Cardioprotection Through S-Nitros(yl)ation of Macrophage Migration Inhibitory Factor

Running title: Lüdike et al.; Cardioprotection by S-nitros(yl)ation of MIF

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Abstract:

**Background** - Macrophage migration inhibitory factor (MIF) is a structurally unique inflammatory cytokine that controls cellular signaling in human physiology and disease through extra- and intracellular processes. MIF has been shown to mediate both disease-exacerbating and beneficial effects, but the underlying mechanism(s) controlling these diverse functions are poorly understood.

**Methods and Results** - Here, we have identified an S-nitros(yl)ation modification of MIF which regulates the protective functional phenotype of MIF in myocardial reperfusion injury. MIF contains three cysteine (Cys) residues; using recombinant wtMIF and site-specific MIF mutants, we have identified Cys-81 to be modified by S-nitros(yl)ation, whereas the CXXC-derived Cys residues of MIF remained unaffected. The selective S-nitrosothiol (SNO) formation at Cys-81 led to a doubling of the oxidoreductase activity of MIF. Importantly, SNO-MIF formation was measured both *in vitro* and *in vivo* and led to a decrease in cardiomyocyte apoptosis in the reperfused heart. This was paralleled by an SNO-MIF- but not Cys81Ser-MIF mutant-dependent reduction of infarct size in an *in vivo* model of myocardial ischemia/reperfusion (I/R) injury.

**Conclusions** - S-nitros(yl)ation of MIF is a pivotal novel regulatory mechanism, providing enhanced activity resulting in increased cytoprotection in myocardial reperfusion injury.

**Key words:** S-nitros(yl)ation, cardioprotection; myocardial infarction; nitric oxide; reperfusion injury
MIF is a structurally unique inflammatory cytokine that plays an important role as an upstream regulator of innate and acquired immunity as well as in cellular redox signaling. Owing to its inflammatory activities, MIF is a mediator of a number of acute and chronic inflammatory diseases.\textsuperscript{1-6} This includes an exacerbating role in atherosclerosis, a major underlying cause for cardiovascular disease in the Western world.\textsuperscript{7-10} However, accumulating evidence indicates that the role of MIF in cardiovascular pathology is more complex. MIF regulates key functions in myocardial I/R injury with an overall cardioprotective activity profile.\textsuperscript{11-14} Mechanistically, this property involves protection against c-Jun N-terminal kinase (JNK)-mediated apoptosis and against oxidative cell stress which prominently occurs during the early phase of reperfusion in myocardial infarction. Protection conferred by MIF against redox stress has been attributed to its intrinsic thiol-protein oxidoreductase (TPOR) activity.\textsuperscript{1,5,11,15}

MIF’s role in redox regulation is due to its structural properties which are unique within the protein family of cytokines and plays a critical role in cellular redox homeostasis and apoptosis inhibition.\textsuperscript{1,11,15} The TPOR activity of MIF is mediated through a conserved Cys\textsuperscript{57}-Ala-Leu-Cys\textsuperscript{60} motif\textsuperscript{15} resembling the CXXC motif of TPORs like thioredoxin (TRX). Moreover, residue Cys-60 is crucial for the interaction interface between MIF and c-Jun N-terminal activation domain binding protein-1 (JAB1/CSN5), an interaction partner of intracellular MIF.\textsuperscript{16} MIF engages in several defined protein-protein interactions with both extra- and intracellular binding partners. The third cysteine in MIF, Cys-81, has been suggested to mediate conformational effects.\textsuperscript{17} However, posttranslational modifications that could serve to control the action and magnitude of such interactions have been unknown.

Protein $S$-nitros(yl)ation constitutes a large part of the nitric oxide (NO$^*$)-mediated effects throughout physiology as well as in a broad spectrum of human diseases.\textsuperscript{18} $S$-nitros(yl)ation is
the coupling of an NO\(^{•}\) moiety to a reactive Cys thiol to form an SNO. That could occur by endogenous NO\(^{•}\)-generating agents such as N\(_2\)O\(_3\), by transition metal-catalyzed addition of NO\(^{•}\) or by trans-nitros(yl)ation from low molecular-weight SNOs, such as Cys-NO.\(^{19}\) Furthermore, \(^{•}\)NO\(_2\) possibly mediates an one-electron oxidation of thiols with subsequent reaction of thyl radicals with NO\(^{•}\). This protein modification has emerged as an important mechanism for dynamic, posttranslational regulation of most or all main classes of proteins.\(^{19}\) S-nitros(yl)ation of selective Cys thiol groups can modulate the action of specific target proteins and thus regulates their function and properties.\(^{20-21}\) There is accumulating evidence that S-nitros(yl)ation of proteins is intimately linked with myocardial I/R injury mediating cardioprotection.\(^{22}\) A number of studies demonstrated that NO\(^{•}\) is generated from both NO synthases (NOS)-dependent and NOS-independent pathways.\(^{23-27}\) During I/R, nitrite reduction to NO\(^{•}\) is an important source of NO\(^{•}\) that can be utilized for S-nitros(yl)ation.\(^{28}\)

In this study, we set out to test whether S-nitros(yl)ation of MIF may regulate the protective functional phenotype of MIF in myocardial reperfusion injury. We reasoned that if MIF is posttranslationally modified by S-nitros(yl)ation, S-nitros(yl)ated MIF could play a fundamental role in myocardial I/R injury.

**Methods**

**Recombinant wtMIF and site-specific mutants of MIF**

Recombinant wild-type human MIF (wtMIF) as well as the Cys mutants Cys57Ser-MIF, Cys60Ser-MIF, and Cys81Ser-MIF used in this study were expressed, purified, and folded as described previously.\(^{29}\)

**Reductive gas phase chemiluminescence**
The amount of SNO-MIF formation after S-nitros(yl)ation of wtMIF or the Cys-Ser substitution mutants was determined by reductive cleavage of the SNO bond with an iodide/triiodide-containing reaction mixture and subsequent determination of the NO released into the gas phase by its chemiluminescence reaction with ozone within a chemiluminescence detector as described previously.30,31

**Synthesis of S-nitros(yl)ated MIF**

S-nitros(yl)ation of wtMIF was achieved by reduction of nitrite to NO from deoxygenated myoglobin (deoxyMb).27,28 This approach prevents false positive chemiluminescence signals from conventionally applied NO donors in the incubation solution, e.g. via Cys-NO. In brief, horse heart Mb was dissolved in PBS to a final concentration of 200 μM. Following deoxygenation by gassing with argon for 15-20 min, deoxyMb was quantified spectrophotometrically (λ = 542 nm; ε_{542} = 13.9 cm⁻¹ mM⁻¹). A 5 mM nitrite solution was anoxically prepared and added at a final concentration of 25 μM to the deoxyMb solution. NO formation was confirmed by chemiluminescence. Incubation of 400 nM wtMIF with 25 μM nitrite and 200 μM deoxyMb was carried out in the dark for 1 h at 37°C. To specify the distinct S-nitros(yl)ation site within MIF, the Cys-Ser replacement mutants Cys57Ser-, Cys60Ser- or Cys81Ser-MIF were used instead of wtMIF. After 1 h, SNO measurement was performed via chemiluminescence. Alkylation of Cys residues via pre-incubation of wtMIF with 100 mM NEM prior to S-nitros(yl)ation or omitting deoxyMb in the incubation tube served as negative controls.

**HED transhydrogenase assay**

Catalytic redox activity of wtMIF and SNO-MIF was measured by the HED transhydrogenase assay which was performed as described previously.32 Briefly, reduction of HED by reduced glutathione was measured in a MIF-catalyzed reaction. Oxidation of NADP+ by oxidized
glutathione was then recorded photometrically in a coupled step (λ = 340 nm). For each measurement, 25 μg wtMIF dissolved in 20 mM sodium phosphate buffer (pH 7.2) was used. SNO-MIF formation was achieved by incubating wtMIF with 250 μM CysNO for 30 min at 37°C in the dark as described previously.\textsuperscript{33} The transhydrogenase reaction was started by adding wtMIF or SNO-MIF to 450 μl of the reaction mixture (final volume: 600 μl; final MIF concentration: 3.4 μM). Equal volumes of 20 mM sodium phosphate buffer and sodium phosphate buffer with 250 μM CysNO (pH 7.2) served as controls. Reactions were recorded for 12 min and MIF activity calculated from the linearized slope of the curve (Δ 340 nm). Values represent the mean±SD of three independent assays with at least duplicate incubations each.

**Detection of S-nitros(yl)ated proteins by biotinylation (biotin-switch assay) and Western blotting**

The biotin-switch assay was essentially performed as described previously with minor modifications.\textsuperscript{34} *Homogenization and blocking step:* Excised and perfused mouse hearts were weighed and homogenized immediately in 20 volumes of ice-cold HEN buffer (250 mM Hepes, 10 mM EDTA, 2 mM NEM, 0.1 mM neocuproine) to block free thiols, by using a Wheaton glass-glass homogenizer immersed in an ice/water bath. The homogenate was centrifuged at 70,000 g for 1 h at 4 °C. The supernatant was incubated for 30 min at room temperature in the dark. *Biotinylation step:* The supernatant was precipitated twice with 5 volumes of ice-cold acetone to remove unreacted NEM and the pellet was resuspended in HENS buffer (Hepes 250 mM, EDTA 1 mM, neocuproine 0.1 mM and 1% SDS). Subsequently, proteins were incubated with 1/3 volume of 4 mM N-(6-(biotinamido)hexyl)-3’-(2’-pyridyldithio)propionamide (biotin-HPDP, Pierce). Ascorbate and copper (1 mM/10 nM) were added to reduce SNO bonds and the mixture was incubated for 1 h at room temperature in the dark. After another ice-cold acetone
precipitation to remove unreacted biotin-HPDP, the pellet was re-suspended in HENS buffer.

**Western blot analysis:** For the specific detection of S-nitros(yl)ated MIF, proteins were immobilized after the biotinylation step via neutravidin resin-coated spin columns and subsequently pulled down according to the manufacturer instructions (Pierce, Pull-Down Biotinylated Protein:Protein Interaction Kit, USA). After pull-down, affinity-purified biotinylated proteins from heart homogenates and MIF were revealed by Western blotting using the MIF-specific antibody Ka565 (1:1000) as described. MIF bands were quantified using Quantity One 1-D analysis software and normalized to the signal of a recombinant MIF band. Amber tubes were used in all these procedures and the reactions were carried out in the dark to avoid unspecific photolysis of SNO bonds from light.

**Myocardial I/R injury in vivo protocol**

Wild-type (WT) and MIF-deficient (Mif<sup>-/-</sup>) mice were anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazin (10 mg/kg) and intubated. Mechanical ventilation parameters were set to a tidal volume of 2.1-2.5 ml and a respiratory rate of 140 breaths per min. Deep anesthesia was maintained by adding 2 vol% isoflurane to the ventilation gas. The chest was opened through a midline sternotomy and the left coronary artery (LCA) was ligated for 30 min. SNO formation was induced by injection of 1.67 μmol/kg nitrite (in 50 μl 0.9% sodium chloride) into the left ventricular cavity 5 min before reperfusion. Sodium chloride injection (50 μl) served as control treatment. The wtMIF (10 μg) and the Cys81Ser-MIF mutant (10 μg), respectively, were injected into the Mif<sup>-/-</sup> mice 5 min prior to onset of ischemia. For detection of SNO-MIF and investigation of apoptosis respectively, hearts were excised after 5 min or 4 h of reperfusion and treated as described in the Method section of the online-only Data Supplement. For analysis of
infarct size, mice were sacrificed after 24 h of reperfusion; hearts were excised and perfused with PBS (for TTC staining see Method section of the online-only Data Supplement).

**Supplemental Methodology**

For a detailed explanation of experimental animals and the methods relative to organ harvest for SNO analysis, myocardial area at risk (AAR) and infarct size measurements, determination of myocardial apoptosis, co-immunoprecipitation (Co-IP) of JAB1/CSN5 and Cys81Ser-MIF, identification of S-nitros(yl)ation sites by mass-spectrometry, please see the Method section of the online-only Data Supplement.

**Statistical Analysis**

The results are presented as mean±SD unless stated otherwise. Data were analyzed by one-way ANOVA and post-hoc Bonferroni’s multiple comparison correction (for all pairwise tests) with GraphPad Prism 5 software to compare differences between multiple groups and Student’s unpaired t-test when analyzing two groups. Testing of main effects and two specific interactions was performed using two-way ANOVA with post-hoc Dunn-Šidák multiple comparison. A P-value <0.05 was considered to be statistically significant.

**Results**

**MIF is selectively S-nitros(yl)ated at Cys-81**

Cysteine residues are highly reactive groups and more recently have been identified to be targeted by posttranslational modifications such S-nitros(yl)ation, serving critical regulatory purposes in cellular signaling and redox homeostasis. We investigated whether the MIF Cys residues are accessible to S-nitros(yl)ation. Reduction of nitrite to NO\(^*\) was employed to synthesize SNO-MIF. Nitrite reduction was achieved by the nitrite reductase activity of
deoxyMb.27 SNO formation was measured by gas phase chemiluminescence.31 MIF was reproducibly and robustly modified by NO* and quantification showed a ratio of 1.4±0.4 nmol SNO/nmol wtMIF, indicating that likely only one of the three Cys residues in MIF was modified (Figure 1A). To confirm the specificity of the detected SNO signal, the thiol groups of the MIF Cys were alkylated with N-ethyimaleimide (NEM) prior to S-nitros(yl)ation. This resulted in a complete ablation of MIF S-nitros(yl)ation (Figure 1A). Similarly, omission of nitrite reduction in the reaction also prevented the formation of SNO-MIF. These data provided clear evidence that MIF can be S-nitros(yl)ated in vitro. To identify the specific Cys residue(s) that is/are S-nitros(yl)ated in MIF, SNO-MIF synthesis was performed using the site-specific MIF mutants Cys57Ser-MIF, Cys60Ser-MIF, and Cys81Ser-MIF in which one of the three Cys residue is isostERICALLY substituted by a serine (Ser) residue (Figure 1B).15 Strikingly, SNO-MIF formation was completely abolished in Cys81Ser-MIF, whereas the Cys57Ser and Cys60Ser mutants showed S-nitros(yl)ation ratios that were comparable to that of wtMIF (1.3±0.6 and 0.9±0.4 nmol SNO/nmol MIF) (Figure 1C). These findings clearly demonstrated that MIF is a target for S-nitros(yl)ation in vitro and that this modification selectively targets Cys-81. Figure 1D illustrates the position of Cys-81 in the structure of the MIF monomer and trimer and indicates where the non-modifiable Cys-57 and Cys-60 are located.

Myocardial SNO-MIF formation in vivo

After having demonstrated that MIF can be modified by S-nitros(yl)ation in vitro, we next investigated whether SNO-MIF formation can be detected in I/R in vivo. We used the biotin-switch technique34 to evaluate whether SNO-MIF formation occurs in the reperfused mouse heart under in vivo conditions. WT mice underwent occlusion of the LCA for 30 min and either saline or nitrite was administered 5 min prior to reperfusion (Figure 2A). After 5 min of reperfusion,
hearts were excised, homogenized and subjected either to gas phase chemiluminescence for the
detection of total SNO content (Figure 2B) or to the biotin-switch assay for specific detection of
SNO-MIF in myocardial tissue. This experiment revealed that nitrite treatment led to a dramatic
increase of both total SNO content as well as specific formation of SNO-MIF in the post-
ischemic heart in vivo (Figure 2C). Quantitative analysis showed that SNO-MIF levels increased
~10-fold after myocardial I/R when compared to basal level (Figure 2D). Pharmacological
inhibition of NOS-mediated NO\(^\bullet\) generation prior to I/R did not affect basal SNO-MIF level
(Supplemental Figure 1), indicating that the SNO-MIF levels as observed in the experiment
were not mainly dependent on NOS-generated NO\(^\bullet\). Thus, we have demonstrated for the first
time that SNO-MIF formation occurs in the heart in vivo and that SNO-MIF levels become
markedly elevated by increased concentrations of S-nitros(yl)ation-active groups during
myocardial I/R.

\textbf{S-nitros(yl)ation of MIF is site-specific in vivo}

SNO formation at Cys thiols depends on precise biochemical conditions that an NO\(^\bullet\) group could
encounter such as O\(_2\) concentration, hydrophobicity, nucleophilicity, and the redox environment
surrounding the target thiol – conditions which could dramatically change during I/R in the
myocardium. Beside these ‘ambient’ conditions, protein characteristics itself such as the ultra-
structural accessibility of a given Cys residue can change during I/R and might determine
whether a particular thiol in a given protein is subjected to S-nitros(yl)ation or not.\(^{36}\) Based on
this background, we examined whether the specific S-nitros(yl)ation of Cys-81 in the MIF
sequence which we had observed in vitro could also be detected in the myocardium in vivo
during I/R. To determine the site of MIF S-nitros(yl)ation in vivo, we used an LC-MS/MS
approach.\(^{37}\) Prior to the biotin-switch assay and subsequent MS analysis, mice underwent
transient LCA occlusion followed by a reperfusion (Figure 2A). Following trypsinolysis of the biotinylated proteins, MS sequencing revealed that the MIF peptide LLC\(^{81}\)GLLSDR was biotinylated at Cys-81, whereas no other biotinylated MIF peptide fragments were detected (Figure 3). Since no unmodified Cys-81 peptide was identified, an efficiency of S-nitros(yl)ation of almost 100% after nitrite treatment can be assumed. However, if the unmodified MIF peptide LLC\(^{81}\)GLLSDR behaved different on the LC material this might lead to non-identification of this particular fraction. This finding confirmed our in vitro observations and unanimously demonstrated that MIF is selectively S-nitros(yl)ated at Cys-81 in the reperfused heart in vivo. In fact, this is the first demonstration of a post-translational modification of the MIF protein in vivo, which moreover appears to be linked to critical physiological and pathophysiological conditions.

**S-nitros(yl)ation of MIF modulates MIF activities in vitro and in myocardial I/R injury in vivo**

Within the TPOR family of redox-regulatory proteins, S-nitros(yl)ation of the non-catalytic Cys residues can critically contribute to an alteration of the functions of these proteins, including their CXXC-dependent oxidoreductase activity.\(^{21}\) The TPOR activity of MIF reduces small molecular weight disulfides such as 2-hydroxyethyldisulfide (HED) and this activity is dependent on the presence of Cys-60 and in part on Cys-57.\(^{15}\) Applying the HED transhydrogenase assay\(^{15,17}\) and comparing the HED activity of wtMIF with that of SNO-MIF, we next asked whether the observed SNO modification would affect the catalytic TPOR activity of MIF. SNO-MIF was synthesized by preincubation of wtMIF with S-nitroso-Cys (Cys-NO).\(^{33}\) Cys-NO led to an S-nitros(yl)ation of Cys-81 of 88±19% (\(n=3\)) (Supplemental Figure 2) and had no influence on assay kinetics (Supplemental Figure 3). SNO-MIF catalyzed the formation of HED at a markedly faster rate (Figure 4A) being twice (205±15%) as active as wtMIF.
(100%) (Figure 4B). This indicated that modification of Cys-81 resulted in a conformational change that promoted the redox activity of MIF. Interestingly, this observation was in line with prior findings showing that alkylation of Cys-81 promoted the redox activity of MIF.\textsuperscript{15} Taken together, these results demonstrate that S-nitros(yl)ation of MIF at Cys-81 increases the inherent catalytic oxidoreductase activity of this protein. Since activity as well as protein-protein interactions of the active site of MIF have been linked to reduction of apoptosis,\textsuperscript{1,16} we next asked whether S-nitros(yl)ation also causally affects the activities of MIF \textit{in vivo}. We were particularly interested in the effect that MIF S-nitros(yl)ation may have on the cardioprotective properties of MIF during myocardial I/R. Protection of cardiomyocytes from apoptosis has been identified as an important mechanism related to MIF’s cardioprotective activity. We compared mouse heart apoptotic cell numbers between WT mice and mice genetically deficient in MIF following I/R and treatment with nitrite. Hearts were harvested 4 h after the reperfusion interval was started. After subsequent immunohistochemical preparation of the excised hearts, cardiomyocyte apoptosis was determined and quantified by TUNEL staining. Visual inspection of the TUNEL stains (Figure 4C) already indicated that the lowest number of TUNEL-positive cells was seen in hearts from WT mice treated with nitrite, whereas the highest number of TUNEL-positive cells was observed in $Mif^{-/-}$ cells that did not receive nitrite. Quantification of the TUNEL analysis in relation to the AAR confirmed this notion and showed that SNO formation in WT mice led to a markedly decreased number of apoptotic nuclei (2.1±1.0%) as compared to control treatment of these mice (8.0±3.5%; Figure 4D). These observations were in line with prior findings from others.\textsuperscript{38} I/R in $Mif^{-/-}$ mice which did not receive nitrite treatment resulted in a markedly increased myocardial apoptosis rate compared to the control-treated WT mice (14.5±1.0% vs. 8.0±3.5%, $P<0.05$; Figure 4D), confirming that MIF exhibits anti-apoptotic
functions in the reperfused heart. Intriguingly, SNO-mediated inhibition of apoptosis after I/R was attenuated in Mif−/− mice (11.3±4.3%; Figure 4D). Of note, the relative reduction in apoptosis after SNO formation was 75% in WT mice (8.0±3.5% vs. 2.1±1.0%) whereas the attenuation of apoptotic nuclei was only 22% in Mif−/− mice (14.5±1.0% vs. 11.3±4.3%; Figure 4D). The anti-apoptotic effect of MIF in the reperfused heart has previously been suggested to be mediated through an inhibition of the JNK pathway.14 We had observed that S-nitrosylation of MIF at Cys-81 led to an alteration of the Cys-60-dependent TPOR activity of MIF. Because Cys-60 is critically involved in the interface between MIF and JAB1 and because MIF/JAB1 interaction blocks JNK activity,16 we investigated whether MIF/JAB1 binding activity could be affected by Cys-81. Co-IP analysis revealed that the Cys81Ser substitution markedly decreased the binding between MIF and JAB1 (100% vs. 31±25%; P<0.001) (Supplemental Figure 4). Importantly, modification of Cys-81 by S-nitrosylation in vivo also led to a marked decrease in the interaction between MIF and JAB1 as revealed by Co-IP following LCA occlusion/reperfusion and nitrite-mediated SNO formation in vivo. These in vitro and in vivo results suggest that S-nitrosylation increases the cellular availability of MIF in the early phase of reperfusion by decreased JAB1 trapping39 and thus mediates increased cardioprotection (Supplemental Figure 5).

**S-nitrosylation at Cys-81 regulates the cardioprotective properties of MIF in vivo**

We have demonstrated that MIF is selectively S-nitrosylated at Cys-81 and that this post-translational modification regulates the anti-apoptotic activity of MIF during I/R.

To elucidate whether the selective SNO modification of MIF is central to MIF-mediated cardioprotection in myocardial I/R, we compared infarct size in Mif−/− mice following myocardial I/R upon wtMIF application and SNO generation (Figure 5A). Injection of wtMIF
into the left ventricular cavity of $Mif^{-/-}$ hearts reduced infarct size (35±5% vs. 30±2%, $P<0.05$; Figure 5B) to the level of WT mice, which naturally show smaller infarct size compared to $Mif^{-/-}$ (Supplemental Figure 6). Application of wtMIF undergoing SNO formation further reduced infarct size to 8±2% in $Mif^{-/-}$ mice. Infarct size reduction in $Mif^{-/-}$ hearts undergoing SNO formation without wtMIF reconstitution was less pronounced (17±3%; Figure 5B). Since SNO-MIF formation was found to selectively represent S-nitrosylation at Cys-81, we applied the Cys81Ser-MIF mutant control instead of wtMIF. Determination of the resulting infarct sizes revealed that SNO-mediated cardioprotection was significantly less pronounced when Cys81Ser-MIF was injected instead of wtMIF (16±4% vs. 8±2%, $P<0.05$; Figure 5B). Injection of the Cys81Ser-MIF mutant without nitrite led to an infarct size of 33±3% (Figure 5B).

In summary, these results demonstrate that MIF is selectively S-nitrosylated at Cys-81 and that this previously unrecognized posttranslational modification regulates MIF function and increases the cardioprotective properties of MIF in vivo.

Discussion

During the early phase of myocardial reperfusion, S-nitrosylation modulates the activity of various proteins involved in apoptosis and oxidative stress. Myocardial MIF functions as an endogenous protection mechanism against lethal I/R injury. It appears that cardioprotection by MIF is especially relevant within the first minutes of reperfusion. However, if this critical cardioprotective activity of MIF may be modulated or “switched on”, by post-translational modifications has not been addressed. Here, we have identified S-nitrosylation as the first described post-translational modification of MIF and provide evidence that S-nitrosylation of MIF is critical for its cytoprotective properties in the reperfused myocardium in vivo.
Protein Cys residues are intrinsically nucleophilic and reactive under physiological pH conditions. Their reactivity renders them amenable to engage in diverse biochemical functions such as disulfide formation, protein-protein interaction, redox catalysis, or redox signaling.\textsuperscript{40} The MIF sequence contains three Cys residues, which have been found to contribute to several of MIF’s functional properties. Cys-57 and Cys-60 form a CXXC motif that has catalytic TPOR activity \textit{in vitro} and is a distinct hallmark of the TPOR protein family. Best characterized is the role of Cys-60, which has been shown to contribute to MIF functions such as inhibition of apoptosis, JAB1 and NM23-H1 binding.\textsuperscript{1,16,41,42} The role of the third, non-CXXC Cys residue in MIF, Cys-81, has hardly been studied, with Cys-81 suggested to contribute to the interaction between MIF and p53.\textsuperscript{43} However, Cys-81 is critically situated in an \( \alpha \)-helix of the MIF structure, where it contributes to the hydrophilic surface area.\textsuperscript{17} Yet, Cys-81 is surrounded by mostly hydrophobic amino acid residues. Consequently, substitution or modification of Cys-81 may alter hydrophobic interactions in this region and substitution of Cys-81 has been suggested to mediate conformational effects.\textsuperscript{17} MIF binding of JAB1 inhibits JNK activation and subsequent c-Jun phosphorylation. Furthermore, MIF blocks JNK activation and cellular apoptosis elicited by thiol starvation.\textsuperscript{1} Importantly, Qi \textit{et al.} demonstrated that MIF-mediated inhibition of the JNK pathway during myocardial reperfusion decreased cardiomyocyte apoptosis in the reperfused myocardium, representing a major axis of the cardioprotective MIF pathway.\textsuperscript{14} Our present findings demonstrate that, although Cys-60 of MIF is part of the binding interface between MIF and JAB1, the MIF/JAB1 interaction critically depends on Cys-81, as deletion of this amino acid significantly attenuated JAB1 binding. An involvement of Cys-81 in the protein-protein interaction between MIF and JAB1 was surprising at first sight because this residue is remotely
situated from the binding interface encompassing residues 50-65. However, modification of
distant regulatory Cys residues by S-nitros(yl)ation has been described to be essential previously.
For example, S-nitros(yl)ation of non-active site Cys is known to regulate the function of
proteins like TRX or G-protein-coupled receptor kinase \(^{21,44}\) and at current, numerous
potentially functional Cys residues in the mammalian proteome have remained unidentified.\(^{40}\) In
fact, MIF can be \(S\)-nitros(yl)ated as demonstrated herein and Cys-81 clearly turned out to be the
only target site for this post-translational modification. Thus, it was of high interest, if this
residue might be a novel regulatory locus which upon modification may affect MIF’s functions
under redox stress.

As discussed above, MIF is a regulator of the cellular redox state and potently inhibits
apoptosis during myocardial I/R.\(^{11,12,14}\) Interestingly, Koga et al., in a mouse model \textit{in vivo},
established a direct link between MIF-mediated ROS quenching and protection of the heart from
I/R injury.\(^{11}\) This effect was attributed to the TPOR activity of MIF’s CXXC motif. We
investigated whether the TPOR activity of MIF would be affected by the Cys-81-dependent \(S\)-
nitros(yl)ation of MIF. Notably, reduction of the small molecule disulfide HED by MIF was
significantly enhanced after \(S\)-nitros(yl)ation.

It was of great interest to ask, whether the \(S\)NO formation at Cys-81 observed \textit{in vitro}
was also detectable under \textit{in vivo} conditions during myocardial I/R. Nitrite reduction is an
important source of \(NO^*\) during I/R that enables \(S\)-nitros(yl)ation and thus attenuates I/R
injury.\(^{28,45,46}\) Accordingly, \(S\)NO levels increased after nitrite application during I/R in our \textit{in vivo}
model. Importantly, we were able to detect \(S\)NO-MIF in the reperfused heart applying the biotin-
switch technique, and demonstrated the specificity of \(S\)NO-formation at Cys-81 by LC-MS/MS
analysis.
$S$-nitros(yl)ation has been shown to regulate the function of several cardioprotective proteins such as TRX, G-protein-coupled receptor kinase 2, or caspase 3. Here we have identified MIF as an important novel target for $S$-nitros(yl)ation during I/R in vivo. MIF is a protein mediator which both acts as an inflammatory cytokine/chemokine, but also exhibits cardioprotective effects due to its intracrine/paracrine activity. This raised the question if the post-translational $S$-nitros(yl)ation of MIF impacted on its cardioprotective properties. We found significantly increased rates of cardiomyocyte apoptosis in mice that lacked SNO-MIF and that had been subjected to I/R in vivo. Since these results were based on the genetic deletion of MIF, we investigated whether replenishment of SNO-MIF in the $\text{Mif}^{-/-}$ was able to reverse the observed phenotype. In fact, administration of wtMIF, which also led to a reinstallation of SNO-MIF levels, led to a marked reduction in infarct sizes in these mice, confirming the importance of MIF and demonstrating the impact of SNO-MIF in reperfusion injury of the myocardium. Thus, these data extended previous findings of Young et al. who could show that application of wtMIF into $\text{Mif}^{-/-}$ hearts reconstitutes WT conditions ex vivo. Of note, the Cys81Ser-MIF mutant failed to reinstall the cardioprotective properties of MIF in $\text{Mif}^{-/-}$ mouse hearts, underscoring the role of SNO-MIF and the specific function of S-nitros(yl)ation at Cys-81 in cardioprotection.

In summary, we have identified a novel post-translational modification of the MIF protein. We could demonstrate that key functions of MIF are regulated by its $S$-nitros(yl)ation with fundamental effects on myocardial I/R injury in vivo. $S$-nitros(yl)ation thus might provide a new therapeutic strategy for regulating MIF’s functions during the early phase of reperfusion.

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Conflict of Interest Disclosures: None

References:


**Figure Legends:**

**Figure 1.** Identification of Cys-81 as selective S-nitrosylation site of MIF. SNO-formation was measured and quantified using reductive gas-phase-chemiluminescence. (A) S-nitrosylation of wtMIF yielded a formation of 1.4±0.4 nmol SNO-MIF/nmol wtMIF (black bar). Omitting nitrite reductase or blocking free thiol (SH) groups with NEM abolished SNO-MIF formation. (B) Schematic illustration of the mutation site within the mutants Cys57Ser, Cys60Ser and Cys81Ser of human MIF in comparison to wtMIF illustrating potential S-nitrosylation sites. (C) S-nitrosylation of wtMIF, Cys57Ser-MIF and Cys60Ser-MIF yielded equal amounts of SNO-MIF formation (black and grey bars). S-nitrosylation of Cys81Ser-MIF yielded no SNO signal (red line); (black and grey bars, n=5, *P*=NS). (D) Three dimensional structure of the MIF monomer and trimer respectively, illustrating the localization of the target Cys-81 (red) and the active-site Cys-57 and Cys-60 (blue).
Figure 2. MIF is S-nitrosoylated in the reperfused heart in vivo. (A) Experimental protocol. Wild-type mice underwent 30 min of LCA occlusion followed by 5 min reperfusion in vivo. 5 min prior to reperfusion, SNO formation was induced by nitrite application (1.67 μmol/kg) and SNO-MIF formation was detected by the biotin-switch technique. (B) Enhanced myocardial SNO content after nitrite application was confirmed by gas-phase-chemiluminescence (mean±SD, n=4-5, ***P<0.001). (C) Original Western blot after biotin-switch assay revealed increased band density after SNO-formation, demonstrating dramatically increased SNO-MIF content in the reperfused heart. Band intensities were normalized to a control band of recombinant MIF (rMIF). (D) Quantitative analysis revealed that amounts of SNO-MIF are significantly increased after nitrite treatment (mean±SD, n=4, **P<0.02).

Figure 3. Characterization of SNO binding site in vivo. LC-MS/MS spectrum of the biotin-HPDP-labeled MIF peptide LLC81GLLSDR; targeted precursor mass 709.372 m/z (2+), corresponding [M+H]Re+ mass 1417.737 Da. Peptide breakage at the amide bound occurring after collision induced dissociation (CID) are labeled in the spectrum in red for b ions, representing N-terminus-containing fragments of a given peptide, and in blue for y ions, representing C-terminus-containing fragments.

Figure 4. S-nitros(yl)ation increases activity and affects anti-apoptotic properties of MIF. (A) Linearized oxidoreductase activity kinetic. Activity is expressed as a decrease of absorbance over time. SNO-MIF catalyzed reduction of HED in the HED-transhydrogenase assay (2.48±0.52 nkat) was markedly increased compared to wtMIF (1.28±0.28 nkat) (mean±SEM; n=3, **P<0.02). (B) Repetitive experiments revealed that S-nitros(yl)ation significantly increased the
activity of SNO-MIF (205±15%) compared to wtMIF (mean±SD, n=3, **P<0.02). (C) TUNEL staining of heart sections from WT and Mif<sup>−/−</sup> mice after 30 min myocardial ischemia and 4 h reperfusion in vivo. Exemplary pictures show increased apoptotic cells in Mif<sup>−/−</sup> compared to WT. SNO formation in WT hearts decreased apoptosis markedly what was less pronounced in SNO-MIF deficient hearts. (D) Quantitative analysis of TUNEL stained heart sections, displayed as apoptotic cells per AAR. Bars show significantly decreased apoptosis level after SNO formation in WT mice (black bars). Lack of SNO-MIF markedly attenuates SNO mediated cardioprotection in Mif<sup>−/−</sup> mice (grey bars) (mean±SD, n=4, *P<0.05).

**Figure 5.** S-Nitros(yl)ation at Cys-81 increases cardioprotective properties of MIF after myocardial I/R injury in vivo. (A) Experimental protocol. Mif<sup>−/−</sup> mice underwent transient regional ischemia followed by reperfusion and subsequent analysis of infarct size per area at risk (AAR). Recombinant mouse MIF (wtMIF) or the non-S-nitros(yl)able Cys81Ser-MIF mutant was applied in Mif<sup>−/−</sup> mice. Nitrite was applied to mediate S-nitros(yl)ation. (B) Reconstitution of wtMIF in Mif<sup>−/−</sup> mice decreased infarct size (35±5% to 30±2%, *P<0.05). Nitrite reduced infarct size to 17±3%. Reconstitution of wtMIF following SNO formation by nitrite reduced infarct size in Mif<sup>−/−</sup> mice to 8±2%. This cytoprotective effect was less pronounced upon application of the non-S-nitros(yl)able Cys81Ser-MIF mutant in conjunction with nitrite (35±5% to 16±4%) or the Cys81Ser-MIF mutant solely (35±5% to 33±3%). The cytoprotective effect mediated by SNO-MIF in comparison to the non-S-nitros(yl)able Cys81Ser-MIF is displayed black shaded (*P<0.05) (mean±SD, n=5-9, *P<0.05, ***P<0.001).
SNO-MIF (nmol SNO/nmol MIF)

Nitrite
Nitrite reductase
NEM

<table>
<thead>
<tr>
<th>MIF</th>
<th>wt</th>
<th>Cys57Ser</th>
<th>Cys60Ser</th>
<th>Cys81Ser</th>
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<tbody>
<tr>
<td>SNO-MIF</td>
<td></td>
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<tr>
<td>(nmol SNO/nmol MIF)</td>
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</tr>
</tbody>
</table>
A

Normalized change of absorbance (340nm) over time (min)

- wtMIF
- SNO-MIF

B

Bar graph showing percentage of wtMIF:

- wtMIF
- SNO-MIF

C

Images of WT and Mif−/− cells:

- DAPI
- TUNEL
- Merge

CTRL, nitrite, CTRL, nitrite

D

Bar graph showing percentage of apoptotic cells/AAR (%):

- CTRL
- nitrite
- CTRL
- nitrite

WT, Mif−/−
A murine in vivo myocardial I/R protocol

LCA-occlusion regional ischemia

30 min

24 hours

5 min
CTRL or rMIF (10 µg) or Cys81Ser MIF (10 µg)

5 min
CTRL or Nitrite (48 nmol)

B

Infarct per AAR (%)

Nitrite

rMIF

Cys81Ser MIF

- - - + + + + -

- + - - + - - -

- - - - + + + +
Cardioprotection Through S-Nitros(yl)ation of Macrophage Migration Inhibitory Factor
Peter Lüdiike, Ulrike B. Hendgen-Cotta, Julia Sobierajski, Matthias Totzeck, Marcel Reeh, Manfred Dewor, Hongqi Lue, Christoph Krisp, Dirk Wolters, Malte Kelm, Jürgen Bernhagen and Tienush Rassaf

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SUPPLEMENTAL MATERIAL

Cardioprotection through S-nitros(yl)ation of Macrophage Migration Inhibitory Factor

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Supplemental Methods

Experimental Animals

MIF deficient (Mif⁻) mice were on a C57BL/6 background and were bred at RWTH Aachen University Hospital. The Mif gene knock out has been described before¹ and the mice had been backcrossed in the C57BL/6 background 10 times. Male C57BL/6 wild-type (WT) mice, which served as controls, and Mif⁻ mice were at 14±2 weeks of age with an average body weight of 26±4 g at the time of the experiments. Mice were kept on a standard rodent chow before experimental use. All experiments were approved by the local ethics committee and all investigations were performed according to the „European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe Treaty Series No. 123).

Organ harvest for SNO analysis

Excision of hearts was performed for analysis of infarct size via TTC staining, determination of myocardial apoptosis or SNO analysis with the biotin-switch assay. Hearts designated for SNO analysis underwent adjusted perfusion. After thoracotomy, a catheter was inserted into
the infrarenal part of the abdominal aorta, and organs were flushed free of blood by retrograde in situ perfusion with ice-cold PBS supplemented with NEM/EDTA (10/2.5 mM) at a rate of 10 ml/min. The superior vena cava was cut directly after the aortic cannulation to provide an exit port for blood and buffer. The addition of NEM/EDTA ensured SH group blocking in situ and inhibited transition metal-catalyzed trans-nitrosation reactions. After 2 min of perfusion, the heart was harvested and immediately snap-frozen in liquid nitrogen and stored at −80°C until SNO analysis.

Co-immunoprecipitation (Co-IP) of JAB1/CSN5 and Cys81Ser-MIF

MIF-deficient mouse embryonic fibroblasts (MEFs) were lysed with cold Co-IP buffer (50 mM Tris-HCl, 150 mM NaCl, 15 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 1 mM sodium azide, 10% glycerol, 0.5 mM sodium vanadate, 1 mM dithiothreitol, 25 mM sodium fluoride, 1% proteinase inhibitor cocktail). After centrifugation, the cell lysates were incubated with wtMIF or Cys81Ser-MIF (1 µg/10 µl) on a tube rotator for 1 h at 4°C. Then, 2 µg of mouse monoclonal anti-JAB1 (2A10.8, Gene Tex/Biozol, Eching, Germany), were added to the mixtures and incubated for 30 min at 4°C. The JAB1/MIF complex was pulled-down by incubation with protein G immobilized on magnet dynabeads (Invitrogen, Darmstadt, Germany) by rotating at 4°C for another 30 min. Proteins were eluted from the beads by heating at 95°C for 5 min in 1x SDS loading buffer and analyzed by SDS-PAGE. Western blots were developed with rabbit anti-JAB1 (FL334, Santa Cruz Biotechnology, Heidelberg, Germany) or with anti-MIF antiserum (Ka565, rabbit).

Pharmacological inhibition of NOS

Inhibition of NOS-mediated NO* generation was achieved by repetitive intraperitoneal (i.p.) administration of the NOS inhibitor N5-(1-iminoethyl)-L-ornithine, dihydrochloride (L-NIO) every 45 min as described previously. WT mice received 5 injections of 100 mg/kg L-NIO
before I/R injury was performed. Repetitive applications of sodium chloride served as control treatment.

Quantification of SNO-MIF formation after CysNO incubation

SNO-MIF formation was achieved by incubating 19.2 µM wtMIF with 250 µM CysNO for 30 min at 37°C in the dark and SNO measurement was performed via chemiluminescence (CLD). The amount of incubated MIF was 8.64 nmol (corresponding to ~25.92 nmol of cysteines, since the MIF structure has 3 cysteines per MIF molecule) and that of CysNO was ~112.5 nmol. After the incubation, SNO-MIF was purified over a 3,000 kDa cut-off filter (Millipore®, Amicon Ultra-0.5 mL Centrifugal Filters) and purified SNO-MIF was analyzed.

Myocardial AAR and infarct size measurements

For analysis of infarct size, hearts were excised and perfused with PBS over 5 min. After perfusion, the LCA was re-ligated in the same location as before. Evans blue dye (1 ml of a 1% solution) was injected into the aorta and coronary arteries for delineation of the ischemic AAR from the non-ischemic zone. The tissue was wrapped in a clear food wrap and stored for one hour in a –20°C freezer. The heart was then serially sectioned perpendicularly to the long axis in 1 mm slices, and each slice was weighed. The sections were incubated in 1% TTC for 5 min at 37°C for demarcation of the viable and non-viable myocardium within the risk zone. Infarction, AAR, and non-ischemic left ventricle were assessed with computer-assisted planimetry by an observer blinded to sample identity. The size of the myocardial infarction was expressed as a percentage of the AAR.

Determination of myocardial apoptosis

Four hours after reperfusion which followed 30 min of ischemia, in situ-perfused hearts were excised and fixed with 4% (w/v) paraformaldehyde. Wax-embedded sections were examined by TUNEL analysis. To standardize the analysis, all consecutive transverse 5 µm slices were
cut from the same region, beginning 1 mm above the cardiac apex. The “In situ Cell Death Detection Kit” (Roche) was used according to the manufacturer’s instructions. Briefly, tissue sections were deparaffinized and permeabilized with 20 µg/ml proteinase K (Roche) at 37°C for 30 min. After rinsing with PBS, the tissue was incubated with enzyme solution (TdT, terminal deoxynucleotidyl transferase) and labeled nucleotide mix for 60 min at 37°C in a dark humid chamber. Negative controls lacked TdT treatment; positive controls were produced by incubation with 10 U/ml DNAse (Roche). Ten sections from each myocardial sample were selected and at least 5 fields per section were randomly evaluated. Microscope image acquisition: Nikon ECLIPSE TE2000-E inverted research microscope, magnification 40x, temperature –30.1°C, Cool Snap HQ2 camera, exposure time 500 ms. TUNEL-positive cardiomyocytes in the ischemic myocardium were evaluated under blinded conditions. The ratio of TUNEL-positive cardiomyocytes to the total number of cardiomyocytes was calculated.

**Mass spectrometry: LC-MS/MS**

Homogenized HDPD-biotin-switch assay-treated mouse heart was resuspended in a 30% methanol solution. Trypsin digestion overnight, followed by a 2 h chymotrypsin digestion, both in a 100 to 1 protein/enzyme ratio, was performed to obtain peptides of interest. Peptides were loaded offline onto a 33 cm multidimensional protein identification technology fused silica column (inner diameter = 100 µm). The column consists of 25 cm Luna C18 reversed phase material (Phenomenex, Germany) followed by 5 cm strong cation exchange material (Phenomenex, Germany) and again 3 cm Luna C18 reversed phase. The column was placed in a column heating block with an internal temperature of 45°C. Peptides were eluted with an Accela HPLC (Thermo Fisher Scientific, San Jose; California), which was connected to the Thermo Fisher LTQ Orbitrap Velos mass spectrometer. The effective flow rate was 130 nl/min. Peptides on the multidimensional protein identification technology fused silica column were eluted using an eight-step acetonitrile gradient program containing salt.
steps of 10, 20, 40, 60, 80 and 100% of 250 mM and 100% 1.5 mM ammonium acetate. A parent mass list was implemented in the X-calibur method file which contains masses of singly, doubly and triply charged modified and unmodified Cys-containing MIF peptides in the mass range of 400–2000 m/z. MS/MS analyses were predominantly triggered on the parent masses, but also on the top 20 most intense signals in the survey scan, when the parent masses were not detected. Acquired MS/MS spectra were interpreted with the SEQUEST algorithm implemented in the Thermo Fisher Scientific Proteome Discoverer software. The spectra were searched against the mouse (Mus musculus) Swiss-Prot database release 15.6/57.6. All accepted mouse peptides had a false discovery rate of less than 0.01 using a reversed database search and a precursor mass accuracy of 10 ppm. For fragment ions, a tolerance of 1 amu was used. Biotinylation and thiomethylation of Cys residues and oxidation of methionine were set as possible modifications.

**Determination of MIF/JAB1 interaction *in vivo***

WT mice underwent 30 min of LCA occlusion followed by 5 min reperfusion *in vivo*. 5 min prior to reperfusion, SNO formation was induced by nitrite application (1.67 µmol/kg) and MIF/JAB1 interaction was determined by Co-IP of whole heart homogenates during the early phase of reperfusion.
Supplemental Figure 3

Normalized change of absorbance (340nm)

- Buffer
- Buffer + CysNO

Time (min)

0  5  10  15
Supplemental Figure 4

A

JAB1  
MIF  

40 kD  
12 kD  

wtMIF  
Cys81Ser  

B

Relative density (%)  

wtMIF  
Cys81Ser

***
Supplemental Figure 5

A
Murine in vivo myocardial I/R protocol
LCA-occlusion regional ischemia  reperfusion
30 min 5 min 5 min
CTRL or Nitrite (48 nmol)

B

JAB1
MIF

Control
Nitrite

C

Relative density (%)

Control Nitrite

40 kD 12 kD

*
Supplemental Figure 6

A

Murine in vivo myocardial I/R protocol

LCA-oclusion regional ischemia

30 min

reperfusion

24 hours

5 min
CTRL

wtMIF (10 μg)

or

B

Infarct per AAR (%)

WT

Mif^{-/-}

Mif^{-/-} + rMIF

rMIF

- - +
Figure Legends

Supplemental Figure 1. NO synthase (NOS) has no influence on S-nitros(yl)ation of MIF after myocardial I/R in vivo. (A) Wild-type mice underwent transient regional ischemia/reperfusion (I/R) injury and NOS inhibition was achieved by repetitive intraperitoneal (i.p.) application of N5-(1-iminoethyl)-L-ornithine, dihydrochloride (L-NIO) (n=4). The control group received equal volumes of sodium chloride (NaCl) (n=4). Hearts were harvested after 5 min of reperfusion and the biotin-switch assay was performed for analysis of SNO-MIF. (B) Exemplary original Western blot of two of the analyses after biotin-switch assay shows equal band intensity after NOS inhibition and control treatment, revealing no difference of SNO-MIF heart content after I/R injury.

Supplemental Figure 2. Incubation of MIF with CysNO leads to formation of SNO-MIF. Left y-axis shows total amount of Cys-81 residues in the wtMIF solution (nmol Cys81/nmol MIF). Right y-axis shows the total proportion of S-nitros(yl)ated Cys81 residues per incubated MIF (nmol SNO/nmol MIF). Comparison of total available Cys-81 residues (left column) and SNO-MIF (right column) shows that incubation of 19.2 µM MIF with 250 µM Cys-NO leads to an S-nitros(yl)ation of Cys-81 of 88±19% (n=3).

Supplemental Figure 3. Cys-NO has no influence on HED assay kinetic. Influence of buffer composition on basal HED assay kinetic. The NO donor Cys-NO showed no influence on basal assay kinetic compared to the plain assay buffer.

Supplemental Figure 4. Cys81 is crucial for the interaction between MIF and JAB1/CSN5. Mif−/− mouse embryonic fibroblast lysates were incubated with wtMIF or Cys81Ser-MIF. Subsequently, JAB1 pull-down and subsequent anti-MIF or anti-JAB1
Western blot analysis were performed. (A) CoIP and subsequent Western blotting revealed a markedly decreased amount of co-precipitated Cys81Ser-MIF. (B) Quantitative analysis showed that the interaction between Cys81Ser-MIF and JAB1 (31±25%) is significantly impaired compared to wtMIF (set as 100%) (mean±SD, n=6, *P<0.0001).

**Supplemental Figure 5. S-nitros(yl)ation leads to increased cellular availability of MIF through decreased binding to JAB1 in the early phase of I/R injury in vivo.** (A) Experimental protocol. Wild-type mice underwent 30 min of LCA occlusion followed by 5 min reperfusion in vivo. 5 min prior to reperfusion, SNO formation was induced by nitrite application (1.67 µmol/kg) and MIF/JAB1 interaction was determined by Co-IP of whole heart homogenates. (B+C) Bar graph diagram quantified results from original Western blots after Co-IP of MIF/JAB1 showing a reduced MIF/JAB1 interaction after application of nitrite, resulting in an increased cellular availability of cardioprotective MIF protein. (n=3-4, *P<0.05, Student’s unpaired t-test).

**Supplemental Figure 6. Application of rMIF reconstitutes wildtype conditions in Mif−/− mice.** (A) Experimental protocol. Wild-type (WT) and MIF deficient mice (Mif−/−) underwent in vivo I/R. Infarct size was determined using TTC staining. In some experiments, wtMIF was applied in Mif−/− mice to reconstitute WT conditions. (B) Mif−/− mice showed a significantly greater infarct size compared to WT mice (35±5% vs. 30±2%). Of note, the size of the infarct seen in the Mif−/− mice reconstituted with wtMIF injected into the left ventricular cavity 5 min prior to ischemia was similar to that observed in WT mice (30±2% vs. 30±2%) what is illustrated by exemplary original TTC stained heart sections (C). (mean±SD, n=5-8, *P<0.05).
Supplemental References


대식세포의 Migration Inhibitory Factor를 S-Nitros(yl)ation 함으로써 심장 보호효과가 나타난다

한 기 혼 교수 서울아산병원 심장내과

Summary

배경
대식세포의 migration inhibitory factor(MIF)는 구조적으로 특이한 염증성 사이토카인(cytokine)으로 세포내 또는 세포외 과정을 거쳐 인간의 생리적 또는 병적 반응에 나타나는 세포신호를 제어한다. 대식세포의 MIF는 병적인 그리고 질병에 대항하는 효과들을 모두 중재한다고 알려져 있으나, 이러한 다양한 기능을 설명하는 기전은 잘 이해되지 않고 있다.

방법 및 결과
본 연구에서는 S-nitrosylation을 통한 MIF의 변형이 심근의 재관류 손상에서 예방 효과를 보이는 MIF의 성질을 매개함을 규명하고자 한다. 대식세포 MIF의 구조에는 cysteine(Cys)이 포함되어 있는바 이들에 대한 site-directed mutation을 통하여 Cys-81이 S-nitrosyl)ation을 일으키는 반면, CXXC-derived Cys residues는 영향이 없었다. Cys-81의 선택적인 S-nitrosothiol 형성으로 oxidoreductase 활성이 배가 되었음을 나타내었다. 이러한 S-nitrosothiol-MIF의 형성은 실험적인 또는 생체실험 모두에서 증명되었는데, 이들은 재관류된 심장에서 심근세포의 세포자멸사를 감소시키는 효과를 유도하였다. 이러한 현상은 S-nitrosothiol-MIF와 상관성이 있었으며, Cys81 serine(Ser)–MIF 돌연변이에서는 관찰되지 않았다.

결론
MIF의 S-nitrosylation은 전기적인 새로운 활성 조절기전이며, 이의 기능이 함정되었을 때 심근 재관류 손상에서 세포 보호효과를 야기하는 활성이 유도된다.
Commentary

MIF의 구조는?
크기가 12.5kDa인 MIF는 trimer로 존재하고 있으며, 하나의 subunit에는 두 개의 antiparallel alpha helix를 가지고 있다. 이들은 중앙의 channel을 중심으로 빙 둘러싼 모습을 하고 있다. MIF에는 크게 두 부분의 catalytic activity를 가지고 있는 효소 기능부위가 있다. 하나는 2-carboxy-2,3-dihydroindole-5,6-quinone(dopachrome)을 5,6-dihydroxyindole-2-carboxylic acid(DHICA)로 전환시키는 N-terminal 근처의 phenylpyruvate tautomerase 부위이며, 하나는 residue 57-60 부위의 Cys이 풍부한(Cys-Ala-Leu-Cys) disulfide reductase의 기능을 이루는 부위이다. C-terminal 부위는 직접적으로 효소로서의 작용은 없으나, 단백질의 안정을 유지하며 catalysis를 조절하는 기능이 있는 것으로 추정된다.

MIF의 기능은?
복잡한 구조에 걸맞게 매우 다양한 기능들이 보고되고 있다. 1966년에 T cell과 관련된 사이토카인으로 보고된 이후 endothelial cell, eosinophil, epithelial cell, lymphocyte, macrophage, neutrophil 등에서 발현하며 이들이 관여하는 매우 넓은 영역의 염증성 질환에 관여하는 것으로 기술되고 있다.

1. 효소로서의 작용
원래 MIF는 구조적으로 bacterial isomerase의 구조와 유사성을 가지고 있다. 따라서 상기 기술한 바와 같이 다양한 효소로서의 작용을 보인다고 할 수 있다. 최근에는 thio-protein oxidoreductase로서의 역할을 하여 세포의 산화스트레스를 제어하는 것으로 알려져 있으며, 특히 neuron 등의 catecholamine 등에 의한 독성을 방지하는 것으로 추정된다. 이는 57-60 염기서열의 Cys-Ala-Leu-Cys가 주로 일으키는 것으로 보고된다.

2. 염증성 인자로서의 작용
전통적으로 chemokine은 최초 두 개의 Cys 위치에 따라 CC, CXC, CX3C 등의 다양한 그룹으로 분류되어 그 성질이 기술되어 왔다. MIF는 이러한 구조적인 특징이 없이도 chemokine receptor를 활성화시키며, 그 폭이 매우 넓은 것이 특징이라 할 수 있다. 그러하여 이를 noncanonical 또는 micro-chemokine이라고 명명하기도 한다. 즉, CXCR2, CXCR4 등과 같은 수용체들을 활성화하여 monocytes/macrophages의 recruitment, 혈관내피 세포, 평활근세포의 활성을 변화시키며, 세포사멸사를 예방한다.

또한, MIF는 CD74와 높은 친화도로 접합하게 된다. CD74는 cytoplasmic tail이 매우 짧아 그 자체가 세포 신호를 일으키지는 못하지만, CD44와 complex를 이루며 PLA2, COX-2 등의 활성화를 Erk-1/2의 인산화를 경유하여 유도한다. 최근에는 Cys-60가 c-Jun N-terminal activation domain binding protein-1(JAB1/CSN5)과 상호작용을 하는 것으로 보고되고 있기도 하다.

본 논문을 통한 MIF의 기능적 의미
기존의 보고에서는 Cys-81이 conformational change에 기여하는 것으로 알려져 있어, 주로 MIF의 구조적인 유지에 역할을 하는 것으로 알려져 있었다. 본 논문에서는 figure1에서 보듯이 57, 60, 81의 Cys을 차례로 제거시켜 그 기능을 평가하는 논리적인 접근을 이루어졌다. S-nitrosylation이란, NO*(nitric oxide) moiety가 reactive Cys thiol과 coupling을 이루어 SNO(s-nitrosothiol)을
형성하는 과정으로 모든 염기단백에서 발생할 수 있다. 
Figure 1에서 보듯이 이러한 과정이 Cys-81의 존재하에서 발생하는 것이 증명되었으며, figure 2에서 보듯이 심근 재관류 시 크게 항진됨을 확인할 수 있다. 이후의 실서 발생하는 것이 증명되었으며, figure 2에서 보듯이 심근 보호효과가 MIF를 제거한 상태에서 일어나지 않는 것으로 증명되어, Cys-81의 존재하에 형성하는 과정으로 모든 염기단백에서 발생할 수 있다.
본 논문은 그동안 연구가 드물었던 Cys-81의 심근 보호효과를 증명하고, 그 기전을 규명하는 데 높은 가치가 존재한다. S-nitroso(yl)ation에 의한 심근 보호효과는 TRX, G protein–coupled receptor kinase 2, 또는 caspase 3 등에 보고된 바 있으며, 본 연구로 MIF의 S-nitroso(yl)ation에 의한 심근 보호효과가 추가로 증명되었다.

Figure 1. Identification of Cys–81 as selective S-nitroso(yl)ation site of MIF. S-nitrosothiol formation was measured and quantified using reductive gas-phase chemiluminescence.

A, S-nitrosylation of wtMIF yielded a formation of 1.4±0.4 nmol SNO-MIF/nmol wtMIF (black bar). Omitting nitrite reductase or blocking free thiol (SH) groups with NEM abolished SNO-MIF formation. B, Schematic illustration of the mutation site within the mutants Cys57Ser, Cys60Ser, and Cys81Ser of human MIF in comparison to wtMIF illustrating potential S-nitrosylation sites. C, S-nitrosylation of wtMIF, Cys57Ser-MIF, and Cys60Ser-MIF yielded equal amounts of SNO-MIF formation (black and gray bars). S-nitrosylation of Cys81Ser-MIF yielded no SNO signal (red line) (black and gray bars, n=5, P<NS). D, Threedimensional structure of the MIF monomer and trimer respectively, illustrating the localization of the target Cys-81 (red) and the active site Cys-57 and Cys-60 (blue).

SNO, S-nitrosothiol; MIF, macrophage migration inhibitory factor; SH, free thiol; wt, wild type.
Enhanced myocardial cellular apoptosis was induced by nitrite application (1.67 μmol/kg), and SNO-MIF formation was detected by the biotin-switch technique. Band intensities were normalized to a control band of recombinant MIF. We compared mouse heart apoptotic cell death with that of WT mice (11.3 ± 1.0%) versus 8.0 ± 1.0%) whereas the attenuation of apoptotic nuclei was only 22% in Mif−/− mice that did not receive nitrite. Quantitative analysis revealed that amounts of SNO-MIF are significantly increased after nitrite treatment (mean ± SD, n = 4, **P < 0.02).

**References**

Cardioprotection Through S-Nitros(yl)ation of Macrophage Migration Inhibitory Factor

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**Background**—Macrophage migration inhibitory factor (MIF) is a structurally unique inflammatory cytokine that controls cellular signaling in human physiology and disease through extra- and intracellular processes. Macrophage migration inhibitory factor has been shown to mediate both disease-exacerbating and beneficial effects, but the underlying mechanism(s) controlling these diverse functions are poorly understood.

**Methods and Results**—Here, we have identified an S-nitros(yl)ation modification of MIF that regulates the protective functional phenotype of MIF in myocardial reperfusion injury. Macrophage migration inhibitory factor contains 3 cysteine (Cys) residues; using recombinant wtMIF and site-specific MIF mutants, we have identified that Cys-81 is modified by S-nitrosylation whereas the CXXC-derived Cys residues of MIF remained unaffected. The selective S-nitrosothiol formation at Cys-81 led to a doubling of the oxidoreductase activity of MIF. Importantly, S-nitrosothiol-MIF formation was measured both in vitro and in vivo and led to a decrease in cardiomyocyte apoptosis in the reperfused heart. This decrease was paralleled by a S-nitrosothiol-MIF– but not Cys81 serine (Ser)–MIF mutant–dependent reduction of infarct size in an in vivo model of myocardial ischemia/reperfusion injury.

**Conclusions**—S-nitrosylation of MIF is a pivotal novel regulatory mechanism, providing enhanced activity resulting in increased cytoprotection in myocardial reperfusion injury. (Circulation. 2012;125:1880-1889.)

**Key Words:** myocardial infarction ■ nitric oxide ■ reperfusion injury

Myocardial inhibitory factor (MIF) is a structurally unique inflammatory cytokine that plays an important role as an upstream regulator of innate and acquired immunity as well as in cellular redox signaling. Because of its inflammatory activities, MIF is a mediator of a number of acute and chronic inflammatory diseases.1–6 This includes an exacerbating role in atherosclerosis, a major underlying cause of cardiovascular disease in the Western world.7–10 However, accumulating evidence indicates that the role of MIF in cardiovascular pathology is more complex. MIF regulates key functions in myocardial ischemia/reperfusion (I/R) injury with an overall cardioprotective activity profile.11–14 Mechanistically, this property involves protection against c-Jun N-terminal kinase (JNK)–mediated apoptosis and against oxidative cell stress which prominently occurs during the early phase of reperfusion in myocardial infarction. Protection conferred by MIF against redox stress has been attributed to its intrinsic thiol-protein oxidoreductase (TPOR) activity.1,5,11,15

MIF’s role in redox regulation is due to its structural properties which are unique within the protein family of cytokines and plays a critical role in cellular redox homeostasis and apoptosis inhibition.1,11,15 The TPOR activity of MIF is mediated through a conserved Cys37-Ala-Leu-Cys60 motif15 resembling the CXXC motif of TPORs like thioredoxin (TRX). Moreover, residue Cys-60 is crucial for the interaction interface between MIF and c-Jun N-terminal activation domain binding protein-1 (JAB1/CSN5), an interaction partner of intracellular MIF.16 MIF engages in several defined protein-protein interactions with both extra- and intracellular binding partners. The third cysteine in MIF, Cys-81, has been suggested to mediate conformational effects.17 However, posttranslational modifications that could serve to control the action and magnitude of such interactions have been unknown.

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Protein S-nitrosylation constitutes a large part of the nitric oxide (NO\(^*-\))-mediated effects throughout physiology as well as in a broad spectrum of human diseases.\(^{18}\) S-nitrosylation is the coupling of an NO\(^*\) moiety to a reactive Cys thiol to form SNO. That could occur by endogenous NO\(^*\)-generating agents such as N\(_2\)O\(_3\), by transition metal-catalyzed addition of NO\(^*\) or by transnitrosylation from low–molecular-weight SNOs, such as S-nitrosocysteines.\(^{19}\) Furthermore, NO\(^*\) possibly mediates an one-electron oxidation of thiols with subsequent reaction of thiyl radicals with NO\(^*\). This protein modification has emerged as an important mechanism for dynamic, posttranslational regulation of most or all main classes of proteins.\(^{19}\) S-nitrosylation of selective Cys thiol groups can modulate the action of specific target proteins and thus regulates their function and properties.\(^{19-31}\) There is accumulating evidence that S-nitrosylation of proteins is intimately linked with myocardial I/R injury mediating cardioprotection.\(^{22}\) A number of studies demonstrated that NO\(^*\) is generated from both NO synthases (NOS)–dependent and NOS-independent pathways.\(^{23-27}\) During I/R, nitrite reduction to NO\(^*\) is an important source of NO\(^*\) that can be used for S-nitrosylation.\(^{28}\)

In this study, we set out to test whether S-nitrosylation of MIF may regulate the protective functional phenotype of MIF in myocardial reperfusion injury. We reasoned that if MIF is posttranslationally modified by S-nitrosylation, S-nitrosylated MIF could play a fundamental role in myocardial I/R injury.

**Methods**

**Recombinant Wild-Type Human MIF and Site-Specific Mutants of MIF**

Recombinant wild-type human MIF (wtMIF) and the Cys mutants Cys57Ser-MIF, Cys60Ser-MIF, and Cys81Ser-MIF used in this study were expressed, purified, and folded as described previously.\(^{29}\)

**Reductive Gas-Phase Chemiluminescence**

The amount of S-nitrosothiol (SNO)-MIF formation after S-nitrosylation of wtMIF or the Cys-ser substitution mutants was determined by reductive cleavage of the SNO bond with an iodide/triiodide-containing reaction mixture and subsequent detection of the NO\(^*\) released into the gas phase by its chemiluminescence reaction with ozone within a chemiluminescence detector as described previously.\(^{30,31}\)

**Synthesis of S-Nitrosylated MIF**

S-nitrosylation of wtMIF was achieved by reduction of nitrite to NO\(^*\) from deoxygenated myoglobin (deoxygenMb)\(^{29,30}\). This approach prevents false-positive chemiluminescence signals from conventionally applied NO\(^*\) donors in the incubation solution (eg, via S-nitroso-Cys). In brief, horse heart Mb was dissolved in PBS to a concentration of 25 \(\mu\)mol/L. After deoxygenation by gassing with argon for 15 to 20 minutes, deoxyMb was quantified spectrophotometrically (\(\lambda = 542\) nm; \(e_{542} = 13.9\) \(\text{cm}^{-1} \cdot \text{mM}^{-1}\)). A 5 \(\mu\)mol/L nitrite solution was anoxically prepared and added at a final concentration of 25 \(\mu\)mol/L to the deoxyMb solution. NO\(^*\) formation was confirmed by chemiluminescence. Incubation of 400 \(\mu\)mol/L wtMIF with 25 \(\mu\)mol/L nitrite and 200 \(\mu\)mol/L deoxyMb was carried out in the dark for 1 hour at 37°C. To specify the distinct S-nitrosylation site within MIF, the Cys-ser replacement mutants Cys57Ser-, Cys60Ser- or Cys81Ser-MIF were used instead of wtMIF. After 1 hour, SNO measurement was performed via chemiluminescence. Alkylation of Cys residues via preincubation of wtMIF with 100 \(\mu\)mol/L N-ethylmaleimide (NEM) before S-nitrosylation or omitting deoxyMb in the incubation tube served as negative controls.

**Hydroxyephedrine Transhydrogenase Assay**

Catalytic redox activity of wtMIF and SNO-MIF was measured by the 2-hydroxyethylsulfide (HED) transhydrogenase assay which was performed as described previously.\(^{32}\) Briefly, reduction of HED by reduced glutathione was measured in a MIF-catalyzed reaction. Oxidation of NADPH by oxidized glutathione was then recorded photometrically (in a coupled step (\(\lambda = 340\) nm). For each measurement, 25 \(\mu\)mol/L wtMIF dissolved in 20 mmol/L sodium phosphate buffer (pH 7.2) was used. S-nitrosothiol–MIF formation was achieved by incubating wtMIF with 250 \(\mu\)mol/L S-nitroso-Cys for 30 minutes at 37°C in the dark as described previously.\(^{33}\) The transhydrogenase reaction was started by adding wtMIF or SNO-MIF to 450 \(\mu\)L of the reaction mixture (final volume: 600 \(\mu\)L; final MIF concentration: 3.4 \(\mu\)mol/L). Equal volumes of 20 mmol/L sodium phosphate buffer and sodium phosphate buffer with 250 \(\mu\)mol/L S-nitroso-Cys (pH 7.2) served as controls. Reactions were recorded for 12 minutes, and MIF activity was calculated from the linearized slope of the curve (\(\Delta 340\) nm). Values represent the mean±SD of 3 independent assays with at least duplicate incubations each.

**Detection of S-Nitrosylated Proteins by Biotinylation (Biotin-Switch Assay) and Western Blotting**

The biotin-switch assay was essentially performed as described previously with minor modifications.\(^{34}\) Homogenization and blocking step: Excised and perfused mouse hearts were weighed and homogenized immediately in 20 volumes of ice-cold HEN buffer (250 \(\mu\)mol/L HEPES, 10 \(\mu\)mol/L EDTA, 2 \(\mu\)mol/L NEM, 0.1 \(\mu\)mol/L neocuproine) to block free thiols, by using a Wheaton glass–glass homogenizer immersed in an ice/water bath. The homogenate was centrifuged at 70,000 \(g\) for 1 hour at 4°C. The supernatant was incubated for 30 minutes at room temperature in the dark. Biotinylation step: The supernatant was precipitated twice with 5 volumes of ice-cold acetone to remove unreacted NEM, and the pellet was resuspended in HENS buffer (250 \(\mu\)mol/L HEPES, 10 \(\mu\)mol/L EDTA, 0.1 \(\mu\)mol/L neocuproine, 1% SDS). Subsequently, proteins were incubated with 1/3 volume of 4 \(\mu\)mol/L N-(6-(biotinamido)hexyl)-3'-(2'-pyridyldithio)propionamide (biotin-HPD, Pierce). Ascorbate and copper (1 \(\mu\)mol/L \(\cdot\)10 \(\mu\)mol/L \(\cdot\) \(\text{L}^{-1}\)) were added to SNO bonds and the mixture was incubated for 1 hour at room temperature in the dark. After another ice-cold acetone precipitation to remove unreacted biotin-HPD, the pellet was resuspended in HENS buffer. Western blot analysis: For the specific detection of S-nitrosylated MIF, proteins were immobilized after the biotinylation step via neutravidin resin-coated spin columns and subsequently pulled down according to the manufacturer instructions (Pierce, Pull-Down Biotinylated Protein: Protein Interaction Kit). After pull-down, affinity-purified biotinylated proteins from heart homogenates and MIF were revealed by Western blotting using the MIF–specific antibody Ka565 (1:1000) as described.\(^{35}\) Macrophage migration inhibitory factor bands were quantified using Quantity One 1-D analysis software and normalized to the signal of a recombinant MIF band. Amber tubes were used in all these procedures, and the reactions were carried out in the dark to avoid unspecific photolysis of SNO bonds from light.

**Myocardial I/R Injury In Vivo Protocol**

Wild-type (WT) and MIF-deficient (\(\text{MIF}^{-/-}\)) mice were anesthetized by ip injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and intubated. Mechanical ventilation parameters were set to a tidal volume of 2.1 to 2.5 mL and a respiratory rate of 140 breaths per min. Deep anesthesia was maintained by adding 2 vol% isoflurane to the ventilation gas. The chest was opened through a midline sternotomy, and the left coronary artery (LCA) was ligated for 30 minutes. S-nitrosothiol formation was induced by injection of 1.67 \(\mu\)mol/kg nitrite (in 50 \(\mu\)L 0.9% sodium chloride) into the left
ventricular cavity 5 minutes before reperfusion. Sodium chloride injection (50 μL) served as control treatment. The wtMIF (10 μg) and the Cys81Ser-MIF mutant (10 μg), respectively, were injected into the Mf+/− mice 5 minutes before onset of ischemia. For detection of SNO-MIF and investigation of apoptosis respectively, hearts were excised after 5 minutes or 4 hours of reperfusion and treated as described in the Methods section of the online-only Data Supplement. For analysis of infarct size, mice were euthanized after 24 hours of reperfusion; hearts were excised and perfused with PBS (for TTC staining see Methods section of the online-only Data Supplement).

Supplemental Methodology
For a detailed explanation of experimental animals and the methods relative to organ harvest for SNO analysis, myocardial area at risk and infarct size measurements, determination of myocardial apoptosis, coimmunoprecipitation of JAB1/CSN5 and Cys81Ser-MIF, identification of S-nitrosylation sites by mass spectrometry, please see the Methods section of the online-only Data Supplement.

Statistical Analysis
The results were presented as mean ± SD unless stated otherwise. Data were analyzed by 1-way ANOVA and posthoc Bonferroni multiple comparison correction (for all pairwise tests) with GraphPad Prism 5 software to compare differences between multiple groups and Student unpaired t test when analyzing 2 groups. Testing of main effects and 2 specific interactions was performed using 2-way ANOVA with posthoc Dunn-Šidák multiple comparison. A P value <0.05 was considered statistically significant.

Results
MIF Is Selectively S-Nitrosylated at Cys-81
Cysteine residues are highly reactive groups and more recently have been identified to be targeted by posttranslational modifications such as S-nitrosylation, serving critical regulatory purposes in cellular signaling and redox homeostasis. We investigated whether the MIF Cys residues are accessible to S-nitrosylation. Reduction of nitrite to NO* was employed to synthesize SNO-MIF. Nitrite reduction was achieved by the nitrite reductase activity of deoxyMb.27 S-nitrosothiol formation was measured by gas-phase chemiluminescence.11 Macrophage migration inhibitory factor was reproducibly and robustly modified by NO*, and quantification showed a ratio of 1.4±0.4 mmol SNO/nmol wtMIF, indicating that likely only 1 of the 3 Cys residues in MIF was modified (Figure 1A). To confirm the specificity of the detected SNO signal, the thiol groups of the MIF Cys were alkylated with NEM before S-nitrosylation. Reduction of nitrite to NO* also prevented the formation of SNO-MIF. These data provided clear evidence that MIF can be S-nitrosylated in vitro. To identify the specific Cys residue(s) that is/are S-nitrosylated in MIF, SNO-MIF synthesis was performed using the site-specific MIF mutants Cys57Ser-MIF, Cys60Ser-MIF, and Cys81Ser-MIF in which 1 of the 3 Cys residues is isosterically substituted by a Ser residue (Figure 1B).15 Strikingly, SNO-MIF formation was completely abolished in Cys81Ser-MIF whereas the Cys57Ser and Cys60Ser mutants showed S-nitrosylation ratios that were comparable to that of wtMIF (1.3±0.6 and 0.9±0.4 nmol SNO/nmol MIF) (Figure 1C). These findings clearly demonstrated that MIF is a target for S-nitrosylation in vitro and that this modification selectively targets Cys-81.

Figure 1D illustrates the position of Cys-81 in the structure of the MIF monomer and trimer and indicates where the nonmodifiable Cys-57 and Cys-60 are located.

Myocardial SNO-MIF Formation In Vivo
After having demonstrated that MIF can be modified by S-nitrosylation in vitro, we next investigated whether SNO-MIF formation can be detected in I/R in vivo. We used the biotin-switch technique14 to evaluate whether SNO-MIF formation occurs in the reperfused mouse heart under in vivo conditions. Wild-type mice underwent occlusion of the LCA for 30 minutes, and either saline or nitrite was administered 5 minutes preceding reperfusion (Figure 2A). After 5 minutes of reperfusion, hearts were excised, homogenized, and subjected either to gas-phase chemiluminescence for the detection of total SNO content (Figure 2B) or to the biotin-switch assay for specific detection of SNO-MIF in myocardial tissue. This experiment revealed that nitrite treatment led to a dramatic increase of total SNO content as well as specific formation of SNO-MIF in the postischemic heart in vivo (Figure 2C). Quantitative analysis showed that SNO-MIF levels increased ∼10-fold after myocardial I/R when compared with the basal level (Figure 2D). Pharmacological inhibition of NOS-mediated NO* generation before I/R did not affect basal SNO-MIF level (online-only Data Supplement Figure 1), indicating that the SNO-MIF levels as observed in the experiment were not primarily dependent on NOS-generated NO*. Thus, we have demonstrated for the first time that SNO-MIF formation occurs in the heart in vivo and that SNO-MIF levels become markedly elevated by increased concentrations of S-nitrosylation-active groups during myocardial I/R.

S-Nitroso(yl)ation of MIF Is Site Specific In Vivo
S-nitrosothiol formation at Cys thiols depends on precise biochemical conditions that an NO* group could encounter, such as O2 concentration, hydrophobicity, nucleophilicity, and the redox environment surrounding the target thiol, conditions that could dramatically change during I/R in the myocardium. Besides these ambient conditions, protein characteristics themselves, such as the ultrastructural accessibility of a given Cys residue, can change during I/R and might determine whether a particular thiol in a given protein is subjected to S-nitrosylation or not.6 On the basis of this background, we examined whether the specific S-nitrosylation of Cys-81 in the MIF sequence that we had observed in vitro could also be detected in the myocardium in vivo during I/R. To determine the site of MIF S-nitrosylation in vivo, we used an LC-MS/MS approach.37 Before the biotin-switch assay and subsequent MS analysis were performed, mice underwent transient LCA occlusion followed by a reperfusion (Figure 2A). After trypsinization of the biotinylated proteins, MS sequencing revealed that the MIF peptide LLC81GLLSDR was biotinylated at Cys-81 whereas no other biotinylated MIF peptide fragments were detected (Figure 3). Because no unmodified Cys-81 peptide was identified, an efficiency of S-nitrosylation of almost 100% after nitrite treatment can be assumed. However, if the unmodified MIF peptide LLC81GLLSDR reacted differently to the LC material this
might lead to nonidentification of this particular fraction. This finding confirmed our in vitro observations and unambiguously demonstrated that MIF is selectively S-nitrosylated at Cys-81 in the reperfused heart in vivo. In fact, this is the first demonstration of a posttranslational modification in vivo of the MIF protein, which moreover appears to be linked to critical physiological and pathophysiological conditions.

**S-Nitrosylation of MIF Modulates MIF Activities In Vitro and in Myocardial I/R Injury In Vivo**

Within the TPOR family of redox-regulatory proteins, S-nitrosylation of the noncatalytic Cys residues can critically contribute to an alteration of the functions of these proteins, including their CXXC-dependent oxidoreductase activity. The TPOR activity of MIF reduces small–molecular-weight disulfides such as HED, and this activity is dependent on the presence of Cys-60 and in part on Cys-57. Applying the HED transhydrogenase assay and comparing the HED activity of wtMIF with that of SNO-MIF, we next asked whether the observed SNO modification would affect the catalytic TPOR activity of MIF. S-nitrosothiol–MIF was synthesized by preincubation of wtMIF with S-nitroso-

Cys. S-nitroso-Cys led to an S-nitrosylation of Cys-81 of 88±19% (n=3) (online-only Data Supplement Figure II) and had no influence on assay kinetics (online-only Data Supplement Figure III). S-nitrosothiol–MIF catalyzed the formation of HED at a markedly faster rate (Figure 4A) being twice (205±15%) as active as wtMIF (100%) (Figure 4B). This faster rate indicated that modification of Cys-81 resulted in a conformational change that promoted the redox activity of MIF.

Interestingly, this observation was in line with prior findings showing that alkylation of Cys-81 promoted the redox activity of MIF. Taken together, these results demonstrate that S-nitrosylation of MIF at Cys-81 increases the inherent catalytic oxidoreductase activity of this protein. Because activity as well as protein–protein interactions of the active site of MIF has been linked to reduction of apoptosis, we next asked whether S-nitrosylation also causally affects the activities of MIF in vivo. We were particularly interested in the effect that MIF S-nitrosylation may have on...
the cardioprotective properties of MIF during myocardial I/R. Protection of cardiomyocytes from apoptosis has been identified as an important mechanism related to the cardioprotective activity of MIF. We compared mouse heart apoptotic cell numbers between WT mice and mice genetically deficient in MIF after I/R and treatment with nitrite. Hearts were harvested 4 hours after the reperfusion interval was started. After subsequent immunohistochemical preparation of the excised hearts, cardiomyocyte apoptosis was determined and quantified by TUNEL staining. Visual inspection of the TUNEL stains (Figure 4C) already indicated that the lowest number of TUNEL-positive cells was seen in hearts from WT mice treated with nitrite, whereas the highest number of TUNEL-positive cells was observed in Mif / mice that did not receive nitrite. Quantification of the TUNEL analysis in relation to the area at risk confirmed this notion and showed that SNO formation in WT mice led to a markedly decreased number of apoptotic nuclei (2.1 ± 1.0%) compared with control treatment of these mice (8.0 ± 3.5%; Figure 4D). These observations were in line with prior findings from others.38 Ischemia/reperfusion in Mif / mice that did not receive nitrite treatment resulted in a markedly increased myocardial apoptosis rate compared with the control-treated WT mice (14.5 ± 1.0% versus 8.0 ± 3.5%, P < 0.05; Figure 4D), confirming that MIF exhibits antiapoptotic functions in the reperfused heart. Intriguingly, SNO-mediated inhibition of apoptosis after I/R was attenuated in Mif / mice (11.3 ± 4.3%; Figure 4D). Of note, the relative reduction in apoptosis after SNO formation was 75% in WT mice (8.0 ± 3.5% versus 2.1 ± 1.0%) whereas the attenuation of apoptotic nuclei was only 22% in Mif / mice (14.5 ± 1.0% versus 11.3 ± 4.3%; Figure 4D). It has previously been suggested that the antiapoptotic effect of MIF in the reperfused heart is mediated through an inhibition of the JNK pathway.14 We had observed that S-nitros(y)lation of MIF at Cys-81 led to an alteration of the Cys-60-dependent TPOR activity of MIF. Because Cys-60 is critically involved in the interface between MIF and JAB1 and because MIF/JAB1 interaction blocks JNK activity,16 we investigated whether MIF/JAB1 binding activity could be affected by Cys-81. Coimmunoprecipitation

Figure 2. Macrophage migration inhibitory factor is S-nitros(y)lated in the reperfused heart in vivo. A, Experimental protocol. Wild-type mice underwent 30 minutes of LCA occlusion followed by 5 minutes reperfusion in vivo. Five minutes before reperfusion, SNO formation was induced by nitrite application (1.67 μmol/kg), and SNO-MIF formation was detected by the biotin-switch technique. B, Enhanced myocardial SNO content after nitrite application was confirmed by gas-phase chemiluminescence (mean ± SD, n = 4–5, **P < 0.001). C, Original Western blot after biotin-switch assay revealed increased band density after SNO formation, demonstrating dramatically increased SNO-MIF content in the reperfused heart. Band intensities were normalized to a control band of recombinant MIF. D, Quantitative analysis revealed that amounts of SNO-MIF are significantly increased after nitrite treatment (mean ± SD, n = 4, **P < 0.02). I/R indicates ischemia/reperfusion; LCA, left coronary artery; CTRL, control; SNO, S-nitrosothiol; and rMIF, recombinant macrophage migration inhibitory factor.
analysis revealed that the Cys81Ser substitution markedly decreased the binding between MIF and JAB1 (100% versus 31±25%; P <0.001) (online-only Data Supplement Figure IV). Importantly, modification of Cys-81 by S-nitros(yl)ation in vivo also led to a marked decrease in the interaction between MIF and JAB1 as revealed by coimmunoprecipitation after LCA occlusion/reperfusion and nitrite-mediated SNO formation in vivo. These in vitro and in vivo results suggest that S-nitros(yl)ation increases the cellular availability of MIF in the early phase of reperfusion by decreased JAB1 trapping and thus mediates increased cardioprotection (online-only Data Supplement Figure V).

S-Nitros(yl)ation at Cys-81 Regulates the Cardioprotective Properties of MIF In Vivo

We have demonstrated that MIF is selectively S-nitros(yl)ated at Cys-81 and that this posttranslational modification regulates the antiapoptotic activity of MIF during I/R. To elucidate whether the selective SNO modification of MIF is central to MIF-mediated cardioprotection in myocardial I/R, we compared infarct size in Mif−/− mice after myocardial I/R on wtMIF application and SNO generation (Figure 5A). Injection of wtMIF into the left ventricular cavity of Mif−/− hearts reduced infarct size (35±5% versus 30±2%, P <0.05; Figure 5B) to the level of WT mice, which naturally show smaller infarct size compared with Mif−/− (online-only Data Supplement Figure VI). Application of wtMIF undergoing SNO formation further reduced infarct size to 8±2% in Mif−/− mice. Infarct size reduction in Mif−/− hearts undergoing SNO formation without wtMIF reconstitution was less pronounced (17±3%; Figure 5B). Because SNO-MIF formation was found to selectively represent S-nitros(yl)ation at Cys-81, we applied the Cys81Ser-MIF mutant control instead of wtMIF. Determination of the resulting infarct sizes revealed that SNO-mediated cardioprotection was significantly less pronounced when Cys81Ser-MIF was injected instead of wtMIF (16±4% versus 8±2%, P <0.05; Figure 5B). Injection of the Cys81Ser-MIF mutant without nitrite led to an infarct size of 33±3% (Figure 5B).

In summary, these results demonstrate that MIF is selectively S-nitros(yl)ated at Cys-81 and that this previously unrecognized posttranslational modification regulates MIF function and increases the cardioprotective properties of MIF in vivo.

Discussion

During the early phase of myocardial reperfusion, S-nitros(yl)ation modulates the activity of various proteins involved in apoptosis and oxidative stress. Myocardial MIF functions as an endogenous protection mechanism against lethal I/R injury. It appears that cardioprotection by MIF is especially relevant within the first minutes of reperfusion. However, whether this critical cardioprotective activity of MIF may be modulated or switched on by posttranslational modifications has not been addressed. Here, we have identified S-nitros(yl)ation as the first-described posttranslational modification of MIF and provide evidence that S-nitros(yl)ation of MIF is critical for its cytoprotective properties in the reperfused myocardium in vivo.

Protein Cys residues are intrinsically nucleophilic and reactive under physiological pH conditions. Their reactivity renders them amenable to engage in diverse biochemical functions such as disulfide formation, protein–protein interaction, redox catalysis, or redox signaling. The MIF sequence contains 3 Cys residues, which have been found to contribute to several of the functional properties of MIF. Cysteine-57 and Cys-60 form a CXXC motif that has catalytic TPOR activity in vitro and is a distinct hallmark of the TPOR protein family. Best characterized is the role of Cys-60, which has been shown to contribute to MIF functions such as inhibition of apoptosis, JAB1, and NM23-H1 bind-
The role of the third, non-CXXC Cys residue in MIF, Cys-81, has hardly been studied, with Cys-81 being thought to contribute to the interaction between MIF and p53.43 However, Cys-81 is critically situated in an α-helix of the MIF structure where it contributes to the hydrophilic surface area.17 Yet Cys-81 is mostly surrounded by hydrophobic amino acids. Consequently, substitution or modification of Cys-81 may alter hydrophobic interactions in this region, and substitution of Cys-81 has been thought to mediate conformational effects.17

Macrophage migration inhibitory factor binding of JAB1 inhibits JNK activation and subsequent c-Jun phosphorylation. Furthermore, MIF blocks JNK activation and cellular apoptosis elicited by thiol starvation.1 Importantly, Qi et al demonstrated that MIF-mediated inhibition of the JNK pathway during myocardial reperfusion decreased cardiomyocyte apoptosis in the reperfused myocardium, representing a major axis of the cardioprotective MIF pathway.14 Our present findings demonstrate that, although Cys-60 of MIF is part of the binding interface between MIF and JAB1, the MIF/JAB1 interaction critically depends on Cys-81, because deletion of this amino acid significantly attenuated JAB1 binding. An involvement of Cys-81 in the protein–protein interaction between MIF and JAB1 was surprising at first sight because this residue is remotely situated from the binding interface encompassing residues 50 to 65. However, modification of distant regulatory Cys residues by S-nitrosylation has previously been shown to be essential. For example, S-nitrosylation of nonactive site Cys is known to regulate the function of proteins like TRX or G-protein–coupled receptor kinase 2,21,44 and currently numerous potentially functional Cys residues in the mammalian proteome have remained unidentified.40 In fact, MIF can be S-nitrosylated, as demonstrated herein, and Cys-81 clearly turned out to be the only target site
for this posttranslational modification. Thus, it was of high interest whether this residue might be a novel regulatory locus that on modification may affect MIF’s functions under redox stress.

As discussed above, MIF is a regulator of the cellular redox state and potently inhibits apoptosis during myocardial I/R.\(^\text{11,12,14}\) Interestingly, Koga et al, in a mouse model in vivo, established a direct link between MIF-mediated reactive oxygen species quenching and protection of the heart from I/R injury.\(^\text{11}\) This effect was attributed to the TPOR activity of the CXXC motif of MIF. We investigated whether the TPOR activity of MIF would be affected by the Cys-81–dependent S-nitrosylation of MIF. Notably, reduction of the small molecule disulfide HED by MIF was significantly enhanced after S-nitrosylation.

It was of great interest to ask whether the SNO formation at Cys-81 observed in vitro was also detectable under in vivo conditions during myocardial I/R. Nitrite reduction is an important source of NO\(^*\) during I/R that enables S-nitrosylation and thus attenuates I/R injury.\(^\text{28,45,46}\) Accordingly, SNO levels increased after nitrite application during I/R in our in vivo model. Importantly, we were able to detect SNO-MIF in the reperfused heart applying the biotin-switch technique and demonstrated the specificity of SNO formation at Cys-81 by LC-MS/MS analysis.

S-nitrosylation has been shown to regulate the function of several cardioprotective proteins such as TRX, G-protein–coupled receptor kinase 2, or caspase 3.\(^\text{20,46,47}\) Here we have identified MIF as an important novel target for S-nitrosylation during I/R in vivo. Macrophage migration inhibitory factor is a protein mediator that both acts as an inflammatory cytokine/chemokine and exhibits cardioprotective effects because of its intracrine/paracrine activity. This effects of MIF raised the question whether the posttransla-

tional S-nitrosylation of MIF affected its cardioprotective properties. We found significantly increased rates of cardiomyocyte apoptosis in mice that lacked SNO-MIF and that had been subjected to I/R in vivo. Because these results were based on the genetic deletion of MIF, we investigated whether replenishment of SNO-MIF in the Mif\(^/-\) mice was able to reverse the observed phenotype. In fact, administration of wtMIF, which also led to a reinstallation of SNO-MIF levels, led to a marked reduction in infarct sizes in these mice, confirming the importance of MIF and demonstrating the impact of SNO-MIF in reperfusion injury of the myocardium. Thus, these data extended previous findings of Young et al who could show that application of wtMIF into Mif\(^/-\) hearts reconstitutes WT conditions ex vivo.\(^\text{14}\) Of note, the Cys81Ser-MIF mutant failed to reinstall the cardioprotective properties of MIF in Mif\(^/-\) mouse hearts, underscoring the role of SNO-MIF and the specific function of S-nitrosylation at Cys-81 in cardioprotection.

In summary, we have identified a novel posttranslational modification of the MIF protein. We could demonstrate that key functions of MIF are regulated by its S-nitrosylation, with fundamental effects on myocardial I/R injury in vivo. S-nitrosylation thus might provide a new therapeutic strategy for regulating the functions of MIF during the early phase of reperfusion.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Macrophage migration inhibitory factor (MIF) is the first identified cytokine, discovered almost half a century ago by David, Bloom, and Bennett. Molecular knowledge about this protein mediator has recently grown tremendously, and a pivotal role has been attributed to MIF as a key regulator of innate and acquired immunity, as well as numerous pathophysiological functions involving its inflammatory cytokine and chemokine. There is emerging evidence that MIF also plays a central role in cardiovascular diseases and myocardial ischemia and reperfusion (I/R) injury. Despite an improved understanding of the pathophysiology of I/R injury and numerous preclinical trials, most of the clinical trials to prevent I/R injury have been disappointing. The present study now provides the first evidence that protein modification by means of S-nitrosylation of MIF increases the cardioprotective properties of this factor in the early phase of I/R injury. This reveals the first-described posttranslational regulatory modification of MIF and thus opens up a novel role of this ancient protein. This study further identifies the precise site of this modification and uncovers a key function of the reactive cysteine 81 residue. S-nitrosylation distinctly affects MIF properties and leads to decreased apoptosis and infarct size in vivo. Beyond the background that the precise pathophysiology of I/R injury is still not entirely understood, targeted regulation of MIF functions by S-nitrosylation might deliver a new therapeutic approach in the treatment of myocardial infarction.