Journal of Cardiovascular Pharmacology and Therapeutics
2021, Vol. 26(6) 702-713

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# Remote Ischemic Perconditioning Ameliorates Myocardial Ischemia and Reperfusion-Induced Coronary Endothelial Dysfunction and Aortic Stiffness in Rats

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## Abstract

**Background:** Vascular stiffness and endothelial dysfunction are accelerated by acute myocardial infarction (AMI) and subsequently increase the risk for recurrent coronary events. **Aim:** To explore whether remote ischemic perconditioning (RIPerc) protects against coronary and aorta endothelial dysfunction as well as aortic stiffness following AMI. Methods: Male OFA-1 rats were subjected to 30 min of occlusion of the left anterior descending artery (LAD) followed by reperfusion either 3 or 28 days with or without RIPerc. Three groups: (1) sham operated (Sham, without LAD occlusion); (2) myocardial ischemia and reperfusion (MIR) and (3) MIR + RIPerc group with 3 cycles of 5 minutes of IR on hindlimb performed during myocardial ischemia were used. Assessment of vascular reactivity in isolated septal coronary arteries (non-occluded) and aortic rings as well as aortic stiffness was assessed by wire myography either 3 or 28 days after AMI, respectively. Markers of pro-inflammatory cytokines, adhesion molecules were assessed by RT-qPCR and ELISA. **Results:** MIR promotes impaired endothelial-dependent relaxation in septal coronary artery segments, increased aortic stiffness and adverse left ventricular remodeling. These changes were markedly attenuated in rats treated with RIPerc and associated with a significant decline in P-selectin, IL-6 and TNF-$\alpha$ expression either in infarcted or non-infarcted myocardial tissue samples. **Conclusions:** Our study for the first time demonstrated that RIPerc alleviates MIR-induced coronary artery endothelial dysfunction in non-occluded artery segments and attenuates aortic stiffness in rats. The vascular protective effects of RIPerc are associated with ameliorated inflammation and might therefore be caused by reduced inflammatory signaling.

## Keywords

ischemia-reperfusion injury, heart disease, endothelium, acute myocardial infarction

## Introduction

ST-elevation myocardial infarction (STEMI) is one of the leading causes of mortality and morbidity worldwide.[1] Although timely reperfusion of the occluded coronary artery is necessary for salvage of cardiomyocytes, reperfusion of the jeopardized myocardium results in a cascade of harmful events, referred to as reperfusion injury.[2] Besides the cardiomyocyte damage, endothelial dysfunction is also a consequence of acute myocardial infarction (AMI), particularly during the first 24 to 72 hours[3,4] and may predict infarct extension, adverse left ventricular (LV) remodeling and subsequently recurrent AMI. Accordingly, previous studies demonstrated that AMI

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**Manuscript submitted:** March 17, 2021; **accepted:** June 20, 2021.

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accelerates microvascular obstruction within the remote area[5] and endothelial dysfunction in the non-occluded coronary vessel,[3] respectively. The underling signaling mechanism of endothelial dysfunction in remote vascular beds after AMI are not completely understood, although the upregulation of inflammatory and adhesion molecules like vascular cell adhesion molecule 2 (VCAM2), P-selectin and platelet-endothelial adhesion molecules has been described.[3] Another important pathophysiological aspect of AMI is the development and progression of aortic stiffness, which is also identified as a risk factor for recurrent acute coronary events.[6] In line with this, the increase in pulse wave velocity in patients with AMI independently predicts infarct size reduction. This has been assessed by cardiac magnetic resonance, revealing a pathophysiological link between aortic stiffness and adverse infarct healing and remodeling.[7] Thus, the exploration of effective therapeutic strategies to reduce recurrent acute coronary events by considering vascular endothelial dysfunction and aortic stiffness are desirable clinical goals.

It is well established that ischemic pre-, post- and remote conditioning protects the myocardium against ischemia and reperfusion (IR)-injury,[8-10] respectively. Beyond infarct size reduction, remote ischemic conditioning (RIC) evokes vascular protective effects. Recent studies have demonstrated that RIC attenuates endothelial IR injury initiated by form arm IR.[11] In addition, Manchurov et al[12] showed that RIC markedly improved brachial artery flow-mediated dilation after 2nd and 7th day in patients with AMI following primary percutaneous coronary intervention (PCI). Other investigators found that RIC treatment may improve arterial stiffness[13] and coronary microvascular function in patients with PCI.[14] Nevertheless, there is lacking evidence whether RIC protects endothelial function in the non-occluded coronary artery and/or ameliorates vascular stiffness following AMI.

Therefore, the objective of this study was to investigate whether remote ischemic perconditioning (RIPerc) mitigates myocardial IR-induced endothelial dysfunction and vascular stiffness in a rat model of myocardial ischemia/reperfusion (MIR)-injury.

## Methods and Materials

### Animals

Male OFA-1 rats (10-12 weeks old, Department for Laboratory Animal Science and Genetics, Himberg, Austria) were used. The experimental protocol was approved by the Ethics Committee for Laboratory Animal Experiments at the Medical University of Vienna and the Austrian Ministry of Science and Research (BMWF-66.009/0023-WF/V/3b/2016) and 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).

### Rat Model of Myocardial Ischemia and Reperfusion

The experimental protocol is depicted in Figure 1. Myocardial ischemia was induced by 30 min of left anterior descending artery (LAD) occlusion followed by either 3- or 28-days reperfusion as described previously.[15] Briefly, rats were anaesthetized by intraperitoneal injection of a mixture of Xylazine (4 mg/kg; Bayer, Germany) and Ketamine (100 mg/kg; Dr E. Gräub AG, Switzerland), intubated (14-gauge tube) and ventilated (0.9 ml/kg body weight, 75-85 stroke/min). To induce myocardial ischemia, the heart was exposed via a left thoracotomy and a ligature was placed around the left coronary artery 2-3 mm away from the origin. Rectal temperature was measured and maintained at 37.5-38.5°C by a heated operating table. Myocardial ischemia was associated with pallor of the myocardial area at risk and ST-elevation on ECG signal. Reperfusion was initiated following the 30 min of LAD occlusion by removal of the snare. Analgesia was initiated by intraperitoneal injection of Piritramide (0.1 ml/kg body weight) preoperatively and Piritramide in drinking water was applied as a postoperative analgesic regimen (2 ampules of Piritramide with 30 ml of Glucose 5% in 250 ml water).

In part I of the investigation, we aimed to investigate the impact of RIPerc on myocardial inflammation and septal artery vascular reactivity. Rats were allocated to the following groups (1) MIR ($n = 6$); (2) RIPerc with 3 cycles of 5 minutes of IR of a hindlimb performed during myocardial ischemia (MIR + RIPerc, $n = 6$) as described previously[15,16] and sham operated animals (without LAD occlusion; $n = 5$, Sham). MIR was induced by 30 min ligation of LAD followed by reperfusion.

In part II of this study, we aimed to investigate the effects of RIPerc on aortic stiffness and left ventricular remodeling. Rats were allocated to (1) MIR ($n = 7$); (2) RIPerc with 3 cycles of 5 minutes of IR of a hindlimb performed during myocardial ischemia (MIR + RIPerc, $n = 6$). Sham operated animals (Sham, without LAD occlusion; $n = 5$). MIR was induced by 30 min ligation of LAD followed by 4 weeks reperfusion. In order to test the effect of TNF-$\alpha$ on septal artery segments additional 6 rats were used.

### Organ Harvest

Three or 28 days after the reperfusion, blood, heart and abdominal aorta were collected. In briefly, the rat was placed on the operation table (under anesthesia as described above) then the thorax was opened and 0.2-0.3 ml heparin was injected into the vena cava inferior. Blood samples were collected for further investigation. For the short-term experiment (3 days) the hearts and in the long-term experiment (28 days) the abdominal aorta was harvested and placed into ice-cold Krebs solution.

### Preparation of Vascular Segments

The segments of the aorta abdominalis were prepared in cold and oxygenated (5% $CO_2$ and 95% $O_2$) Krebs solution containing (in mM/L) 119 NaCl, 4.7 KCl, 2.5 $CaCl_2 \cdot 2\,H_2O$, 1.17

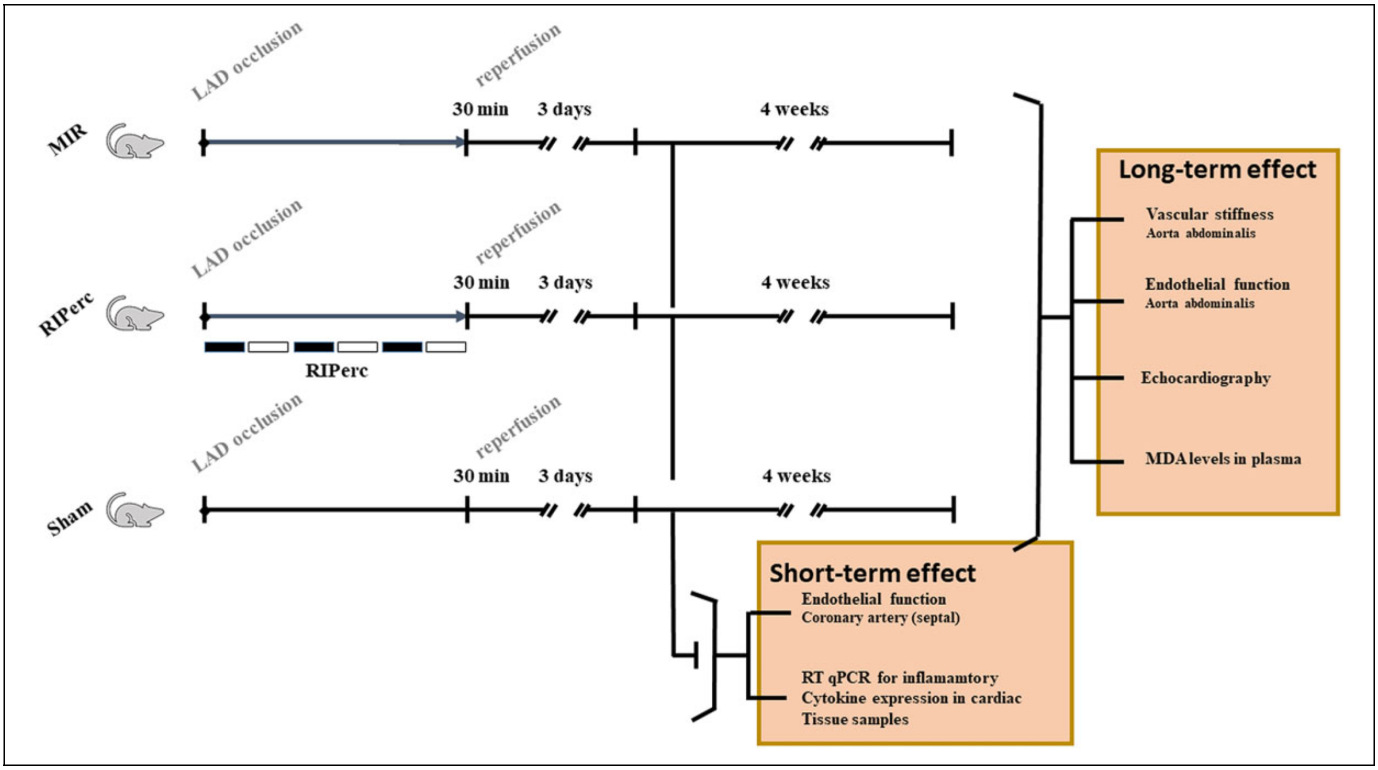


**Figure 1.** Experimental protocol. Rats were allocated to (1) Sham group, in which rats underwent sham operation without any intervention; (2) MIR group, in which rats underwent 30 minutes LAD ischemia followed by 3 days or 4 weeks reperfusion with no conditioning therapy; (3) remote ischemic perconditioning (RIPerc) group, in which 3 cycles of 5 minutes of IR of a hindlimb performed during myocardial ischemia. Cardiac and vessel segments (septal artery and aorta) were collected 3 days (short-term) or 4 weeks (long-term) after reperfusion, respectively. Cardiac function was assessed by transthoracic echocardiography 4 weeks after LAD occlusion. To measure the levels of oxidative stress, plasma MDA was measured by high performance liquid chromatography. LAD indicates left anterior descending artery; MIR, myocardial ischemia/reperfusion; MDA, malondialdehyde.

$MgSO_4 \cdot 7\,H_2O$, 20 $NaHCO_3$, 1.18 $KH_2PO_4$, 0.027 EDTA, 10.5 glucose. The segments were cleaned from connective tissue and blood, subsequently the segments contained only tunica adventitia, smooth muscle layer (tunica media) and the intact endothelium. This procedure was performed as fast as possible to avoid damage of the endothelial layer. In addition, these procedures were done under a Zeiss stereo preparation microscope. After cleaning the piece of aorta abdominalis, the segment was cut into 2 mm pieces and mounted onto a multichambered isometric myograph system (Model 620 M, Danish Myo Technology, Aarhus, Denmark). Similar to the preparation of aorta abdominalis the animal hearts were also prepared in cold Krebs buffer solution and the septal artery was cleaned and mounted onto wire myograph system. In general, the rats have 3 major coronary artery beds; the left coronary artery bed, right coronary artery bed and septal artery bed, which invariably branches off either from the proximal part of the left coronary artery or the right coronary artery.[17]

## Assessment of Vascular Function and Stiffness

*Vascular reactivity assessment.* Aortic rings (2-3 mm) and septal artery segments were mounted onto a multichamber isometric myograph system (Model 620 M, Danish Myo Technology, Aarhus, Denmark) as descried previously.[18] The organ chambers of the myograph were filled with heated (37°C) and oxygenated Krebs solution and the individual chambers were further heated and bubbled during the whole procedure. Segments were allowed to equilibrate for 45 minutes and the resting tension was continuously adjusted during this period as described previously.[18] Reference contractions were elicited by hyperkalemic (124 mM, KCl) solution. Precontraction of the aorta and septal coronary segments was achieved by Phenylephrine (PE, 1 nM-10 μM, Sigma Aldrich) and Thromboxane A2 analogue, U-46619 (1 μM, Cayman Chemical), respectively. Endothelial dependent and independent relaxation was tested by the cumulative dosage of Acetylcholine (ACh, 1 nM-10 μM, Sigma Aldrich) and Sodium Nitroprusside (SNP, 0.1nM-1 μM, Merck), respectively. The data were continuously recorded using the software program LabChart Pro (ADInstruments). To further evaluate the deleterious role of inflammatory cytokine on endothelial dysfunction, rat septal artery segments were incubated (2 hours) with recombinant human TNF-α (rh TNF-α; 1000 pg/ml, Sigma Aldrich) and vascular reactivity was assessed as described above.

*Aorta stiffness assessment.* In addition, the vascular stiffness of the aorta was calculated with data obtained from the vascular reactivity assessment as described previously.[19] Briefly, diameter–tension relationships were determined by stepwise stretching of the tissue, increasing its passive diameter by increasing the distance between the wires passing through the lumen. At each step, both the force and the internal circumference of the vessel were recorded, which was transformed into vessel diameter in μm. The tension is plotted on the y-axis, the calculated diameter on the x-axis. The estimated diameter at 100 mmHg was calculated from the diameter–tension relationship and the Laplace equation (Tension = [pressure * radius]/thickness) using the DMT normalization module (LabChart software, ADInstruments). For each vessel segment, a linear regression was calculated from the diameter–tension relationship. The steeper slope indicates the greater amount of stiffness.

### Assessment of Inflammatory Cytokines Expression by RT-qPCR

*Total RNA isolation.* Total RNA was isolated from rat myocardium tissue samples using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the respective instruction. Collected RNA was quantified using Sunrise absorbance plate reader (Tecan, SparkControl Magellan V 2.2).

*Reverse transcription and quantitative real-time polymerase chain reaction.* cDNA was prepared using QuantiTect reverse transcription kit (Qiagen Qiagen, Hilden, Germany). Samples were analyzed in duplicates with 20 μl each using Mastercycler Realplex (Eppendorf) with the corresponding software. Denaturation was performed at 95°C for 3 minutes followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 50°C and 30 seconds at 72°C. Melting curve analysis was performed to ensure primer specificity. Relative gene expression was calculated using ΔΔCt method. 18 S ribosomal RNA was used as an internal control. The primers used in this study are shown in Supplemental Table 1.

### Assessment of Plasma TNF-α by ELISA

Rat TNF-α ELISA kit from Elabscience (E-EL-R0019 96 T) was used. The ELISA kit was used according to the manufacturer's protocol in order to assess plasma TNF-α expression in RIPerc and MIR groups 3 days after MI.

### Transthoracic Echocardiography

Transthoracic echocardiography was performed on the anesthetized animal (isoflurane 2%-3%) using a Vivid7 system GE Healthcare echocardiography machine equipped with an 11.5 MHz 10 S sector transducer as described previously.[20] Rats were placed in a supine position after and shaving and gel application an echocardiography probe was placed in gentle contact with the chest. Parasternal short axis views of the LV cavity were collected at the level of the papillary muscle. M-mode echocardiography was performed. Left ventricular ejection fraction (LVEF), LV fractional shortening (FS), LV end-systolic (LVESD) and LV end-diastolic diameter (LVEDD) were assessed in 3 consecutive beats and averaged.

### Assessment of Plasma Malondialdehyde Levels

Plasma levels of malondialdehyde (MDA) (28 days after reperfusion) was determined in principal according to a previously described HPLC method by Pilz et al.[21] After derivatization with 2,4-dinitrophenylhydrazine (DNPH). Briefly, for alkaline hydrolysis of protein bound MDA 25 μL of 6 mol/L sodium hydroxide was added to 0.125 mL of EDTA plasma (1.5 mL Eppendorf tubes) and incubated at 60° (Eppendorf heater) for 30 min. The hydrolyzed sample was deproteinized with 62.5 μL 35% (v/v) perchloric acid. 125 μL supernatant obtained after centrifugation (14000 g; 2 min) was mixed with 12.5 μL DNPH solution and incubated for 10 min. This reaction mixture, diluted derivatisized standard solutions (0.625 nmol/mL-10 nmol/mL) and reagent blanks, was injected into the HPLC system (injection volume: 40 μL). The MDA standard was prepared by dissolving 25 μL 1,1,3,3-tetramethoxypropane (TMP) in 100 mL bidistilled $H_2O$ (stock solution: 1 mmol/L). The hydrolysis was performed with 200 μL TMP stock solution in 10 mL 1% sulfuric acid and incubation for 2 h at room temperature (Esterbauer H, 1984). The resulting MDA standard of 20 nmol/mL were father diluted with 1% sulfuric acid to the final concentrations. The DNPH derivates (hydrazones) were isocratically separated on a 5-μm ODS hypersil column (150 × 4.6 mm) guarded by a 5-μm ODS hypersil column (10 × 4.6 mm; Uniguard holder) with a mobile phase consisting of a 0.2% (v/v) acetic acid solution (bidistilled water) containing 50% acetonitrile (v/v). The HPLC separations were performed with an L-2200 autosampler, a L-2130 HTA pump a L-2450 diode array detector (all: VWR Hitachi Vienna; Austria). Detector signals (absorbance at 310 nm) were recorded and program EZchrom Elite (VWR) was used for data requisition and analysis.[22]

### Statistical Analysis

Data are presented as means ± SEM. Vascular relaxation to ACh was expressed as percentage of contraction to U46619 or PE, respectively. Differences in concentration-dependent relaxations induced by ACh were analyzed using two-way ANOVA followed by Bonferroni's test when appropriate. Differences between two groups were performed using unpaired 2-tailed t-test, while differences among multiple groups were analyzed using one-way ANOVA followed by Bonferroni's test. The number of experimental observations (n) refers to the number of animals. All analysis was calculated using GraphPad Prism. Statistical significance was accepted when $P < 0.05$.

## Results

### Vascular Function of Septal Artery Following Myocardial Ischemia and Reperfusion

To investigate whether RIPerc exerts vascular protective effects, the vascular reactivity was assessed in septal coronary

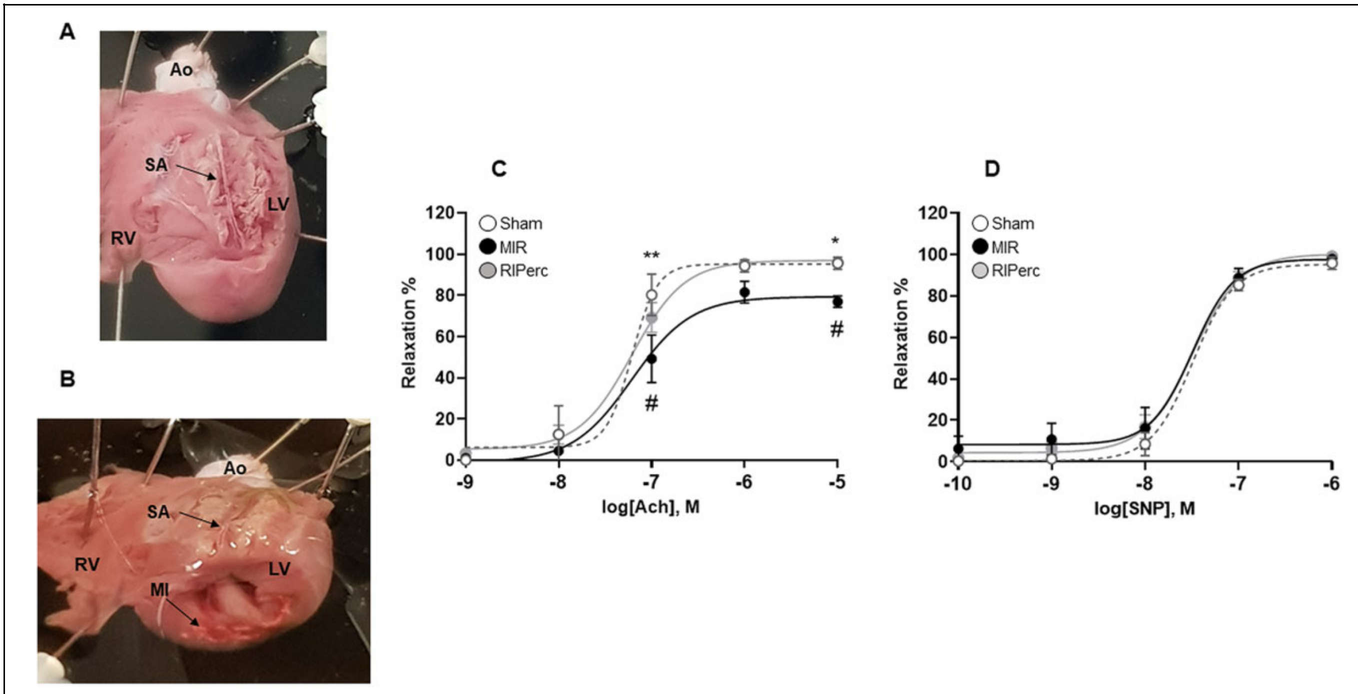


**Figure 2.** Septal artery vascular function following myocardial ischemia and reperfusion. Representative pictures about the septal coronary artery preparation without (A) and with (B) myocardial ischemia and reperfusion in rat hearts. Graph showing (C) endothelium-dependent relaxation in response to ACh and (D) endothelium independent relaxation induced by sodium nitroprusside (SNP) in septal artery rings from wt and Sham, MIR and RIPerc rats. Segments were collected 3 days after the induction of myocardial ischemia. Data are expressed as mean $\pm$ SEM, n = 4-6/group, $^*P < 0.05$ and $^*P < 0.01$ Sham vs MIR; $^\#P < 0.05$ MIR vs RIPerc using two-way ANOVA. Ach indicates acetylcholine; SNP, sodium nitroprusside (SNP); Ao, aorta; RV, right ventricular; LV, left ventricular; MIR, myocardial ischemia/reperfusion; SA, septal artery. White circle represent Sham group, black circles represent MIR group, and gray circles represent RIPerc group.

arteries segments 3 days after reperfusion. These selected segments showed a significantly reduced response to cumulative dosage of ACh in the MIR group compared to the Sham group ($P < 0.01$, Figure 2C), revealing endothelial dysfunction in septal artery following MIR. The endothelium independent relaxation elicited by cumulative dosage of SNP was not affected by MIR (Figure 2D). Of importance, the impairment of endothelial function was markedly reversed by RIPerc, underlining the vascular protection of the septal artery (Figure 2C, $P < 0.05$ vs MIR).

### *Inflammatory Cytokine Expression, Adhesion Molecule, and eNOS Expression After MIR*

In line with previous findings, we found that rats with RIPerc treatment show a marked reduction in pro-inflammatory cytokines expression within infarcted and non-infarcted cardiac tissue (Figure 3). Accordingly, we observed that MIR triggered the upregulation of several inflammatory cytokines such as monocyte chemoattractant protein 1 (MCP-1), interleukin 6 (IL-6) and particularly TNF-$\alpha$ not only within the infarcted zone but also in the remote area compared to the Sham animals (MIR vs. Sham, Figure 3A, B, and D). RIPerc markedly attenuated the upregulation of MCP-1 and IL-6 and showed a strong decreasing tendency of TNF-$\alpha$ in both area (Figure 3A, B, and C). Cell adhesion molecule, P-selectin expression was markedly increased in the remote cardiac tissue samples after MI compared to the Sham group ($P < 0.05$, Figure 3H) and this upregulation was significantly decreased by RIPerc ($P < 0.05$, RIPerc vs. MIR, Figure 3H). In addition, endothelial nitric oxide synthase (eNOS) expression was markedly declined in infarcted tissue samples in rats with MIR in comparison to Sham group ($P < 0.05$, Figure 3E). Similar to this, a reduction in eNOS expression without significant changes was observed in non-infarcted area (Figure 3J). However, eNOS expression did not show difference between MI and RIPerc group (Figure 3E and J).

We sought to further investigate the role of inflammation in the development of endothelial dysfunction in non-occluded coronary artery, focusing on TNF-$\alpha$, a known regulator of endothelial dysfunction. First, plasma levels of TNF-$\alpha$ protein declined in rats with RIPerc in comparison to MIR group (Figure 4A, $P < 0.05$). In addition, to further elucidate the mechanistic significance of endothelial dysfunction in septal artery, we tested the effects of TNF-$\alpha$. After the incubation with rh TNF-$\alpha$, vessel segments showed a significant impaired endothelium-dependent relaxation (Figure 4B). This result confirms that TNF-$\alpha$ at least partially contributes to coronary artery damage following MIR in rats.

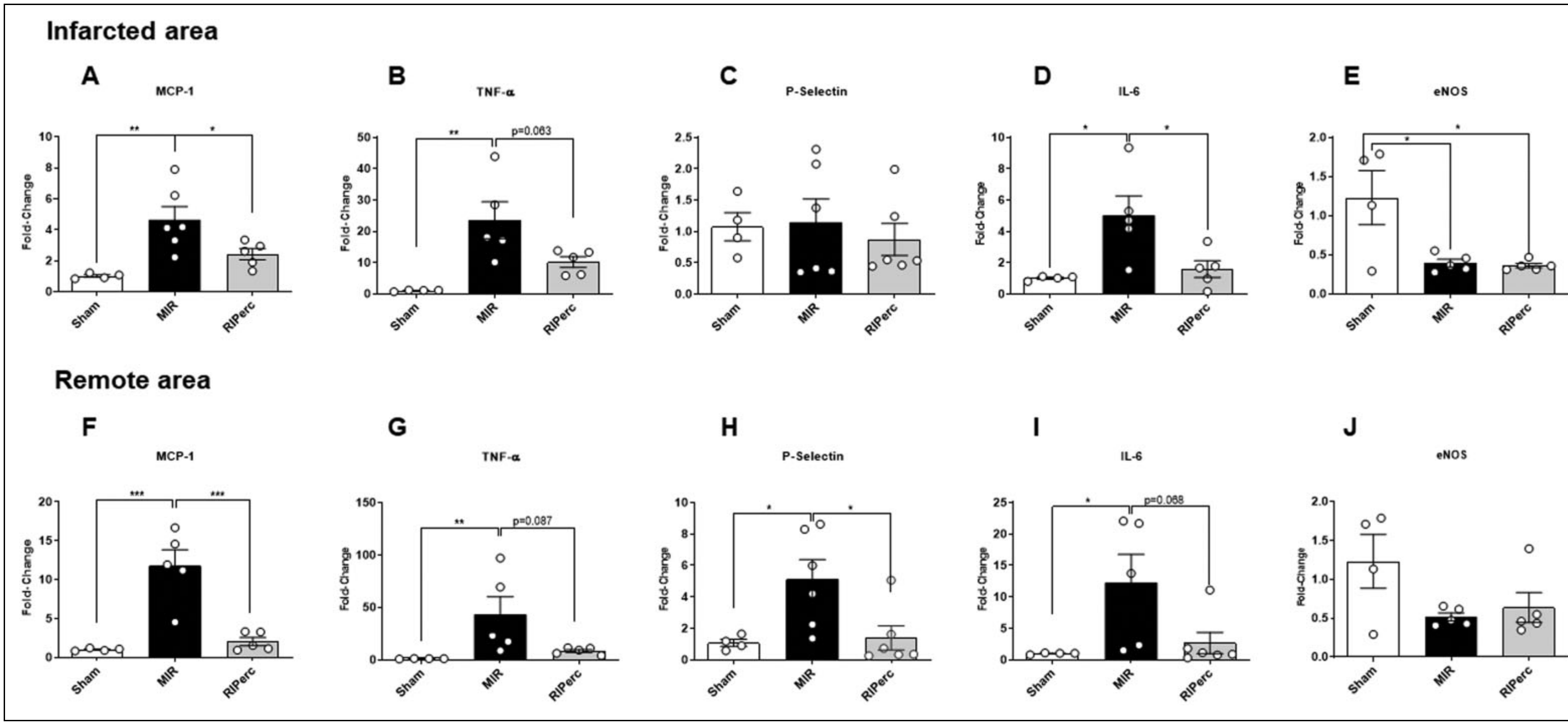


**Figure 3.** Expression of inflammatory cytokines, P-selectin and eNOS in infarcted and non-infarcted cardiac tissue. RT-qPCR data of LV infarcted and non-infarcted tissue samples from Sham, MIR, and RIPerc rats. Samples were collected 3 days after myocardial infarction. Upper panel showing the mRNA expression MCP-1 (A), TNF-$\alpha$ (B), P-selectin (C), IL-6 (D), and eNOS (E) in infarcted cardiac tissue samples. Lower panel showing the mRNA expression MCP-1 (F), TNF-$\alpha$ (G), P-selectin (H), IL-6 (I), and eNOS (J) in non-infarcted cardiac tissue samples. Data are expressed as mean $\pm$ SEM, n = 5-6/group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ using one-way ANOVA. MCP-1 indicates monocyte chemoattractant protein 1; TNF-alpha, tumor necrosis factor alpha; IL-6, Interleukin 6; eNOS, endothelial nitric oxide synthase; MIR, myocardial ischemia/reperfusion; RIPerc, remote ischemic perconditioning.

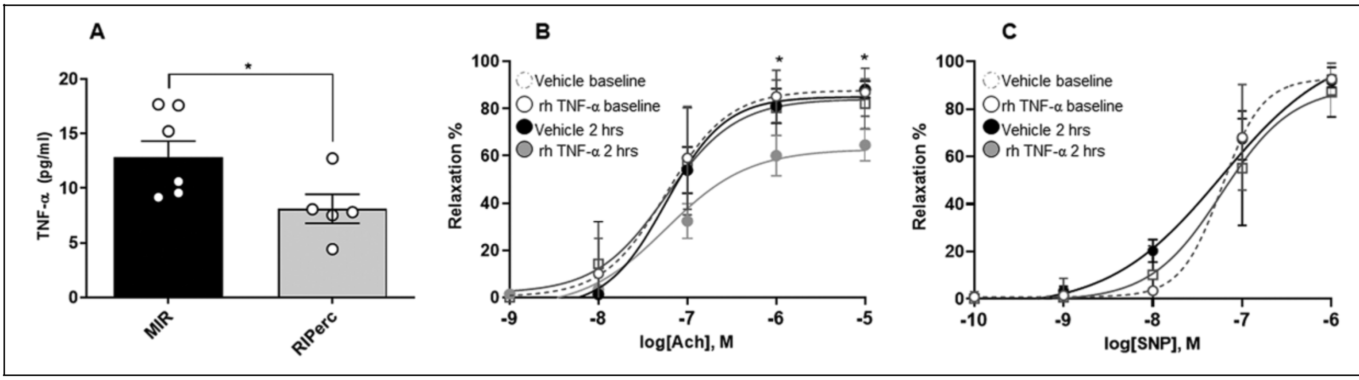


**Figure 4.** Plasma levels of TNF-α 3 days after myocardial reperfusion and the effect of TNF-α on septal artery vascular function. (A) Plasma levels of TNF-α in MIR and RIPerc group 3 days after the initiation of reperfusion. Graph showing the reactivity of septal artery segments to (B) ACh in the absence or presence of rh TNF-α for (1000 pg/ml; 2 hours incubation period) and to (C) SNP. Data are expressed as mean ± SEM; n = 5-6/group; *$P < 0.05$, using unpaired Student's t-test (A) and two-way ANOVA (B and C), respectively. Ach indicates acetylcholine; TNF-α, tumor necrosis factor alpha; MIR, myocardial ischemia/reperfusion; RIPerc, remote ischemic perconditioning. Dotted circle represent vehicle group (baseline); black circles represent vehicle treatment group, white circle represent rh TNF-α group (baseline); and grey represent rh TNF-α (2 hours incubation) treated group.

### *Endothelial Function and Vascular Stiffness in Segments of Aorta Abdominalis*

To further investigate the vascular-protective effects of RIPerc, endothelial (dys)function and aorta stiffness were assessed in segments of the aorta abdominalis. Samples were collected 4 weeks after the imitation of myocardial reperfusion. The aorta segments from MIR rats showed a significant decrease in the response to cumulative dosage of ACh compared to the Sham group ($P < 0.05$, MIR vs Sham, Figure 5A). In the RIPerc group, endothelial function was ameliorated, but did not reach statistically significance in comparison to MIR group (Figure 5A). Interestingly, we observed a decreased response to SNP in the aorta segments from the MIR rats compared to Sham group, indicating vascular smooth muscle cell (VSMC) dysfunction (Figure 5B). This effect was slightly improved by RIPerc (Figure 5B). Furthermore, the linear regression analysis of the plotted tension and diameter values showed a significant steeper slope in case of the MI rat aorta segments compared to segments of RIPerc group revealing a greater vascular stiffness ($slope_{MI} = 0.030$ vs. $slope_{RIPerc} = 0.0269$, Figure 5C). Having established oxidative stress as a potential regulator of aorta stiffening, we further investigated whether RIPerc could influence plasma MDA levels as marker of oxygen radical-induced lipid peroxidation. MIR led to a significant increase in plasma MDA levels (Figure 5D, $P < 0.001$ vs Sham). However, RIPerc failed to decrease MDA levels when compared to MIR (Figure 5D).

### *Cardiac Function Assessed by Transthoracic Echocardiography*

To evaluate the effect of MIR and RIPerc on cardiac function, transthoracic echocardiography was performed. LVEF and FS were significantly decreased in MIR rats compared to the Sham group ($P < 0.0001$, Figure 6A and B). In contrast, rats in RIPerc group showed significantly preserved LVEF and FS as compared to rats in MIR group ($P < 0.01$ and $P < 0.05$ respectively, Figure 6A and B). MIR also caused changes in the heart geometry as LVESD and LVEDD were significantly increased, indicating dilation of LV ($P < 0.001$, Figure 6C and D), whereas RIPerc showed preserved LV morphology (LVESD $P < 0.05$ and LVEDD, $P = 0.08$ vs MIR, Figure 6C and D).

## Discussion

In this study, we have to the best of our knowledge for the first time shown that RIPerc improves vascular endothelial dysfunction in the septal artery and aorta stiffness in a rat model of myocardial ischemia and chronic reperfusion. Moreover, endothelial function improvement is in parallel with significant decline in pro-inflammatory cytokines and adhesion molecules expression. More specifically, the vascular protection of RIPerc is associated with a reduction in TNF-α plasma levels. In addition, vascular (endothelium) protective effects of RIPerc have been shown in isolated rat septal artery segments after MIR-injury. In this context, previous clinical studies have demonstrated higher aortic stiffness in the early phase of post AMI. In consequence this higher aortic stiffness leads to impairment of ventricular-arterial coupling resulting in LV remodeling despite successful revascularization,[7] suggesting the detrimental role of large elastic vessel dysfunction in the progression of post-MI adverse LV remodeling and cardiac dysfunction. Our findings demonstrate that RIPerc markedly improves aortic stiffness in comparison to MIR rats. This improvement is associated with preserved cardiac function and LV morphology. No difference is found in plasma MDA levels between the RIPerc and MIR groups.

A remarkable progress in clinical management over the past years has resulted in a significant prevention of death at the time of the first AMI. However, survivors are at a particularly high risk—nearly 6 times higher compared to individuals

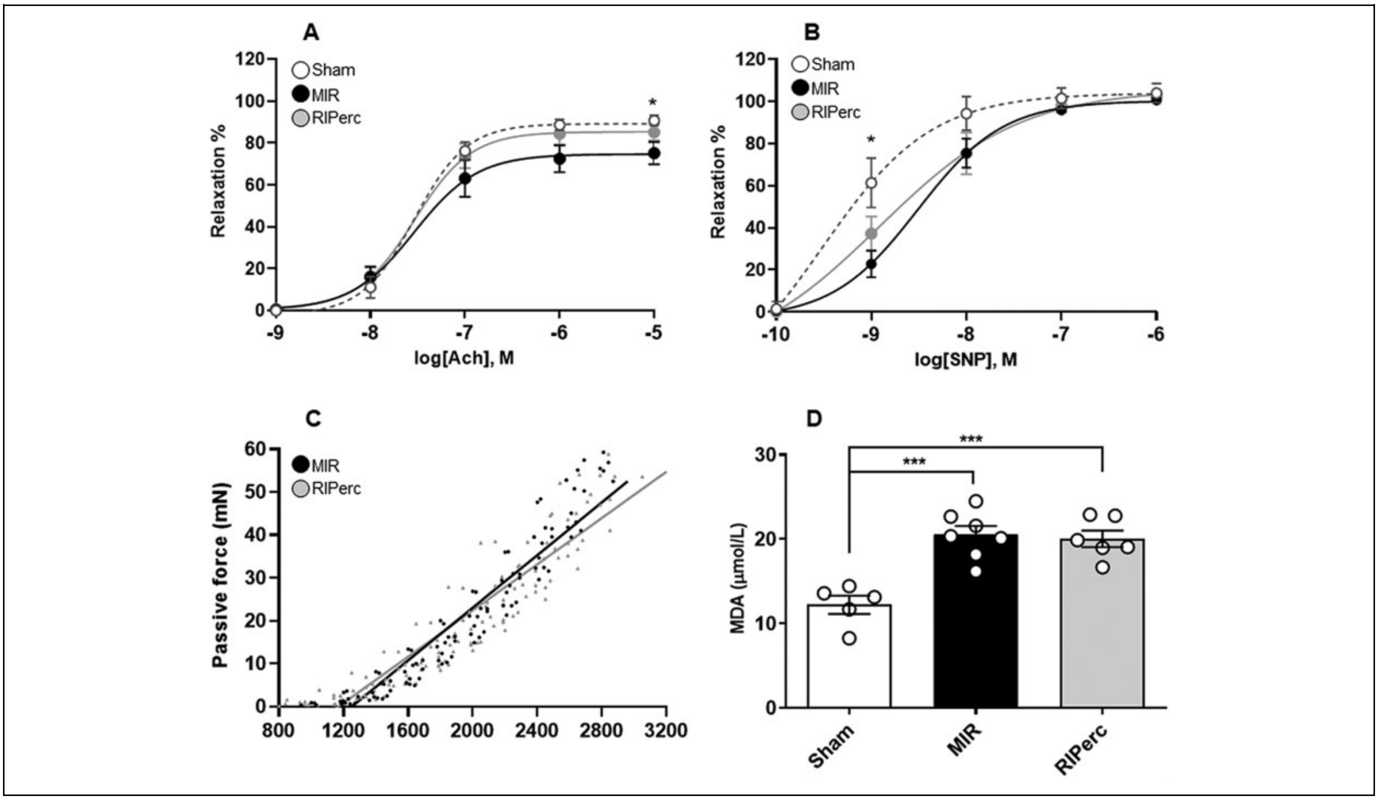


**Figure 5.** Aortic vascular function and stiffness and levels of malondialdehyde in plasma. Vascular reactivity and aortic stiffness were assessed by using wire myograph in aorta segments were taken 4 weeks after myocardial infarction. Graph showing (A) endothelium-dependent relaxation in response to ACh and (B) endothelium-independent vasorelaxation by sodium nitroprusside (SNP) in aortic rings from wt and Sham, MIR and RIPerc rats. (C) Aortic stiffness was assessed by using wire myograph and the passive property of vessel segments was used to measure the aortic stiffness. (D) Levels of MDA in plasma in Sham, MIR and RIPerc group 4 weeks after myocardial infarction. Data are expressed as mean $\pm$ SEM, n = 4-7/group, *$P < 0.05$ Sham vs MIR, and ***$P < 0.001$ Sham vs MIR and RIPerc, using one-way ANOVA and two-way ANOVA, respectively. Ach indicates acetylcholine; MDA, malondialdehyde; MIR, myocardial ischemia/reperfusion; RIPerc, remote ischemic perconditioning. White circle represent Sham group; black circle represent MIR group and grey circle represent RIPerc group.

without MI history—for recurrent AMI.[23] A major cause for the increased risk of recurrent infarctions may be accelerated atherosclerosis that is triggered upon AMI.[24] Nevertheless, the mechanisms by which atherosclerosis progression is enhanced post-MI remain largely elusive. More recently, the relationship between endothelial dysfunction in non-occluded coronary artery and increase risk for atherothrombotic events following AMI has been explored extensively.[3] Mocetti et al[3] showed that NADPH-oxidase (NOX) upregulation triggers the signaling mechanism referring to endothelial dysfunction, such as upregulation of pro-inflammatory cytokines and adhesion molecules like P-selectin and VCAM expression in mice with advanced atherosclerosis. Here, we investigated whether RIPerc protects against MIR-induced endothelial dysfunction in segments of the septal coronary artery. In line with the findings by Mocetti et al,[3] our study also confirmed that MIR triggers endothelial dysfunction in remote vessel segments 3 days post-MI in association with a massive upregulation of pro-inflammatory cytokines within the infarcted myocardial tissue even in absence of atherosclerosis. Furthermore, P-selectin mRNA expression was significantly increased in the non-infarcted (septum) cardiac tissue samples following MIR. RIPerc attenuated these effects of MIR. In a previous study, Duda et al[25] demonstrated that ischemic preconditioning in isolated guinea-pig heart prevents endothelial dysfunction in association with a reduction in P-selectin and neutrophil adhesion expression in the early phase of reperfusion (15-30 min). Another study[8] demonstrated that ischemic postconditioning ameliorates P-selectin mediated endothelial dysfunction on coronary endothelium isolated from the left anterior descending coronary artery (LAD; occluded coronary artery). However, this study failed to show endothelial dysfunction on the right coronary artery segments after 3 hours reperfusion. Besides P-selectin, other adhesion molecules such as VCAM and ICAM as well as platelet-leukocytes interactions certainly contribute and trigger endothelial dysfunction after MI. In our study, we only measured P-selectin expression. However, further studies are needed to confirm whether the vascular protective effects of RIPerc involves signaling mechanisms acts on a reduction in VCAM and ICAM expression. In addition, eNOS plays a significant role in vascular homeostasis[26] as well as number of experimental and clinical studies show that nitric oxide (NO) plays a role in the cardioprotective effect of ischemic conditioning.[27-29] In contrast to previous

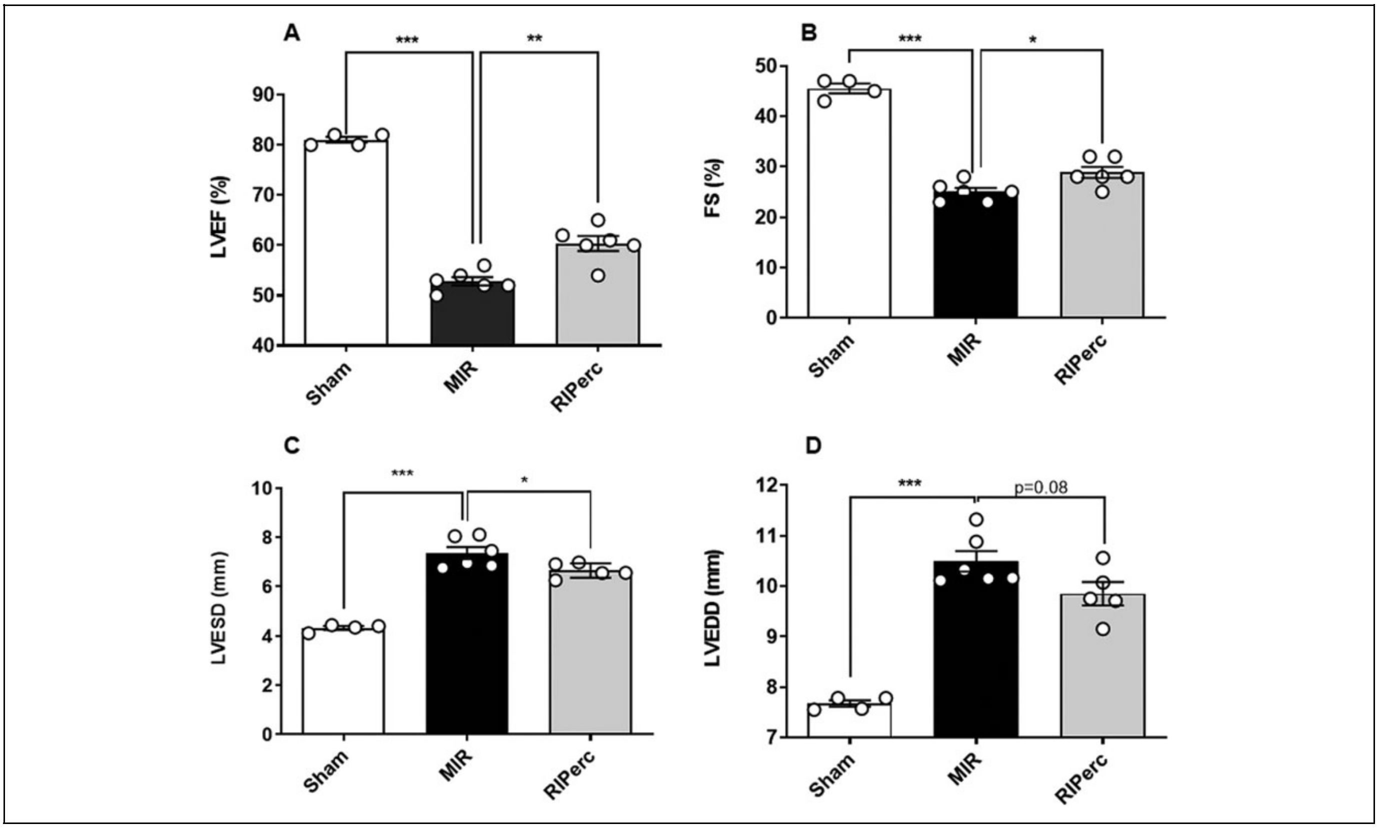


**Figure 6.** Left ventricular function and morphology. RIPerc significantly improves LVEF, FS and LV dilation. Graphs showing (A) left ventricular ejection fraction, (B) fractional shortening, (C) left ventricular end-systolic, and (D) end-diastolic diameter following 4 weeks after myocardial infarction. Data are expressed as mean $\pm$ SEM, n = 4-6/group, *$P < 0.05$, **$P < 0.001$, and ***$P < 0.0001$, using one-way ANOVA. LVEF indicates left ventricular ejection fraction; FS, fractional shortening; LVESD, ventricular end-systolic diameter; LVEDD, ventricular end-diastolic diameter; MIR, myocardial ischemia/reperfusion; RIPerc, remote ischemic perconditioning.

findings,[27-29] eNOS expression did not show a significant upregulation in cardiac tissue samples were obtained from RIPerc rats in comparison to MIR group. The discrepancy between the previous results and our findings may be explained by the different time points (after conditioning stimulus vs 3 days MI) for the measurement of eNOS and/or NO levels.

Pro-inflammatory cytokines also trigger endothelial and cardiac dysfunction following AMI.[30] In our study, we observed a marked upregulation of TNF-$\alpha$ mRNA expression in cardiac tissue samples by MIR and a trend toward their decrease by RIPerc. These results led us to investigate TNF-$\alpha$ levels in plasma as well as the effects of TNF-$\alpha$ on coronary endothelial function. We found that TNF-$\alpha$ levels was markedly declined in rats with RIPerc in comparison to the MIR group. These findings are in line with previous studies demonstrating that RIC has a strong anti-inflammatory effect.[15,31] Furthermore, rh TNF-$\alpha$ incubation resulted in an impaired endothelium-dependent relaxation on septal coronary artery segments similar to previously findings.[32] In accordance with our findings, recent clinical studies in healthy male volunteers have demonstrated that RIC has protective effects against forearm IR-injury.[11,33]

In conjunction with the beneficial effects of RIPerc on coronary endothelial dysfunction following MIR, we also observed long-term cardiovascular protective effects. Other investigators found that RIC treatment improves arterial stiffness[13] and coronary microvascular function in patients with elective PCI.[14] Notably, either one or two cycles of bilateral brachial cuff inflation (vascular conditioning) within 48 h post-PCI in STEMI-patients resulted in a facilitating reverse LV remodeling in association with a decline in carotid-femoral pulse wave velocity as a surrogate marker of vascular stiffness.[27] In our study, we also assessed the impact of MIR on peripheral vascular endothelial function and stiffness as well as long-term vascular protective effects. Accordingly, our study clearly demonstrates that AMI (4 weeks) initiates endothelial dysfunction in aorta as described previously.[34,35] In contrast to our findings on septal artery segments during early reperfusion, RIPerc only show a tendency to improve peripheral vascular endothelial dysfunction 4 weeks after MI. Importantly, we did observe a significantly decreased sensitivity to response for SNP in aorta segments from the MIR rats as compared to Sham group, suggesting VSMC dysfunction. Data from previous report also demonstrated that MI-induced VSMC dysfunction,[36] however the underling signaling mechanism as well as the contribution of VSMC dysfunction to the development of aortic stiffness are not fully known.

It is important to mention that endothelial dysfunction after AMI is likely due to upregulation of oxidative stress, inflammation and substantially reduction in NO bioavailability. However, we demonstrated that RIPerc reduced inflammatory cytokine and adhesion molecule expression 3 days after reperfusion, presumably contributing to endothelial function preservation. To further characterize whether oxidative stress could be attenuated by RIPerc, we extended our analysis to plasma MDA levels. Although plasma MDA levels were markedly increased even 4 weeks after reperfusion, RIPerc failed to reduce MDA levels. Interestingly, a previous study by Naya et al[37] demonstrated that IL-6 and TNF-α rather than MDA (oxidative stress) were identified as independent predictors of coronary endothelial dysfunction in hypertensive patients, suggesting inflammation plays a putative role in the progression of coronary endothelial dysfunction.

Next, we investigated whether RIPerc could affect aortic stiffness. Mechanistically, aortic stiffening, which occurs with various pathologic states such as AMI has important consequences on cardiovascular pathophysiology, including excessive penetration of pulsatile energy into the microvasculature of target organs that operate at low vascular resistance, and abnormal ventricular-arterial interactions that promote adverse LV remodeling.[38] The potential benefit of vascular conditioning on aortic stiffness in STEMI patients has been demonstrated previously, however it was measured at baseline (within 48 h after PCI) and 40 min after onset of RIC.[27] In our study, we also could demonstrate the favorable effect of RIPerc on aortic stiffness which was associated with the attenuation of post-infarct LV remodeling (4 weeks after reperfusion). Nevertheless, we cannot exclude that other cardioprotective mechanisms triggered by RIC also alleviates adverse remodeling.[15,31] Aortic stiffness is determined by multiple mechanisms including reduced elastin/collagen ratio, production of elastin cross-linking, reactive oxygen species, induced inflammation, activation of the renin-angiotensin aldosterone system, VSMC stiffness, and endothelial dysfunction.[39] Our study was not aimed to clarify the exact signaling mechanism of aortic stiffness and its reduction by RIPerc. However, we are tempting to speculate that inflammatory signaling may play a major role in this context. Future studies that test this hypothesis more directly are warranted.

## Conclusions

In conclusion, the growing prevalence and associated risk of coronary endothelial dysfunction and arterial stiffness in recurrent AMI provide a major thrust to better understand the underlying mechanisms and test novel strategies for reducing cardiovascular death. In our study we demonstrate for the first time that RIPerc could alleviate MIR-induced endothelial dysfunction in septal artery segments and aortic stiffness in rats. The vascular protective effects of RIPerc are associated and might occur via the reduction of inflammatory signaling.


## Acknowledgments

We would like to thank Mr. Milat Inci (Ludwig Boltzmann Institute for Cardiovascular Research, Vienna, Austria) for helping to perform in vivo experiments.


## Author Contributions

Petra Lujza Szabó and Christopher Dostal contributed equally to the study.


## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by funding from the Ludwig Boltzmann Society (REM 2017-20) and Austria-Hungary Action Foundation (92öu8).



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## Supplemental Material

Supplemental material for this article is available online.

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