# STRESS ADAPTATION OF THE HEART: ROLE OF HYPERLIPIDEMIA AND MMP-2

Ph.D. Thesis

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# 1. LIST OF PUBLICATIONS

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

I. Lalu MM, Csonka C, **Giricz Z**, Csont T, Schulz R, Ferdinandy P. Preconditioning decreases ischemia/reperfusion-induced release and activation of matrix metalloproteinase-2. Biochem. Biophys. Res. Commun. 2002; 296(4):937-41. [IF: 2.904]

II. **Giricz Z**, Csonka C, Onody A, Csont T, Ferdinandy P. Role of cholesterol-enriched diet and the mevalonate pathway in cardiac nitric oxide synthesis. Basic Res. Cardiol. 2003; 98(5):304-10. [IF: 3.009]

III. Puskas LG, Nagy ZB, **Giricz Z**, Onody A, Csonka C, Kitajka K, Hackler L Jr, Zvara A, Ferdinandy P. Cholesterol diet-induced hyperlipidemia influences gene expression pattern of rat hearts: a DNA microarray study. FEBS Lett. 2004; 562(1-3):99-104. [IF: 3.843]

IV. **Giricz Z**, Lalu MM, Csonka C, Bencsik P, Schulz R, Ferdinandy P. Hyperlipidemia attenuates the infarct-size limiting effect of ischemic preconditioning: role of matrix metalloproteinase-2 inhibition. J. Pharmacol. Exp. Ther. 2005; in press. [IF: 4.335]

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

V. 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. 2003; 58(3):663-70. [IF: 4.575]

VI. Soti C, Nagy E, **Giricz Z**, Vigh L, Csermely P, Ferdinandy P. Heat shock proteins as emerging therapeutic targets. Br. J. Pharmacol. 2005; in press. [IF: 3.325]

VII. Csont T, Csonka C, **Giricz Z**, Ferdinandy P. A szív iszkémiás stressz-adaptációs képességének csökkenése kórélettani állapotokban: pathomechanizmus és terápiás konzekvenciák Praxis 2005; 14(10):11-21.

# CONTENTS

## 2. ABBREVIATIONS

| | |
|---|---|
| NO | nitric oxide |
| HMG-CoA | 3-hydroxy-3-methyl-glutaryl-CoA |
| NOS | NO synthase |
| MMP | matrix metalloproteinase |
| PKC | protein kinase C |
| ROS | reactive oxygen species |
| cGMP | cyclic guanosine 3':5'-monophosphate |
| TIMP | tissue inhibitor of metalloproteinases |
| $ONOO^-$ | peroxynitrite |
| TTC | triphenyltetrazolium-chloride |
| LDH | lactate dehydrogenase |
| DETC | diethyl-dithio-carbamate |
| NBT | nitro blue tetrazolium |
| QRT-PCR | quantitative polymerase chain reaction |
| SSC | saline sodium citrate |
| ANOVA | one way analysis of variance |
| EST | expressed sequence tag |
| START domain | steroidogenic acute regulatory protein-related lipid transfer domain |
| CEACAM9 | carcinoembryonic antigen-related cell adhesion molecule 9 |
| CLIC | chloride intracellular channel |

## 3. SUMMARY

Cardiac stress adaptation, *i.e.* preconditioning, is deteriorated in hyperlipidemia, but the underlying mechanisms are still undiscovered. Bioavailability of a major effector of preconditioning, nitric oxide (NO), is decreased in hyperlipidemia, however, inhibition of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, a key enzyme of the mevalonate pathway (cholesterol synthesis), increases the endothelial NO synthase (eNOS) mRNA level. Release and activation of matrix metalloproteinases (MMPs) contribute to myocardial injury after ischemia/reperfusion, but their role in preconditioning and in the loss of preconditioning in hyperlipidemia is not known. Therefore, here we have studied the role of dietary and pharmacological inhibition of the mevalonate pathway on cardiac NO metabolism in normal and hyperlipidemic animals; and we have looked at the activation and release of MMPs in preconditioning. Moreover, since hyperlipidemia-induced gene expression changes of the rat heart have not been investigated to date, we performed a cDNA microarray study as well. Rats were fed with 2% cholesterol-enriched or normal diet for 9 weeks. Normal and hyperlipidemic animals were treated with farnesol, a major metabolite of the mevalonate pathway, or with lovastatin, a HMG-CoA reductase inhibitor. In separate experiments, isolated hearts of normal and hyperlipidemic rats were subjected to a preconditioning protocol (3×5 min ischemia and reperfusion) followed by 30 min ischemia and 2 h reperfusion, or a time matched non-preconditioning protocol. We have found that hyperlipidemia decreased cardiac NO level, however, dietary or pharmacological inhibition of the mevalonate pathway did not influence cardiac NO metabolism. Neither hyperlipidemia, nor farnesol, or lovastatin affected cardiac NOS activity or eNOS protein level. Preconditioning decreased infarct size and deteriorated release and activation of MMP-2 in normal but not in hyperlipidemic animals. A reduction of infarct size in non-preconditioned hearts from both control and hyperlipidemic group was produced by the MMP inhibitor ilomastat. DNA microarray analysis of 3200 genes revealed that dietary hyperlipidemia up-regulated cardiac expression of 26 genes, while 25 showed down-regulation. Taken together, hyperlipidemia blocks preconditioning-induced cardioprotection, in which enhanced post-ischemic myocardial activation and release of MMP-2 play a significant role. Furthermore, we conclude that pharmacological inhibition of MMPs is a valid option to mimic the cardioprotective effect of preconditioning in both normal and hyperlipidemic animals. Although some genes, expression of which is altered by hyperlipidemia, have been suspected to be related to cardiovascular diseases, none of them have been previously shown to be involved in cardiac effects of the hyperlipidemia. Therefore, our present findings may open new directions in the research of the cardiac effects of hyperlipidemia.

# 4. INTRODUCTION

## 4.1. Ischemic preconditioning

Ischemic heart diseases are the leading cause of death in developed countries, *e.g.* they were responsible for the 23% of deaths in Hungary in 2003 [1]. Moreover, prevalence of factors that increase the risk of ischemic heart diseases such as obesity, smoking and diabetes is also a huge problem in these societies. Therefore, investigation of cardioprotective mechanisms is of great importance.

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 reviews) [2, 3]. A great number of mechanisms have been shown to play a role in preconditioning. For example, activation of protein kinase C (PKC) isoforms [4], opening of sarcolemmal and mitochondrial ATP sensitive potassium channels [5, 6], and prostaglandin-related pathways [7] is involved in the cardioprotective mechanism of preconditioning.

Although preconditioning confers a remarkable cardioprotection in a variety of species including humans, it seems that its effectiveness is attenuated in several disease states such as hyperlipidemia, diabetes, heart failure, nitrate tolerance etc. (see for reviews) [8, 9]. However, most studies show that hyperlipidemia inhibits the cardioprotective effect of preconditioning (when looking at end-points such as ST-segment elevation or cardiac function), but there is a controversy whether the most relevant factor of the preconditioning, the infarct-size limiting effect is lost in hyperlipidemia (see for review) [9]. The discrepancies can be attributed to the substantial differences in hyperlipidemia models (species, duration of hyperlipidemic diet, presence of significant coronary sclerosis). Therefore, we have chosen Wistar rats to study the direct cardiac effects of dietary cholesterol, as this species shows moderate increase in serum cholesterol level due to a high-cholesterol diet without substantial development of atherosclerosis [10-12].

Mechanisms by which hyperlipidemia abrogates the protective effect of preconditioning are not exactly known, however, several mechanisms have been proposed. A variety of mechanisms, *i.e.* inhibition of heat shock response [13], and expression of oxidized low-density lipoprotein receptors which induces apoptosis [14, 15] have been shown to play a role in the cardiac effects of hyperlipidemia. Thus, it appears that the hyperlipidemia-induced loss of preconditioning is based on a direct effect of hyperlipidemia on the myocardium rather than an indirect effect through coronary sclerosis [10, 16].

### 4.2. NO and hyperlipidemia

NO plays an important role in the regulation of coronary circulation, contractile function, and ischemic adaptation of the heart [17] by modulation of cardiac muscle contractile function, and by inhibiting oxygen consumption [18, 19]. In physiological conditions, NO is generated by isoforms of NOS which catalyzes the conversion of L-arginine to NO and L-citrulline (see for review) [20]. NOS belongs to a family of P-450 cytochrome oxidoreductase enzymes. In normal hearts, NO is synthesized by $Ca^{2+}$-dependent NOS isoforms in cardiac myocytes, vascular, and endocardial endothelium (eNOS) and in specific cardiac neurons (nNOS). Under pathological conditions (*e.g.* during inflammation or bacterial infection), temporary high level of NO is produced after the expression of a $Ca^{2+}$-independent, inducible NOS (iNOS). Bioavailability of NO is decreased in several diseases due to excess of reactive oxygen species (ROS) possibly produced by malfunctioning oxidative enzymes such as NAD(P)H oxidases or xanthine oxidoreductase [21, 22]. Thus, formation of NO-related ROS *e.g.* peroxynitrite ($ONOO^-$) could increase, which lead to deterioration of several mechanisms and oxidative modification of numerous macromolecules (see for review) [23].

Increasing number of evidence accumulated in recent years showing that high-cholesterol diet impairs NO - cyclic guanosine 3':5'-monophosphate (cGMP) signaling in both endothelial and non-endothelial cells [10, 24, 25]. We have previously shown that cardiac NO level is significantly decreased in rats with experimental hyperlipidemia [10]. In contrast to the aforementioned findings, inhibition of HMG-CoA reductase, the key enzyme of the endogen cholesterol synthesis, by statins has been shown to increase mRNA level of eNOS in human endothelial cells [26, 27]. It is not known, however, if dietary, or pharmacological inhibition of HMG-CoA reductase affects NO synthesis significantly in the heart tissue, and if the mevalonate pathway plays a role in the regulation of cardiac NO metabolism.

### 4.3. Preconditioning and MMPs

We have previously reported that $ONOO^-$, a highly reactive product of NO and superoxide, is involved in preconditioning [28], and that hyperlipidemia leads to enhanced formation of $ONOO^-$ [29]. A significant cellular target of $ONOO^-$ is oxidative activation of the MMPs [30].

MMPs are a large family of over 20 $Zn^{2+}$-containing endopeptidases synthesized as proenzymes by various tissues. MMPs are subdivided into 5 groups, namely matrilysins, collagenases, stromelysins, membrane-type MMPs, and gelatinases. Each subfamily has unique domain sequence and, therefore, substrate specificity. MMPs are responsible for long-term degradation and remodeling of the extracellular matrix, and play a significant role in numerous

physiological and pathological processes such as angiogenesis [31], and remodeling after acute myocardial infarction [32]. Activity of MMPs is additionally regulated by their endogen inhibitors, tissue inhibitors of metalloproteinases (TIMPs). MMP proenzymes are usually activated via limited proteolysis by the cleavage of an autoinhibitory pro-peptide domain. However, it has been demonstrated that oxidizing agents can activate MMPs via a non-proteolytic oxidative mechanism by oxidizing the sulphydryl bond between a cystein residue of the pro-peptide domain and the $Zn^{2+}$ catalytic center, resulting in fully active proenzymes without a change in molecular weight (Fig. 1) [30].

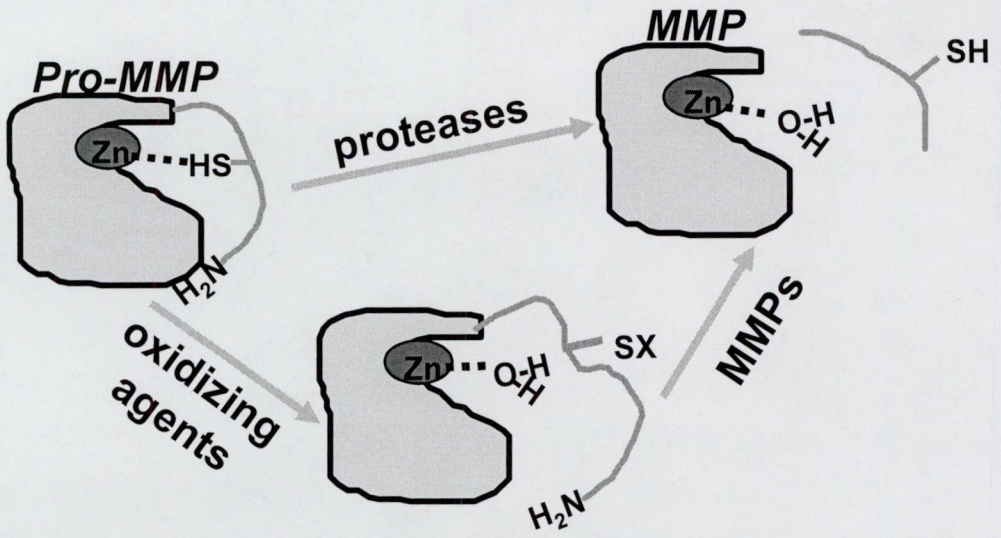



**Figure 1.** Activation of MMPs.

Recently, it has been demonstrated that MMPs play a role not only in long-term extracellular matrix remodeling, but also in acute regulation of cardiac function. For example, MMP-7 promotes vasoconstriction in rat mesenteric arteries [33] and ischemia-induced activation and release of gelatinase-A (MMP-2) is a major effector of acute mechanical dysfunction after ischemia-reperfusion in rat hearts [34-36]. Furthermore, signaling pathways involved in the mechanism of ischemic preconditioning influence the expression or activation of MMPs. For example, activation of PKC-ζ and θ subtypes increases expression of MMP-2 in rat cardiac fibroblast culture [37]. Phosphatidylinositol 3-kinase-dependent upregulation of membrane-type 1-MMP expression modulates MMP-2 activity in injured pig coronary arteries [38]. However, it is not known if MMPs and their endogenous inhibitors, the TIMPs, play a role in cardioprotection produced by preconditioning. Furthermore it is unknown if inhibition of MMPs plays a role in the loss of preconditioning-induced cardioprotection in hyperlipidemia.

We hypothesized that hyperlipidemia blocks the infarct size limiting effect of preconditioning due to the loss of inhibition of MMP activation and release induced by

preconditioning. Furthermore, we speculated that an imbalance between MMP activity and TIMPs may be involved in this mechanism.

### 4.4. Does hyperlipidemia modulate cardiac gene expression?

Underlying molecular mechanisms of direct effects of cholesterol diet-induced hyperlipidemia on the myocardium have been addressed by a few studies, but the exact biochemical mechanisms are still a question of debate. We suspected several possible mechanisms, such as alteration of NO bioavailability and increased oxidative stress; however, traditional biochemical and pharmacological approaches seem to be insufficient so far to explore key cellular events in the heart in hyperlipidemia. Recent studies have attempted to identify gene activity changes in atherosclerotic plaques in human and animal blood vessel samples [39, 40], but gene expression pattern of the heart in response to hyperlipidemia induced by chronic cholesterol-enriched diet is not known. Hence, performing cDNA microarray studies on the heart may help us identifying new cellular pathways involved in the direct cardiac effects of hyperlipidemia induced by dietary cholesterol.

## 5. AIMS

Aims of our present studies were:

Study 1: to investigate the effect of the modulation of the mevalonate pathway by dietary cholesterol and by the HMG-CoA reductase inhibitor lovastatin on cardiac NO level and NOS activities in rat hearts.

Study 2: to assess the infarct size limiting effect of preconditioning in normal and cholesterol-enriched chow-fed rats; to measure activation and release of cardiac MMP-2, and its inhibitors, TIMP levels. Furthermore, we studied the possible infarct size limiting effect of pharmacological inhibition of MMPs in control chow and cholesterol-enriched chow-fed rats in order to prove the link between MMP inhibition and cardioprotection.

Study 3: to profile the gene expression pattern of the heart associated with hyperlipidemia using cDNA microarrays in the hope of identifying new cellular pathways involved in the direct cardiac effects of hyperlipidemia induced by dietary cholesterol.

# 6. MATERIALS AND METHODS

## 6.1. Induction of hyperlipidemia

Eighteen-week old male Wistar rats were fed normal or 2% cholesterol-enriched diet for 9 weeks. Body weights of the animals after the diet period were 420-500 g and there was no significant difference between control and cholesterol-fed groups. Wistar rats were chosen for the study since they show moderate increase in serum cholesterol level due to high-cholesterol diet without substantial atherosclerosis [12]. The 9-week cholesterol-enriched diet increased serum cholesterol from 1.45±0.05 to 1.92±0.10 (n=6 in each group, $p<0.05$) and serum triglyceride from 0.52±0.04 to 1.25±0.14 mM (n=6-12, $p<0.05$).

## 6.2. Experimental design

### 6.2.1. Study 1:

Animals on normal or cholesterol-rich diet were treated either with the HMG-CoA reductase inhibitor lovastatin (3×5 mg×kg$^{-1}$ *per os* for 3 days at the end of diet), or farnesol, a major metabolite of the mevalonate pathway (2×2.2 mg×kg$^{-1}$ *i.p.*, 6 and 1 hours before isolation of hearts, n=5-6 in each group).

Rats were then anesthetized, heparin (500 U×kg$^{-1}$ *i.v.*) was given, and the heart was isolated and perfused for 1 min at 37 °C in Langendorff mode with Krebs-Henseleit buffer to wash out blood. Ventricular tissue was frozen for further measurements of NOS activities, and NOS protein.

### 6.2.2. Study 2:

At the end of the diet-period, rats were anesthetized with diethyl ether, heparin (500 U×kg$^{-1}$ *i.v.*) was administered, and hearts were isolated and perfused with Krebs-Henseleit buffer in Langendorff mode for 15 min at 37 °C. Hearts from cholesterol-fed or control rats were subjected to a no-flow ischemia-induced preconditioning protocol (3×5 min ischemia and 5 min reperfusion) or a time matched non-preconditioning protocol each followed by test ischemia/reperfusion (30 min global normothermic ischemia followed by 120 min reperfusion) (Fig. 2). In separate experiments, ventricular tissue was freeze-clamped and crushed at liquid $N_2$ temperature after the initial 15 min perfusion or at the 5th min of reperfusion for zymography and western blot studies. Coronary perfusate samples were collected before preconditioning for 5 min and during the first 5 min of reperfusion after test ischemia for zymographic analysis and for coronary flow measurement (Fig. 2). Heart rate was monitored as previously described [41].

In separate experiments, after determination of a suitable concentration of ilomastat for MMP-2 inhibition (see below), hearts from control and cholesterol-fed groups were perfused with Krebs-Henseleit buffer containing 0.1 or 0.25 μM MMP-inhibitor ilomastat (GM-6001; CHEMICON, Temecula, CA) throughout the perfusion protocol and subjected to test ischemia/reperfusion (30 min global normothermic ischemia, followed by 120 min reperfusion) without preceding preconditioning (Fig. 2).

Fig. 1

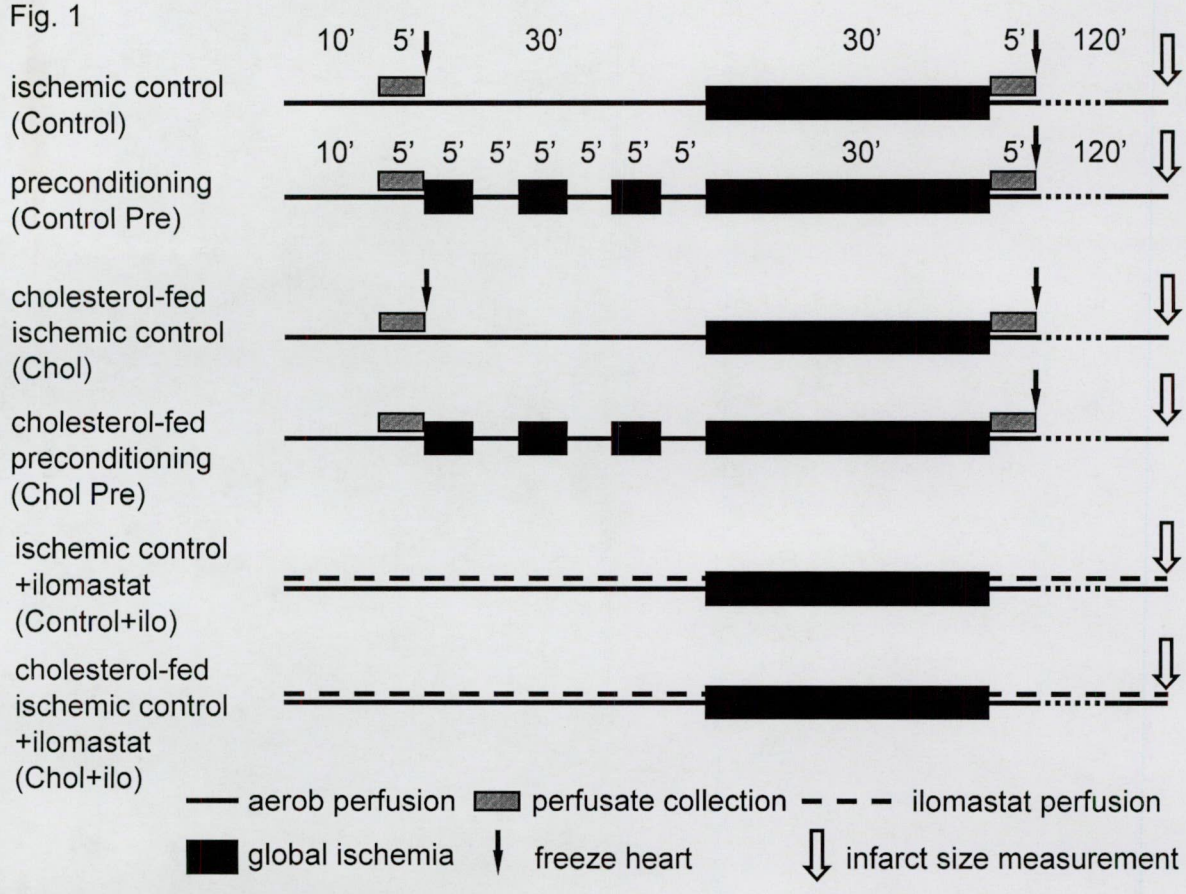



**Figure 2.** Experimental protocol - study 2.
Control and 2% cholesterol-enriched chow-fed animals were subjected to either 3×5-5 min ischemia-reperfusion (Control Pre, Chol Pre), or time-matched aerobic perfusion without preconditioning (Control, Chol) followed by 30 min test ischemia and 120 min reperfusion. Coronary perfusate was collected before the first preconditioning occlusion and corresponding control periods, as well as upon the first 5 min of reperfusion after test ischemia. Infarct size measurement was performed at the 120th min of reperfusion. In separate experiments, left ventricles were frozen in liquid nitrogen after the initial 15 min perfusion or at the 5th min of reperfusion after test ischemia for MMP measurements. Hearts of normal or cholesterol-enriched chow-fed animals were subjected to nonpreconditioning protocol in the presence of 0.1 or 0.25 μM MMP-inhibitor ilomastat (Control+ilo, Chol+ilo) as well.

### 6.2.3. Study 3:

Animals were anesthetized and 500 $U \times kg^{-1}$ heparin was given. Hearts from normal and hyperlipidemic rats (n=8 in each group) were then isolated and perfused in Langendorff mode

with Krebs-Henseleit buffer for 5 min. Subsequently, the perfusion was switched to a working mode [42, 43] to measure cardiac mechanical functional and hemodynamic parameters including heart rate, coronary flow, aortic flow, left ventricular developed pressure and its first derivatives ($+dP/dt_{max}$, $-dP/dt_{max}$), and left ventricular end-diastolic pressure as described [44]. Lactate dehydrogenase release (LDH) was measured from coronary effluent collected for 5 min at the beginning of the perfusion [42]. At the end of the perfusion protocols, ventricles of hearts from both groups were cut out, immediately frozen, and powdered with a pestle and mortar in liquid nitrogen for RNA preparation.

### 6.3. Measurement of cardiac NO by electron spin resonance spectroscopy

To measure NO level in cardiac tissue directly, in vivo spin-trapping of NO was applied, followed by ESR analysis of left ventricular tissue samples as described [42, 43, 45, 46]. The spin trap diethyl-dithio-carbamate (DETC, 200 $mg \times kg^{-1}$), 50 $mg \times kg^{-1}$ $FeSO_4$ and 200 $mg \times kg^{-1}$ sodium citrate were slowly administered intravenously into the femoral vein under ether anesthesia. DETC dissolved in distilled water was injected separately from $FeSO_4$ and sodium citrate in 0.5 ml total volume to avoid precipitation of $Fe^{2+}(DETC)_2$. $FeSO_4$ and sodium citrate were dissolved in distilled water, the pH was set to 7.4 with 1 M NaOH, and brought to 1 ml volume before injection. Five minutes after DETC, $FeSO_4$, and citrate treatment, hearts were isolated and perfused in Langendorff mode for 1 minute to eliminate blood, and approximately 150 mg tissue samples of the left ventricles were placed into quartz ESR tubes, frozen in liquid nitrogen until assayed for ESR spectra of the NO-$Fe^{2+}(DETC)_2$ complex. $Fe^{2+}(DETC)_2$ has high affinity for NO while forming NO-$Fe^{2+}(DETC)_2$. The specific triplet signal of the NO-$Fe^{2+}(DETC)_2$ is superimposed on the dominant background spectra of $Cu^{2+}(DETC)_2$. The detection limit of NO by this ESR method is 0.05 nM [47]. ESR spectra was recorded with a Bruker ECS106 (Rheinstetten, Germany) spectrometer operating at X band with 100 kHz modulation frequency at a temperature of 160 K, using 10 mW microwave power to avoid saturation. Scans were traced with 2.85 G modulation amplitude, 340 G sweep width, and 3356 G central field as described [47]. After subtraction of the background signal of $Cu^{2+}(DETC)_2$, analysis of NO content was performed with double integration. Values were expressed in arbitrary units.

### 6.4. Measurement of NOS activities

NOS activities in cardiac homogenate of frozen ventricular tissue were determined from the rate of conversion of L-[$^{14}$C]arginine to L-[$^{14}$C]citrulline as described [43, 48]. Briefly, 20 µl supernatant of freshly homogenized heart sample was incubated in 100 µl reaction solution

containing 50 mM $KH_2PO_4$, adjusted to pH 7.2 with 2N KOH; 1.2 mM $MgCl_2$, 0.24 mM $CaCl_2$, 50 mM L-valine, 10 μM 6(R,S)-5,6,7,8-tetrahydrobiopterin-2 HCI, 1 mM dithiotreitol, 100 μM NADPH, 18 μM L-arginine, 1 mM L-citrulline, 2 μM L-[$^{14}$C]arginine, in the presence or absence of either EGTA (1 mM) or EGTA plus $N^G$-monomethyl-L-arginine (1 mM). After 25 min incubation at 37 °C, reaction was terminated with the addition of 1ml 50% slurry AG 50W-X8 activated cation-exchanger resin (Bio-Rad Laboratories, Hercules, CA). Supernatant was vortexed thoroughly with Opti Phase HiSafe-3 (0.7:2 v/v, PerkinElmer, Wellesley, MA) in scintillation tube, and counted by Tri-Carb 2100TR liquid scintillation counter (PerkinElmer, Wellesley, MA) in direct DPM mode. $Ca^{2+}$-dependent and -independent NOS activities were expressed in pmol×min$^{-1}$×mg$^{-1}$ protein.

### 6.5. Measurement of superoxide production

To assess if hyperlipidemia leads to increased cardiac superoxide formation, in separate experiments, superoxide production in freshly minced ventricles was assessed by lucigenin-enhanced chemiluminescence in normal and hyperlipidemic control groups [43, 19]. 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 [43]. 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 superoxide scavenger nitro blue tetrazolium (NBT, 200 μM). NBT-inhibitable chemiluminescence was considered an index of myocardial superoxide generation.

### 6.6. Measurement of infarct size

After test ischemia/reperfusion, 5 ml of 1% triphenyltetrazolium-chloride (TTC) dissolved in phosphate buffer (pH 7.4) was slowly injected for 5 min into the aortic root to stain the myocardium. TTC-stained hearts were frozen (-20 °C), cut into approximately 3 mm thick slices, and scanned between glass plates. TTC-stained red and unstained pale areas of images were quantified by planimetry (Adobe Acrobat Professional, Adobe, San Jose, CA). Infarct size was represented as a percentage of total heart volume.

### 6.7. Measurement of LDH release

LDH release of hearts was measured using a LDH-P kit (Diagnosticum, Budapest, Hungary) from coronary perfusates collected for 5 min upon reperfusion after test ischemia. LDH release was expressed as mU×min$^{-1}$×g$^{-1}$wet heart weight.

### 6.8. Zymographic analysis of MMP activity

To measure myocardial MMP-2 activity and its release into the perfusate, we collected heart tissue and coronary perfusate samples 10 min after starting perfusion protocol for 5 min, and for 5 min after test ischemia (Fig. 2). Gelatinolytic activities of MMPs were examined as previously described [34]. Briefly, 8% polyacrylamide gels were copolymerized with 2 $mg \times ml^{-1}$ gelatin and a constant amount of protein per lane (coronary perfusate, 15 μg; ventricular homogenate, 40 μg) 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 3×15 min, and incubated for 24-48 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, St. Louis, MO) in a mixture of methanol:acetic acid:water (2.5:1:6.5, v/v) and destained in aqueous mixture of 4% methanol and 8% acetic acid (v/v). Since MMP-2 digests its substrate the gelatin in the zymographic gel, gelatinolytic activities are detectable as transparent bands against the dark blue background at the appropriate molecular weight (75, 72 and 68 kDa). Zymograms were digitally scanned and band intensities were quantified using Quantity One software (BioRad Laboratories, Hercules, CA) and expressed as a ratio to the internal standard. Band density was expressed as arbitrary $units \times mg^{-1}$ protein to exclude a possible nonspecific release of MMP into the perfusate during ischemia.

### 6.9. Selection of ilomastat concentration for appropriate inhibition of cardiac MMP-2 activation

The concentration-response relationship of the MMP-2 inhibitory effect of ilomastat in rat heart tissue homogenate was determined *in vitro*. Hearts (n=3) from control rats were aerobically perfused for 15 min to wash out blood, then left ventricles were rapidly frozen, homogenized, and zymography was performed as described above. The incubation buffer of zymography contained 0, 0.01, 0.1, 0.25, 0.5, 1 or 10 μM ilomastat during the first 3 h of the 24 h incubation. Three hours incubation in the presence of ilomastat was selected as the perfusion protocol in the *ex vivo* heart was 3 hours in duration.

### 6.10. Western blot analyses

To assess cardiac TIMP-2 and TIMP-4 protein levels, cardiac tissue was frozen 5 min after test ischemia. Cardiac eNOS protein was determined from samples frozen after the initial 5 min aerobic perfusion. Cardiac tissue was then homogenized and diluted to load 15 μg of total protein on 6 or 12% polyacrylamide gel. Electrophoresis (150 V, 1.5 h) and blotting onto

nitrocellulose membrane (35 V, 1 h) was performed. Nitrocellulose membranes were then blocked overnight in Tris-buffered saline solution containing 0.1% Tween-20 and 5% skimmed milk powder. Membranes were then incubated with mouse monoclonal anti-TIMP-2 antibody (Calbiochem IM56L, Merck, Darmstadt, Germany), or with rabbit polyclonal anti-TIMP-4 antibody (Chemicon AB816, CHEMICON, Temecula, CA), or with mouse monoclonal anti-eNOS antibody (Transduction Laboratories Lexington, KY) for 1.5 h. Rabbit anti-mouse IgG or goat anti-rabbit IgG secondary antibodies (P0161 and P0448 respectively, DakoCytomation, Glostrup, Denmark) were used for incubation at 37 °C for 2 h, as appropriate. Membranes were developed with an enhanced chemiluminescence kit (ECL Plus, Amersham Biosciences, Uppsala, Sweden), exposed to X-ray film and scanned. Band density was calculated by integrating the area (in pixels×intensity, expressed in arbitrary units).

### 6.11. Cardiac nitrotyrosine measurement

As a marker of baseline cardiac $ONOO^-$ formation, we have measured cardiac nitrotyrosine level by enzyme-linked immunosorbent assay (Cayman Chemicals, Ann Arbour, MI) from heart tissue homogenate of control and cholesterol-fed groups sampled after the initial 15 min perfusion (Fig. 2) according to the manufacturer's instructions. Nitrotyrosine content was normalized to protein content of cardiac homogenate and expressed as $pg \times mg^{-1}$ protein.

### 6.12. Protein assay

Protein concentrations were measured by the bicinchoninic acid assay (Sigma, St. Louis, MO) with bovine serum albumin as a standard.

### 6.13. RNA preparation

Total RNA was purified (25-25 mg tissue from each heart) with NucleoSpin RNA purification kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions as described [49]. The quantity and quality 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=4, randomly selected from each group) and used in replica experiments. Total RNA was used for microarray analysis as well as for reverse transcription quantitative polymerase chain reaction (QRT-PCR).

### 6.14. Microarrays and probes

Construction and use of microarrays were performed as described [50, 51]. Briefly, 3200 amplified cDNA inserts from different mouse 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% dimethylsulfoxide/water and arrayed in duplicate on FMB cDNA slides (Full Moon Biosystems, Sunnyvale, CA, USA) using a Micro-Grid Total Array System spotter (BioRobotics, Cambridge, UK) with 16 pins in a 4×4 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 [46, 48]. 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 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 samples were mixed, reconstituted in 16 μl hybridization buffer (50% formamide, 5×saline sodium citrate (SSC), 0.1% SDS, 100 μg/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 1×SSC, 0.2% SDS, 1% bovine serum albumin for 30 min at 45°C, rinsed in water and dried. The slide was covered by a 22 mm×22 mm coverslip, and sealed with DPX Mountant (Fluka, Buchs, Switzerland) in order to prevent evaporation. Slides were incubated at 42°C for 20 h in a humid hybridization chamber. After hybridization the mountant was removed and the arrays were washed by submersion and agitation for 10 min in 1×SSC with 0.1% SDS, for 10 min in 0.1×SSC with 0.1% SDS and for 10 min in 0.1×SSC at room temperature, then rinsed briefly in water and dried. 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, USA) 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) [49]. Scanned output files were analyzed using the GenePix Pro 3.0 software (Axon Instruments, Foster City, CA, USA). Each spot was defined by automatic positioning of a grid of circles over the image. The average and median pixel intensity ratios calculated from both channels and the local background of each spot were determined. An average expression ratio (MeaR, denotes the average of local background corrected pixel intensity ratios) was determined for each spot. Normalization was performed by the global Lowess method [53]. Those data were flagged and excluded where the replicate spots from a different site of the same array or results from the replicate experiments were significantly different. Data analysis was done by the significance analysis of microarrays method [54] and visualization of scatter images was performed with the Microsoft EXCEL

software. The cholesterol-regulated genes were determined by calculating the average fold change between heart samples from untreated and cholesterol-fed animals. From two biological replicates and two hybridizations altogether four data points were gathered from each gene. Genes for which the average change (increase or decrease) of the four data points was at least 1.9-fold were considered genes regulated by cholesterol diet.

### 6.15. Real-time QRT-PCR

Confirmatory real-time QRT-PCR was performed on a RotorGene 2000 instrument (Corbett Research, Sydney, Australia) with gene-specific primers and SYBR Green protocol to confirm the gene expression changes observed by DNA microarrays as described [49]. In brief, 10 μg of total RNA from each pool was reverse transcribed in the presence of oligo(dT) primer in a 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, 1×Bio-Rad SYBR Green buffer, Bio-Rad, 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 ignoring data from cycles close to baseline. Relative expression ratios were normalized to L-actin and calculated with the Pfaffl method [55]. Primers were designed using the ArrayExpress software (Applied Biosystems). All the PCRs were performed four times in separate runs.

### 6.16. Statistical analysis

Results were expressed as mean±SEM and analyzed by SigmaStat software (Jandel Scientific, Costa Madre, CA) as appropriate. Student's T-test, or one way analysis of variance (ANOVA) followed by Tukey post-hoc test was used to evaluate differences between groups, as appropriate. Differences were considered significant at $p<0.05$.

# 7. RESULTS

## 7.1. Study 1

### 7.1.1. Cardiac NO content

After an 9-week cholesterol-enriched diet, myocardial NO level was significantly decreased in cholesterol-fed animals when compared to normal rats as assessed by electron spin spectroscopy after *in vivo* spin trapping. To test if the decreased NO level in cholesterol-fed rats is due to inhibition of the mevalonate pathway, cholesterol-fed rats were treated with farnesol, a major metabolite of the mevalonate pathway. Farnesol treatment did not influence the attenuated NO level in the cholesterol-fed group or the basal NO level in the normal group. To further test if a direct inhibition of HMG-CoA reductase changes NO content, normal and cholesterol-fed rats were treated with lovastatin. Lovastatin failed to change cardiac NO when compared to nontreated normal or cholesterol-fed groups, respectively (Fig. 3).

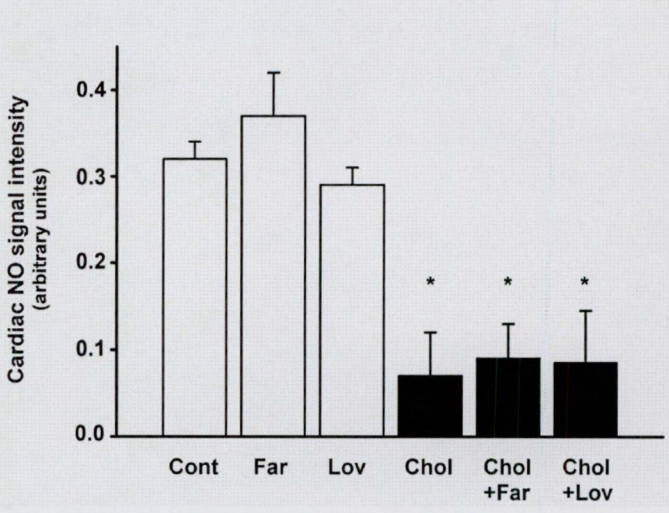



**Figure 3.** Cardiac NO level measured by electron spin resonance spectroscopy in rat hearts.
Rats were fed 2% cholesterol-enriched (Chol) or normal diet (Cont) for 9 weeks. Normal and cholesterol-fed animals were treated with farnesol (Far; Chol+Far), a major metabolite of the mevalonate pathway (2.2 mg×kg$^{-1}$ *i.p.*), or with the HMG-CoA reductase inhibitor lovastatin (Lov; Chol+Lov, 3×5 mg×kg$^{-1}$ *per os* for 3 days, n=5-6 in each group). Data are mean ± SEM. *$p$<0.05 vs. controls.

### 7.1.2. Cardiac NOS activities and eNOS protein levels

To test if changes in cardiac NO level is due to different rate of NO synthesis, NOS activities and NOS protein contents were assayed. Cardiac $Ca^{2+}$-dependent NOS activity was not influenced by cholesterol-enriched diet when compared to normal controls. Neither farnesol, nor lovastatin influenced cardiac NOS activities (Fig. 4A). Similarly to NOS activities, cardiac eNOS protein content was not influenced by the different treatments (Fig. 4B). As cardiac calcium-independent iNOS activity was below the detection limit in all groups, cardiac iNOS protein content was not measured.

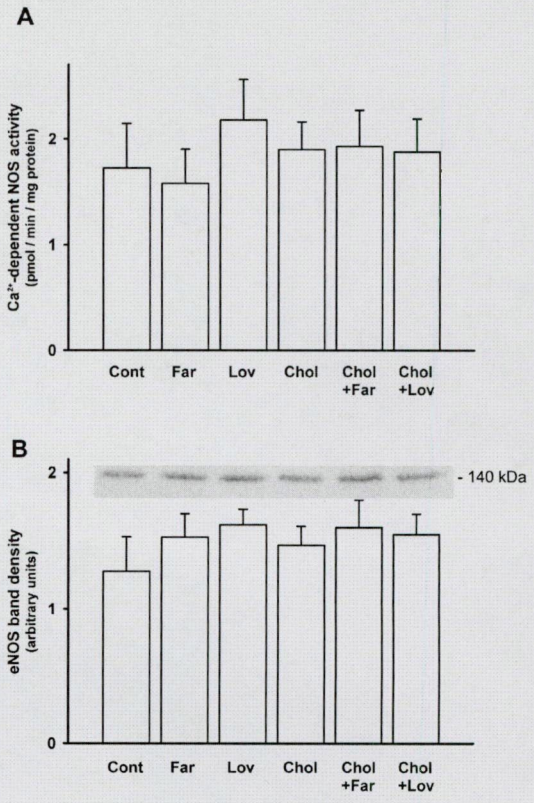



**Figure 4.** Cardiac eNOS activity assessed by a $^{14}$C-citrulline assay (A) and cardiac eNOS protein content measured by western blotting (B).
Rats were fed 2% cholesterol-enriched (Chol) or normal diet (Cont) for 9 weeks. Normal and cholesterol-fed animals were treated with farnesol (Far; Chol+Far), a major metabolite of the mevalonate pathway (2.2 mg×kg$^{-1}$ *i.p.*), or with the HMG-CoA reductase inhibitor lovastatin (Lov; Col+Lov, 3×5 mg×kg$^{-1}$ *per os* for 3 days, n=5-6 in each group). Data are mean ± SEM.

### 7.1.3. Cardiac superoxide production

To investigate if cardiac NO level is decreased in cholesterol-fed animals due to increased free radical formation, we measured superoxide-anion production in hearts of normal and cholesterol-fed animals by lucigenin-enhanced chemiluminescence assay. Cholesterol-enriched diet significantly increased superoxide production (control: 146.9±9.4, hyperlipidemic: 366.4±35.0 cpm×mg$^{-1}$ wet weight, $p<0.05$, n=6 and 7).

## 7.2. Study 2

### 7.2.1. Effect of preconditioning on infarct size and LDH release in control and cholesterol-fed groups

To assess the cardioprotective effect of preconditioning, we measured infarct size after test ischemia/reperfusion. In control hearts, preceding preconditioning significantly decreased infarct size as compared to nonpreconditioned hearts. In hearts of cholesterol-fed animals, preconditioning failed to significantly decrease infarct size (Fig. 5A).

LDH release was measured in coronary perfusate to detect ischemic injury. Thirty min test ischemia followed by 5 min reperfusion resulted in a significant LDH release in the nonpreconditioned control group. In the preconditioned control group, LDH release was significantly attenuated. In the cholesterol-fed group, preconditioning failed to decrease LDH release (Fig. 5B).

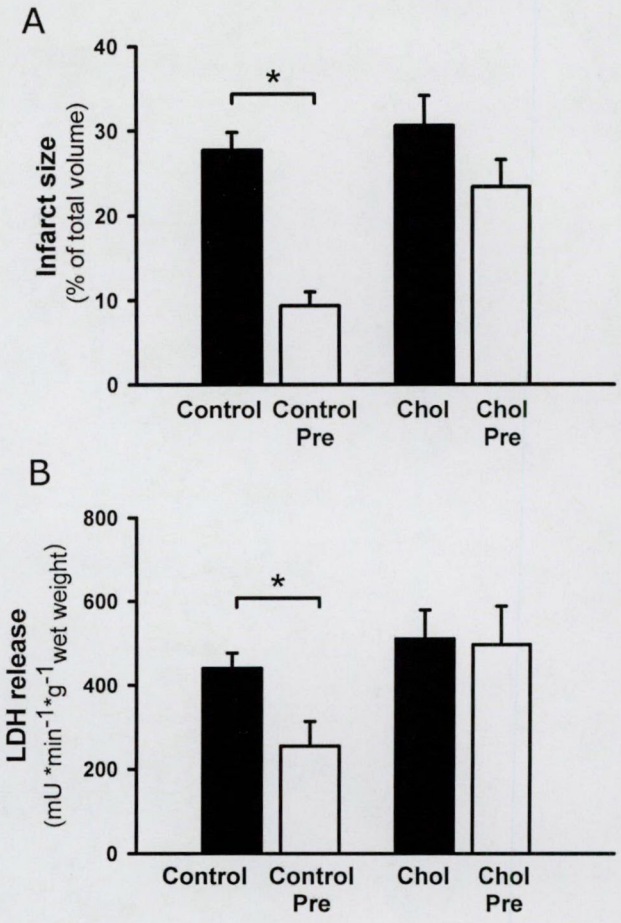



**Figure 5.** Infarct size and LDH release. Preconditioning significantly decreased infarct size (panel A) and lactate dehydrogenase release (LDH, panel B) in control chow-fed rats (Control Pre) as compared to nonpreconditioned controls (Control). Preconditioning did not significantly decrease infarct size and LDH release in hyperlipidemic rats (Chol and Chol Pre). n=6-8; *$p<0.05$ vs. Control.

### 7.2.2. Effect of preconditioning on myocardial MMP-2 activation and its release into the perfusate in control and cholesterol-fed groups

To assess myocardial MMP-2 activation and release, zymographic analysis was performed in cardiac tissue and coronary perfusate samples (pre and post ischemia/reperfusion, see Fig. 2). In the control chow-fed nonpreconditioned and the cholesterol-fed nonpreconditioned groups, a loss of cardiac 72 and 62 kDa MMP-2 activities was noted following ischemia/reperfusion (Fig. 6A). This loss was accompanied by an increase in 72 and 62 kDa activities in the coronary perfusate (Fig. 6B). In the control chow-fed group, preconditioning attenuated the release of 72 and 62 kDa MMP-2 by 17.6±3.4% ($p<0.05$) and 21.9±7.9% ($p<0.05$), respectively, as compared to the control nonpreconditioned group. In the cholesterol-fed group, however, preconditioning failed to inhibit the ischemia/reperfusion-induced release of cardiac MMP-2 activity when compared to the cholesterol-fed nonpreconditioned group (Fig. 6B).

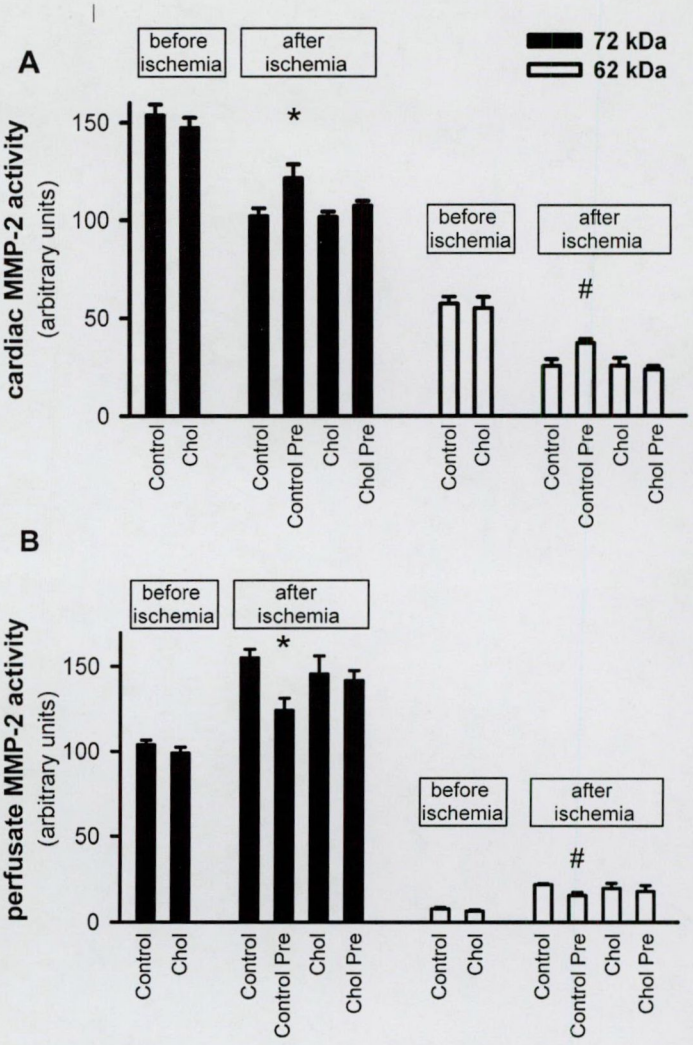



**Figure 6.** Cardiac and perfusate MMP activities.
Panel A: Thirty min ischemia induced a loss of myocardial MMP-2 (both 72 and 62 kDa forms), in normal (Control) and cholesterol-fed (Chol) groups. In control hearts, ischemia-induced loss of cardiac MMP-2 was reduced by preconditioning as compared to nonpreconditioned control group (Control vs. Control Pre). Preconditioning in cholesterol-fed rats failed to inhibit the ischemia-induced loss of MMP-2 activity (Chol vs. Chol Pre). n=6 in each group; *, # $p<0.05$ vs. Control-after ischemia.
Panel B: Thirty min ischemia increased myocardial release of MMP-2 into the perfusate (Control), which was attenuated by preconditioning in hearts from control chow-fed rats (Control Pre). However, in the hyperlipidemic group preconditioning failed to attenuate myocardial MMP-2 release into the coronary perfusate (Chol vs. Chol Pre). n=6 in each group; *$p<0.05$ vs. Control-after ischemia.

### 7.2.3. TIMP-2 and 4 western blot

To measure cardiac protein level of endogenous MMP inhibitors, we performed TIMP-2 and TIMP-4 western blots from cardiac tissue samples collected 5 min after test ischemia. Western blots show that neither cholesterol diet nor preconditioning influenced TIMP-2 or TIMP-4 protein level after 30 min test ischemia followed by 5 min reperfusion in the isolated rat heart (Fig. 7A-B).

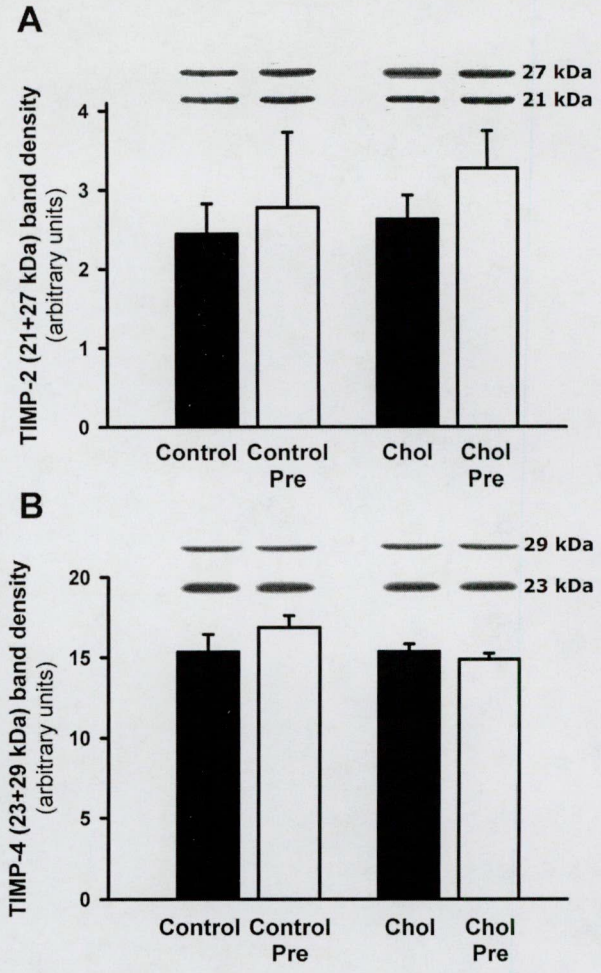
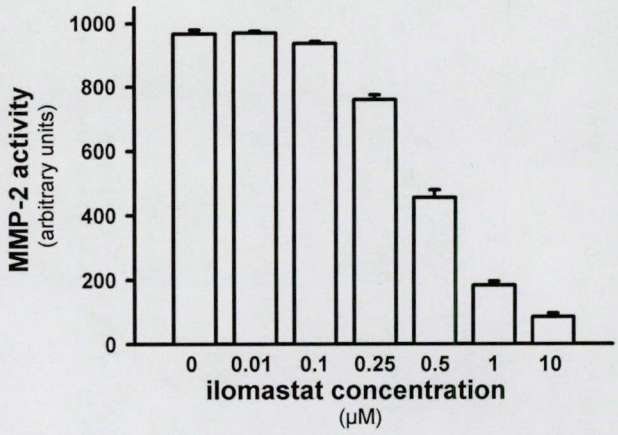



**Figure 7.** TIMP-2 and TIMP-4 western blots. Representative western blots show 27 and 21 kDa bands for TIMP-2, as well as 29 and 23 kDa bands for TIMP-4, known as glycosylated and unglycosylated proteins, respectively. Neither hyperlipidemia, nor test ischemia, or preconditioning significantly affected cardiac total TIMP-2 (A), or total TIMP-4 (B) protein level as assessed by western blots. n=6 in each group.

### 7.2.4. Effect of ilomastat on infarct size and LDH-release

To prove the causative relationship between MMP inhibition and limitation of infarct size, we mimicked the MMP inhibitory effect of preconditioning by a pharmacological inhibition of MMP-2 activity by approximately 20%, as observed in preconditioned control hearts. Therefore, first we determined the concentration of ilomastat that inhibits cardiac MMP-2 by approximately 17-22% *in vitro* (Fig. 8). We found that ilomastat inhibited myocardial MMP-2 activity by 3.0±0.8% at 0.1 μM and 19.1±1.4% at 0.25 μM. Therefore, we chose 0.25 μM and 0.1 μM for further heart perfusion studies. After test ischemia/reperfusion 0.1 μM ilomastat failed to significantly decrease infarct size and LDH release in hearts of both control and cholesterol-fed animals, however 0.25 μM ilomastat significantly decreased infarct size and LDH release both in control and cholesterol-fed group also (Fig. 9A-D). Ilomastat did not influence heart rate and coronary flow in either control or cholesterol-fed group (data not shown). These results demonstrate that 0.25 μM ilomastat protected hearts against ischemic injury not only in hearts from control chow-fed rats, but also in those from cholesterol-fed animals.

**Figure 8.** Effect of ilomastat on MMP-2 activity in vitro.
MMP-2 activity in nontreated heart tissue homogenate was assessed by zymography in the presence of 0, 0.01, 0.1, 0.25, 0.5, 1 and 10 μM ilomastat for 3 hours in the incubation buffer. n=3 in each group.



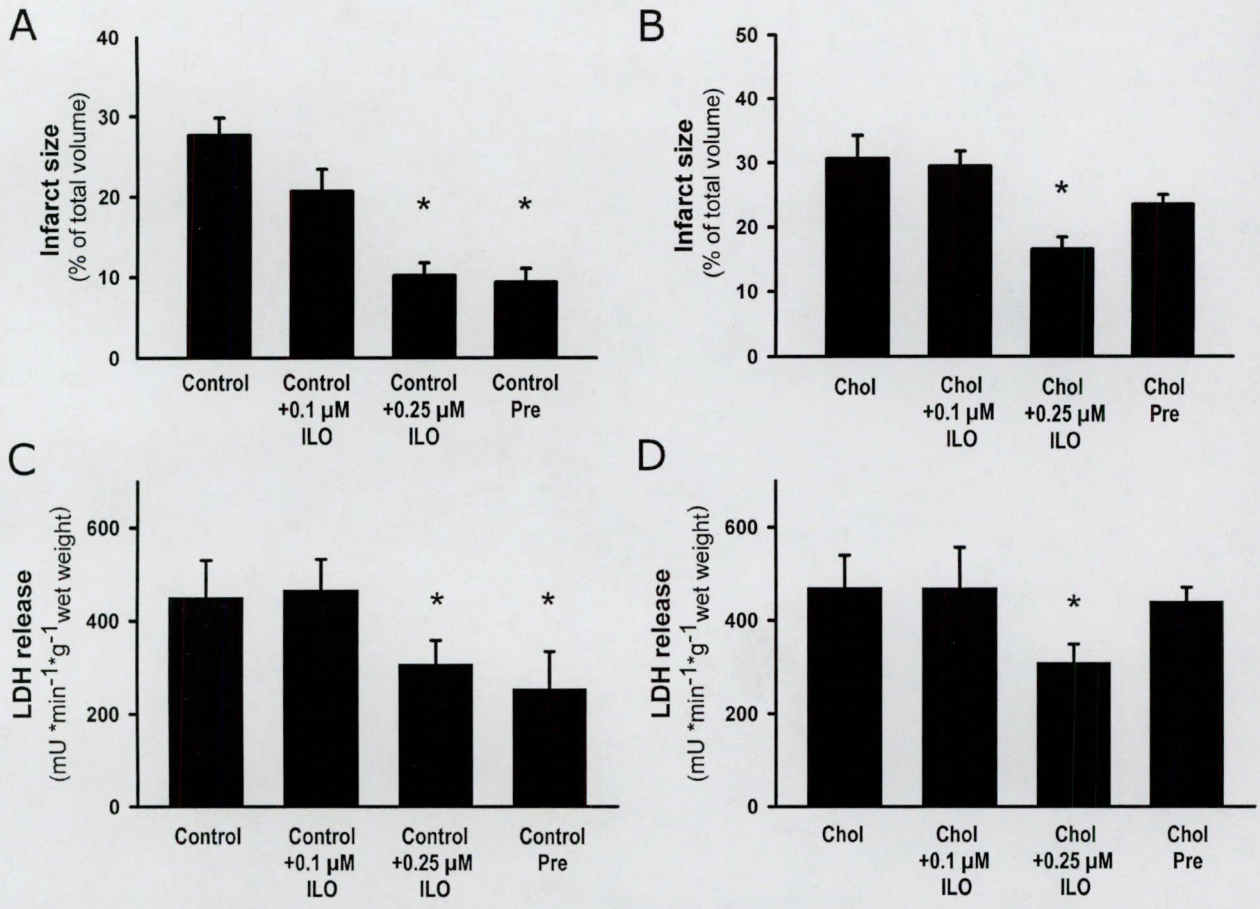




**Figure 9.** Effects of ilomastat on infarct size and LDH release in control and cholesterol-fed group.
Ilomastat (0.1 μM and 0.25 μM) dose-dependently decreased infarct size and LDH release both in control (panel A and C) and cholesterol-fed rats (panel B and D). n=5-8 in each group; *$p<0.05$ vs. Control or Chol.

### 7.2.5. Effect of hyperlipidemia on cardiac nitrotyrosine level

In order to investigate whether hyperlipidemia influences cardiac formation of $ONOO^-$, we have measured cardiac nitrotyrosine level and found that hyperlipidemia significantly increased nitrotyrosine formation in the heart, as compared to controls (Fig. 10).

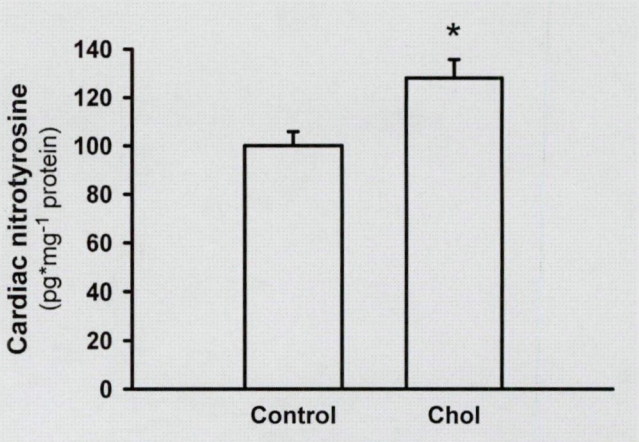



**Figure 10.** Effects of hyperlipidemia on cardiac nitrotyrosine.
Hyperlipidemia enhanced baseline cardiac nitrotyrosine formation as compared to controls (n=4 in each group; *$p$<0.05 vs. Control).

## 7.3. Study 3

### 7.3.1. Effect of hyperlipidemia on cardiac functions

Similarly to our previous results [10, 29], left ventricular end diastolic pressure was significantly increased in the hyperlipidemic group showing a mechanical dysfunction of the heart (Table 1). Other hemodynamic parameters including coronary flow were not changed by hyperlipidemia (Table 1) and no lactate dehydrogenase release was detected (data not shown) indicating that hyperlipidemia did not result in restriction of coronary circulation and development of myocardial ischemia. Therefore, gene expression changes observed in this study can be attributed to a direct effect of chronic hyperlipidemia on the myocardium.

| | HR | CF | AF | LVDP | $+dP/dt_{max}$ | $-dP/dt_{max}$ | LVEDP |
|---|---|---|---|---|---|---|---|
| **Control** | 273±6 | 23.0±0.5 | 44.4±2.0 | 18.5±0.4 | 844±46 | 456±31 | 0.51±0.05 |
| **Chol** | 271±8 | 21.9±0.5 | 45.1±1.3 | 18.9±0.5 | 935±50 | 478±43 | 0.87±0.05* |

**Table 1.** Cardiac functional parameters in control and cholesterol-fed groups
Heart rate (HR, beats×min$^{-1}$); Coronary flow (CF, ml×min$^{-1}$); Aortic flow (AF, ml×min$^{-1}$); Left ventricular developed pressure (LVDP, kPa); Left ventricular end-diastolic pressure (LVEDP, kPa); +d$P/dt_{max}$ (kPa×s$^{-1}$); -d$P/dt_{max}$ (kPa×s$^{-1}$). Values are means ± SEM (n=8 in each group). *$p$<0.05 shows significant difference compared to control by Student's t-test.

### 7.3.2. Effect of hyperlipidemia on cardiac gene expression

Relative gene expression changes in rat hearts in response to cholesterol diet-induced hyperlipidemia were compared to the expression profiles of rats on a normal diet. Changes of 3200 genes were followed by mouse-specific cDNA microarrays. Among the 3200 genes examined in the present study an average of 1324 showed significant intensity and 4.0% (51 genes) showed altered expression: 26 genes exhibited significant up-regulation (Table 2A) and 25 were down-regulated (Table 2B) after 9 weeks of high-cholesterol diet. Out of the 51 genes, 43 genes of known function and eight expressed sequence tags (ESTs) or hypothetical protein

genes with unknown function were detected. The gene expression changes ranged from -4.4-fold to +5.5- fold (Table 2).

| Function | Gene Product | Acc. no. | Microarray (average-fold) | SD |
|---|---|---|---|---|
| **Structural proteins** | Cofilin/destrin (actin depolymerizing factor) | W17549 | 3.15 | 0.21 |
| | Calsarcin-1, myozenin-like 2 | XM_215692 | 2.35 | 0.52 |
| | Procollagen, type III, alpha 1 | W89883 | 2.14 | 0.50 |
| | | | | |
| **Regulatory proteins** | Protein phosphatase 1, regulatory subunit 9A | AA087542 | 4.80 | 1.52 |
| | Transcription repressor p66 | XM_227388 | 3.38 | 0.80 |
| | Sim. to developmentally reg. prot | AA259357 | 3.52 | 0.39 |
| | Pleiotropic regulator 1 | AA286018 | 2.77 | 0.20 |
| | Glycogen synthase kinase-3 | NM_032080 | 1.95 | 0.04 |
| | Protein kinase C inhibitor, iota | NM_022192 | 1.90 | 0.03 |
| | | | | |
| **Adhesion molecules, membrane proteins** | p120 catenin isoform 4B | AW545658 | 5.48 | 1.84 |
| | SH3-containing protein SH3P4 | W34672 | 5.11 | 0.74 |
| | Clara cell phospholipid-binding prot. | W36838 | 4.51 | 0.71 |
| | NIPSNAP2 protein | W15931 | 3.00 | 0.34 |
| | Tensin | U26310 | 1.97 | 0.19 |
| | | | | |
| **Stress proteins** | Glutathione S-transferase | AA231621 | 3.26 | 0.20 |
| | Metallothionein II | H32024 | 1.97 | 0.06 |
| | Heat shock Protein, Hsp86 | AW536140 | 1.95 | 0.14 |
| | | | | |
| **Others** | Engulfment and cell motility 2 | AA087542 | 4.80 | 0.34 |
| | Disintegrin, metalloprotease domain 10 | AA267983 | 3.21 | 0.32 |
| | Mad-related protein Smad7 | AA068440 | 2.70 | 0.28 |
| | Hom. to ribosomal RNA processing 4 | NM_144886 | 2.58 | 0.20 |
| | Synaptic vesicle protein 2B | AF372834 | 1.96 | 0.09 |
| | | | | |
| **Hypothetical** | Hypothetical protein | AA245492 | 4.57 | 0.56 |
| | KIAA0719 protein | AW545304 | 3.70 | 0.93 |
| | Hypothetical | XM_220393 | 2.27 | 0.41 |
| | H. sapiens HSPC137 | AW545388 | 1.92 | 0.18 |

**Table 2A.** Up-regulated genes due to high-cholesterol diet-induced hyperlipidemia in rat hearts
SD – standard deviation

| Function | Gene Product | Acc. no. | Microarray (average-fold) | SD |
|---|---|---|---|---|
| **Energy metabolism** | Sarcomeric mit. creatine kinase | XM_226693 | -3.58 | 0.81 |
| | Enolase 3, beta | W11965 | -3.15 | 0.27 |
| | NADH-ubiquinone oxidored. | W83085 | -2.59 | 0.26 |
| | Muscle form glycogen phosphorylase | W16286 | -2.34 | 0.26 |
| | ND5 resp. NADH dehydrog. | S46798 | -2.08 | 0.08 |
| | ATP synthase subunit c | D13123 | -2.07 | 0.09 |
| | | | | |
| **Ion channels, Receptors** | Fibronectin receptor beta-chain | AW544628 | -3.29 | 0.53 |
| | Sodium/potassium ATPase beta | AW544502 | -2.02 | 0.34 |
| | Chloride intracellular channel 4 | NM_031818 | -2.00 | 0.12 |
| | Intracell. chloride channel pr. | AW539790 | -1.95 | 0.20 |
| | | | | |
| **Protein degradation, Folding (chaperons)** | Proteasome component C9 | AA277958 | -3.60 | 0.92 |
| | Ubiquitin-like protein FUBI | AA239437 | -3.47 | 0.57 |
| | 105-kDa heat shock protein | AW544862 | -2.71 | 0.32 |
| | Calreticulin | AW545345 | -2.26 | 0.35 |
| | Chaperonin subunit 5, epsilon | AA955792 | -2.00 | 0.08 |
| | | | | |
| **Cholesterol synthesis, transprot** | START domain containing 7 | W36450 | -3.61 | 0.43 |
| | Farnesyltransferase beta subunit | AA259357 | -2.05 | 0.09 |
| | CEA-related cell adhesion mol. 9 | AW545543 | -1.98 | 0.12 |
| | | | | |
| **Others** | Fibroblast inducible secreted pr. | W36541 | -2.68 | 0.02 |
| | Basigin | AW544934 | -2.26 | 0.43 |
| | CD81 antigen | NM_013087 | -2.09 | 0.35 |
| | | | | |
| **Hypothetical** | Hypothetical protein | AA403436 | -4.45 | 0.51 |
| | mKIAA0475 protein | AA274981 | -2.33 | 0.99 |
| | EST | W44032 | -2.33 | 0.21 |
| | EST | AA068436 | -2.17 | 0.37 |

**Table 2B.** Down-regulated genes due to high-cholesterol diet-induced hyperlipidemia in rat hearts. SD – standard deviation

### 7.3.3. Confirmation of microarray results by QRT-PCR

In order to confirm the differential expression of genes revealed by microarray analysis of rat hearts after cholesterol diet, several genes were analyzed by RT-PCR. We have selected 12 genes differentially expressed in hearts from cholesterol-fed animals for real-time RT-PCR analysis (Table 3). Results for 11 genes were in agreement with the microarray data, while in case of glycogen synthase kinase 3K gene, the moderate alteration in its expression (a statistically borderline case) could not be confirmed. Genes encoding procollagen type III K1, synaptic vesicle protein 2B, tensin, heat shock protein Hsp86 and metallothionein II had very

significant rises in transcription rate. In all these cases, more pronounced induction could be detected by QRT-PCR than with the microarray technique. A higher degree of change was also observed in the repression of CD81 antigen. Gene expression changes obtained by QRT-PCR for genes encoding ATP synthase subunit c, chloride intracellular channel 4, protein kinase C inhibitor S, ND5 respiratory NADH dehydrogenase, and chaperonin subunit 5O were repressed and values were very similar to those obtained in the microarray measurements.

| Gene Product | Accession Number | Microarray (average-fold) | Real-time PCR (average-fold) | Average Ct values | Confirmed by real-time PCR |
|---|---|---|---|---|---|
| Procollagen, type III, alpha 1 | W89883 | 2.14 | 4.15 | 15.24 | Yes |
| Synaptic vesicle protein 2B | AF372834 | 1.96 | 3.86 | 14.32 | Yes |
| Tensin | U26310 | 1.97 | 2.76 | 16.40 | Yes |
| Heat shock protein, Hsp86 | NM_175761 | 1.95 | 2.45 | 16.43 | Yes |
| Protein kinase C inhibitor, iota | NM_022192 | 1.90 | 1.74 | 14.95 | Yes |
| Glycogen synthase kinase-3 alpha | NM_032080 | 1.95 | -1.60 | 12.65 | No |
| Metallothionein II | H32024 | 1.97 | 2.46 | 16.74 | Yes |
| ATP synthase subunit c | D13123 | -2.07 | -1.92 | 10.52 | Yes |
| Chloride intracellular channel 4 | NM_031818 | -2.00 | -1.74 | 16.14 | Yes |
| ND5 resp. NADH dehydrog. | S46798 | -2.08 | -2.16 | 8.56 | Yes |
| Chaperonin subunit 5, epsilon | AA955792 | -2.00 | -2.20 | 16.98 | Yes |
| CD81 antigen | NM_013087 | -2.09 | -3.44 | 14.35 | Yes |

**Table 3.** Confirmation of gene expression changes due to high cholesterol diet-induced hyperlipidemia by real-time PCR in rat hearts. Ct – threshold cycle.

# 8. DISCUSSION

## 8.1. New findings

1. Hyperlipidemia-induced decrease in cardiac NO level is not associated with changes in cardiac NOS activities and NOS protein content.
2. HMG-CoA reductase inhibitor lovastatin, or farnesol, a major metabolite of the mevalonate pathway, fail to restore cardiac NO level in hyperlipidemic rats.
3. Neither farnesol, nor lovastatin affects cardiac NO level, NOS activities, and NOS protein in normal rats.
4. Cardiac superoxide generation is enhanced in hyperlipidemic rats.
5. Hyperlipidemia blocks the inhibition of ischemia-induced activation and release of myocardial MMP-2 brought about by preconditioning and therefore attenuates the infarct size-limiting effect of preconditioning.
6. Preconditioning-induced inhibition of ischemia-induced MMP-2 activation and release is not mediated by a change in cardiac TIMP-2 or 4 levels.
7. Pharmacological inhibition of MMPs markedly reduces infarct size in both control and hyperlipidemic rats.
8. Nine weeks of cholesterol-enriched diet up-regulate cardiac expression of 26, and down-regulate that of 25 genes out of the examined 3200.

## 8.2. Changes in NO bioavailability by modulation of the mevalonate pathway

A number of evidences suggest that HMG-CoA reductase inhibitors (statins) affect vascular NO production in different experimental models. Inhibition of HMG-CoA reductase by statins blocks hypoxia-mediated down-regulation of eNOS and increases eNOS mRNA level in the mouse aorta [26, 56] and in hypercholesterolemic rabbits [57]. Endres *et al.* [58] found that simvastatin increased the activity of calcium-dependent NOS in the mouse aorta by 2- to 3-fold. Increased NO level has been reported in cultured bovine aortic endothelial cells after simvastatin treatment [59]. Although these findings suggest the role of the mevalonate pathway in the regulation of vascular NO production, the exact mechanisms remained unknown.

In contrast to the aforementioned findings in vascular tissue, we have found here that in the heart tissue, neither hyperlipidemia nor inhibition of HMG-CoA reductase by lovastatin affected NOS activities and NOS protein content. This may show that the regulation of NOS activities

and NOS protein synthesis is not affected significantly by the mevalonate pathway in the heart tissue.

It is well known that high cholesterol level influences NO-cGMP signaling pathway. Lefer and Ma observed a reduced NO release from rabbit aorta in hypercholesterolemia [25]. Deliconstantinos *et al.* showed that incorporation of high concentration of cholesterol into endothelial cell membranes caused a down-regulation of NO synthesis in cultured bovine aortic endothelial cells [60]. Feron *et al.* found that in cultured bovine aortic endothelial cells, experimental hypercholesterolemia decreased NO production but did not affect eNOS protein content [61]. Further studies in this model showed that hypercholesterolemia-induced deterioration of NO production, measured in a single cell by NO selective electrode, was due to increased production of caveolin-1 [62]. In contrast to these findings, increased eNOS level and NO production were found in cholesterol treated cultured bovine aortic endothelial cells [63]. We have found in the present study that cholesterol diet-induced hyperlipidemia although decreases NO level, but it does not change NOS activity and NOS protein content in the rat heart. This shows that decreased cardiac NO level is not due to impaired NO synthesis, however, it suggests that breakdown of cardiac NO is increased due to hyperlipidemia. The mechanism of decreased cardiac NO level in hyperlipidemia remains unknown, however, it is well known that hyperlipidemia leads to increased production of ROS in the vasculature which leads to formation of $ONOO^-$ [64-69]. We have found here an increased superoxide production in hyperlipidemic hearts, so it is plausible to speculate that elevated ROS production is responsible for decreased NO level in hyperlipidemic myocardium. The source of increased superoxide formation in hyperlipidemic hearts has not been determined in the present studies. It has been previously shown that hypercholesterolemia leads to increased angiotensin II levels and angiotensin receptor subtype 1 expression, which up-regulates NADPH oxidase activity, a major source of superoxide in the vasculature [70, 71]. Therefore, it is plausible to speculate that increased NADPH oxidase activity due to hyperlipidemia is a major source of increased superoxide production in hyperlipidemic hearts.

### 8.3. Loss of preconditioning in hyperlipidemia

We have shown here that the protective effect of preconditioning was lost in hearts from cholesterol-fed rats, as preconditioning failed to reduce infarct size and LDH release in the hyperlipidemic group. This is in agreement with Ueda *et al.*, who found that the infarct size limiting effect of preconditioning was significantly attenuated in rabbits fed 1% cholesterol-enriched diet for 16 weeks [72]. Moreover, Juhasz *et al.* found a significant increase in infarct

size in preconditioned hyperlipidemic rabbits compared to nonpreconditioned ischemic control [73]. In contrast to these studies, in apolipoprotein E/low density lipoprotein receptor double knockout mice fed a 0.15% cholesterol- and 21% fat-enriched diet for 8 months [74] or in rabbits fed a cholesterol-enriched diet for 8 weeks [75], or cholesterol and coconut-oil rich chow for 4 weeks [76], the infarct size limiting effect of preconditioning was preserved. The discrepancies can be attributed to substantial differences in experimental hyperlipidemia (species, duration and composition of diet) leading to differences in the severity of coronary atherosclerosis. To exclude the effect of coronary sclerosis-induced disturbances of coronary perfusion we used hearts of male Wistar rats to study the direct cardiac effect of dietary cholesterol, since this species shows a moderate increase in serum cholesterol level due to a cholesterol-enriched diet without substantial development of atherosclerosis. Most of the studies to date including our present study show that the cardioprotective effect of preconditioning is reduced in hyperlipidemic animals and in humans as well (see for reviews) [8, 77, 78].

Mechanisms by which hyperlipidemia affects preconditioning are not exactly known. A few studies demonstrated structural and functional alterations in the myocardium due to hypercholesterolemia [79, 80]. Ecto-5'-nucleotidase activity [72], inhibition of the mevalonate pathway [41], as well as enhanced apoptotic cell death [35] have been shown to contribute to the increased ischemia-reperfusion injury and to the loss of preconditioning in hyperlipidemic animal models. We have previously reported that diminished NO bioavailability plays an important role in the loss of pacing-induced preconditioning in hyperlipidemia [10]. Furthermore, we have shown that hyperlipidemia leads to increased formation of $ONOO^-$ in the heart [29]. As it was shown that $ONOO^-$ oxidatively activates MMPs [30], and that MMPs, as downstream targets of $ONOO^-$, contribute to ischemia-induced cardiac injury [34], here we hypothesized that altered activation of MMP-2 and its release into the perfusate might contribute to preconditioning-induced cardioprotection and to the loss of preconditioning in hyperlipidemia.

### 8.4. Role of MMPs in preconditioning

To test this hypothesis, in the present study we measured MMP-2 activity in the heart and in the coronary perfusate, as well as its physiological inhibitors TIMP-2 and TIMP-4 proteins in cardiac tissue. The most abundant MMP in the rat heart is MMP-2. MMP-2 appears as both 72 kDa (oxidatively activated pro-MMP-2) and 62 kDa (proteolytically activated MMP-2) gelatinolytic bands. A 75 kDa glycosylated MMP-2 activity has also been identified in rat hearts (personal communication, Chris Overall, University of British Columbia). The 75 kDa activity

appeared only intermittently, therefore, we excluded it from data analysis. As previously reported [34, 35], MMP-9 (92 kDa and 84 kDa) was not detectable by zymography in cardiac tissue or perfusate samples in our present studies, since MMP-9 is predominantly synthesized by leucocytes. Here we have shown that activation of MMP-2 in the heart as a result of ischemia-reperfusion injury results in its enhanced release into the perfusate during reperfusion which is accompanied by a decrease in its activity in the myocardium. Our data suggest that ischemia-induced release of cardiac MMP-2 is not a passive necrosis-related mechanism, but an active process, since we have found increased MMP-2 normalized to total protein release. Our previous studies with MMP inhibitors also showed an active contribution of MMP-2 release and activation to cardiac dysfunction upon reperfusion [34]. While cardiac MMP-2 was released immediately after test ischemia into the perfusate, the level of its inhibitors, TIMP-2 and TIMP-4, was not changed in the heart in the present study. This shows that MMP-2 is released without its main inhibitors, the TIMPs, therefore it might be activated. We have found in the present study that preconditioning failed to attenuate ischemia-induced activation and release of MMP-2 in hearts of hyperlipidemic rats, however, the cardiac protein level of the most abundant endogenous MMP-2 inhibitors, TIMP-2 or TIMP-4, were unaffected in either group. This shows that preconditioning inhibits ischemia/reperfusion-induced activation and release of MMP-2 independently of TIMP-2 and TIMP-4 and that hyperlipidemia blocks the effect of preconditioning on MMP-2 activation and release.

The mechanism by which hyperlipidemia interferes with MMP activation and release during preconditioning is unknown. Enhanced MMP-1 activity was found in the plasma and in aortic rings from hyperlipidemic pigs [81], which was reduced by oral administration of antioxidant vitamin C and E. This suggests that hyperlipidemia may induce oxidative activation of MMPs. Pro-MMP-2 may be activated not only through proteolytic cleavage yielding 62 kDa MMP-2, but also through oxidant-induced conformational change. Indeed, the powerful oxidant $ONOO^-$ was shown to directly activate MMPs by S-glutathiolation of a cystein residue in the autoinhibitory pro-peptide domain, allowing the conformational change which results in a fully active 72 kDa pro-MMP-2 [30, 82]. Moreover, we have previously demonstrated that infusion of $ONOO^-$ into isolated rat hearts induces myocardial MMP-2 activation and its release into coronary perfusate, followed by a subsequent loss in cardiac contractile function, the latter blocked by treating the heart with a MMP inhibitor [35]. Furthermore, we have also shown that preconditioning attenuates the activation and release of myocardial MMP-2, thereby protecting the heart from ischemic injury. Thus, it is plausible to speculate that the loss of preconditioning-induced inhibition of MMP-2 activation and release, which is independent of TIMPs but might

be related to increased oxidative stress, is involved in the loss of cardioprotective effects of preconditioning in hyperlipidemia. Indeed, similarly to our previous studies, here we have demonstrated an increased baseline $ONOO^-$ formation in the hyperlipidemic heart, however, it did not result in increased baseline MMP activation. This suggests that the link between oxidative stress and MMP activation is rather complex in the heart. Further studies are necessary to follow changes in formation of reactive oxygen species and MMP activation during preconditioning in both normal and hyperlipidemic heart to reveal the mechanism by which hyperlipidemia interferes with preconditioning-induced MMP activation and release.

To test if the inhibition of MMP-2 activation and release is a key mechanism in the infarct size limiting effect of preconditioning, first we selected a concentration of the MMP inhibitor ilomastat that shows a similar extent of MMP-2 inhibition to that of preconditioning, *i.e.* 18%. Then we tested if 18% inhibition of MMP-2 by ilomastat reduces infarct size in normal and hyperlipidemic rats. We found here that acute, approximately 18% inhibition of MMPs by 0.25 μM ilomastat decreased infarct size similarly to the effect of preconditioning in normal hearts. This strongly suggests that inhibition of MMPs is an effector mechanism in the cardioprotective mechanism of preconditioning. Moreover, we have found that ilomastat reduced infarct size in hyperlipidemic rat hearts as well. These findings show that the lack of MMP inhibition contributes to the loss of preconditioning in hyperlipidemia, as pharmacological inhibition of MMP attenuates ischemia/reperfusion injury even in hearts from rats fed a cholesterol-enriched diet.

### 8.5. Cardiac expression of gene families down-regulated by hyperlipidemia

We have found that hyperlipidemia significantly affected cardiac energy metabolism and the ATP generating machinery at the level of gene expression, as sarcomeric mitochondrial creatine kinase, enolase 3L, ND5 respiratory NADH dehydrogenase, NADH-ubiquinone oxidoreductase, muscle form glycogen phosphorylase, and ATP synthase subunit c were markedly down-regulated as evidenced by microarray data and in the case of ATP synthase subunit c by RT-PCR results as well. These data are consistent with recent observations showing that hypercholesterolemia leads to an increase in mitochondrial damage in cardiovascular tissues [83]. Reduced energy metabolism and ATP synthesis may lead to functional deterioration of hyperlipidemic hearts as observed in our present and previous studies [10, 29].

We have observed down-regulation of genes involved in cholesterol synthesis and transport in the heart after a high cholesterol diet. Steroidogenic acute regulatory protein-related lipid transfer (START) domains can bind sterol and perform critical functions in moving the sterol

substrate to the mitochondrial inner membrane, and stimulate steroidogenesis [84]. The marked down-regulation of a START domain in the present study suggests that excess exogenous cholesterol inhibits intracellular cholesterol transport and steroidogenesis in the heart. The down-regulation of farnesyltransferase L subunit can be attributed to inhibition of the mevalonate pathway (the pathway for cholesterol synthesis) due to excess exogenous cholesterol [24]. We have also observed a moderate repression of carcinoembryonic antigen-related cell adhesion molecule 9 (CEACAM9). Members of the CEACAM family of proteins play a role in the biliary cholesterol crystallization, promoting low-density protein-lipid complex [85], and serve as a potent angiogenic factor and a major effector of vascular endothelial growth factor [86]. These data suggest that dietary cholesterol reduces cardiac cholesterol synthesis and transport, and confirm our previous assumptions that high-cholesterol diet inhibits the mevalonate pathway thereby reducing protein prenylation and ubiquinone synthesis [41].

Genes encoding ion transport proteins and ion channels such as sodium/potassium ATPase L, chloride intracellular channel 4 (CLIC4), and intracellular chloride channel genes were also repressed. Decreased activity of sodium/potassium ATPase increases cardiac contractility, and may therefore be an adaptive response to attenuate hyperlipidemia-induced loss of cardiac function [87]. The role of the CLIC family of proteins is poorly understood, and no data are available on the role of CLIC in the heart; however, cellular volume control, cellular motility, and apoptosis have been suspected [88-90]. The possible role of CLIC in hyperlipidemia in the heart is an entirely new observation that needs further study. Another gene encoding a membrane-bound protein, fibronectin receptor L chain gene, was also markedly down-regulated. Integrins are heterodimeric receptors that couple the extracellular matrix to intracellular signaling pathways and the cytoskeleton. Integrins have been suggested to play a role in cardiac development and several cardiovascular disorders [91, 92]. Our present study is the first to suggest that down-regulation of fibronectin receptor plays a role in the cardiac effects of hyperlipidemia.

### 8.6. Hyperlipidemia-induced cardiac overexpression of genes

The most significantly up-regulated genes were membrane proteins, p120 catenin isoform 4B, SH3-containing protein SH3P4 (endophilin), Clara cell phospholipid binding protein, and NIPSNAP2 protein coding genes. The exact role of these proteins in the heart is not known. P120 catenin affects cell-cell adhesion, as it controls internalization and degradation of classical cadherins in endothelial cells [93], and it is likely to have additional roles in the nucleus [94]. It is plausible to speculate that the expression changes of these membrane protein genes may be

partly the consequence of the possible alterations in membrane composition of the heart due to cholesterol diet-induced hyperlipidemia.

Several regulatory protein genes were induced after cholesterol treatment: a protein kinase, a pleiotropic regulator, the regulatory subunit 9A of the protein phosphatase 1, and a transcriptional repressor. Among the regulatory protein coding genes, the protein kinase C inhibitor S gene was slightly induced, which was confirmed by QRT-PCR as well. Induction of this gene could have a dramatic effect on apoptosis of cardiomyocytes, as protein kinase C inhibitor S is the critical downstream target of Bcr-Abl, which mediates the anti-apoptotic effects of Bcr-Abl [95]. Moreover, Wang *et al.* have shown that diet-induced hypercholesterolemia in rabbits was associated with a markedly increased activation of caspase-3 and an increased myocardial Bcl-2/Bax ratio (markers of apoptosis) within the ischemic myocardium, showing the increased extent of cardiomyocyte apoptosis [96].

We have previously shown that transcription of hsp70 was significantly higher in hearts of rats fed a 2% cholesterol-enriched diet compared to normal controls, although the HSP70 protein level was not different [13]. We have also shown that hyperlipidemia inhibits expression of cardiac Hsp70 in response to heat stress and ischemia [13]. Here, we have detected extensive changes in the expression of stress proteins due to hyperlipidemia. The induction of heat stress proteins is well known in response to myocardial, renal, and cerebral ischemia [97-99], but this is the first demonstration that hsp86 and the antioxidants metallothionein II and glutathione S-transferase can be induced by cholesterol diet. However, other stress proteins, such as chaperonin subunit 5O, 105-kDa heat shock protein, calreticulin, and a ubiquitin-like protein were significantly down-regulated. Mechanisms that underlie the opposite regulation of these stress proteins due to high-cholesterol diet would be interesting to clarify, as still little is known about the function of these proteins in the heart [100, 101].

Opposite changes in transcription of structural protein genes have been observed in the present study. Genes encoding a fibrogenic gene (fibroblast-inducible secreted protein) was down-regulated, while genes for cofilin/destrin (actin depolymerizing factor), calsarcin-1, myozenin-like 2, and procollagen III K1 were induced by high-cholesterol diet. Function of these proteins in the heart might be some remodeling process in response to hyperlipidemia. However, very little is known about the exact role of these genes in the heart.

Activity of numerous genes with diverse function was also significantly altered in hearts of rats fed cholesterol-rich diet as shown in Table 2. For example, we have observed overexpression of Mad-related protein Smad7. Recent reports have implicated Smad7 as a crucial regulator of transforming growth factor L activity in human disease [102] and found that

Smad6 and Smad7 constitute a novel class of MAD-related proteins, termed vascular MADs, which are induced by fluid mechanical forces and can modulate gene expression in response to both humoral and biomechanical stimulation in vascular endothelium [103].

These findings show that various cellular mechanisms are involved in direct cardiac effect of hyperlipidemia induced by cholesterol-enriched diet, and emphasize that much more investigations should be carried out in order to thoroughly describe mechanisms underlying the hyperlipidemia in the heart.

## 9. CONCLUSIONS

We demonstrated that modulation of the mevalonate pathway does not change bioavailability of NO in heart. Furthermore, hyperlipidemia-induced decrease in NO bioavailability in the heart is not dependent on the mevalonate pathway. Therefore, to restore NO level in the heart in hyperlipidemia, pharmacological manipulation of the mevalonate pathway does not seem to be a valid option.

Our results show that hyperlipidemia blocks preconditioning-induced moderate inhibition of cardiac MMP-2 activation and release and therefore attenuates the infarct size limiting effect of preconditioning; preconditioning-induced inhibition of MMP-2 activation and release is independent of an effect of TIMP-2 or TIMP-4, and that pharmacological inhibition of MMPs by ilomastat reduces infarct size in both control and hyperlipidemic rats. These results strongly suggest that pharmacological inhibition of MMPs may be a powerful cardioprotective mechanism in acute ischemic challenge.

Furthermore, we conclude that cholesterol-enriched diet-induced hyperlipidemia leads to significant changes in expression of several genes in rat hearts. As hyperlipidemia does not lead to coronary sclerosis and myocardial ischemia in our rat model, gene expression changes can be attributed to a direct effect of hyperlipidemia on the myocardium. The role of most of the genes we have found to be regulated by hyperlipidemia is not exactly known in the heart, therefore, our present findings open new directions in the research of the cardiac effects of hyperlipidemia.

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