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Ribose Treatment Reduced the Infarct Size and Improved Heart Function after Myocardial Infarction in Rats

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Key Words

Myocardial infarction • Ribose • Infarct size • Heart function • Cytokine expression

Abstract

Objective: In this study the effect of ribose on heart function and infarct-size was analyzed 6 h after myocardial infarction (MI) in rats. Methods: Continuous i.v.-infusion of NaCl or ribose (200 mg/kg/h) was started one day prior to induction of MI in female Sprague-Dawley rats which was done by ligation of the left coronary artery. Six hours after MI heart function was measured with 3F tip catheter, cardiac output by thermodilution method. Thereafter the ischemic area was delineated by Evans Blue infusion, and the infarct area was visualized by triphenyltetrazolium chloride staining. The mRNA expression of interleukin (IL)-1β, IL-6, matrix-metalloproteinase (MMP)-8, and -9 was measured by ribonuclease protection assay. Results: Heart function was severely depressed 6 hours after coronary artery occlusion, but recovered significantly under the influence of ribose. Left ventricular (LV) systolic pressure (LVSP) and contractility ($LVdP/dt_{max}$) were restored to the normal levels of sham-operated animals, while parameters of LV relaxation ($LVdP/dt_{min}$ and time constant of relaxation τ) were impaired compared to sham-operated animals, but significantly improved by ribose treatment compared to sham-treated MI-rats. Moreover, the infarct size was significantly smaller in the ribose treated animals despite a comparable ischemic area at risk in all MI-rats. The cytokine mRNA expression after MI was significantly reduced after ribose treatment, while there were no differences regarding MMP expression. Conclusion: MI size was significantly reduced and LV function significantly improved by ribose treatment at 6 h after MI. This seemed to be based on slowing the velocity of the necrotic wave front across the LV wall after MI resulting in smaller infarcts.

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Introduction

Myocardial ischemia and infarction result in severe reduction of overall cardiac pump function due to loss of contractile mass [1]. This leads to numerous molecular, structural, and metabolic alterations. These include changes in the expression of components of the extracellular matrix like collagens, colligin, and matrix-metalloproteinases (MMPs) as well as of cytokines like interleukins and TGF-β isoforms [2, 3]. The metabolic alterations include a reduction in ATP content and mitochondrial ATP synthase, glycogen depletion, formation of lactic acid, accumulation of fatty acids, inhibition of β-oxidation, and depression of mitochondrial function [4-6]. The imbalance between the rate of oxidative phosphorylation and the utilization of high energy phosphates results in a progressive decrease of ATP levels in the myocardium [7]. The degradation products finally diffuse out of the myocytes with myocardial perfusion [4]. This is deleterious, because high energy phosphates are essential for myocardial contraction, relaxation and maintenance of cellular integrity [8]. Alterations of myocardial energy phosphate levels, accumulation of extracellular Ca^{2+} and reactive oxygen species further contribute to cell death by necrosis and apoptosis [9]. Even when ischemia ends and although fuels for generation of ATP by oxidative phosphorylation like glycogen and fatty acids are usually available, restoration of ATP levels takes several days and is costly, since it depends on de novo synthesis, due to the loss of ATP precursors from the myocytes [4, 10].

ATP is synthesized from blood supplied glucose that can be converted to 5-Phosphoribosyl-1-pyrophosphate (PRPP) through the pentose phosphate pathway (PPP). The relevance of this pathway has recently been demonstrated by increased myocardial dysfunction after ischemia/reperfusion in mice lacking Glucose-6-Phosphate Dehydrogenase [11]. The activity of Glucose-6-Phosphate Dehydrogenase and Phosphogluconat Dehydrogenase limit the rate of the PRPP production. The pentose sugar ribose bypasses the rate-limiting steps of the PRPP synthesis and stimulates the salvage as well as the de novo synthesis of nucleotides by increasing PRPP [12]. In cardiac myocytes, ribose increases ATP and decreases NAD content without influences on the adenosine content [13]. Furthermore, ribose has no influence on coronary blood flow, myocardial oxygen consumption, and hemodynamics in the normal heart. Therefore, ribose has been suggested to be an attractive nutraceutical supplement for the metabolic support of the heart in numerous cardiac disorders [14-18].

Previous studies have shown that ribose administration increases the PRPP pool [16, 19] and improves ventricular function in different pathophysiological states that are accompanied by ATP reduction [20, 21]. In experimental in vivo models in rats such as in the overloaded or the catecholamine-stimulated heart, the normalization of the metabolic situation by ribose administration was accompanied by an improvement of global heart function [20]. Also after temporary local ischemia, ribose accelerated the replenishment of the adenine nucleotide pool and improved the return of function [22]. Furthermore, ribose pretreatment significantly elevated the heart's energy stores (glycogen), and delayed the onset of irreversible ischemic injury [23]. In spontaneously hypertensive rats, however, ribose had no effect on ischemic tolerance, but improved left ventricular function [23]. Additionally, application of ribose in combination with adenine and verapamil decreased Ca^{2+} level and increased level of high energy phosphates after global ischemia in isolated rat hearts [24].

Therefore, we hypothesized that treatment before and during ischemia with ribose might reduce the ischemic injury and also prevent LV dysfunction in an acute stage of MI. Accordingly, the objective of the study was to evaluate the effects of i.v. infusion of ribose 24 h prior to permanent coronary artery ligation on infarct size, ventricular function, and expression of cytokines in rats.

Materials and Methods

Animal model

A total of 73 female Sprague-Dawley rats (3.5 months of age and 251±6 g of body weight) were used in this study. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the appropriate Federal State Agency. The rats were anesthetized with 2 % isoflurane and randomized to receive either intravenous infusion (i.v.) of 0.9% sodium chloride (NaCl) or D-Ribose (200 mg/kg/h). All substances were administered as continuous i.v. infusion via a catheter (Vygon) placed in the left jugular vein. Each catheter was connected to a 50 ml syringe placed in an infusion pump (Infors) and the infusion rate was set to 4 ml/kg/h as previously described [20]. After implantation of the infusion catheter, the animals were allowed to recover from anesthesia in individuals cages with free access to water and rat chow.

After 24 hours of i.v. infusion, myocardial infarction was induced by ligation of the left anterior descending coronary artery (LAD) under ether anesthesia as previously described [3, 25]. Briefly, the fourth intercostal space was opened, the

heart was exteriorized, and the pericardium was cut. The LAD was ligated between the left auricle and the pulmonary outflow tract with a monofil thread (Ethicon USP 6/0, Johnson+Johnson) while holding the apex of the heart with forceps. Thereafter, the chest was closed and the rats were allowed to recover. Due to the mortality after acute MI (overall 29%), not all animals completed the study (n=10 for MI+NaCl and n=18 for MI+R). In an additional group, MI was induced in rats without any treatment (MI-CTRL, n=11). Sham-operated animals underwent the same procedure except that no ligation was performed (sham, total n=18, each n=6 for sham-CTRL, sham+NaCl and sham+R, respectively).

Hemodynamic measurements

Heart function was measured 6 h after surgery in closed-chest spontaneously breathing rats anesthetized with thiopental sodium (Trapanal® 60 mg/kg i.p., Byk Gulden) using ultraminiature catheter pressure-transducers (3 F, Millar Instruments Inc.) as previously described [2, 3]. Briefly, the LV catheter (model SPR-249) was placed in the right carotid artery and advanced upstream to the aorta and into the LV. Heart rate (HR), right and left ventricular (RV and LV, respectively) pressure and the rate in rise and fall of ventricular pressure (LV and RVd*P*/dt, respectively) were recorded continuously on a PC at a sampling rate of 2 kHz using DASYLab V7.0 software (National Instruments) for 10 - 15 min. During the hemodynamic measurements, also the ECG was recorded via needle electrodes to verify successful induction of MI. Cardiac output was measured by the thermodilution method (Cardiomax IIR, Columbus Instruments).

Tissue collection and infarct size measurement

After the hemodynamic measurements had been obtained, Evans blue solution was infused to delineate the ischemic area at risk (AAR). Thereafter, the hearts were removed, cut into slices of about 2 mm and incubated in 1% triphenyltetrazolium chloride (TTC) in 0.1 M phosphate buffer, pH 7.4, at 37°C for 15 min. Then, stained slices were fixed with 10% formaldehyde overnight and scanned as previously described [26]. The AAR and the infarct size were determined via planimetry by using image analyzer software (Image Pro Plus 4.5), and the infarct area was calculated as the percentage of the AAR.

In additional experiments the hearts were rapidly excised after the hemodynamic measurements. Myocardial infarction was determined by the inspection of the pale zone on the LV free wall. The RV free wall was trimmed away and the infarct area was cut from the non-infarcted LV additionally leaving a border zone of about 2 mm in width. The tissue pieces were snap frozen in liquid nitrogen for RNA isolation.

RNase Protection Assay (RPA)

Total RNA was isolated using the Trizol®-Reagent (GibcoBRL) according to the protocol supplied by the manufacturer. For the RNase protection assay (RPA), 5µg or 7.5µg of total RNA were used for the expression analysis of matrix metalloproteinases (MMP) or cytokines, respectively [2, 3]. The cytokine probe template set rCK1 was obtained from BD PharMingen while the MMP template set was generated by RT-PCR as previously described [2]. Each probe template set was labeled with [α-^{32}P]-UTP (3000 Ci/mmol, Amersham) by means of RiboQuant® In Vitro Transcription Kit (BD PharMingen) as described by the manufacturer. After hybridization (final concentration: $8x10^3$ cpm/µl for each probe in template set) at 56°C for 12-16 h the unhybridized riboprobe was digested with a mixture of RNases A and T1 (RiboQuant® RPA Kit, BD PharMingen) according to the manufacturer's instructions. Protected probes were electrophoresed on a denaturing gel containing 5 % polyacrylamide/8 M urea and visualized and quantified using the Molecular Imager®FX and QuantityOne4.4 software (BioRad). The signals of specific mRNAs were normalized to those of GAPDH mRNA.

Statistical analysis

The results were expressed as mean ± SEM. Statistical significance was evaluated by one way ANOVA for multiple samples subsequently utilizing multigroup comparison procedure according to Newman-Keuls using SigmaStat2.0 (Jandel). A value of $p<0.05$ was considered statistically significant, but the significance level was adjusted according to the number of groups.

Results

Effects of ribose on Left Ventricular Function

Heart function was severely impaired 6 h after coronary artery occlusion (Fig. 1). Left ventricular systolic pressure (LVSP) as well as the maximal rates of rise and fall in ventricular pressure (LVd*P*/dt_{max} and LVd*P*/dt_{min}, respectively) were greatly reduced while left ventricular end-diastolic pressure (LVEDP) and the time constant of early relaxation τ were significantly increased. This impairment in LV function was comparable between sham-treated animals (MI+NaCl) and MI rats without any infusion (MI-CTRL). Infusion of ribose (MI+R), however, restored LVSP and LVdP/dt_{max} to the level of sham-operated controls (Fig. 1). Also LVd*P*/dt_{min} and τ improved after ribose treatment, but remained significantly impaired compared to sham-operated controls. Enhanced global pump function was also evident by significantly increased cardiac output in the ribose treated rats relative to sham-operated and sham-treated animals (Fig. 2). Total peripheral resistance (TPR) increased after the infusion of both, NaCl or ribose, but reached statistical significance only in the ribose treated rats. The heart rate (HR) was comparable between all groups (Fig. 2).

Effects of ribose on infarct size

The ischemic area at risk was comparable between all MI-groups, but treatment with ribose significantly

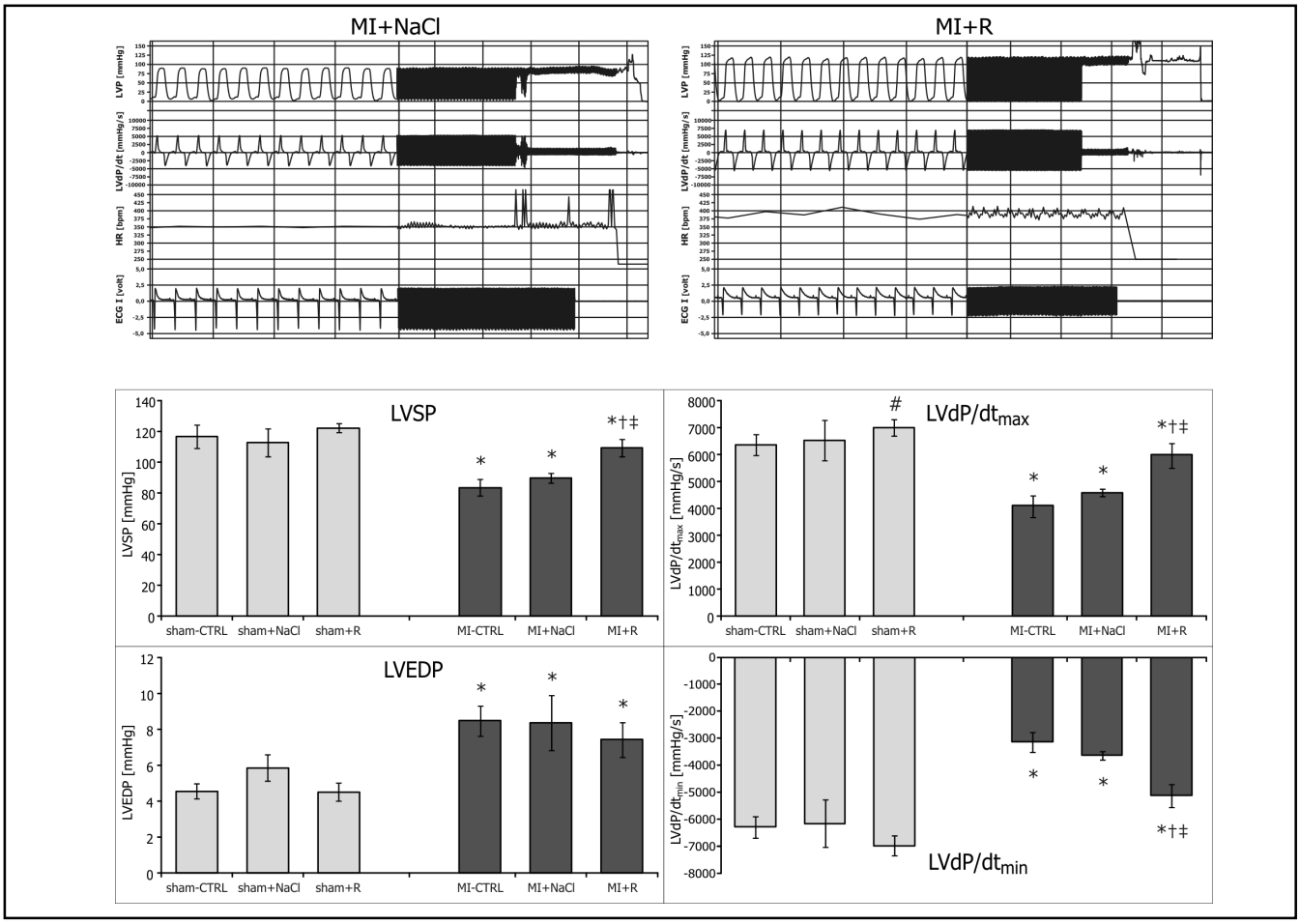


Fig. 1. Upper panels: Original recordings of hemodynamic measurements 6 h after MI+NaCl (left) of MI+R (right). Lower panels: Quantitative summary of left ventricular (LV) systolic pressure (LVSP), end-diastolic pressure (LVEDP), and rates of rise and fall of ventricular pressure (LVd*P*/dt$_{max}$ and LVd*P*/dt$_{min}$, respectively) 6 h after sham operation (sham, n = 6 for each sham group), untreated MI (MI-CTRL, n = 11), MI treated with NaCl (MI+NaCl, n = 10) or MI treated with ribose (MI+R, n = 18); # $p < 0.05$ *vs* sham-CTRL; * $p < 0.05$ *vs* corresponding sham; † $p < 0.05$ *vs* MI-CTRL; ‡ $p < 0.05$ *vs* MI+NaCl.

reduced the resulting infarct size as compared to untreated or sham-treated MI rats (Fig. 3).

Effects of ribose on mRNA expression

At 6 h post MI, IL-6 and IL-1β significantly increased in the infarct area of MI and MI+NaCl groups as compared with sham-operated hearts. This increase was significantly attenuated in the hearts of ribose treated animals (Fig. 4). Also the expression of MMP-8 and 9 increased after MI as compared with sham-operated controls (Fig. 5). This increase was significantly pronounced in both groups of MI animals that received an infusion of either NaCl or ribose ($p<0.05$ *vs* MI-CTRL). There were, however, no differences in the expression of MMP-8 and 9 between MI+NaCl and MI+R (Fig. 5).

Discussion

The present study shows that i.v. infusion of ribose from 24 h prior to and up to 6 h after permanent coronary artery ligation in rats reduces the infarct size, prevents systolic and diastolic ventricular dysfunction and the increase of cytokine expression.

Myocardial infarction induced severe systolic and diastolic dysfunction (Figs. 1 and 2) and increased the expression of pro-inflammatory cytokines and MMPs (Figs. 4 and 5). At 6 h after MI, LV dysfunction was characterized by a decline of systolic pressure, contractility, and cardiac output and by an increase in LVEDP and a delayed relaxation rate. The main difference between untreated (MI-CTRL) and sham-treated MI rats (MI+NaCl) was the small increase in MMP expression

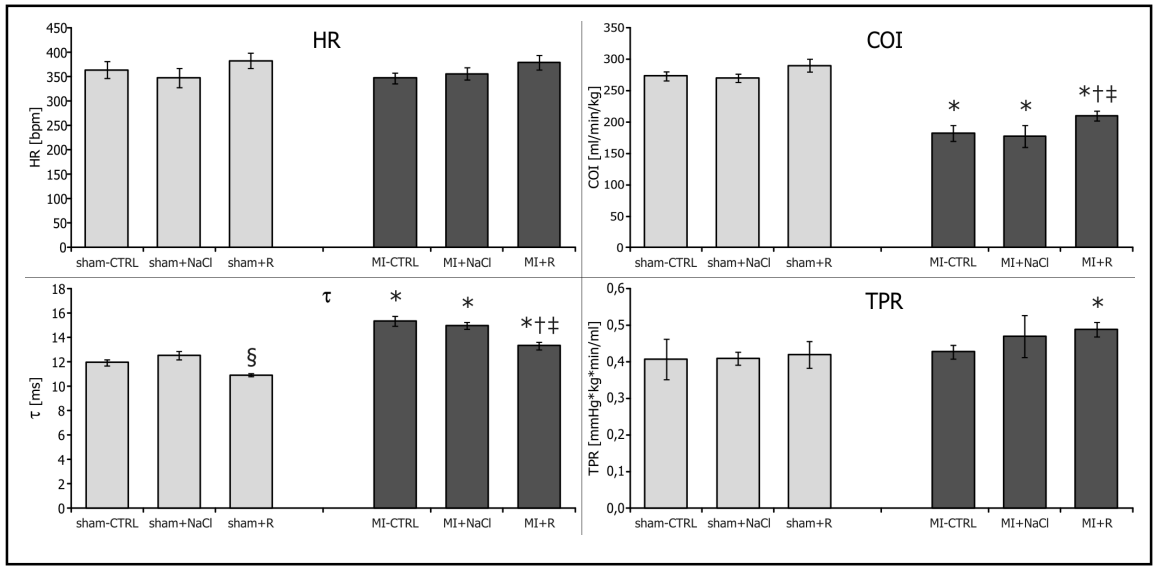
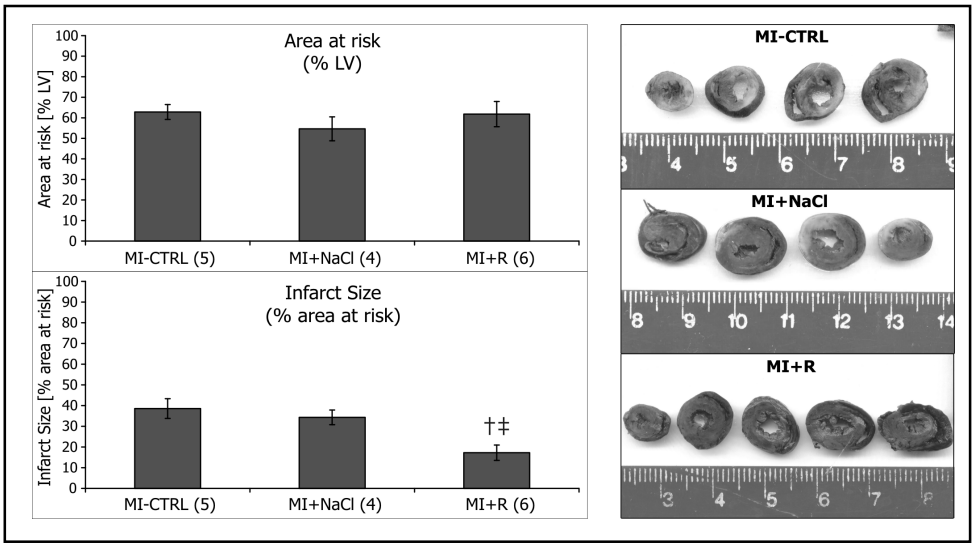


Fig. 2. Quantitative summary of heart rate (HR), time constant of relaxation tau (τ), cardiac output index (COI) and total peripheral resistance (TPR) 6 h after sham operation (sham), untreated MI (MI-CTRL), MI treated with NaCl (MI+NaCl) or MI treated with ribose (MI+R); § $p < 0.05$ *vs* sham-CTRL and sham+NaCl; * $p < 0.05$ *vs* corresponding sham; † $p < 0.05$ *vs* MI-CTRL; ‡ $p < 0.05$ *vs* MI+NaCl.

Fig. 3. Left: Planimetric measurements of ischemic area (area at risk) and the infarct area (% of area at risk) 6 h after untreated MI (MI-CTRL), NaCl treated MI (MI+NaCl) or ribose treated MI (MI+R); † $p < 0.05$ *vs* MI-CTRL; ‡ $p < 0.05$ *vs* MI+NaCl. Right: Representative photographs of Evans-Blue/TTC stained heart sections 6 h after MI-CTRL (upper), MI+NaCl (middle) and MI+R (bottom).



in the latter group probably induced by the considerable volume of the infusion. The infusion of ribose significantly reduced the infarct size and restored systolic pressure and contractility essentially to the normal level of sham-operated controls (Fig. 1). Furthermore, the relaxation rate ($LVdP/dt_{min}$ and τ; Figs. 1 and 2, respectively) was improved and the reduction in cardiac output normalized to body weight (COI, Fig. 2) was attenuated by ribose treatment. The rather moderate increase in LVEDP after 6 h may indicate that bulging of the non-infarcted heart did not occur at this time; only later, LVEDP elevations have been regularly observed [2, 3, 20]. The volume of the infusion is unlikely to contribute to changes in the LVEDP, since there were no differences in LVEDP between treated and untreated (MI-CTRL) rats and, furthermore, the volume was the same for ribose and NaCl. The improvement in the relaxation rate may follow the favorable effect of ribose on contractility.

Interestingly, the reduction in infarct size and the improvement in heart function by ribose after MI were accompanied by attenuation of the IL-1β and IL-6 expression (Fig. 4). Although the role of both cytokines after acute myocardial injury it is still not clear, it has been shown that the myocytes in the ischemic area are a major source of their expression [27]. Therefore, it may be suggested that the attenuation of their expression after

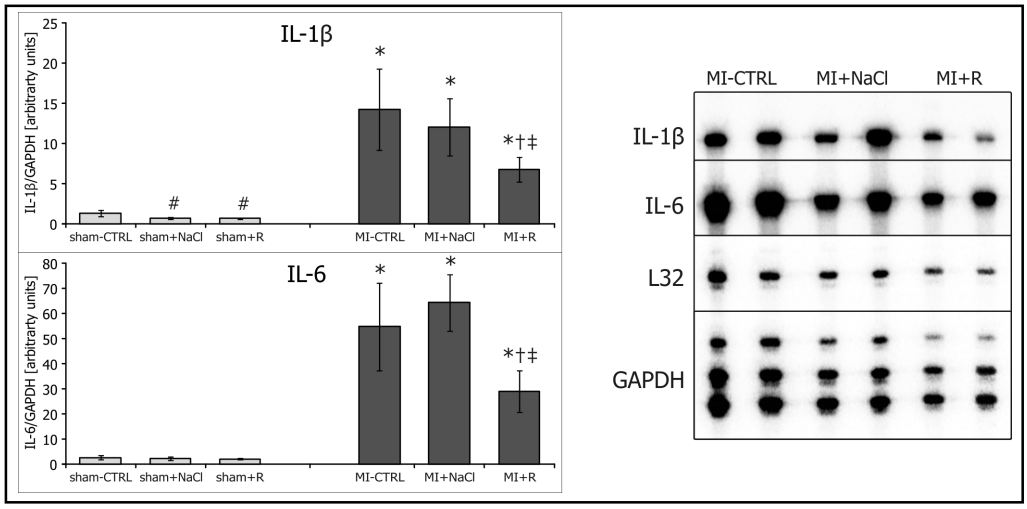


Fig. 4. Left: Quantitative summary of mRNA-Expression of interleukin (IL)-1β and -6 6 h after sham operation (sham), untreated MI (MI-CTRL), MI treated with NaCl (MI+NaCl) or MI treated with ribose (MI+R); # $p < 0.05$ *vs* sham-CTRL; * $p < 0.05$ *vs* corresponding sham; † $p < 0.05$ *vs* MI-CTRL; ‡ $p < 0.05$ *vs* MI+NaCl. Right: Representative ribonuclease protection assay (RPA) of mRNA-Expression of IL-1β and -6.

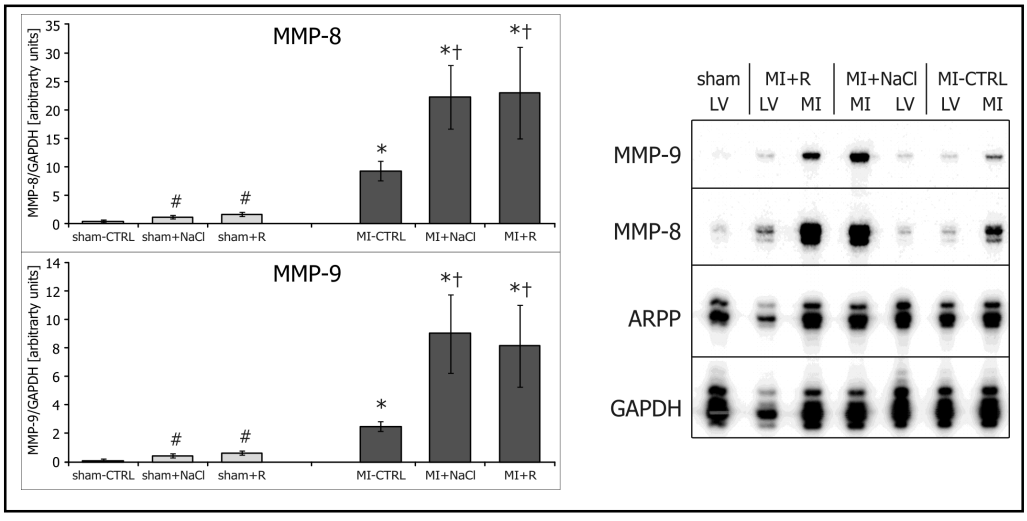


Fig. 5. Left: Quantitative summary of mRNA-Expression of matrix-metalloproteinases (MMP)-8 and -9 6 h after sham operation (sham), untreated MI (MI-CTRL), MI treated with NaCl (MI+NaCl) or MI treated with ribose (MI+R); # $p < 0.05$ *vs* sham-CTRL; * $p < 0.05$ *vs* corresponding sham; † $p < 0.05$ *vs* MI-CTRL. Right: Representative ribonuclease protection assay (RPA) of mRNA-Expression of MMP -8 and -9.

MI by ribose may reflect a less severe ischemic injury. However, it cannot be decided whether attenuation of IL-1β and IL-6 expression contributes to the prevention of infarct extension or whether it may just be a secondary effect of infarct size reduction. Most MMPs, on the other hand, are usually not expressed in normal tissue. Particularly MMP-8 and -9 are mainly produced by neutrophils or macrophages, respectively. This would indicate that the post-ischemic infiltration of the myocardium was not influenced by ribose treatment. However, also an effect of the volume load has to be considered (Fig. 5), but both aspects were not studied in further detail.

Previous studies showed that myocytes have alternative means of producing energy that would allow reversing the imbalance between oxygen supply and demand, as it occurs in a situation of ischemia [28]. It is, however, a characteristic metabolic feature of the myocyte to very slowly restore their adenine nucleotide pool after it has been depleted [29]. It has been shown that myocytes find two alternative pathways for ATP production under these conditions that would allow them reversing the ventricular dysfunction [14]. The first one would involve adenosine, inosine, and adenine (salvage pathway); and the second would be by stimulating adenine nucleotide biosynthesis. The latter can be achieved by means of ribose. This pentose sugar can permeate the cell membrane and enhance the adenine nucleotide biosynthesis by bypassing the rate-limiting enzymatic steps in the PPP through conversion to ribose-5-phosphate and formation of 5-phosphoribosyl-1-pyrophosphate [30]. That this is a specific function of ribose has been shown in that glucose did not have any of the ribose effects [17, 31]. The current study substantiates the hypothesis that i.v. infusion of ribose from 24 h prior to permanent coronary artery ligation and during permanent ischemia protects the heart from irreversible injury after 6 h of myocardial infarction.

The effect of ribose administration has been assessed in several experimental models with depressed heart

function associated with high energy phosphate loss [14, 15, 22, 32-35]. Administration of ribose increased the synthesis of adenine nucleotides and prevented the drop of cardiac function caused by isoproterenol infusion [14, 15]. It has, furthermore, been demonstrated that continuous i.v. administration of ribose in rats with MI attenuated the fall of the ATP levels in the non-ischemic myocardium and accelerated their replenishment [20]. This was accompanied by a reduction of LVEDP after 2 and 4 d of ribose administration. Further cardioprotective effects of a bolus ribose pretreatment have also been described in global ischemia [23]. Also in clinical trials in ischemic heart disease, ribose administration displayed favorable effects by improving diastolic function and quality of life and, of note, tolerance to ischemia [17, 18, 36].

In the current study, it is hard to differentiate between the effects on the ischemic and the non-ischemic myocardium, since heart function was measured globally, the mRNA expression levels were measured in the macroscopic ischemic area, whereas ATP levels were not measured. However, it has been shown that ribose increased adenine nucleotide levels in the previously ischemic heart after reperfusion over a period of 72 h [22] and in the non-infarcted heart even over 4 d [20]. It may thus be inferred that in the present experimental situation, pre-ischemic ribose infusion for 24 h may have brought the later ischemic myocardium into a more favorable metabolic condition, i.e. increase in adenine nucleotide content so that it could sustain ischemia better. On the other hand, continuing ribose infusion for 6 h after coronary artery ligation may have enabled the non-ischemic part of the heart to dampen the wave-like expansion of the ischemic area. The improved function recorded at that time (Figs. 1 and 2) does actually confirm this notion.

It has, however, also to be considered that both, the necrotic wave front and its detectability develop dynamically, but little data are available in rats. In dogs, which develop collaterals, it appears to take up to 6 h until the wave front of cell death beginning at 15 to 20 minutes after the onset of ischemia from the subendocardial region reaches the less ischemic subepicardial region and the final transmural extent of the infarct is established [37]. This, on the other hand, also depends on time, rate of oxygen consumption, collateral flow, mode of ischemia, and on the species investigated [38]. Furthermore, the lack of reperfusion prolongs the time after which TTC staining would allow a clear differentiation between living and necrotic areas. Recent studies indicate that an observation period as short as 2 h is sufficient for a reliable detection of the infarct area in a model of ischemia/ reperfusion in mice [39]. Therefore, 6 h may be sufficient in a rat model of permanent coronary artery occlusion. It should, finally, also be mentioned that positive TTC staining does not verify that these myocytes would function normally, since positive TTC staining may also occur in stunning or hibernating myocardium. Therefore, the attenuation of LV dysfunction after MI by pretreatment with ribose may be attributed to both, its effects on non-infarct zone as well as on the infarct area. The current study indicates that ribose has cardioprotective effects on the ischemic myocytes. Further studies will be needed to investigate if ribose may be beneficial in a more clinically relevant setting to treat acute myocardial infarction and reperfusion injury.

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