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# Short term protective effects of iron in a murine model of ischemia/reperfusion

Bernhard Metzler[1], Johannes Jehle[1], Igor Theurl[2], Susanne Ludwiczek[2], Peter Obrist[3], Otmar Pachinger[1] & Günter Weiss[2,*]
[1]*Division of Cardiology, Medical University, A-6020, Innsbruck, Austria;* [2]*Department of General Internal Medicine, Clinical Immunology and Infectious Diseases, Medical University, Anichstrasse 35, A-6020, Innsbruck, Austria;* [3]*Departement of Pathology, Landeskrankenhaus Linz, Linz, Austria; *Author for correspondence (E-mail: guenter.weiss@uibk.ac.at)*

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**Abstract**

The role of iron in the pathogenesis of cardio-vascular disorders is still controversial. We studied the effects of iron perturbations on myocardial injury upon temporary ischemia/reperfusion. C57BL/6J male mice were injected with iron dextran for 2 weeks while controls received saline. Mice were then subjected to 30 min of myocardial ischemia and subsequent reperfusion for 6–24 h. Tissue damage was quantified histologically and by troponin T determination. The expressions of tumor necrosis factor-α (TNF-α), superoxide dismutase (SOD) and inducible nitric oxide synthase (iNOS) were investigated in non-ischemic and ischemic regions of both groups. After myocardial ischemia and reperfusion, troponin T levels, as a marker of myocardial damage, were significantly reduced in iron-treated mice as compared to control mice ($P < 0.05$). Under the same conditions the infarction area and damage score were significantly lower in iron-treated animals. In parallel, TNF-α and SOD expressions were increased in infarcted regions of iron-treated mice as compared to controls, whereas myocardial iNOS expression was significantly lower in iron-treated mice. Although, iron challenge increased radical formation and TNF-α expression *in vivo*, this did not result in myocardial damage which may be linked to the parallel induction of SOD. Importantly, iron treatment inhibited iNOS expression. Since, an increased nitric oxide (NO) formation has been linked to cardiac damage after acute myocardial infarction, iron may exert short time cardio-protective effects after induction of ischemia/reperfusion via decreasing iNOS formation.

## Introduction

Ischemia/reperfusion (I/R) injury is a common clinical problem observed after thrombolysis for acute myocardial infarction, angioplasty or coronary bypass surgery. Manifestations of reperfusion injury include arrhythmia, reversible contractile dysfunction-myocardial stunning, endothelial dysfunction and cell death (Verma *et al.* 2002; Galinanes & Fowler 2004). Therefore, the therapeutic goals of modern cardiology are to design strategies aimed to minimise myocardial necrosis and to optimise cardiac repair following myocardial infarction. One major factor in the pathogenesis of I/R is oxidative stress (Griendling & Alexander 1997) which can be referred to increased formation of reactive oxygen species (ROS) with subsequent tissue injury.

Free iron, which is abundant in states of iron overload can potentiate the production of ROS on the basis of Fenton chemistry. This reaction mostly creates the harmful hydroxyl radical ($OH^{\cdot}$)

which can cause DNA strand brakes, destruction of lipid membranes and proteins (Papanikolaou & Pantopoulos 2005). In addition, part of the toxic action of free radicals may be aggravated by the formation of peroxinitrate ($ONOO^-$), a highly toxic radical which is formed by superoxide anion and nitric oxide (NO) (Ferdinandy & Schulz 2003).

NO is produced by the enzymatic conversion of L-arginine to L-citrulline which is catalysed by the heme-enzyme nitric oxide synthase (NOS) of which three isotypes have been characterised. While neuronal (NOS1) and endothelial (NOS3) are constitutively expressed, NOS2 (iNOS) is transcriptionally induced after stimulation with cytokines and LPS (Nathan & Xie 1994). High output formation of inducible nitric oxide synthase (iNOS) by macrophages and endothelial cells is a key event in the pathogenesis of septic cardiovascular circulation failure and increased iNOS meditated NO formation in the myocardium has been linked to cardiac damage (Saito *et al*. 2002; Chauhan *et al*. 2003). This is of interest, since NO targets the central iron sulphur clusters of several critical cellular enzymes such as mitochondrial aconitase, ribonucleotide reductase or complex I in mitochondrial respiration, thereby leading to their inactivation (Bogdan 2001). Moreover, NO regulates iron homeostasis by a posttranscriptional mechanism involving iron regulatory proteins (IRP) (Weiss *et al*. 1993), while on the other hand iron negatively affects cytokine inducible NO formation by a transcriptional mechanism (Melillo *et al*. 1997; Dlaska & Weiss 1999).

Because of these regulatory networks, disturbances of iron homeostasis in the body may be closely related to the extent of myocardial damage during I/R. Several studies have investigated the epidemiological impact of dietary and hereditary iron overload disorders for the risk for atherosclerosis or myocardial infarction (Baer *et al*. 1994; Liao *et al*. 1994; Kiechl *et al*. 1997; Tuomainen *et al*. 1999; Gaenzer *et al*. 2002) with contrasting results. Accordingly, we were interested in the role of iron in I/R, which is different from the epidemiological debate of long-term iron exposure. Although, the role of iron in I/R was first phenomenologically investigated in animal and human models (Bernard *et al*. 1988; Bolli *et al*. 1990; Turoczi *et al*. 2003), we now concentrated specifically on the interrelationship between iron, inflammatory processes and cardiac injury in a non-hemochromatosis *in vivo* model of I/R. We were aimed to examine the mechanisms underlying a putative regulatory effect of iron in this setting. Thus, in the current study we used a well established *in vivo* murine model of I/R (Michael *et al*. 1995; Metzler *et al*. 2001) and investigated the effects of iron perturbations on tissue necrosis and inflammation following transient left coronary artery ligation with subsequent reperfusion for 6–24 h.

## Materials and methods

### *Mice*

C57BL/6J male mice were injected intraperitoneally with 50 $\mu$l of iron dextran (= 5 mg iron) dissolved in 500 $\mu$l phosphate-buffered saline (PBS) every second day for 2 weeks. The control group received normal saline. All animals were maintained on a light-dark (12 h/12 h) cycle at 24 °C receiving water and food *ad libitum*. All procedures were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### *Surgical procedures*

Surgical intervention was carried out as described (Michael *et al*. 1995). The whole procedure was aided by a microscope (Olympus SZH 10). After removing the pericardial sac and slightly retracting the left auricle, the left descending artery (LAD) became clearly visible. A 1-mm section of a PE-10 tubing was placed on top of the LAD to secure the ligation of the LAD without damaging the artery. The LAD ligation was done with a 8-0 silk and was evident by discoloration of the left ventricle. The retraction sutures were removed, and the chest wall was approximated. To prevent desiccation the chest was covered with a small moist swab. After 30 min of LAD occlusion, the ligature was removed by cutting the knot on top of this PE-10 tube and reperfusion was visually confirmed. Iron-treated ($n$ = 31) and control ($n$ = 30) mice were subjected to 30 min of LAD occlusion and in every of the two groups to 6 h ($n$ = 6) or 24 h ($n$ = 6) of reperfusion, respectively.

*Determination of serum troponin T, myocardial iron and serum iron levels*

From each mouse heparin-blood was collected when they were sacrificed. Serum levels of troponin T (TnT) were determined as an indicator of myocardial damage by using the quantitative TnT rapid assay from Roche Diagnostics (Mannheim, Germany). Serum iron accumulation was estimated upon determination of total iron (TI) and unsaturated iron-binding capacity (UIBC) using commercially available assays (Sigma Diagnostics, Munich, Germany). Myocardial iron accumulation was determined in paraffin embedded tissue slices using Prussian blue staining technique.

*Tissue preparation and damage score*

After reperfusion, the hearts were harvested and cut into two portions 1 mm below the ligation suture. Tissues were fixed in 4% paraformaldehyde at 4 °C overnight and embedded in paraffin. Sections of 5 $\mu$m were cut from the cross area and stained with hematoxilin and eosin (HE) for histological evaluation of tissue damage. For quantitative estimation of tissue damage we used the previously published score system (Zingarelli *et al.* 1998). According to that score, the following criteria in the area of tissue injury were considered: score 0, no damage; score 1 (mild), interstitial edema and focal necrosis; score 2 (moderate), diffuse myocardial cell swelling and necrosis; score 3 (severe), necrosis with the presence of contraction bands and neutrophil infiltrate; and score 4 (highly severe), widespread necrosis with the presence of contraction bands, neutrophil infiltrate, and hemorrhage. Six animals in each group were included and eight sections from each animal heart were evaluated.

The measurement of the infarction or the scar area was done by reviewing the sections with a Bx60 microscope (Zeiss, Jena, Germany) equipped with a Sony 3CCD camera and television monitor. A transmission scanning microscope (Bio-Rad), equipped with a 488 nm argon ion laser and Plan Neofluar $10\times/0.3$ oculars connected with the program START LSM 510 was used to scan the images. The scar was defined as the region between the living myocytes and the cardiac membrane. Areas were measured and recorded in square micrometers. In total, there were eight slices per mouse measured from the point of ligation to the apex and averaged for statistical analysis. The investigator for the analysis was blinded to group assignment.

*Immunohistochemical analysis*

Serial 5 $\mu$m frozen sections were cut from cryopreserved tissue blocks, fixed in a cold 1:1 acetone–chloroform mixture for 10 min, and washed with PBS for 20 min. The sections were subsequently placed in a humidified chamber, where they were over-layed with mouse monoclonal antibodies against superoxide dismutase (SOD) (0.2 $\mu$g/ml, Stressgen Biotechnology, Victoria, Canada) or iNOS (anti-iNOS-antibody, 0.1 $\mu$g/ml, BD Bioscience Erembodegem, Belgium) and subsequently counterstained with anti-rat Ig conjugated with FITC as described (Ludwiczek *et al.* 2004). For peroxynitrite determination sections were incubated with a polyclonal rabbit anti-nitrotyrosin antibody (Upstate Biotechnology, NY, USA) in a dilution of 1:100 for 30 min and counter-stained with haem-alaun. For quantitative determination of nitro-tyrosin expression a score was calculated as the product of a proportion score and an intensity score according to the method described (Gastl *et al.* 2000). The proportion score described the estimated fraction of positive-stained cells (0,none; 1, $<10\%$; 2, 10–50%; 3, 50–80%; 4, $>80\%$). The intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The total score ranged from 0 to 12.

*Real time PCR and Western blot analysis*

RNA was extracted from tissue slices obtained from ischemic and non-ischemic areas of the left ventricle after 6–24 h of reperfusion. The tissue expression of iNOS and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) mRNA was evaluated by TaqMan real-time PCR and normalised for 18S RNA expression exactly as described (Ludwiczek *et al.* 2004). The following primers and TaqMan probes were used: Probes were labeled with the reporter dye 6-carboxyflurescein (FAM) at the 5′-end and with 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3′-end. mu TNF-$\alpha$:5′-TTC TAT GGC CCA GAC CCT CA-3′, 5′-TTG CTA CGA CGT GGG CTA CA-3′, 5′-CTC AGA TCA TCT TCT CAA AAT

CGA GTG ACA AGC-3′, muiNOS: 5′-CAG CTG GGC TGT ACA AAC CTT-3′, 5′-CAT TGG AAG TGA AGC GTT TCG-3′, 5′-CGG GCA GCC TGT GAG ACC TTT GA-3′,

Protein-extracts were prepared from nitrogen frozen ischemic myocardial tissues and Western blotting was carried out exactly as described (Ludwiczek *et al*. 2004) using either anti-iNOS-antibody (0.1 μg/ml, BD Biosience, Erembodegem, Belgien) or anti-Mn SOD-antibody (0.2 μg/ml, Stressgen Biotechnology, Victoria, Canada). However, when using an anti-TNF-α antibody we could never detect a clear band in the Western blot.

*Statistical analysis*

Statistical analysis was performed by using SPSS for Windows (Version 11.5) employing Students' *t*-test. A *P*-value < 0.05 was considered to be statistically significant.

## Results

To see whether or not the administration of iron resulted in iron overload and myocardial iron accumulation, we measured serum iron levels, unsaturated iron-binding capacity (TIBC) and myocardial iron content. Serum tests revealed significantly higher levels of serum iron in iron-treated mice as compared to control animals (Figure 1) whereas UIBC was significantly lower in iron loaded mice (Figure 1). Tissue iron staining demonstrated significant iron accumulation in the myocardium of iron-treated mice (Figure 1C, D).

*Iron overload reduces myocardial necrosis*

From a total of 36 iron-treated animals which were subjected to surgery, 31 (86%) survived and were used for further evaluation. In the control group 30 (86%) out of 35 animals subjected to surgery survived.

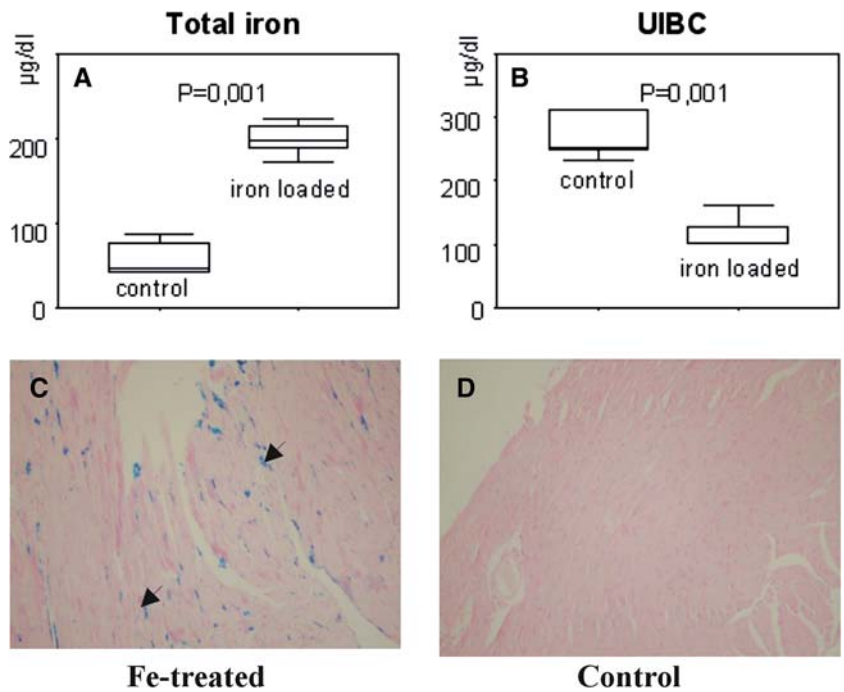


*Figure 1.* Serum iron levels and myocardial iron accumulation. Mice were intraperitoneally injected either with iron or saline (control group) for 2 weeks before the left descending artery was occluded for 30 min and reperfused for up to 24 h. Serum iron levels (A) and UIBC (B) were determined in blood from mice using commercially available assays. Tissue iron content was estimated in paraffin embedded sections of hearts. Prussian blue staining of myocardial iron accumulation. (A) and (B) reflect differences in serum iron and UIBC of iron-treated and control mice ($n = 30$ for iron treatment and $n = 31$ for controls, $p < 0.0001$). (C) and (D) show a representative example iron content in myocardial tissue of iron-treated and control mice stained with Prussian blue (arrows).

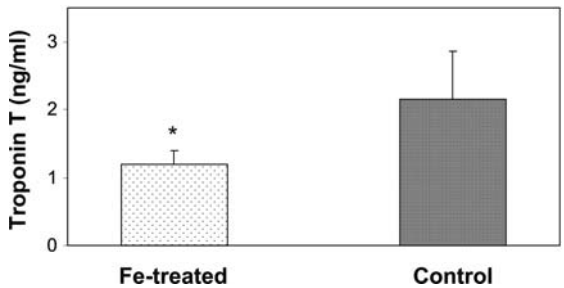


*Figure 2*. Iron-pretreatment reduces Troponin T levels in ischemia/reperfusion (I/R) injury. Mice were either intraperitoneally injected with iron or saline (control group) for 2 weeks before the left descending artery was occluded for 30 min and reperfused for 6 h. TnT levels were determined as described in the Materials and methods. *Marks significant difference from control group mice, $p < 0.05$).

Troponin T (TnT) is a sensitive and specific marker for cardiac damage not only in men but also in mice (Metzler *et al*. 2002). Following thirty minutes of LAD occlusion and six hours of reperfusion a marked increase of serum TnT levels was observed in control mice ($2.16 \pm 0.7$ ng/ml; means $\pm$ SD) as compared to sham operated animals not undergoing LAD occlusion (below detection limit; 0.04 ng/ml). In iron-pretreated mice undergoing this I/R procedure TnT levels were significantly lower than in control mice ($1.19 \pm 0.2$ ng/ml, $P < 0.05$, Figure 2).

To get a more profound insight into the pathological changes following I/R in control and iron loaded mice, a histological examination of myocardial tissue was performed. In control mice I/R resulted in marked myocardial necrosis with the development of contraction bands and neutrophil infiltration while iron-treated mice showed less morphological alterations as compared to control animals (Figure 3) which was confirmed upon applying damage score quantification technique (Zingarelli *et al*. 1998). The evaluation of scar sizes showed a similar pattern as a significantly smaller area of infarction in iron-treated mice while compared to control mice (Figure 3).

### *Expression of iNOS, SOD and TNF-α in ischemic and non-ischemic myocardium following reperfusion*

Since iNOS and TNF-α expression may be involved in the post-ischemic inflammatory response during reperfusion we determined mRNA levels of iNOS and TNF-α in ischemic and non-ischemic areas of hearts from iron-treated and control mice. When investigating ischemic areas after 24 h of reperfusion we found a reduced expression of

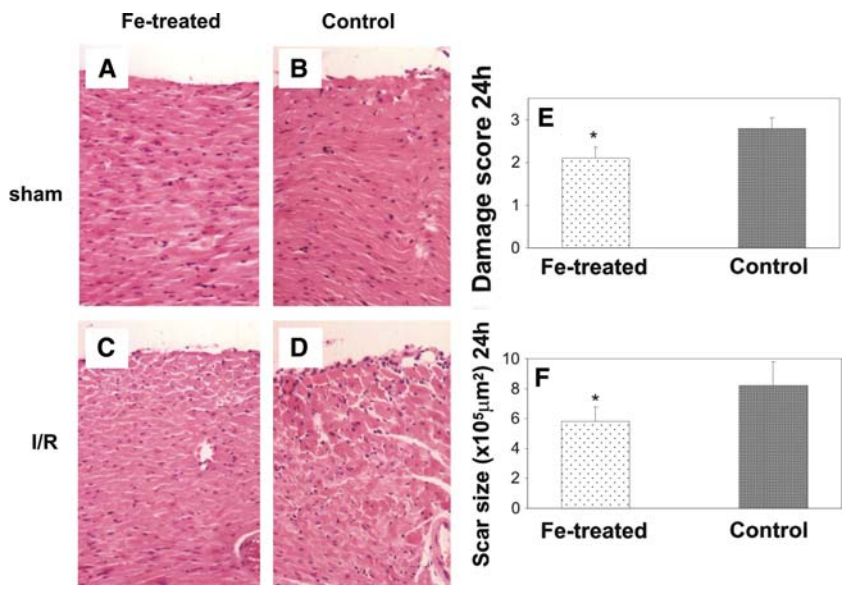


*Figure 3*. Effects of iron-pretreatment on tissue damage in ischemia/reperfusion (I/R). Mice were either intraperitoneally injected with iron or saline (control group) for 2 weeks before the left descending artery was occluded for 30 min and reperfused up to 24 h. Histological sections were taken after 24 h of reperfusion (C, D) or 24 h after sham operation (A, B). The extend of myocardial damage was quantified according to the method described by Zingarelli et al. Note that typically damaged tissues were marked in (D). (E) and (F) reflect differences in damage scores and scar size of the control and iron-treated group undergoing ischemia and reperfusion for 24 h ($n = 6$, *marks significant difference from control group mice, $p < 0.05$).

iNOS mRNA in iron-treated mice as compared to control mice (Figure 4A). This was also true for non ischemic areas. The expression of iNOS mRNA was almost undetectable in sham operated animals and not changed by iron treatment (details not shown).

To see whether these changes are also reflected by protein expression we performed Western blot analyses and immuno-histochemistry for iNOS in ischemic areas. While no iNOS expression was evident in sham operated animals (Figure 5A), iNOS protein was strongly induced in control animals undergoing I/R (Figure 5D), and its expression there was more pronounced than in iron-treated animals following I/R (Figure 5C). Accordingly, this was confirmed by Western blot analysis whereupon iNOS-protein expression was higher in control than in iron-treated mice following I/R (Figure 5E).

Interestingly, in comparison to iNOS, TNF-α mRNA expression showed the inverse picture; following I/R TNF-α mRNA levels were highest in iron-treated mice as compared to control mice in both, ischemic and non ischemic areas (Figure 4B).

Superoxide dismutase (SOD) plays a crucial role in limiting iron-induced free radical myocardial damage and its expression is increased upon oxidative stress. Thus, we determined SOD expression in ischemic areas of hearts from iron-treated and control mice. By means of Western blot and immunohistochemical analysis we found increased expression of SOD in iron loaded mice following I/R as compared to control animals (Figure 6C–E), while no iron dependent changes in SOD protein expression were found in sham operated animals (Figure 6A, B).

Thus, SOD expression paralleled TNF-α mRNA levels in this I/R model which were both found to be highest in iron-treated mice whereas iNOS expression, TnT release and myocardial damage were reduced upon iron pretreatment.

*Nitro-tyrosine expression*

Our experiments suggested that iron may affect cardiac damage by modulating NO formation. Since in I/R ROS are produced in parallel with NO we studied whether or not iron treatment may affect the formation of peroxynitrite ($ONOO^-$). To test this we performed immuno-histochemistry with an antibody detecting nitro-tyrosine residues on proteins, which is indicative for *in vivo* $ONOO^-$ formation (Foster *et al.* 2003). Following ischemia and 24 h of reperfusion, no significant difference in nitro-tyrosine tissue expression, an indicator of ONOO-activity, became obvious. This was confirmed by quantitative scoring of nitro-tyrosin expression showing no difference between control ($6.4 \pm 2.3$) and iron pre-treated mice ($5.8 \pm 1.6$, $P = 0.63$).

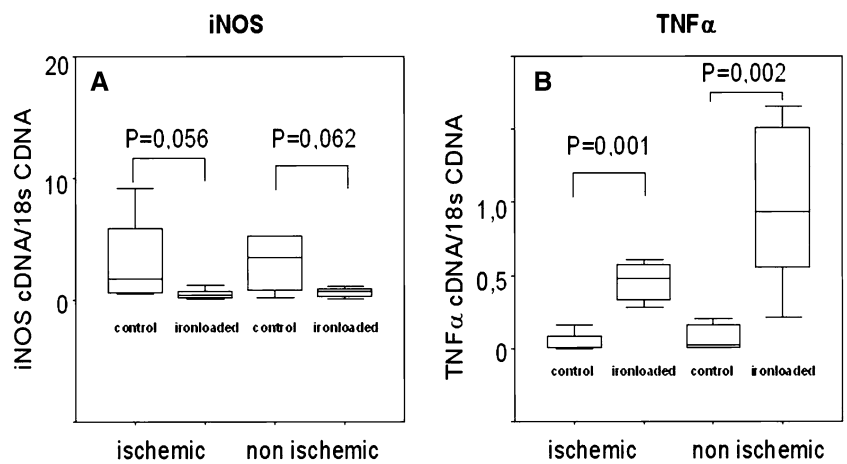


*Figure 4.* Effect of iron on inducible nitric oxide synthase (iNOS) and tumor necrosis factor α (TNF-α) mRNA expression in ischemic and non ischemic areas of the myocardium. Control or iron-preloaded mice were subjected to ischemia/reperfusion (I/R) as detailed in Materials and Methods, and mRNA from ischemic and non ischemic areas was extracted after 24 h of reperfusion. Real-time PCR was performed on these individuals with iNOS and TNF-α primers and probes, and normalized to 18 S rRNA. Measurements were performed in triplicates. The relative abundance of iNOS and TNF-α mRNA normalized to 18 S rRNA is shown. Data are presented as means ± SD for six animals examined in each group.

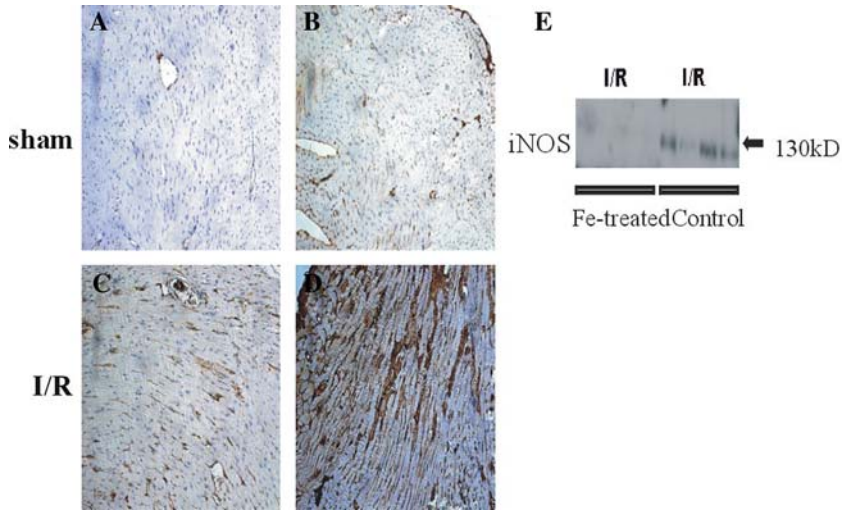


*Figure 5*. Tissue expression of inducible nitric oxide synthase (iNOS) in ischemic regions. Iron-treated and control mice were subjected to ischemia/reperfusion (I/R) for 24 h. Sections of myocardial tissue from ischemic areas were prepared and either immunohistochemically stained for iNOS or used for Western blot analysis as described in the Methods. No iNOS expression was found in sham operated animals of either group (A and B). Representative examples of iNOS expression in ischemic tissues of control (D) and iron-pretreated mice (C) are shown. Positive cells in ischemic areas are stained in dark brown and were enumerated under the microscope as described in the methods ($n = 8$, $p < 0.05$). (E) Reflects differences in iNOS protein levels as determined by Western blot analysis of ischemic areas of iron-treated and control mice after 24 h of reperfusion.

## Discussion

The association between iron and cardio-vascular disease has been controversial. Using a murine model of ischemia/reperfusion (I/R) we herein demonstrate, that tissue iron overload has beneficial short-term effects on the extent of myocardial damage. Following a 30 min ligation of the left coronary artery and subsequent reperfusion for up to 24 h, mice which were injected with iron prior to initiation of I/R, presented with decreased infarction size, damage score and troponin T levels as compared to animals not exposed to iron. This parallels a recent observation made with the iron chelator pyridoxal isonicotinoyl hydrazone which resulted in a drastic increase in troponin T levels in rabbits (Adamcova *et al*. 2002).

A recent study in mice-knocked out for HFE, the gene being mutated in association with hereditary hemochromatosis-showed that iron overload of HFE-/-mice resulted in increased I/R injury (Turoczi *et al*. 2003). However, HFE knock out results in drastic changes in body iron homeostasis but also in alterations of cellular iron transport and tissue iron distribution as it becomes evident especially in macrophages or myocytes (Pietrangelo 2004). Thus, observations made in HFE knock out models do not apply to dietary or experimental iron overload in normal individuals.

When investigating the possible underlying mechanisms we found that in control animals iNOS was drastically increased following I/R while in iron loaded animals tissue expression of iNOS was reduced. This is of importance since iNOS is responsible for high output formation of NO by inflammatory stimuli (Nathan & Xie 1994; Bogdan 2001). NO harbours both beneficial and detrimental effects on endothelial and myocardial cells: NO can result in relaxation of smooth muscle cells while at high concentrations NO attenuates myocyte contraction and catecholamine responses (Morita *et al*. 1994; Moncada 1997). Accordingly, over-expression of iNOS by cardiomyocytes has also been linked to apoptotic cell death (Mungrue *et al*. 2002). The latter can be in part referred to a NO mediated inhibition of iron containing enzymes in mitrochondrial respiration or the Krebs cycle which results in enzyme destruction and apoptosis (Drapier & Hibbs 1988; Weiss *et al*. 1993; Foster *et al*. 2003).

On the other hand, iron negatively affects iNOS transcription by inhibiting the binding affinity of NF-IL6 and hypoxia inducible factor-1 to the iNOS promotor (Weiss *et al*. 1994; Melillo *et al*. 1997; Dlaska & Weiss 1999) which may also

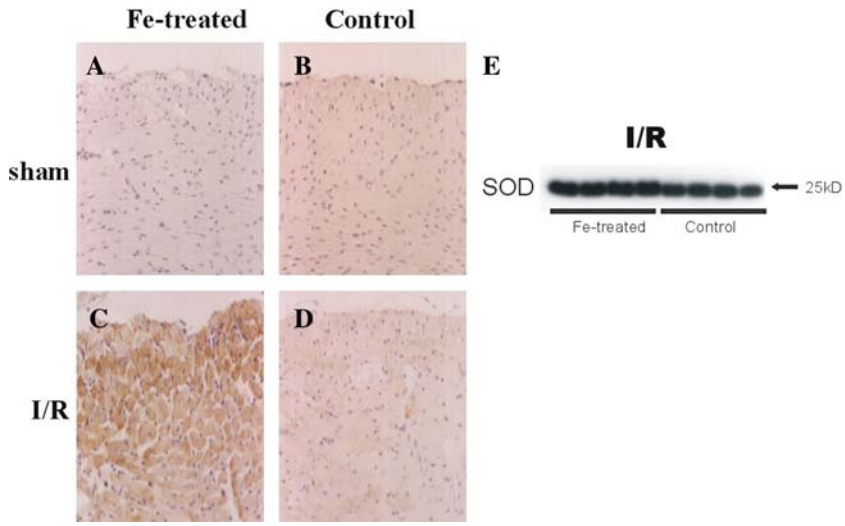


*Figure 6*. Tissue expression of superoxide dismutase (SOD) in ischemic regions. Iron-treated and control mice were subjected to ischemia/reperfusion (I/R) for 24 h and protein expression of SOD was determined by means of Western blots and immuno-histochemistry. Representative examples of SOD expression in tissues of sham operated control (B) and iron-pretreated mice (A), and in ischemic tissues of control (D) and iron-pretreated mice (C) after 24 h of reperfusion are shown. (E) Reflects differences in SOD protein levels as determined by Western blot analysis of ischemic areas of iron-treated and control mice after 24 h of reperfusion.

underlie our observation of decreased iNOS expression with myocardial iron overload in I/R injury (Theurl *et al*. 2005).

In parallel to down-regulation of iNOS and reduction of myocardial damage, iron overload prior to induction of I/R was accompanied by an

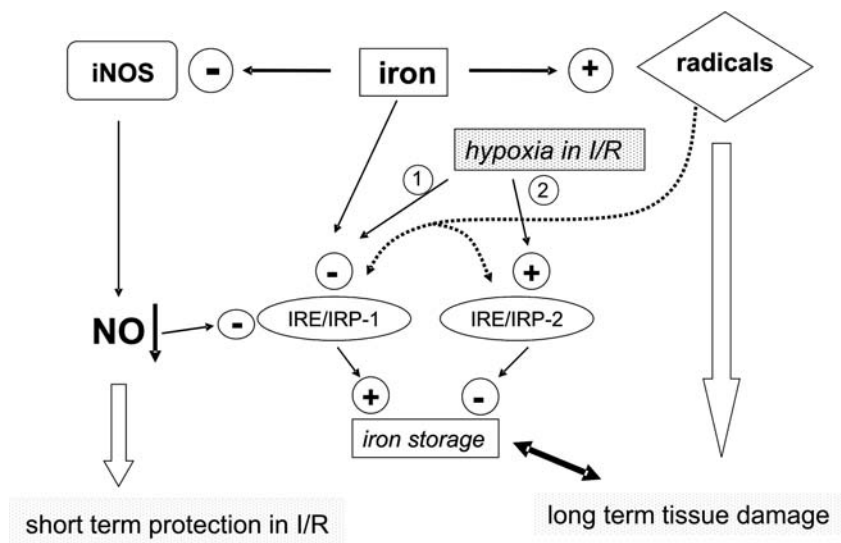


*Figure 7*. Model diagram on short and long-term effects of iron in ischemia/reperfusion injury and the role of IRPs: excess iron inhibits the induction of iNOS and subsequently the formation of NO, thus reducing NO mediated tissue damage in ischemia/reperfusion (I/R). On the other hand, iron catalyzes the formation of toxic radicals leading to long-term tissue damage and progressive organ failure. The regulation of intracellular iron metabolism via IRPs is modified during I/R. In early stages of hypoxia (1) IRP-1 binding affinity is down-regulated, which is possibly also a consequence of reduced NO formation and of an increased iron availability (Schneider & Leibold 2003). This results in iron storage via promotion of ferritin synthesis. In the late phase of hypoxia (2), IRP-2 binding affinity increases resulting in decreased ferritin synthesis. Radicals, such as $H_2O_2$ or $O_2^-$, differently regulate IRP-1 and IRP-2 activities and thus cellular iron homeostasis. Thereby, the bioavailability of catalytically active iron is modified which is critical for long-term tissue damage by the metal. NO: nitric oxide; iNOS: inducible nitric oxide synthase; IRE/IRP: binding affinity of iron regulatory proteins (IRP-1, IRP-2) to target iron responsive elements.

increase of myocardial TNF-α and SOD expression. SOD is scavenging free oxygen radicals which are produced in the course of I/R injury (Naranjan *et al*. 2000). The production of radicals may be further aggravated in the presence of iron, since this metal catalyses the formation of highly toxic hydroxyl-radicals (Papanikolaou & Pantopoulos 2005). Hydroxyl radicals may be involved in the initiation and propagation of the inflammatory cascade in triggering TNF-α release via activation of NF-kB, while on the other hand TNF-α is a strong inducer of radical formation by macrophages, neutrophils or endothelial cells (Strieter & Bone 1993). This would also fit to our observation on an increased expression of TNF-α together with enhanced SOD activity in iron-treated mice.

However, iron overload although being associated with increased formation of TNF-α was clearly associated with a reduced myocardial damage. This may be referred to the following facts:

(i) TNF-α may exert anti-apoptotic effects, thereby preventing myocardial injury and myocyte apoptosis in association with myocardial ischemia (Kurrelmeyer *et al*. 2000; Sack *et al*. 2000).
(ii) With the formation of oxygen radicals and via TNF-α induction the expression of the detoxifying enzyme SOD is up-regulated which can be partly referred to activation of NF-kB or activator protein-1 (AP-1) (Warner & Wispe 1996; Jones & Boss 1997). The induction of Mn-SOD therefore contributes significantly to the protection against myocardial I/R injury.
(iii) Recent evidence also suggests that NO by itself regulates the formation of superoxide under low oxygen concentrations, a condition which is very prevalent in I/R. Thereby, under low oxygen conditions an increased formation of superoxide anion was observed which was diminished by NO synthase inhibition (Palacios-Callender *et al*. 2004). Accordingly, the decrease in NO formation in iron-pretreated animals undergoing I/R would thus result in reduced formation of superoxide anion and in prevention of myocardial damage by these radicals.

We finally investigated the possibility whether or not the reduced expression of iNOS in the presence of iron may also effect the formation of peroxynitrate which is formed by enzymatic conversion of NO and superoxide and which is also believed to play a role in post-ischemic injury (Mungrue *et al*. 2002; Ferdinandy & Schulz 2003; Foster *et al*. 2003). However, we could not find an obvious difference in protein nitro-tyrosine expression between the two groups, although iNOS expression was significantly lower with iron overload. This suggests that at least in our model NO rather than $ONOO^-$ is primarily responsible for tissue damage in I/R injury (Saito *et al*. 2002) and that the decrease of NO formation by iron is not rate-limiting for $ONOO^-$-production. Importantly, the recent observation that iron complexes can prevent myocardial dysfunction in endotoxemic rats by leading to a decomposition of peroxynitrite, also pointing to a protective effect of iron observed herein, strongly support our results described herein (Lancel *et al*. 2004).

In summary, we demonstrate that short-term iron overload can protect the heart from I/R injury. The mechanism of protection by iron appears to be primarily mediated by inhibition of iNOS expression, but may partly be referred to induction of SOD expression and de-toxification of superoxide anion. Although these effects of iron may be relevant to reduce short-term tissue damage in I/R injury one has to be aware of clinical and experimental studies showing that long-term exposure to iron may result in endothelial dysfunction, early arteriosclerotic changes and increases risk for acute cardio-vascular events (Figure 7). Moreover, the regulation of iron homeostasis and the role of IRPs in the setting of hypoxia and reperfusion is a dynamic process and appears to be a central determinant for the net effect of iron in terms of causing tissue damage or protection (Schneider & Leibold 2003; Papanikoleau & Pantopoulos 2005). Future research should focus on the therapeutic potential of modulating iron and iNOS metabolism for cardiac perfusion while trying to accumulate more knowledge on genetic and environmental factors being associated with prolonged iron overload as a potential hazard for cardio-vascular disease.

## Acknowledgements

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