

**Title Page**

**Hyperlipidemia attenuates the infarct-size limiting effect of ischemic preconditioning: role of matrix metalloproteinase-2 inhibition**

Zoltán Giricz, Manoj M. Lalu, Csaba Csonka, Péter Bencsik, Richard Schulz, Péter Ferdinandy

**Primary laboratory of origin:**

Cardiovascular Research Group, Department of Biochemistry, University of Szeged, Hungary

**Affiliations:**

Cardiovascular Research Group, Department of Biochemistry, University of Szeged, Hungary (Z.G., C.C., P.B., P.F.)

Departments of Pharmacology (M.M.L., R.S.) and Pediatrics (R.S.), Cardiovascular Research Group, University of Alberta, Edmonton, Canada

PharmaHungary 2000 Co, Szeged, Hungary [www.cardiovasc.com](http://www.cardiovasc.com) (P.F.)

## **Running Title Page**

Running title: Matrix metalloproteinases in cardioprotection

Corresponding author:

Péter Ferdinandy, MD, PhD, DSc

Cardiovascular Research Group, Department of Biochemistry

University of Szeged,

Dóm tér 9, Szeged, H-6720, Hungary

Phone: (+36) 62 545 755, fax: (+36) 62 545 097

E-mail: peter@biochem.szote.u-szeged.hu

**<http://www.cardiovasc.com>**

Text pages: 29

Tables: 0

Figures: 7

References: 32

Number of words:

Abstract: 246

Introduction: 614

Discussion: 1500

Abbreviations: ONOO<sup>-</sup>, peroxynitrite; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; TTC, triphenyltetrasolium-chloride; LDH, lactate-dehydrogenase

Section assignment: Cardiovascular

## **Abstract**

Hyperlipidemia attenuates the cardioprotective effect of preconditioning via unknown mechanisms. We have previously reported that in normolipidemic rats preconditioning decreased ischemia-induced activation and release of myocardial matrix metalloproteinase-2 (MMP-2) into the coronary perfusate. Here we investigated whether hyperlipidemia interferes with the cardioprotective effect of preconditioning through modulation of MMP-2. Hearts isolated from male Wistar rats fed 2% cholesterol-enriched or control chow for 9 weeks were subjected to a preconditioning protocol (three intermittent periods of ischemia/reperfusion of 5 min duration each) or a time-matched non-preconditioning protocol. This was followed by a test ischemia/reperfusion (30 min ischemia and 120 min reperfusion) in both groups. Preconditioning decreased infarct size in the control but not the cholesterol-fed group. Cardioprotection in the preconditioned control group but not in the cholesterol-fed group was associated with a  $18\pm 3\%$  ( $p < 0.05$ ) inhibition of test ischemia/reperfusion-induced activation and release of myocardial MMP-2 into the perfusate. Myocardial protein levels of tissue inhibitors of MMPs (TIMP-2 and TIMP-4) were not changed in either group. A reduction of infarct size in non-preconditioned hearts from both control and cholesterol-fed group was produced by the MMPs inhibitor ilomastat at 0.25  $\mu\text{M}$ , a concentration producing MMP-2 inhibition comparable to that of preconditioning in the control group. We conclude that: (i) hyperlipidemia blocks preconditioning-induced cardioprotection, (ii) hyperlipidemia abolishes preconditioning-induced inhibition of myocardial MMP-2 activation and release, (iii) preconditioning-induced inhibition of MMP-2 activation and release is not mediated by TIMPs, and (iv) pharmacological inhibition of MMPs produces cardioprotection in both normal and hyperlipidemic rats.

## **Introduction**

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) (Yellon and Downey, 2003; Baxter and Ferdinandy, 2001). Although preconditioning confers 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: Ferdinandy et al., 1998b; Ferdinandy, 2003).

Although most studies show that hyperlipidemia inhibits the cardioprotective effect of preconditioning (when looking at end-points such as ST-segment elevation or cardiac function), there is a controversy whether the infarct-size limiting effect of preconditioning is lost in hyperlipidemia or not (see for review: Ferdinandy, 2003). The discrepancies can be attributed to the substantial differences in hyperlipidemia models (species, duration of hyperlipidemic diet, presence of significant coronary sclerosis). Therefore, here we have used hearts of male 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 (Horton et al., 1995; Roach et al., 1993; Ferdinandy et al., 1997).

The mechanism by which hyperlipidemia abrogates the protective effect of preconditioning is not exactly known, however, several mechanisms have been proposed. It appears that this phenomenon is based on a direct effect of hyperlipidemia on the myocardium rather than an indirect effect through coronary sclerosis (Ferdinandy et al., 1997; Szilvassy et al., 1995). We have previously

reported that peroxynitrite ( $\text{ONOO}^-$ ) is involved in preconditioning (Csonka et al., 2001), and that hyperlipidemia leads to enhanced formation of  $\text{ONOO}^-$  (Onody et al., 2003). A significant cellular target of  $\text{ONOO}^-$  is activation of a large family of zinc-dependent endopeptidases, matrix metalloproteinases (MMPs) via a non-proteolytic oxidative mechanism resulting in fully active proenzymes (Okamoto et al., 2001). It has been demonstrated that MMPs play a role not only in long-term extracellular matrix remodelling, but also in acute regulation of cardiac functions. E.g., MMP-7 promotes vasoconstriction in rat mesenteric arteries (Hao et al., 2004) and ischemia-induced activation and release of MMP-2 is a major effector of acute mechanical dysfunction after ischemia-reperfusion in rat hearts (Cheung et al., 2000; Wang et al., 2002; Lalu et al., 2005). Furthermore, signalling pathways involved in the mechanism of preconditioning influence the expression or activation of MMPs. E.g., activation of protein kinase C- $\zeta$  and  $\theta$  subtypes increases expression of MMP-2 in rat cardiac fibroblast culture (Xie et al., 2004). Phosphatidylinositol 3-kinase-dependent upregulation of membrane-type 1-MMP expression modulates MMP-2 activity in injured pig coronary arteries (Zahradka et al., 2004). We have previously shown that preconditioning inhibits ischemia-induced activation and release of MMP-2 into the perfusate in rat hearts (Lalu et al., 2002), however, it is not known if MMPs and their endogenous inhibitors, the tissue inhibitors of matrix metalloproteinases (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 preconditioning-induced inhibition of MMP activation and release. Furthermore, we speculated that an imbalance between MMP

activity and TIMPs may be involved in this mechanism. Therefore, we here studied the infarct size limiting effect of preconditioning in control chow and cholesterol-enriched chow-fed rats, and measured changes in cardiac MMP-2 activity and TIMP levels as well as activation and release of cardiac MMP-2 into the perfusate. To prove the link between MMP inhibition and cardioprotection, we have also studied the possible infarct size limiting effect of pharmacological inhibition of MMPs in control chow and cholesterol-enriched chow-fed rats.

## **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 it was approved by a local animal ethics committee.

### **Experimental design**

Eighteen-week old male Wistar rats were fed control 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 (Roach, et al., 1993). The 9-week cholesterol-enriched diet increased serum cholesterol from  $1.45 \pm 0.05$  to  $1.92 \pm 0.10$  (n=6 in each group,  $p < 0.05$ ) and serum triglyceride from  $0.52 \pm 0.04$  to  $1.25 \pm 0.14$  (n=6-12,  $p < 0.05$ ) mmol/L, similar to our previous studies (Giricz et al., 2003; Puskas et al., 2004).

At the end of the diet-period, rats were anesthetized with diethyl ether, heparin (500 U/kg *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 x 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 1). In separate experiments, ventricular tissue was freeze-clamped and crushed at liquid N<sub>2</sub> temperature after the initial 15 min perfusion or at the 5<sup>th</sup> 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 1). Heart rate was monitored as previously described (Ferdinandy et al., 1998a).

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  $\mu\text{M}$  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 1).

### **Measurement of infarct size**

After test ischemia/reperfusion, 5 mL of 1% triphenyltetrazolium-chloride (TTC, Sigma, St. Louis, MO) dissolved in phosphate buffer (pH 7.4) was slowly injected for 5 min into the aorta to stain the myocardium. TTC-stained hearts were frozen ( $-20^{\circ}\text{C}$ ), 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.

### **Measurement of lactate-dehydrogenase (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  $\text{mU}\times\text{min}^{-1}\times\text{g}^{-1}$  wet heart weight.

## **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 1). Gelatinolytic activities of MMPs were examined as previously described (Cheung et al., 2000). Briefly, 8% polyacrylamide gels were copolymerized with gelatin (2 mg/mL, type A from porcine skin, Sigma, St. Louis, MO) 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 x 15 min, and incubated for 24-48 h at 37 °C in incubation buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl<sub>2</sub>, and 0.05% NaN<sub>3</sub>, 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 4% methanol:8% acetic acid (v/v). Gelatinolytic activities were detected as transparent bands against the dark blue background. Zymograms were digitally scanned and band intensities were quantified using Quantity One software (BioRad Laboratories, Hercules, CA) and expressed as a ratio to the internal standard. Band density was expressed as arbitrary units/mg protein to exclude a possible nonspecific release of MMP into the perfusate during ischemia.

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

In order to determine the concentration of ilomastat that inhibits MMP-2 activity comparable to the inhibitory effect of preconditioning on MMP-2 activation and its release from the ischemic-reperfused heart (Lalu et al., 2002), we determined the concentration-response relationship of the MMP-2 inhibitory effect of ilomastat in rat heart tissue homogenate *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  $\mu\text{M}$  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.

### **Western blot analysis of TIMP-2 and TIMP-4 proteins**

To assess cardiac TIMP-2 and TIMP-4 protein levels, in separate experiments cardiac tissue was frozen 5 min after test ischemia. Cardiac tissue was then homogenized and diluted to load 15  $\mu\text{g}$  of total protein on 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 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) 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  $\times$  intensity, expressed in arbitrary units).

### **Cardiac nitrotyrosine measurement**

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

### **Protein assay**

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

### **Statistical analysis**

Results were expressed as mean  $\pm$  SEM and analyzed by SigmaStat software (Jandel Scientific, Costa Madre, CA) as appropriate. One way analysis of variance (ANOVA) followed by Tukey post-hoc test was used to evaluate differences between groups. Differences were considered significant at  $p < 0.05$ .

## **Results**

### **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 2A).

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 2B).

Heart rate and coronary flow were not changed significantly by cholesterol diet (data not shown).

### **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 1). 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 3A and C). This loss was accompanied by an increase in 72 and 62 kDa activities in the coronary perfusate

(Fig 3B and D). In the control chow-fed group, preconditioning attenuated the release of 72 and 62 kDa MMP-2 by  $17.6\pm 3.4\%$  ( $p<0.05$ ) and  $21.9\pm 7.9\%$  ( $p<0.05$ ), 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 3B and D).

### **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 4A-B).

### **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 17-22%, 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 5). We found that ilomastat inhibited myocardial MMP-2 activity by  $3.0\pm 0.8\%$  at 0.1  $\mu\text{M}$  and  $19.1\pm 1.4\%$  at 0.25  $\mu\text{M}$ . Therefore, we chose 0.25  $\mu\text{M}$  and 0.1  $\mu\text{M}$  for further heart perfusion studies. After test ischemia/reperfusion 0.1  $\mu\text{M}$  ilomastat failed to significantly decrease infarct size and LDH release in hearts of both control and cholesterol-fed animals, however 0.25  $\mu\text{M}$  ilomastat significantly decreased infarct

size and LDH release both in control and cholesterol-fed group also (Fig 6A-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  $\mu\text{M}$  ilomastat protected hearts against ischemic injury not only in hearts from control chow-fed rats, but also in those from cholesterol-fed animals.

### **Effect of hyperlipidemia on cardiac nitrotyrosine level**

In order to investigate whether hyperlipidemia influences cardiac formation of  $\text{ONOO}^-$ , we have measured cardiac nitrotyrosine level and found that hyperlipidemia significantly increased nitrotyrosine formation in the heart, as compared to controls (Fig. 7).

## **Discussion**

This is the first demonstration that preconditioning decreases infarct size and LDH release and leads to a moderate, approximately 18% inhibition of ischemia-induced activation and release of myocardial MMP-2 in control but not cholesterol-fed rats, while cardiac TIMP-2 and TIMP-4 levels were not affected in either group. Furthermore, we have shown here for the first time that the MMP-inhibitor ilomastat, at a concentration showing an MMP-2 inhibitory effect comparable to that of preconditioning, significantly reduces infarct size in both control and cholesterol-fed groups. These results clearly show that: (i) 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, (ii) preconditioning-induced inhibition of ischemia-induced MMP-2 activation and release are not mediated by a change in cardiac TIMP-2 or 4 level, and (iii) pharmacologic inhibition of MMPs markedly reduces infarct size in both control and hyperlipidemic rats.

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 (Ueda *et al.*, 1999). Moreover, Juhasz *et al.* found a significant increase in infarct size in preconditioned hyperlipidemic rabbits compared to nonpreconditioned ischemic control (Juhasz *et al.*, 2004). 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 (Li *et al.*, 2001) or in rabbits fed a cholesterol-enriched

diet for 8 weeks (Kremastinos et al., 2000), or cholesterol and coconut-oil rich chow for 4 weeks (Jung et al., 2000), 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: Ferdinandy et al., 1998b; Ferdinandy and Schulz, 2003; Ungi et al., 2005).

Mechanisms by which hyperlipidemia affects preconditioning are not exactly known. A few studies demonstrated structural and functional alterations in the myocardium due to hypercholesterolemia (Hexeberg et al., 1993; Senges et al., 1981). Ecto-5'-nucleotidase activity (Ueda et al., 1999), inhibition of the mevalonate pathway (Ferdinandy et al., 1998a), as well as enhanced apoptotic cell death (Wang et al., 2002) 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 preconditioning in hyperlipidemia (Ferdinandy et al., 1997). Furthermore, we have shown that hyperlipidemia leads to increased formation of ONOO<sup>-</sup> in the heart (Onody et al., 2003). As it was shown that ONOO<sup>-</sup> oxidatively activates MMPs (Okamoto et al., 2001), and we have demonstrated that downstream targets of ONOO<sup>-</sup>, the MMPs, contribute to preconditioning-induced cardioprotection via

preconditioning-induced inhibition of their myocardial activation and release (Lalu et al., 2002), here we hypothesized that altered activation of MMP-2 and its release into the perfusate might contribute to the loss of preconditioning in hyperlipidemia.

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 (Lalu et al., 2002). The 75 kDa activity appeared only intermittently, therefore, we excluded it from data analysis. As previously reported (Cheung et al., 2000; Wang et al., 2002; Lalu et al., 2002), MMP-9 (92 kDa and 84 kDa) was not detectable by zymography in cardiac tissue or perfusate samples in our present studies. Here we have shown that the 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 (Cheung et al., 2000). 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 (Orbe et al., 2003), 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<sup>-</sup> was shown to directly activate MMPs by S-glutathiolation of a cysteine residue in the autoinhibitory pro-peptide domain, allowing the conformational change which results in a fully active 72 kDa pro-MMP-2 (Okamoto et al., 2001; Rajagopalan et al., 1996). Moreover, we have previously demonstrated that infusion of ONOO<sup>-</sup> 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 (Wang et al., 2002). Furthermore, we have also shown that preconditioning attenuates the activation and release of myocardial MMP-2, thereby protecting the heart from ischemic injury (Lalu et al., 2002). 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<sup>-</sup> 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% MMP inhibition by 0.25  $\mu$ M 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.

Taken together, our results show that: (i) hyperlipidemia blocks preconditioning-induced moderate inhibition of cardiac MMP-2 activation and release and therefore attenuates the infarct size limiting effect of preconditioning, (ii) preconditioning-induced inhibition of MMP-2 activation and release is independent of an effect of TIMP-2 or TIMP-4, and (iii) 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.

## **References**

- Baxter GF and Ferdinandy P (2001) Delayed preconditioning of myocardium: current perspectives. *Basic Res Cardiol* 96:329-344.
- Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski MW and Schulz R (2000) Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. *Circulation* 101:1833-1839.
- Csonka C, Csont T, Onody A and Ferdinandy P (2001) Preconditioning decreases ischemia/reperfusion-induced peroxynitrite formation. *Biochem Biophys Res Commun* 285:1217-1219.
- Ferdinandy P (2003) Myocardial ischaemia/reperfusion injury and preconditioning: effects of hypercholesterolaemia/hyperlipidaemia. *Br J Pharmacol* 138:283-285.
- Ferdinandy P, Csonka C, Csont T, Szilvassy Z and Dux L (1998a) Rapid pacing-induced preconditioning is recaptured by farnesol treatment in hearts of cholesterol-fed rats: role of polyprenyl derivatives and nitric oxide. *Mol Cell Biochem* 186:27-34.
- Ferdinandy P and Schulz R (2003) Nitric oxide, superoxide and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. *Br J Pharmacol* 138:532-543.
- Ferdinandy P, Szilvassy Z and Baxter GF (1998b) Adaptation to myocardial stress in disease states: is preconditioning a healthy heart phenomenon? *Trends Pharmacol Sci* 19:223-229.

Ferdinandy P, Szilvassy Z, Horvath LI, Csont T, Csonka C, Nagy E, Szentgyorgyi R, Nagy I, Koltai M and Dux L (1997) Loss of pacing-induced preconditioning in rat hearts: role of nitric oxide and cholesterol-enriched diet. *J Mol Cell Cardiol* 29:3321-3333.

Gircz Z, Csonka C, Onody A, Csont T and Ferdinandy P (2003) Role of cholesterol-enriched diet and the mevalonate pathway in cardiac nitric oxide synthesis. *Basic Res Cardiol* 98:304-310.

Hao L, Du M, Lopez-Campistrous A and Fernandez-Patron C (2004) Agonist-induced activation of matrix metalloproteinase-7 promotes vasoconstriction through the epidermal growth factor-receptor pathway. *Circ Res* 94:68-76.

Hexeberg S, Willumsen N, Rotevatn S, Hexeberg E and Berge RK (1993) Cholesterol induced lipid accumulation in myocardial cells of rats. *Cardiovasc Res* 27:442-446.

Horton JD, Cuthbert JA and Spady DK (1995) Regulation of hepatic 7 alpha-hydroxylase expression and response to dietary cholesterol in the rat and hamster. *J Biol Chem* 270:5381-5387.

Juhasz B, Der P, Turoczi T, Bacskay I, Varga E and Tosaki A (2004) Preconditioning in intact and previously diseased myocardium: laboratory or clinical dilemma? *Antioxid Redox Signal* 6:325-333.

Jung O, Jung W, Malinski T, Wiemer G, Schoelkens BA and Linz W (2000) Ischemic preconditioning and infarct mass: the effect of hypercholesterolemia and endothelial dysfunction. *Clin Exp Hypertens* 22:165-179.

Kremastinos DT, Bofilis E, Karavolias GK, Papalois A, Kaklamanis L and Iliodromitis EK (2000) Preconditioning limits myocardial infarct size in hypercholesterolemic rabbits. *Atherosclerosis* 150:81-89.

Lalu MM, Csonka C, Giricz Z, Csont T, Schulz R and Ferdinandy P (2002) Preconditioning decreases ischemia/reperfusion-induced release and activation of matrix metalloproteinase-2. *Biochem Biophys Res Commun* 296:937-941.

Lalu MM, Pasini E, Schulze CJ, Ferrari-Vivaldi M, Ferrari-Vivaldi G, Bachetti T and Schulz R (2005) Ischaemia-reperfusion injury activates matrix metalloproteinases in the human heart. *Eur Heart J* 26:27-35.

Li G, Tokuno S, Tahep IP, Vaage J, Lowbeer C and Valen G (2001) Preconditioning protects the severely atherosclerotic mouse heart. *Ann Thorac Surg* 71:1296-1303.

Okamoto T, Akaike T, Sawa T, Miyamoto Y, van d, V and Maeda H (2001) Activation of matrix metalloproteinases by peroxynitrite-induced protein S-glutathiolation via disulfide S-oxide formation. *J Biol Chem* 276:29596-29602.

Onody A, Csonka C, Giricz Z and Ferdinandy P (2003) Hyperlipidemia induced by a cholesterol-rich diet leads to enhanced peroxynitrite formation in rat hearts. *Cardiovasc Res* 58:663-670.

Orbe J, Rodriguez JA, Arias R, Belzunce M, Nespereira B, Perez-Illzarbe M, Roncal C and Paramo JA (2003) Antioxidant vitamins increase the collagen content and reduce MMP-1 in a porcine model of atherosclerosis: implications for plaque stabilization. *Atherosclerosis* 167:45-53.

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

Rajagopalan S, Meng XP, Ramasamy S, Harrison DG and Galis ZS (1996) Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest* 98:2572-2579.

Roach PD, Balasubramaniam S, Hirata F, Abbey M, Szanto A, Simons LA and Nestel PJ (1993) The low-density lipoprotein receptor and cholesterol synthesis are affected differently by dietary cholesterol in the rat. *Biochim Biophys Acta* 1170:165-172.

Senges J, Weihe E, Brachmann J, Pelzer D, Nimmrich H and Kubler W (1981) Effects of hypercholesterolaemia without ischemia on some electrophysiological and ultrastructural properties of the rabbit heart. *J Mol Cell Cardiol* 13:253-264.

Szilvassy Z, Ferdinandy P, Szilvassy J, Nagy I, Karcsu S, Lonovics J, Dux L and Koltai M (1995) The loss of pacing-induced preconditioning in atherosclerotic rabbits: role of hypercholesterolaemia. *J Mol Cell Cardiol* 27:2559-2569.

Ueda Y, Kitakaze M, Komamura K, Minamino T, Asanuma H, Sato H, Kuzuya T, Takeda H and Hori M (1999) Pravastatin restored the infarct size-limiting effect of ischemic preconditioning blunted by hypercholesterolemia in the rabbit model of myocardial infarction. *J Am Coll Cardiol* 34:2120-2125.

Ungi I, Ungi T, Ruzsa Z, Nagy E, Zimmermann Z, Csont T and Ferdinandy P (2005) Hypercholesterolemia attenuates the anti-ischemic effect of preconditioning during coronary angioplasty. *Chest* in press

Wang W, Sawicki G and Schulz R (2002) Peroxynitrite-induced myocardial injury is mediated through matrix metalloproteinase-2. *Cardiovasc Res* 53:165-174.

Xie Z, Singh M and Singh K (2004) Differential regulation of matrix metalloproteinase-2 and -9 expression and activity in adult rat cardiac fibroblasts in response to interleukin-1beta. *J Biol Chem* 279:39513-39519.

Yellon DM and Downey JM (2003) Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev* 83:1113-1151.

Zahradka P, Harding G, Litchie B, Thomas S, Werner JP, Wilson DP and Yurkova N (2004) Activation of MMP-2 in response to vascular injury is mediated by phosphatidylinositol 3-kinase-dependent expression of MT1-MMP. *Am J Physiol Heart Circ Physiol* 287:H2861-H2870.

## **Footnotes**

### Financial support:

This work was supported by grants from the Hungarian Scientific Research Found (OTKA T046417), Hungarian Ministry of Health (ETT 616/2003), the National Research and Development Program (NKFP 001/2001), and the North Atlantic Treaty Organization Cooperative Linkage grant (NATO, LST.CLG.976650). MML was a graduate trainee of the Alberta Heritage Foundation for Medical Research (AHFMR) and the Canadian Institutes of Health Research, and NATO visiting student at the University of Szeged. RS is a Senior Scholar of the AHFMR. PF holds an István Széchenyi Professorship of the Hungarian Academy of Sciences.

### Reprint requests to:

Péter Ferdinandy

Cardiovascular Research Group, Department of Biochemistry

University of Szeged,

Dóm tér 9, Szeged, H-6720, Hungary

E-mail: peter@biochem.szote.u-szeged.hu

www.cardiovasc.com

### **Legend for figures**

Figure 1. Experimental protocol.

Control and 2% cholesterol-enriched chow-fed animals were subjected to either 3x5-5 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 120<sup>th</sup> min of reperfusion. In separate experiments, left ventricles were frozen in liquid nitrogen after the initial 15 min perfusion or at the 5<sup>th</sup> 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  $\mu\text{mol/L}$  MMP-inhibitor ilomastat (Control+ilo, Chol+ilo) as well.

Figure 2. 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.

Figure 3. 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.

Panel C and D: Representative zymograms of heart tissue and perfusate, respectively.

Figure 4. 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.

Figure 5. 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  $\mu$ M ilomastat for 3 hours in the incubation buffer. n=3 in each group.

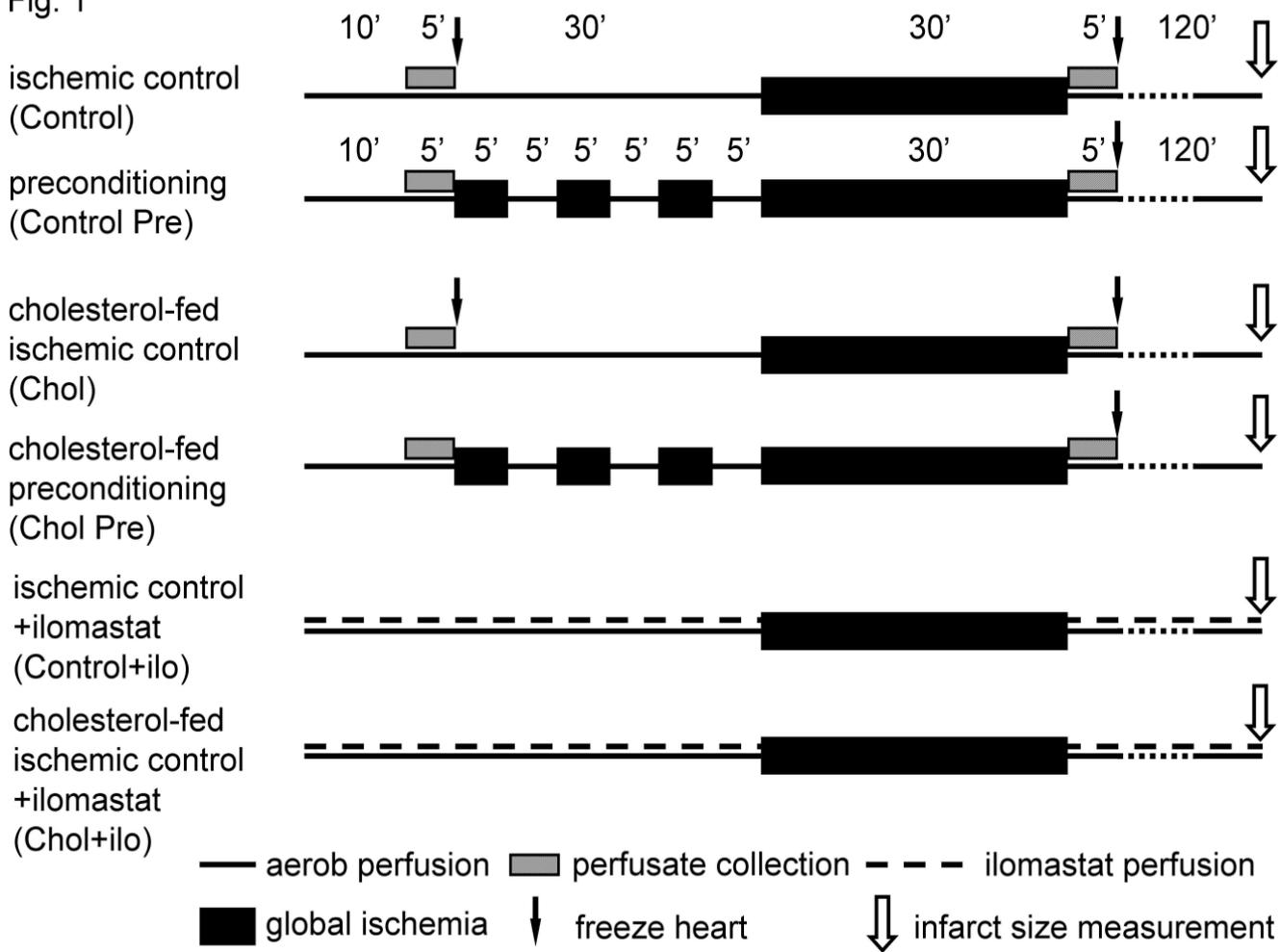
Figure 6. Effects of the MMP inhibitor ilomastat on infarct size and lactate dehydrogenase (LDH) release in control and cholesterol-fed group.

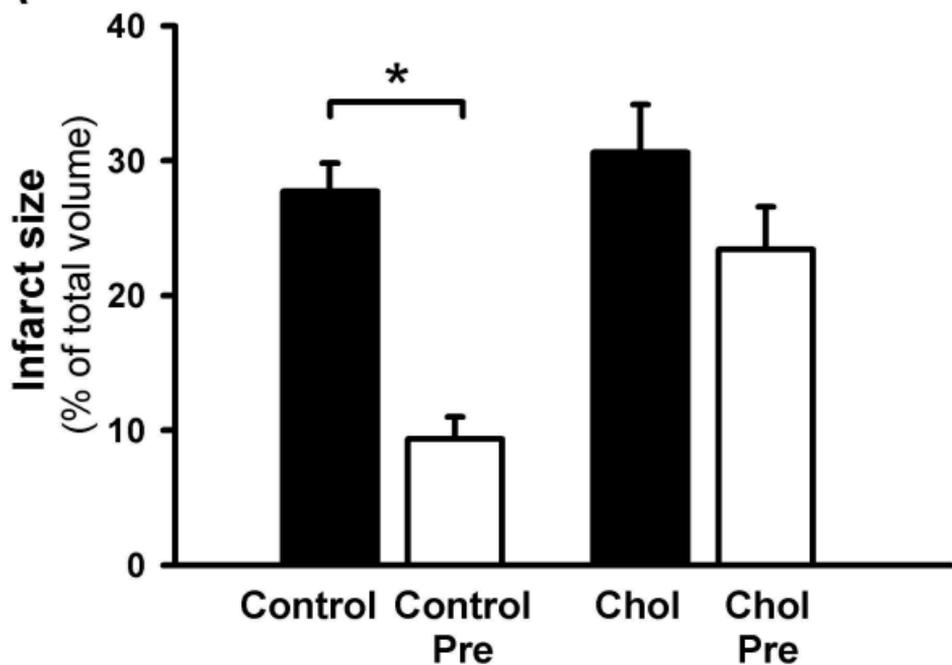
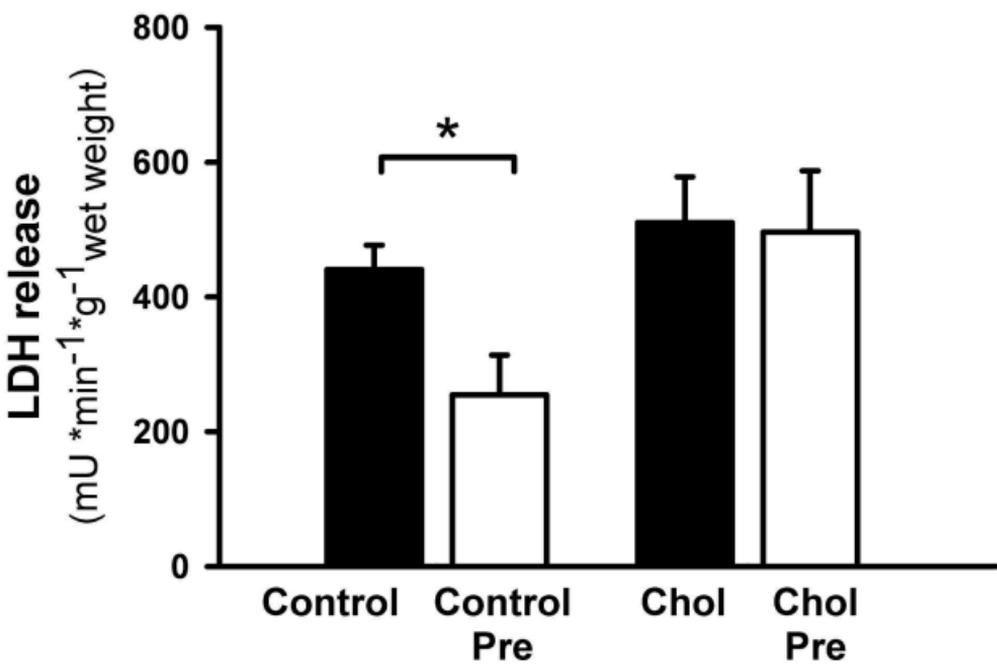
Ilomastat (0.1  $\mu$ M and 0.25  $\mu$ M) dose-dependently decreased infarct size and LDH release both in control (panel A and C) and cholesterol-fed rats (panel B and D). n=5-8 in each group; \*p<0.05 vs. Control or Chol.

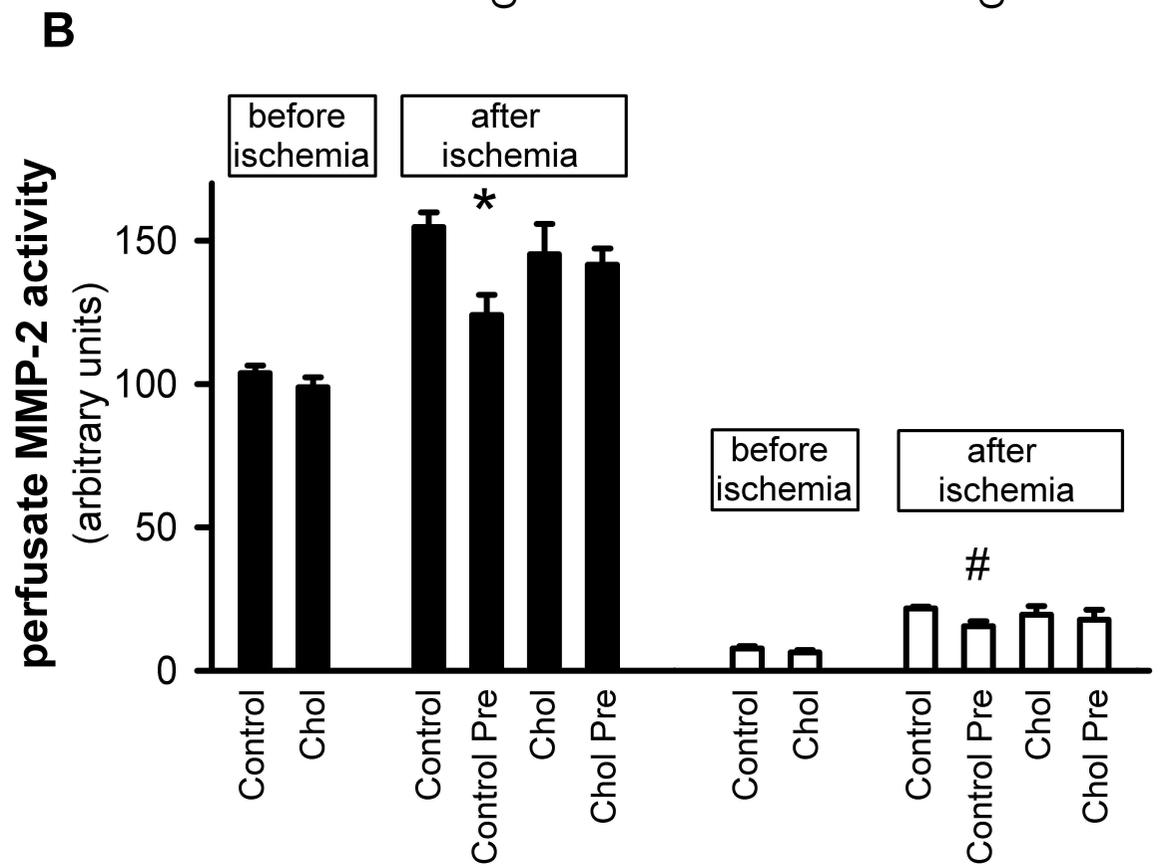
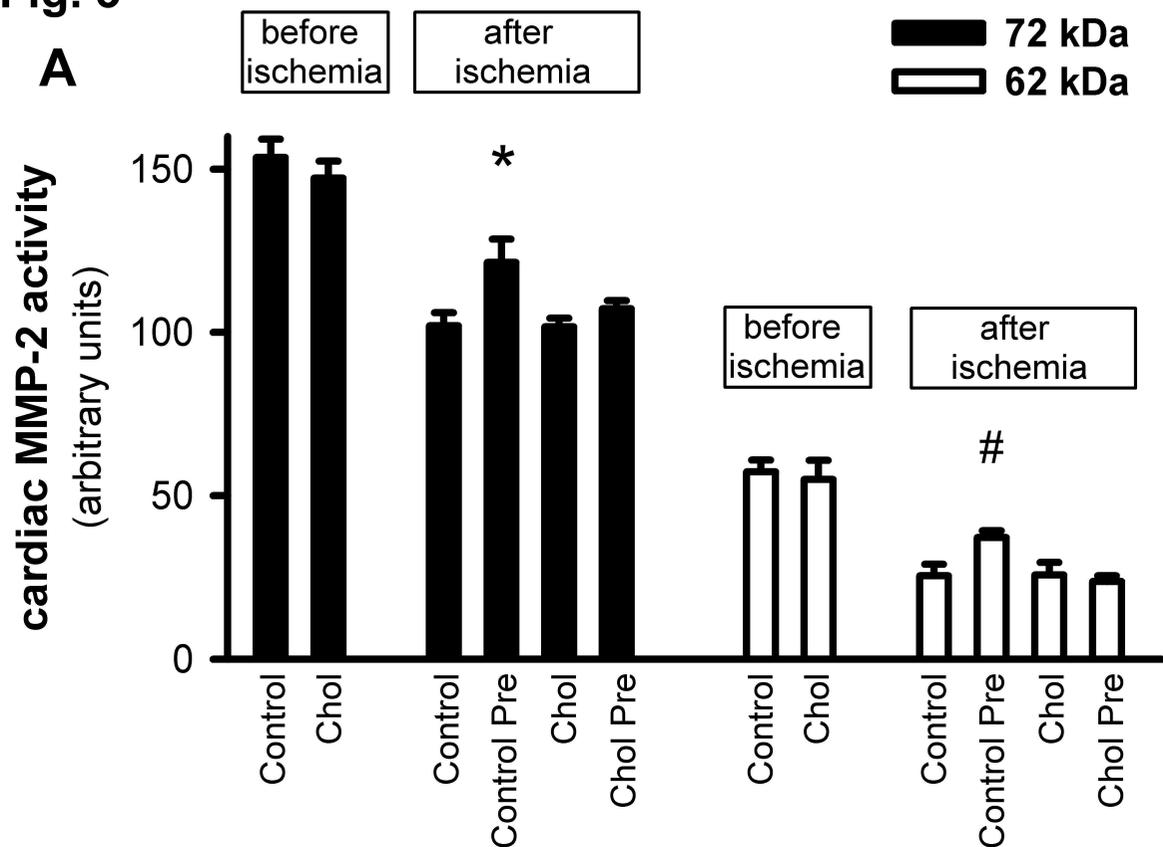
Figure 7. Effects of hyperlipidemia on cardiac nitrotyrosine.

Hyperlipidemia enhanced baseline cardiac nitrotyrosine formation as compared to controls (n=4 in each group; \*p<0.05 vs. Control).

Fig. 1



**Fig. 2****A****B**

**Fig. 3**

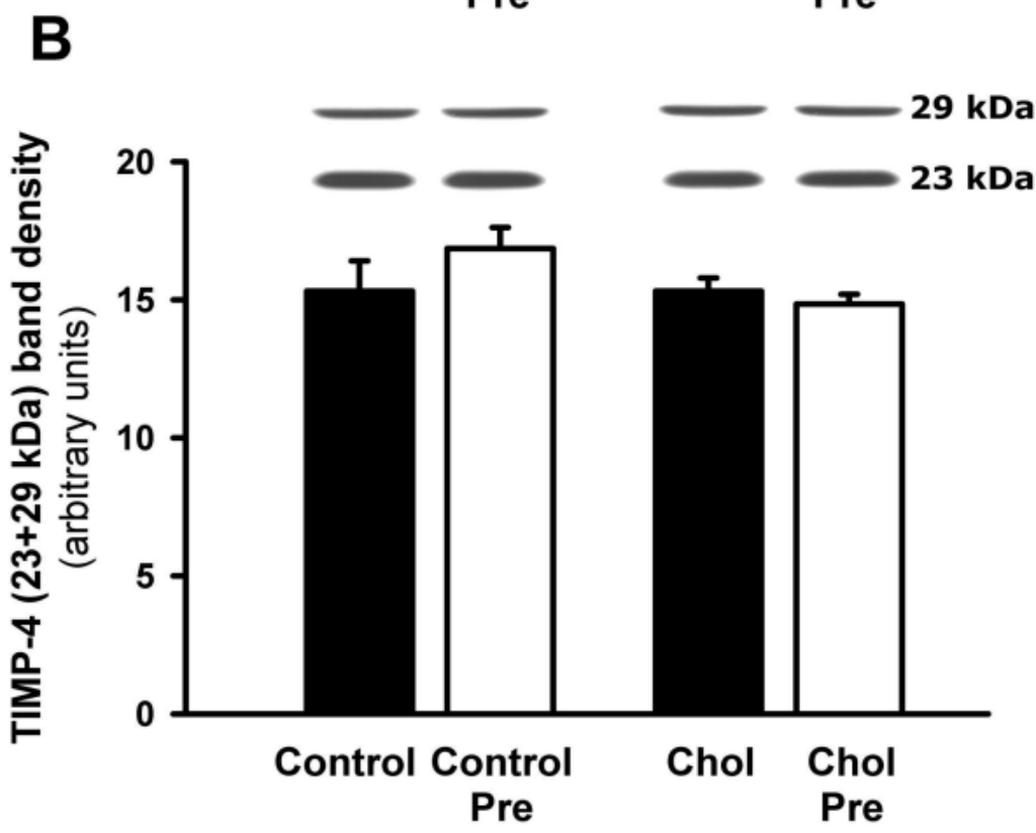
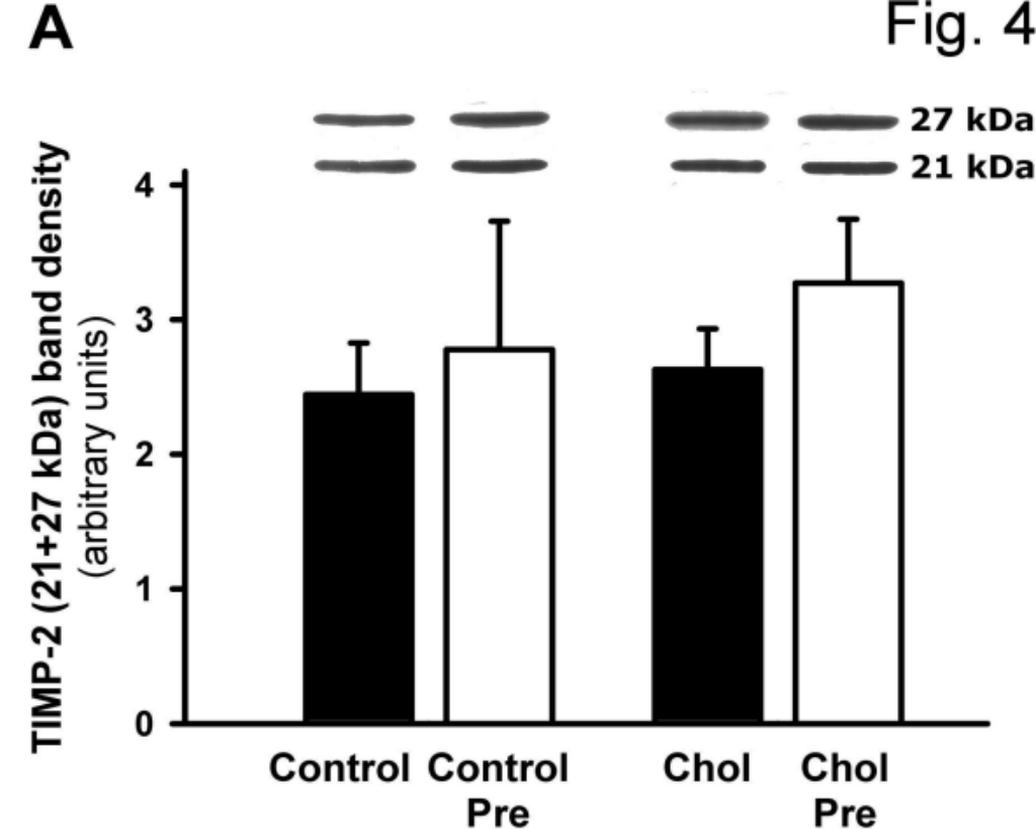
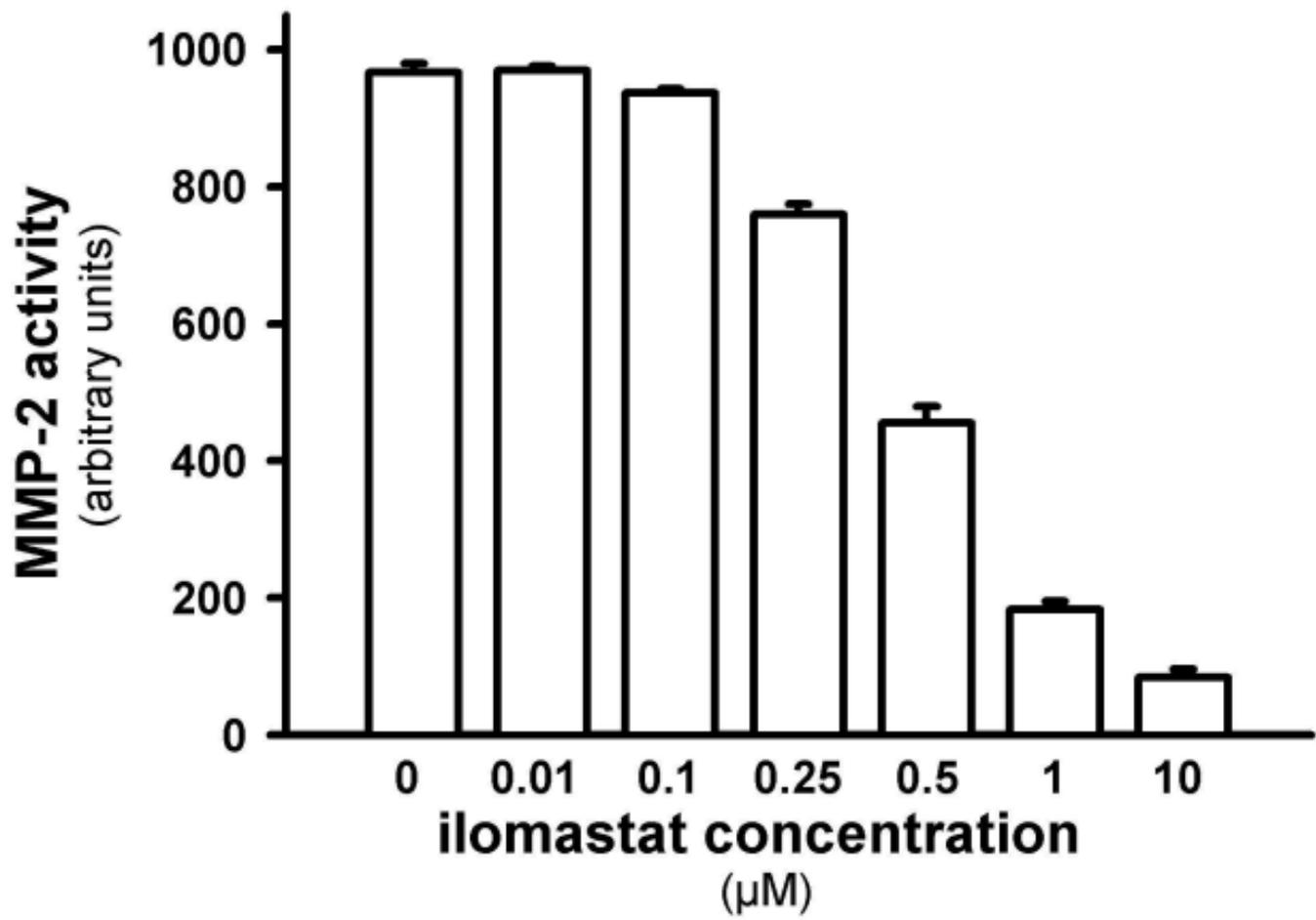
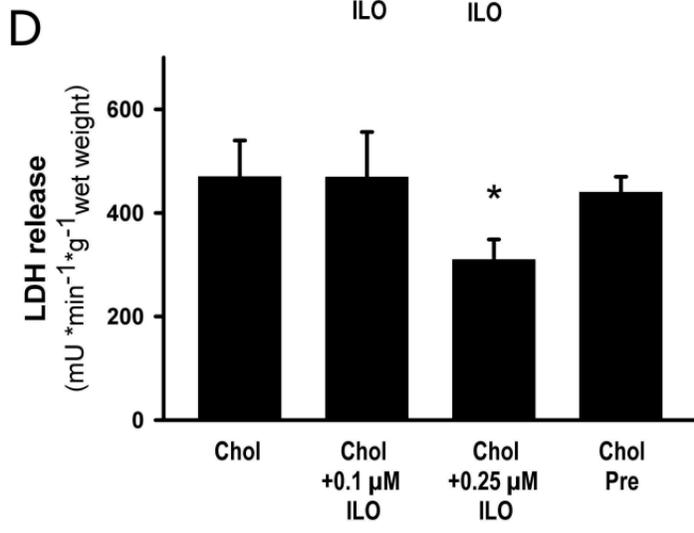
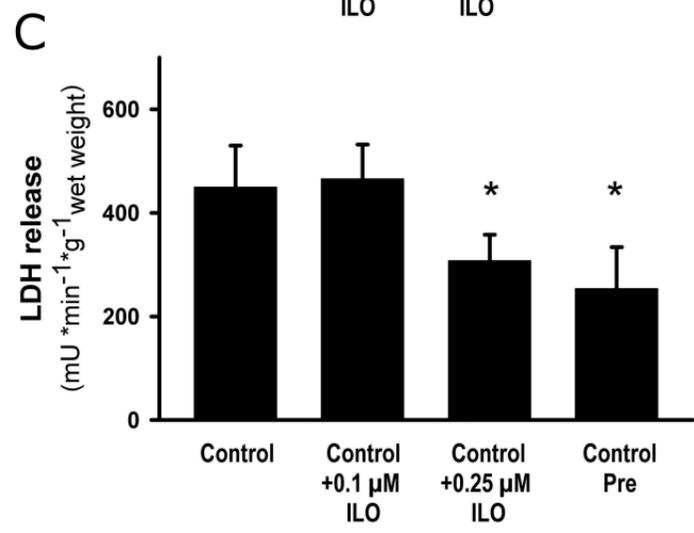
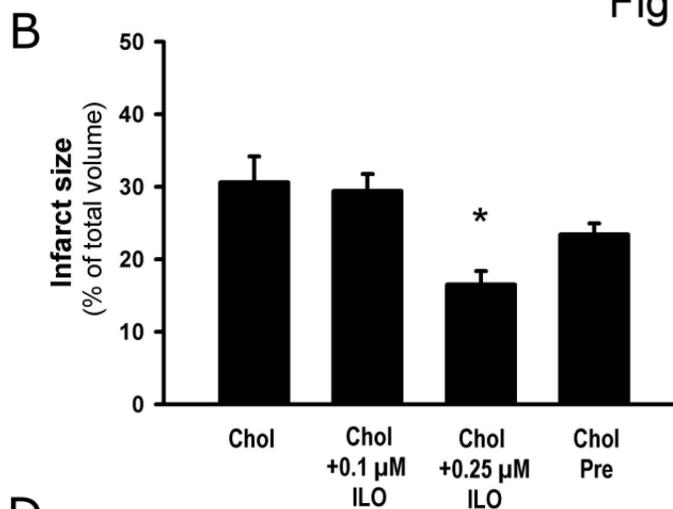
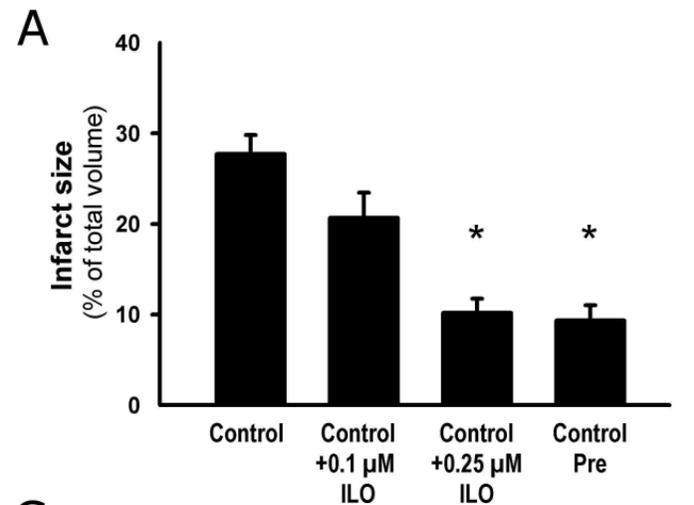


Fig. 5





**Fig. 7**

