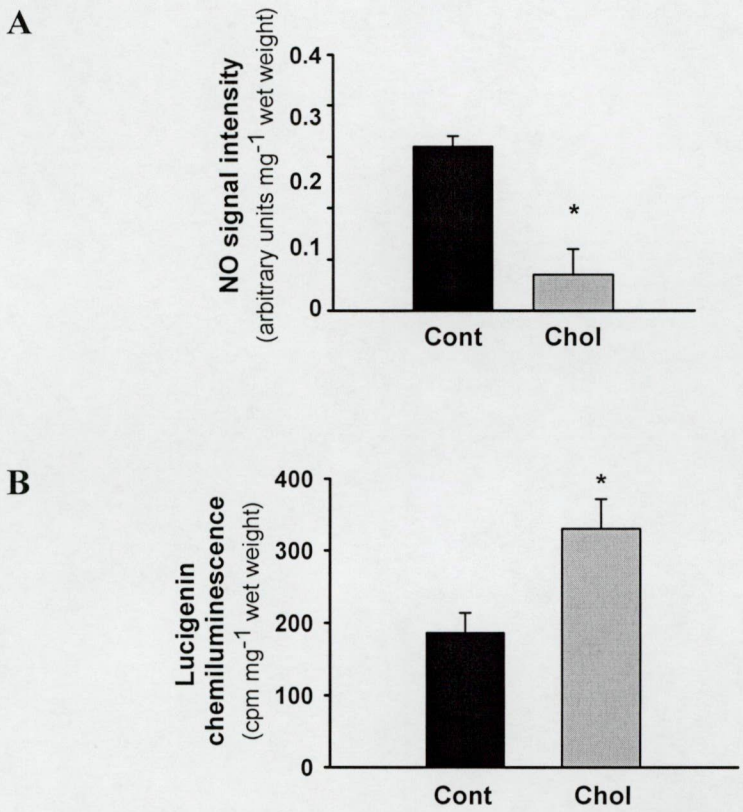


Figure 5. Myocardial NO content (A), cardiac $O_2^{\cdot-}$ production (B) in the control (Cont) and cholesterol-fed (Chol) groups. Results are means±SEM (n=7 in both groups). *P<0.05 vs. control.

7.3.2. Cardiac and systemic ONOO⁻

To test formation of $ONOO^-$ in the heart, isolated hearts obtained from cholesterol-fed and control groups were perfused with a buffer supplemented with 0.3 mM L-tyrosine. Marker of cardiac $ONOO^-$ generation, dityrosine in the coronary effluent, was increased in the cholesterol-fed group as compared to controls. The formation of the other cardiac $ONOO^-$ marker, 3-nitrotyrosine in the coronary effluent, did not statistically significantly increased in the cholesterol-fed group (control 10.48±2.27; cholesterol-fed 14.19±4.56).

We also studied if high-cholesterol diet increased systemic formation of $ONOO^-$ (Fig.6). Therefore, plasma free 3-nitrotyrosine concentration was measured in control and cholesterol-fed groups as a marker for systemic $ONOO^-$ formation. Plasma free 3-nitrotyrosine was increased approximately two-fold in cholesterol-fed rats as compared to controls.

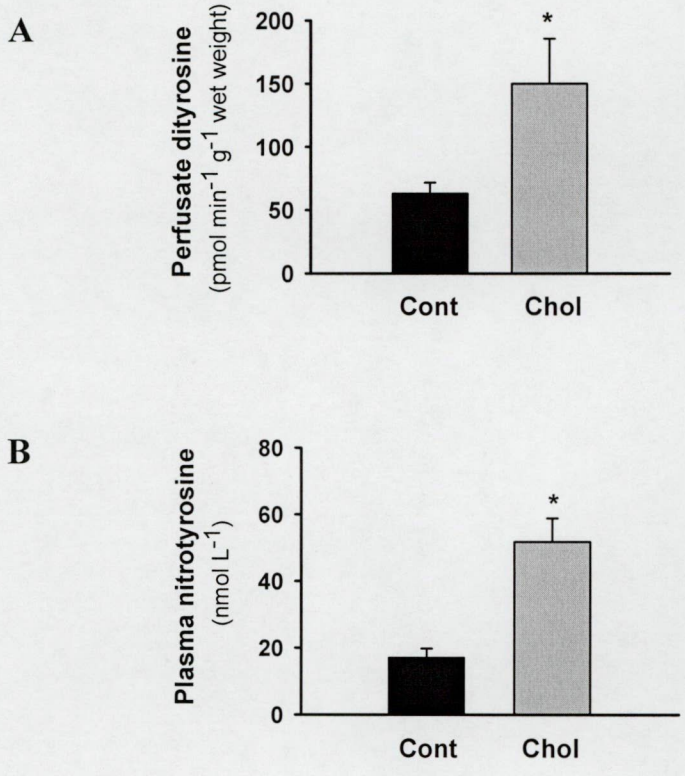


Figure 6. Dityrosine (A) formation in the perfusate, a marker for cardiac peroxynitrite ($ONOO^-$) generation; plasma 3-nitrotyrosine (B) concentration, a marker for systemic $ONOO^-$ generation in

the control (Cont) and cholesterol-fed (Chol) groups. Results are means±SEM (n=7 in both groups). *P<0.05 vs. control.

7.3.3. Cardiac function

To test if an increase in cardiac $ONOO^-$ formation leads to alterations in cardiac performance, cardiac contractile parameters were measured in isolated working hearts.

Table 4. Cardiac functional parameters in the control and cholesterol-fed groups

	HR	CF	AF	CO	LVDP	$+dP/dt_{max}$	$-dP/dt_{min}$	LVEDP
Control	271.0±7.5	22.9±0.5	43.4±2.0	66.3±2.5	18.2±0.4	839.5±45.2	460.0±34.2	0.52±0.05
Chol-fed	270.3±9.4	22.1±0.5	45.3±1.2	67.4±1.6	18.9±0.4	945.0±40.2	483.8±41.6	0.85±0.05*

Heart rate (HR, beats/min); coronary flow (CF, ml/min); aortic flow (AF, ml/min); cardiac output (CO, ml/min); left ventricular developed pressure (LVDP, kPa); left ventricular end-diastolic pressure (LVEDP, kPa); $+dP/dt_{max}$ (kPa/s); $-dP/dt_{max}$ (kPa/s). Values are means ± SEM (n=8 in each group). *P<0.05 shows significant difference compared to control.

LVEDP was significantly increased in the hyperlipidemic group as compared to controls. Other parameters of cardiac performance such as HR, AF, CF, LVDP, $+dP/dt_{max}$, $-dP/dt_{max}$ were not affected significantly by cholesterol-diet when compared to the control group (Table 4).

To further test if hyperlipidemia-induced elevation of LVEDP was due to enhanced $ONOO^-$ formation, hyperlipidemic and normal rats were treated with FeTPPS, a $ONOO^-$ decomposition catalyst. In the hyperlipidemic group, LVEDP was recovered to control values after FeTPPS treatment, however, FeTPPS did not change LVEDP in the normal group (Fig.7).

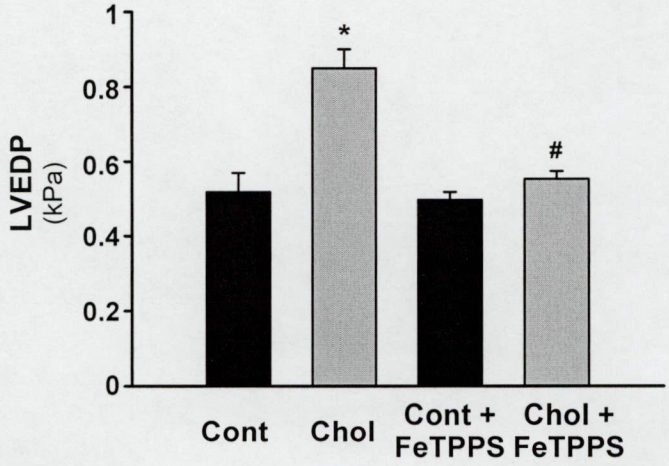


Figure 7. Left ventricular end-diastolic pressure (LVEDP) in control (Cont), cholesterol-fed (Chol), control+FeTPPS treated (Cont+FeTPPS), and cholesterol-fed+FeTPPS treated (Chol+FeTPPS) groups. Results are means±SEM (n=8 in each group). *$P<0.05$ vs. control, $^{\#}P<0.05$ vs. cholesterol-fed.

8. Discussion

8.1. New findings

The results of the present study led to the following novelty:

i. preceding ischemic preconditioning inhibits endogenous cardiac $ONOO^-$ production induced by test ischemia/reperfusion; and subsequent cycles of ischemia/reperfusion result in a gradual decrease in $ONOO^-$ formation

ii. there are a number of genes the expression of which is significantly altered due to ischemia/reperfusion and preconditioning in the heart. The role of these genes in preconditioning has not been shown previously.

iii. cholesterol-enriched diet for 8 weeks increases endogenous myocardial $ONOO^-$ formation which results in a deterioration of cardiac function.

8.2. $ONOO^-$ and ischemia/reperfusion

It is well established that myocardial ischemia leads to an accumulation of NO in the heart which is associated with a deterioration of myocardial mechanical function during reperfusion. Csonka *et al.* (1999) have previously found that ischemic preconditioning markedly decreases NO accumulation during test ischemia/reperfusion, improves postischemic cardiac function, and decreases the release of LDH. As many studies, we have found also that NO-dependent myocardial injury is mediated by $ONOO^-$ (Zweier *et al.*, 1995; Schulz and Wambolt *et al.*, 1995; Yasmin *et al.*, 1997) and that $ONOO^-$ formation deteriorates myocardial mechanical function (Ferdinandy *et al.*, 2000); we hypothesized that this cardiac $ONOO^-$ formation can be attenuated by ischemic preconditioning, thereby the heart is protected against functional damage. Accordingly, we have found here that ischemic preconditioning markedly decreased $ONOO^-$ formation upon ischemia/reperfusion.

Although preconditioning attenuated $ONOO^-$ formation upon test ischemia/reperfusion in our present study, the first brief cycle of preconditioning ischemia/reperfusion significantly enhanced $ONOO^-$ formation; however, after subsequent cycles of brief ischemia/reperfusion $ONOO^-$ formation was reduced. This may show that $ONOO^-$ formed during ischemia/reperfusion

might act as a trigger for preconditioning, but preconditioning in turn decreases formation of $ONOO^-$ upon subsequent cycles of ischemia/reperfusion. As the majority of studies show that NO and oxygen free radicals are both required to elicit preconditioning, it was plausible to speculate that formation of $ONOO^-$ is an important oxidative stimulus to trigger cellular adaptive mechanisms. $ONOO^-$ as a trigger for preconditioning is supported by Altug *et al.* (2000; 2001), who described that brief exposure of isolated rat hearts to 1 μM exogenous $ONOO^-$ was capable of mimicking the beneficial effects of ischemic preconditioning, and this was abolished by the administration of the antioxidant N-2-mercaptopropionylglycine.

8.3. Gene expression studies

8.3.1. Gene expression alteration after ischemia/reperfusion

It is well established that myocardial ischemia leads to a changes of gene expression pattern in the heart. Due to ischemia/reperfusion 28 genes exhibited significant up-regulation and 20 were down-regulated as compared to non-ischemic hearts. Little is known about the possible role of most of these genes in ischemia/reperfusion.

In a recent report, global expression analysis in response to renal ischemia was performed by Yoshida et al. (2002). They found that most of the genes showing altered expression are involved in cell structure, extracellular matrix, tissue repair, and cell division/differentiation. By using an Affymetrix oligonucleotide microarray containing 10,000 gene-specific samples they found 122 genes, the expression of which changed due to ischemia-induced acute renal failure. In our present study, several genes with similar characteristics were altered due to myocardial ischemia, i.e. tubulin, procollagen, glycine-N-acyltransferase, several metabolic enzymes and proteins involved in programmed cell death. We have detected extensive changes in heat shock proteins in our present study. A chaperonin and two heat shock proteins (86 and 105 kDa) were induced by ischemia. The induction of heat-stress proteins are well known in response to myocardial, renal, and cerebral ischemia (Kelly, 2002; Snoeckx *et al.*, 2001; Kitagawa *et al.*, 2001; Papadopoulos *et al.*, 2000), however, this is the first demonstration that chaperonin subunit ε is significantly upregulated due to cardiac ischemia/reperfusion.

Ischemia/reperfusion repressed several genes including some mitochondrial genes and

aconitase, a major enzyme of the citrate cycle. It is well known that ischemia/reperfusion results in mitochondrial damage leading to cell apoptosis or necrosis (Lust *et al.*, 2002; Scarabelli *et al.*, 2002; Jassem *et al.*, 2002), however, little is known about the cellular mechanisms of these phenomena.

Lynn *et al.* (2000) studied gene expression profile of ischemic injury produced left coronary artery occlusion without reperfusion in mice hearts. They used an array with only 588 gene-specific probes and found only a small number of genes affected by ischemia. Genes with altered expression were those coding for proteins implicated in oxidative stress, apoptosis and cardiac muscle development. In a rat infarction model, a detailed gene expression analysis was performed using a microarray containing 7000 cDNAs (Stanton et al., 2000) and several genes encoding proteins involved in cytoskletal architecture, contractility, and metabolism were identified. In accordance with their findings, we have found several genes in the present study which exhibited changes in expression in response to ischemia/reperfusion, i.e. heat-shock proteins, ubiquinone oxidoreductase, ubiquinone binding protein, collagen, tubulin, and atrial natriuretic factor.

8.3.2. Gene expression alteration due to preconditioning

It is also well established that genes exhibiting characteristic changes in expression in the heart due to preconditioning as compared to ischemia/reperfusion-induced gene-expression pattern. Due to classic preconditioning 15 clones were overexpressed and 16 repressed.

Changes in the expression of some genes by preconditioning followed by ischemia/reperfusion were similar to those changed by ischemia/reperfusion alone (overexpressed: a secretory membrane protein, an amino acid transporter, an anion exchanger, a ribosomal RNA and a chaperonin gene; repressed: β-tubulin). Because in the case of preconditioning the control sample was ischemia/reperfusion alone, therefore, the expression of these genes changed more dramatically when compared to nonishemic controls. This suggests that these genes might have significant roles in ischemic adaptation of the heart during a single ischemia without preconditioning as well.

The rest of the genes were specifically and differentially expressed in response to preconditioning and were not altered after a single ischemia/reperfusion. Among these genes, metallothionein, coagulation factor VII, cystein proteinase inhibitor, peroxisome proliferator activator receptor γ (PPARγ) and myosin light chain kinase genes were previously shown to have connections with

ischemia or other heart diseases (Okawa *et al.*, 2002; Emerson *et al.*, 2000; Paschen *et al.*, 1999; Szilvássy *et al.*, 1994; Fruchart *et al.*, 1999; Thiemermann and Wayman, 2001; Tsuchida *et al.*, 1986; Ma *et al.*, 1992; Keller *et al.*, 2000). Hypoxia preconditioning induced the expression of metallothionein in the brain (Emerson *et al.*, 2000). In addition, we have found here that 2'-5' oligoadenylate synthase gene showed overexpression in response to preconditioning. It has been previously shown by others, that the mRNA level of this gene rose more than 2-3-folds after 24 h recovery from ischemia in the rat brain (Paschen *et al.*, 1999). Therefore, it is plausible to speculate that oligoadenylate synthase might have a protective effect on the heart as well. Chaperonin subunit ε and natriuretic peptide precursor type B also exhibited up-regulation due to preconditioning. None of these genes have been previously shown to be involved in preconditioning.

A more dramatic induction was detected in the expression of a cGMP phosphodiesterase (PDE9A1). Alterations in cGMP levels in the heart have been previously shown in response to preconditioning (Szilvássy *et al.*, 1994), however, this is the first demonstration that the expression of a phosphodiesterase gene is altered due to preconditioning.

PPARγ exhibited one of the most pronounced repression due to preconditioning. PPARγ has been shown to be involved in several cardiovascular pathologies including atherosclerosis and ischemic heart disease, however, this is the first demonstration that PPARγ could play a role in ischemic preconditioning. Interestingly, most of the previous studies showed that pharmacological activation of PPARγ protects the ischemic heart (Fruchart *et al.*, 1999; Thiemermann and Wayman, 2001). In contrast, our present study shows that preconditioning leads to a marked repression of PPARγ gene. This suggests that the role of PPARγ in ischemic injury and ischemic adaptation is still unclear.

Degradation of myocardial structural proteins in myocardial infarction has been shown to be reduced by a cystein proteinase inhibitor (Tsuchida *et al.*, 1986). In our present study a 1.59-fold repression has been detected by microarray analysis and a 1.75-fold repression by real-time quantitative PCR. It seems that the activity of cystein proteases are favoured in preconditioning. Another gene related to protein degradation was also repressed: protease 28 subunit had a 1.79-fold repression; this protease subunit has a regulatory function in proteasome for small protein substrate degradation (Ma *et al.*, 1992) and has implications for oxidative stress (Keller *et al.*, 2000).

8.4. $ONOO^-$ and cholesterol-enriched diet

The present results show that cholesterol-enriched diet for 8 weeks markedly reduces cardiac NO level, enhances cardiac formation of $O_2^{\cdot-}$ and their reaction product $ONOO^-$, thereby leading to an increase in LVEDP, which can be prevented by pretreatment with a $ONOO^-$ decomposition catalyst, FeTPPS. This is the first demonstration that high-cholesterol diet leads to enhanced $ONOO^-$ formation in the heart which results in a deterioration of cardiac function.

In accordance with other previous studies (Ferdinandy *et al.*, 1997), we have found in the present study that high-cholesterol diet leads to a decrease in cardiac NO level. It is well known that NO rapidly reacts with $O_2^{\cdot-}$ to form the cytotoxic species $ONOO^-$ (Beckman and Koppenol, 1996). Hyperlipidemia has been shown to increase production of reactive oxygen species including $ONOO^-$ in the vasculature (Szilvássy *et al.*, 2001; Beckman and Koppenol, 1996; White *et al.*, 1996; Miller *et al.*, 1998). Although it is not known if hyperlipidemia leads to increased formation of reactive oxygen species in the heart, it is plausible to speculate that this mechanism is involved in the enhanced breakdown of NO in the myocardium in hyperlipidemia. Therefore, we measured cardiac $O_2^{\cdot-}$ and $ONOO^-$ production. Myocardial $O_2^{\cdot-}$ level was significantly increased due to cholesterol-diet in this study.

In addition to increased myocardial $O_2^{\cdot-}$ formation, we have found here that high-cholesterol diet increases formation of a potential marker of cardiac $ONOO^-$, dityrosine in the perfusate. This is the first demonstration that hyperlipidemia increases $ONOO^-$ formation in the heart. In contrast to dityrosine, perfusate 3-nitrotyrosine was not statistically significantly increased in our present study. This can be explained by recent results showing that at relatively low level of $ONOO^-$, 3-nitrotyrosine formation is suppressed in favour of dityrosine (Hurst, 2002). We have also found that hyperlipidemia increases plasma 3-nitrotyrosine level, a marker for systemic $ONOO^-$ generation. This is in accordance with our previous study showing an increase in serum 3-nitrotyrosine in rabbits with high-cholesterol diet (Szilvássy *et al.*, 2001). The reason why 3-nitrotyrosine level was increased in the plasma but not in the coronary effluent is not clear. However, it is worth mentioning that an increased systemic $ONOO^-$ formation has greater chance to increase 3-nitrotyrosine level in the circulating plasma *in vivo*, whereas cardiac $ONOO^-$ formation has less chance to increase 3-nitrotyrosine significantly in the perfusate as the perfusion

buffer passes through the coronary circulation only once in the Langendorff preparation.

The cytotoxic effects of $ONOO^-$ include lipid peroxidation, nitration of tyrosine residues, oxidation of sulfhydryl groups, DNA-strand breakage (Rubbo *et al.,* 1996), and inhibition of mitochondrial respiration (Beckman and Koppenol, 1996), leading to tissue injury, which manifests itself, e.g. as a depression in myocardial contractile function (Schulz *et al.,* 1997). Many studies show that enhanced formation of $ONOO^-$ in the myocardium is cytotoxic to the heart and contributes to ischemia/reperfusion injury both *in vitro* and *in vivo*, to the spontaneous loss of cardiac function as well as to cytokine-induced myocardial contractile failure in isolated rat hearts and in dogs *in vivo* (Yasmin *et al.,* 1997; Ferdinandy and Schulz, 2001b; Oyama *et al.,* 1998). In addition, a correlation between $ONOO^-$ formation and deterioration of cardiac function was shown (Ferdinandy and Schulz, 2001b). Therefore, here we tested if increased $ONOO^-$ in hearts of cholesterol-fed rats leads to a deterioration of cardiac function. We have found a significant increase in LVEDP in the cholesterol-fed group. LVEDP elevation is the most sensitive parameter of cardiac dysfunction showing that the capability of the heart to relax is deteriorated. This finding is in accordance with a study by Schwemmer *et al.* (2000) who reported a substantial decline in myocardial contractile and relaxation parameters in hypercholesterolemic guinea pig hearts.

To further test if an increase in LVEDP was due to $ONOO^-$ formation, we examined the effect of FeTPPS, a $ONOO^-$ decomposition catalyst, on cardiac performance in cholesterol-fed and control groups. FeTPPS catalyzes the isomerization of $ONOO^-$ to nitrate anion and thereby decreases its decomposition to highly reactive intermediates such as nitrogen dioxide and hydroxyl radical (Misko *et al.,* 1998). Our results show that pretreatment with FeTPPS normalizes LVEDP in the cholesterol-fed group, but it does not change LVEDP in the control group. This finding further suggests that hyperlipidemia induces $ONOO^-$ formation in the rat heart which leads to an increase in LVEDP. As the biochemical measurements were not repeated in the FeTPPS groups, some unspecific effects of FeTPPS on cardiac NO and $O_2^{\cdot-}$ formation may also account for the effect of FeTPPS, which is a limitation of this study.

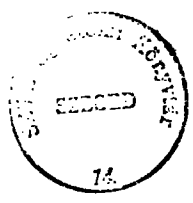
8.5. Limitations of the study

Our studies regarding the assessment of $ONOO^-$ release in heart do not determine the source of $ONOO^-$. Coronary and endocardial endothelial cells, specific cardiac nerves, and cardiac myocytes may all potentially contribute to $ONOO^-$ formation in the heart, since all of these cells are able to synthesize NO and $O_2^{\cdot-}$.

It should be noted that dityrosine and 3-nitrotyrosine have been criticised as being specific for $ONOO^-$, e.g. myeloperoxidase activity in the presence of nitrite may also lead to 3-nitrotyrosine formation (Hurst, 2002; Eiserich *et al.,* 1998). However, both myeloperoxidase activity and nitrite concentration are very low in granulocyte-free, Krebs-perfused hearts. This suggests that the myeloperoxidase pathway does not substantially contribute to 3-nitrotyrosine formation in our present study. Furthermore, biochemical data that suggesting that $ONOO^-$ does not cause tyrosine nitration (Pfeiffer and Mayer, 1998) have been recently refuted (Goldstein *et al.,* 2000). Nevertheless, the $NO_2^{\cdot}$ radical may also contribute to 3-nitrotyrosine formation (Hurst, 2002).

Our studies were performed in crystalloid-perfused isolated rat hearts and the analysis of gene expression was done using cardiac tissue that did not contain components of blood. Therefore, the mechanisms of ischemia/reperfusion injury and preconditioning might be somewhat different in the present *ex vivo* experimental model as compared to *in vivo* situations. Further limitation of the present study is that analysis of cardiac tissue for gene expression pattern has been done at the end of the 2 h reperfusion to allow time for mRNA accumulation and/or degradation. Therefore, the present study cannot distinguish between the 'trigger' and 'mediator' genes of preconditioning (see references for reviews: Baxter and Ferdinandy, 2001; Ferdinandy *et al.,* 1998; Bolli *et al.,* 1998), however, to address this issue rises many technical problems in study design and needs further investigations in the future.

Our present work does not clarify the exact cellular mechanisms by which cholesterol-diet leads to an increased formation of $O_2^{\cdot-}$ and therefore $ONOO^-$. We used an isolated, crystalloid-perfused rat heart model in our present study. In this model, the direct effect of plasma lipids and the effect of atherosclerosis can be excluded, since Wistar rats show moderate increase in serum cholesterol level and no substantial functional atherosclerosis develops due to cholesterol diet (Ferdinandy *et al.,* 1997; Roach *et al.,* 1993; Horton *et al.,* 1995). Therefore, enhancement of



$ONOO^-$ is most likely due to the accumulation of tissue/membrane cholesterol (Hexeberg *et al.*, 1993) rather than the direct acute effects of hyperlipidemia itself.

9. Conclusion

Our results show that (i) although $ONOO^-$ formation during ischemia/reperfusion might act as a trigger for preconditioning, preconditioning in turn decreases formation of $ONOO^-$ upon subsequent cycle(s) of ischemia/reperfusion; (ii) there are a number of genes the expression of which is significantly altered due to ischemia and/or preconditioning in the heart; (iii) sustained exposure to dietary cholesterol leads to an increase in cardiac $ONOO^-$ formation which can be in associated with the deterioration of cardiac function and the endogenous adaptive mechanisms.

These results could provide a basis for the development of new drugs to increase the ability of the heart to adapt to ischemia in diseased states, such as hyperlipidemia.

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