High-Mobility Group Box-1 in Ischemia-Reperfusion Injury of the Heart

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Background—High-mobility group box-1 (HMGB1) is a nuclear factor released by necrotic cells and by activated immune cells. HMGB1 signals via members of the toll-like receptor family and the receptor for advanced glycation end products (RAGE). Although HMGB1 has been implicated in ischemia/reperfusion (I/R) injury of the liver and lung, its role in I/R injury of the heart remains unclear.

Methods and Results—Here, we demonstrate that HMGB1 acts as an early mediator of inflammation and organ damage in I/R injury of the heart. HMGB1 levels were already elevated 30 minutes after hypoxia in vitro and in ischemic injury of the heart in vivo. Treatment of mice with recombinant HMGB1 worsened I/R injury, whereas treatment with HMGB1 box A significantly reduced infarct size and markers of tissue damage. In addition, HMGB1 inhibition with recombinant HMGB1 box A suggested an involvement of the mitogen-activated protein kinases jun N-terminal kinase and extracellular signal-regulated kinase 1/2, as well as the nuclear transcription factor nuclear factor-HB in I/R injury. Interestingly, infarct size and markers of tissue damage were not affected by administration of recombinant HMGB1 or HMGB1 antagonists in RAGE/H11002/H11002 mice, which demonstrated significantly reduced damage in reperfused hearts compared with wild-type mice. Coincubation studies using recombinant HMGB1 in vitro induced an inflammatory response in isolated macrophages from wild-type mice but not in macrophages from RAGE/H11002/H11002 mice.

Conclusions—HMGB1 plays a major role in the early event of I/R injury by binding to RAGE, resulting in the activation of proinflammatory pathways and enhanced myocardial injury. Therefore, blockage of HMGB1 might represent a novel therapeutic strategy in I/R injury. (Circulation. 2008;117:3216-3226.)

Key Words: hypoxia inflammation ischemia myocardial infarction reperfusion

The distal cascade of inflammatory responses that results in organ damage after ischemia/reperfusion (I/R) injury has been studied extensively. However, the extent to which the initial cellular injury contributes to propagation of the inflammatory response and further tissue damage is poorly understood.

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We propose that a key link between the initial damage to cells and the activation of inflammatory signaling involves endogenous danger signals from ischemic cells. Extracellular high-mobility group box-1 (HMGB1) is a potent innate “danger signal” for the initiation of host defense or tissue repair.1 It can be either passively released from necrotic cells or actively secreted by activated immune cells.2 HMGB1 has been found to play a pivotal role in the pathogenesis of inflammatory disorders such as sepsis3 and autoimmune disease.4 Further studies indicated that HMGB1 may mediate inflammatory cell recruitment in acute hepatic necrosis5 and in acute lung injury.6 HMGB1 has been reported to interact with toll-like receptor (TLR) 4 and 91,7 and the receptor for advanced glycation end products (RAGE).8–10 RAGE is a multiligand receptor of the immunoglobulin superfamily inductively expressed in most tissues and present on a wide range of cells where its ligands accumulate.11–13 In addition, RAGE has been ascribed an essential modulatory role in the myocardial metabolism relative to I/R injury in an external reperfusion Langendorff model.14 The aim of this study was to test the hypothesis that HMGB1 is an early mediator of inflammation and cell injury after cardiac I/R injury.
and that the proinflammatory effects of HMGB1 are dependent on the interaction with RAGE. We show that HMGB1 is upregulated by hypoxia in cultured neonatal cardiomyocytes and by I/R injury of the heart in vivo. Treatment with HMGB1 box A, a functional antagonist of extracellular HMGB1 cytokine activity and HMGB1 interaction with RAGE, significantly protects against I/R injury and suppresses the activation of the inflammatory cascade. In addition, RAGE+/− mice undergoing I/R present with significantly reduced tissue damage. Coincubation studies with rHMGB1 in cell culture confirmed an inflammatory response only in isolated macrophages from WT mice in contrast to recombinant HMGB1 (rHMGB1)-stimulated macrophages from RAGE+/− mice.

Methods

Murine Model of I/R Injury
C57BL6 wild-type (WT) mice 10 to 12 weeks old purchased from Charles River (Sulzfeld, Germany) and RAGE−/− mice15–17 were anesthetized and subjected to I/R according to the protocols approved by the Animal Care and Use Committee of the University of Heidelberg. The transient coronary artery occlusion and reperfusion procedure was performed as described previously.18 Mice were placed in a supine position, and endotracheal polyethylene 90 tubing was used to provide ventilation (250 µL, 150 cycles per minute) via a rodent ventilator (model 683, Harvard, South Natick, Mass). The chest was opened, and ligation of the left anterior descending artery was performed using an 8-0 silk suture. Ligation lasted for 30 minutes, and reperfusion was established by loosening the knot. After 48 hours of reperfusion, the area at risk and infarct area were determined as described previously.19 In brief, the chest was reopened, and the area at risk and infarct area were analyzed by perfusion with 1% Evans blue and consequent incubation with 1.5% triphenyltetrazolium chloride. Each heart was digitally photographed at 50%.

HMGB1 Application
Recombinant HMGB1 (10 µg per mouse) was administered intra-peritoneally 1 hour before induction of I/R injury as previously described for liver I/R models.5 When indicated, HMGB1 box A (400 µg per mouse) or vehicle PBS was applied by intraperitoneal injection 1 hour before induction of I/R injury. Expression and purification of the full-length HMGB1 protein and its truncated form (box A) were performed as previously described.21

Echocardiography
Thransthoracic echocardiography was performed as previously described in detail.22

Immunohistochemistry and Histological Evaluation
Hearts were retrieved from indicated animals, and sections were prepared for immunohistochemistry. Hematoxylin and eosin (H&E) staining was performed using standard protocol. Immunohistochemical staining for HMGB1 was performed by an avidin-biotin-alkaline phosphatase (ABC-AP, Dako, Hamburg, Germany) system with AP substrate (Sigma-Aldrich UK, Dorset, UK) and biotinylated donkey anti-rabbit antibodies (1:200, Dianova, Hamburg, Germany) as the secondary reagent (1:250, Abcam, Cambridge, UK) and biotinylated goat anti-rabbit antibodies (1:200, DianoVA, Hamburg, Germany) as the primary reagent (1:250). Rabbit polyclonal anti-HMGB1 as the primary reagent (1:500, Abcam) and that the proinflammatory effects of HMGB1 are dependent on the interaction with RAGE. We show that HMGB1 is upregulated by hypoxia in cultured neonatal cardiomyocytes and by I/R injury of the heart in vivo. Treatment with HMGB1 box A, a functional antagonist of extracellular HMGB1 cytokine activity and HMGB1 interaction with RAGE, significantly protects against I/R injury and suppresses the activation of the inflammatory cascade. In addition, RAGE−/− mice undergoing I/R present with significantly reduced tissue damage. Coincubation studies with rHMGB1 in cell culture confirmed an inflammatory response only in isolated macrophages from WT mice in contrast to recombinant HMGB1 (rHMGB1)-stimulated macrophages from RAGE+/− mice.

Cell Culture and Induction of Hypoxia
Ventricular cardiomyocytes from neonatal hearts were prepared as published in detail.21 Isolation of macrophages was performed by their ability to adhere to culture plates. Purity was >95% as assessed by fluorescent-activated cell sorter analysis.24 In brief, murine macrophages were obtained by peritoneal lavage using 10 mL of RPMI containing 10% heat-inactivated FBS, 100 µg/mL streptomycin sulfate, 100 U/mL penicillin G, and 2 mmol/L glucose. Cells were plated at 5×10^6 cells per well (6-well plate) and incubated for 6 hours at 37°C in a humidified atmosphere of 5% CO2. After 2 washing steps with RPMI medium, adherent macrophages were cultured at 90% confluence up to 12 hours at 37°C. Hypoxia experiments were performed in an O2/CO2 controlled incubator (NUNC, Wiesbaden, Germany) adjusted to 1.5% O2, 5% CO2, and balance N2 at 37°C. Isolated cardiomyocytes and macrophages were stimulated with HMGB1 (10 µg/mL).25

Electrophoretic Mobility Shift Assay
Nuclear proteins were harvested, and 10 µg nuclear protein was assayed for nuclear factor-κB (NF-κB) binding activity with radioactive labeled oligonucleotides for the defined NF-κB consensus sequence (5’-AGT TGA GGC GAC TTT CCC AGG C-3’).26 Binding reaction and separation of the protein-DNA complexes from unbound DNA by electrophoresis were performed as previously described in detail.17,18

Western Blot
Total protein extracts from mouse heart tissue or from cells were prepared as previously described.17 The antibodies and the dilutions were as follows: phosphorylated SAP/Jun NH2-terminal kinase (JNK) No. 9255 (1:1000), phosphorylated extracellular signal-regulated kinase (ERK) (1/2) No. 9101 (1:1000), ERK (1/2) No. 4695 (1:1000), phosphorylated p38 No. 9211 (1:1000), p38 No. 9212 (1:1000) (Cell Signaling Technology, Danvers, Mass), and HMGB1 (1 µg/mL, No. 18256, Abcam Plc, Cambridge, UK). Horseradish peroxidase–coupled rabbit or mouse IgG (1:2000) was used as secondary antibodies.

Real-Time Reverse-Transcription Polymerase Chain Reaction
Total RNA was extracted from each heart into Tripreq reagent (Boehringer Mannheim, Mannheim, Germany). To determine the levels of expression of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and β-actin, 1 µg total RNA was used to performed quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) with LightCycler (Roche, Mannheim, Germany) as described previously.17 The primer sequences were as follows: IL-6 forward, 5’-AGT GAC TCT CAC TCA TGC TTT CTG G-3’; IL-6 reverse, 5’-CCATTCCTTGGCAGTCTTCGTTG-3’; TNF-α forward, 5’-CCATTCCTGAGTGTCTCAGAAG-3’; TNF-α reverse, 5’-GAAATATAAATAGAGGGGGCC-3’; IL-6 forward, 5’-CCCTAAGGCCAACCGTGAAA-3’; and β-actin forward reverse, 5’-ACGACCAAGGCTACATGGGAAC-3’. Standard curves were established with SYBR Green I kit (Roche, Basel, Switzerland). The primers for mouse HMGB1 (No. PPM05059E) were purchased from Super Array (Frederick, Md), and the RT-PCR for mouse HMGB1 was performed according to the manufacturer’s instructions.

Measuremet of Cardiac Troponin T, Lactate Dehydrogenase, IL-6, and TNF-α
Cardiac troponin T (cTnT) was measured quantitatively with electrochemiluminescence technology (third-generation cardiac cTnT, Elecsys 2010, Roche, Mannheim, Germany). A spectrophotometric kit (Sigma-Aldrich UK, Dorset, UK) was used to assess lactate dehydrogenase (LDH) in serum 48 hours after reperfusion or in supernatants of cardiomyocytes exposed to hypoxia. Supernatants from isolated cardiomyocytes and peritoneal macrophages cultured as above were assayed for IL-6 and TNF-α production by ELISA.
(Pharmingen/BD Biosciences, San Diego, Calif) according to the manufacturer’s instructions.

Statistical Analysis
Results are expressed as mean±SD. Data were analyzed using ANOVA with a priori contrasts. Differences were considered significant at values of P<0.05. Figure 2d needed data transformation because of heterogeneity of variances.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Cardiac HMGB1 Expression Is Upregulated in I/R Injury of the Heart
To test whether HMGB1 is involved in heart I/R injury in mice, ischemia was induced by ligation of the left anterior descending artery for 30 minutes, followed by 48 hours of reperfusion (Figure 1). Although sham-operated mice showed intact myocardial fibers with transverse striation (Figure 1a) and only moderate nuclear staining for HMGB1 (Figure 1b and 1c), cardiac sections from mice undergoing I/R injury presented with significant features of infarcted tissue (necrosis, hypereosinophilia, loss of nuclei, and loss of transverse striation, as well as a mixed inflammatory cellular infiltrate) (Figure 1d). Because of the strong expression of HMGB1 on infiltrating leukocytes (Figure 1e and 1f), the number of HMGB1-positive cells was increased compared with sham-treated control (Figure 1b and 1c).

Figure 1. HMGB1 expression in reperfused myocardium vs sham-treated controls. H&E staining (a and d) and corresponding immunohistochemical staining of HMGB1 expression in the myocardium of sham-treated mice (b and c) and from mice after 48 hours of I/R (e and f). Arrows indicate HMGB1-positive cells. Images are representative heart sections from 6 mice per group. H&E staining, magnification ×32 (a and d). Alkaline phosphatase immunohistochemistry for HMGB1, magnification ×32 (b and e) and ×64 (c and f).

HMGB1 Expression Pattern in the Heart In Vivo and In Vitro
We next evaluated whether HMGB1-dependent injury of the myocardium could be explained by changes in HMGB1 expression levels. HMGB1 expression was determined in isolated left ventricles after I/R injury at given time points using real-time RT-PCR. Compared with sham-treated animals, I/R injury of the heart resulted in increased expression of HMGB1 after 30 minutes of ischemia (P<0.03), peaking at 6 hours after reperfusion (P<0.005). It is of note that HMGB1 mRNA expression levels were still significantly increased after 7 days of reperfusion (P<0.005; Figure 2a). We also performed Western blot (WB) analysis on left ventricular tissue lysates from animals subjected to I/R injury (Figure 2b). HMGB1 expression was increased as early as after 30 minutes of ischemia and remained significantly increased up to 7 days later. The early increase in HMGB1 on both RNA and protein levels with sustained increase up to 7 days suggests a combined expression pattern consisting of initial passive release and new synthesis of HMGB1. Because it is known that I/R injury is initiated during hypoxia, we investigated the effect of hypoxia (1.5% O2) on the release of HMGB1 in isolated neonatal cardiomyocytes. Consistent with the in vivo observations, isolated cardiomyocytes showed a time-dependent increase in HMGB1 synthesis and release that peaked after 12 hours (Figure 2c). Primary neonatal cardiomyocytes exposed to normoxia (21% O2) demonstrated a basal level of HMGB1 expression that did not change significantly after up to 24 hours of incubation (data not shown). LDH, measured as a tissue injury parameter in the supernatant of hypoxic cardiomyocytes, was significantly increased after exposure to hypoxia for 6 hours, with a peak after 24 hours of hypoxia, pointing to passive release of HMGB1 from necrotic cells (Figure 2d).

The Role of HMGB1-RAGE Interaction in I/R Injury
Receptor binding studies revealed a high affinity of HMGB1 for RAGE,27,28 a multifunctional receptor medi-
ating the proinflammatory effects of its ligands through the activation of NF-κB and the consequent upregulation of NF-κB–regulated genes. To investigate whether RAGE contributes to HMGB1-mediated ischemic injury in the myocardium, WT (Figure 3a and 3d) and RAGE−/− mice (Figure 3g and 3j) either were left untreated or received HMGB1 box A (Figure 3b, 3e, 3h, and 3k) or rHMGB1 (Figure 3c, 3f, 3i, and 3l), respectively. I/R experiments were performed as above before tissue was taken and examined by H&E staining as summarized in a histopathological score (Figure 3m). Compared with untreated WT mice (Figure 3a and 3d), treatment with HMGB1 box A protected WT mice from I/R-mediated inflammation (histopathological score ≤3) in the reperfused myocardium (P<0.005; n=10; Figure 3c, 3f, and 3m). In contrast, RAGE−/− mice (P<0.001; n=10; Figure 3g and 3j) displayed only slight inflammation resulting from I/R injury that was neither blunted by HMGB box A (Figure 3h, 3k, and 3m) nor significantly increased by rHMGB1 (Figure 3i, 3l, and 3m).

Consistently, both HMGB1 box A–treated WT and RAGE−/− mice demonstrated significantly reduced infarct sizes (21.9±8.1% versus 60.5±8.2%, P<0.005, n=10; and 35.0±6.5% versus 60.5±8.2%, P<0.0001, n=10; Figure 4a) and improved recovery of cardiac function (42.0±1.1% versus 29.0±1.1%, P<0.01, n=10; and 37.0±1.2% versus 29.0±1.1%, P<0.01, n=10; Figure 4b) compared with untreated WT mice undergoing I/R. Furthermore, HMGB1 box A–treated WT mice and RAGE−/− mice demonstrated markedly reduced release of the cardiac necrosis parameter serum
TnT levels (2.9±0.9 versus 7.6±1.4 μg/L, P<0.005, n=10; and 3.9±0.7 versus 7.6±1.4 μg/L, P<0.0001, n=10; Figure 4c). Treatment of WT and RAGE−/− mice with rHMGB1 before I/R injury resulted in increased infarction sizes (81.1±8.4% versus 60.5±8.2%; P<0.005; n=10; Figure 4a), worsened I/R injury (25.0±0.6% versus 29.0±1.1%; P=0.05; n=10; Figure 4b), and increased serum TnT release (9.7±1.4 versus 7.6±1.4 μg/L; P<0.005; n=10; Figure 4c) only in WT mice. In contrast, infarction sizes (Figure 4a), fractional shortening (Figure 4b), and serum TnT levels were not affected by rHMGB1 in RAGE−/− mice (Figure 4a through 4c). Noteworthy, no difference was found in cardiac injury monitored by serum TnT levels between sham-operated mice that were given either vehicle (PBS) or a low dose of rHMGB1 (data not shown).

TNF-α and IL-6 are described as key mediators in the pathophysiology of cardiac I/R injury.31,32 When TNF-α and IL-6 mRNA were determined in reperfused left ventricles using real-time RT-PCR, I/R injury resulted in increased expression of TNF-α and IL-6 mRNA 3 hours after reperfusion (Figure 4d and 4e) compared with sham-treated animals. HMGB1 box A–treated mice demonstrated only minimal
increases in cardiac TNF-α (P<0.0001; n=5) and IL-6 mRNA levels (P<0.001; n=5), whereas rHMGB1-treated mice demonstrated enhanced cardiac TNF-α (P<0.01; n=5) and IL-6 mRNA levels (P<0.005; n=5; Figure 4d and 4e). In contrast, RAGE−/− mice showed reduced cardiac expression of TNF-α (P<0.0001; n=5) and IL-6 (P<0.0001; n=5) mRNA compared with WT mice. However, pretreatment with either HMGB1 box A or rHMGB1 had no effect in RAGE−/− mice (Figure 4d and 4e).

**HMGB1 Box A Modulates Inflammatory Signaling Pathways**

Among the mitogen-activated protein (MAP) kinase family, JNK, ERK1/2, and p38 have been demonstrated to be activated in I/R injury. To study whether the HMGB1-RAGE axis influences MAP kinase activation, we analyzed these MAP kinases by WB analysis (Figure 5a). I/R induced phosphorylation of JNK, ERK1/2, and p38 in isolated left ventricles from WT mice in a time-dependent manner, with a peak at 60 minutes of reperfusion. In HMGB1 box A–treated WT mice, phosphorylation of ERK1/2 (P<0.005; n=6) and JNK (P<0.001; n=6) was decreased compared with untreated WT mice (Figure 5a through 5c). Treatment with HMGB1 box A did not affect total cellular levels of ERK1/2 or JNK (Figure 5a). In line with a protection from I/R injury shown above, reperfused left ventricles isolated from RAGE−/− mice displayed significantly reduced phosphorylation for ERK1/2 (P<0.005; n=6) and JNK (P<0.001; n=6) compared with the control group (Figure 5a through 5c). Of note, activation of p38 MAPK as a result of I/R injury, determined by immunoblotting for Tyr182 phosphorylation, was not affected by either treatment with HMGB1 box A or RAGE deficiency (Figure 5a). One downstream effector of MAP kinase activation is the proinflammatory transcription factor NF-κB known to be activated after I/R injury of the heart. Nuclear extracts of reperfused left ventricles demonstrated a significant increase in NF-κB DNA binding activity compared with sham-operated controls (P<0.001; Figure 5d and 5e). WT mice pretreated with rHMGB1 before I/R injury showed a slightly higher but not significant increase in

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**Figure 4.** WT and RAGE−/− mice were subjected to I/R injury. Infarction volumes (a; *P<0.005), left ventricular function as fractional shortening (b; FS; *P<0.01), and serum levels of TnT (c; *P<0.005) were determined in each group. Cardiac TNF-α (d; *P<0.0001, **P<0.01) and IL-6 mRNA (e; *P<0.001, **P<0.005, ***P<0.0001) levels in reperfused myocardium are shown. Results were obtained with real-time RT-PCR and expressed as relative increase of mRNA level vs sham-operated animals. The mean±SD is reported. Inf/AAR indicates infarcted area at risk.
MAP kinase activation was determined in WT and RAGE\textsuperscript{−/−} mice (a through c). WB analyses of phosphorylated (p) and total (t) ERK1/2, JNK, and p38 were performed. Each blot shown is representative of 3 experiments with similar results. b and c, Summaries of the densitometric analyses of all extracts tested (b, \textsuperscript{*}P<0.005; c, \textsuperscript{*}P<0.001). NF-\textsuperscript{k}B binding activity in isolated left ventricles from WT and RAGE\textsuperscript{−/−} mice (d and e). Specificity of NF-\textsuperscript{k}B binding activity was shown by including a 160-fold molar excess of unlabeled consensus NF-\textsuperscript{k}B oligonucleotide (Cons). The electrophoretic mobility shift assay shown is representative of 3 experiments with similar results. e, Summary of the densitometric analysis of all extracts tested; the mean±SD is reported. \textsuperscript{*}P<0.001.
NF-κB DNA binding activity compared with control, whereas pretreatment with HMGB1 box A resulted in a significant reduction in NF-κB binding activity (P<0.001). Isolated left ventricles from RAGE−/− mice, however, displayed only basal NF-κB binding activity after I/R injury (P<0.001) that did not significantly differ from the NF-κB activation seen in untreated mice or in mice pretreated with rHMGB1 or HMGB1 box A (Figure 5d and 5e).

**Induction of HMGB1-RAGE–Mediated Proinflammatory Response Is Dependent on Macrophages In Vitro**

To characterize direct effects of HMGB1-RAGE interactions in more detail, we performed coinoculation studies with rHMGB1 in isolated cardiomyocytes and macrophages (Figure 6a through 6g). rHMGB1 stimulation of cultured WT cardiac myocytes failed to induce MAP kinases ERK1/2 and JNK (Figure 6a), TNF-α, and IL-6 expression (Figure 6b). In contrast, stimulation of isolated macrophages with rHMGB1 for 1 hour resulted in a strong activation (determined by their phosphorylation status) of MAP kinases ERK1/2 (P<0.01; Figure 6c and 6d) and JNK (P<0.01; Figure 6c and 6e) only in WT mice but not in RAGE−/− macrophages. Furthermore, rHMGB1 stimulation of WT macrophages led to a significant and time-dependent increase in IL-6 and TNF-α at a maximum of 12 hours after coincubation (P<0.0001; Figure 6f), whereas incubation of RAGE−/− macrophages with rHMGB1 resulted in a marginal but not significant increase in both cytokines (Figure 6g).

**Discussion**

It has become apparent in recent years that HMGB1 is instrumental in mediating a response to tissue damage and infection. HMGB1 also is released from necrotic or damaged cells and serves as a signal for inflammation. It has been demonstrated that HMGB1 not only is released in response to proinflammatory stimuli but also induces the production of inflammatory mediators by macrophages and neutrophils.36 HMGB1 has been demonstrated to be of pathogenic relevance in sepsis, pneumonia, and endotoxemia.35,36 On the other hand, exogenous HMGB1 supports regeneration on tissue injury. Recent data indicate that HMGB1 mediates myocardial regeneration after myocardial infarction by inducing resident cardiac c-kit+ cell proliferation and differentiation on local administration into the peri-infarcted left ventricle.37 Moreover, HMGB1 has angiogenic activities; it promotes the migration and growth of endothelial cells of various origins, including endothelial progenitor cells, and their organization in tubules.10 Thus, HMGB1 signaling not only mediates an inflammatory response but also provides for tissue growth and remodeling, depending on HMGB1 concentration and release in the particular pathophysiological situation or the mode of application in the experimental and/or interventional setting. During I/R injury, endogenous HMGB1 is massively released by necrotic and inflammatory cells and, in turn, activates vascular and inflammatory cells to express proinflammatory gene products.1,2,10 In this context, HMGB1-induced cell responses are mediated at least in part by the RAGE.9,10,12,27,28 One of the unique features of RAGE engagement is the sustained activation of NF-κB overwhelming autoregulatory feedback inhibitory loops.26,29 RAGE-dependent sustained NF-κB activation therefore has been implicated in a number of chronic inflammatory diseases.11–13,38 Because RAGE expression is upregulated by NF-κB and ligation of RAGE results in sustained NF-κB activation, endogenous HMGB1 and its binding to RAGE might result in sustained inflammation. Considering the pathogenic role of inflammation in cardiovascular diseases, we hypothesized that binding of excessive endogenous HMGB1 to RAGE might be critical in I/R injury and thereby overrules the described cardioprotective effects of low HMGB1 concentrations.

Here, we demonstrate that (1) HMGB1 is upregulated in cultured neonatal cardiomyocytes by hypoxia and in I/R injury of the heart in vivo; (2) treatment with HMGB1 box A, a functional antagonist of extracellular HMGB1 cytokine activity, and HMGB1 interaction with RAGE significantly protect against I/R injury; and (3) antagonizing HMGB1 suppresses the activation of the inflammatory cascade. We also demonstrate the involvement of the HMGB1–RAGE interaction in the mechanisms of HMGB1-mediated tissue damage and provide evidence that binding of endogenous HMGB1 to RAGE might play a central role in I/R injury of the heart.

Our studies identify endogenous HMGB1 as an early mediator in acute, local organ injury. HMGB1 expression levels were elevated as early as after 30 minutes of hypoxia determined in real-time PCR and WB. To analyze the molecular mechanisms underlying the activation of HMGB1, we investigated MAP kinase and NF-κB signaling pathways known to be involved in HMGB1-dependent signaling1,2 and I/R injury of the heart.33,34 The reduction in ERK1/2 and JNK activation seen with HMGB1 blocking treatment suggests MAP kinase involvement in HMGB1-mediated I/R injury. Interestingly, HMGB1 neutralization did not affect activation of p38 after I/R injury, implying selective actions of HMGB1 in this stress model (Figure 5a through 5c). We also tested for the influence of HMGB1 neutralization on the regulation of NF-κB, a transcription factor activated via the HMGB1-RAGE interaction and involved primarily in inflammatory stress responses.39 NF-κB binding activity was significantly reduced by HMGB1 agonists, pointing to its potential modulatory role in the development of the inflammatory response in I/R injury of the heart (Figure 5d).

HMGB1 is known to interact with TLRs and RAGE.1,7 Receptor binding studies revealed high affinity of HMGB1 for RAGE,27 and recent studies suggest an essential role of HMGB1 as a regulator and inducer of RAGE-dependent inflammation.8–10 Consistently, the deleterious effects of endogenous HMGB1 on I/R injury could be explained at least in part by engagement of RAGE because RAGE−/− mice were significantly protected despite TLR-4 and TLR-9 expression comparable to that of WT mice (data not shown). Furthermore, infarct size and markers of tissue damage in RAGE−/− mice were not changed by administration of rHMGB1 or HMGB1 antagonists. These results imply that the early release of HMGB1 from damaged, necrotic, or inflammatory cells is sufficient to mediate an early inflammatory response by binding to RAGE, which in turn is likely to amplify and
Figure 6. Isolated cardiomyocytes (a and b) and WT and RAGE−/− macrophages (c through g) were stimulated with HMGB1 (10 μg/mL) for 1 or 12 hours, respectively, before cell extracts were assayed in WB. a, Phosphorylated (p) and total (t) ERK1/2 and JNK in cardiomyocytes. b, TNF-α and IL-6 expression in cardiomyocytes stimulated with HMGB1 (10 μg/mL) for 12 hours. c, WB analysis of phosphorylated and total ERK1/2 and JNK in WT and RAGE−/− macrophages. Densitometric quantification (relative arbitrary units, x-fold increase) for phosphorylated ERK (d) and JNK (e). The mean±SD is reported. *P<0.01. f and g, TNF-α and IL-6 expression in isolated macrophages from WT (f) and RAGE−/− (g) mice. All experiments were performed in triplicate with similar results.
sustain the initial inflammation. Consistently, HMGB1 activation of isolated macrophages depends on the presence of RAGE and is absent in RAGE−/− macrophages (Figure 6). The in vitro studies further point to the importance of HMGB proteins, and holds a patent application on the use of Dr Bianchi is part owner of HMGBiotech, a company specializing in HMGB1 stimulation (Drs Bierhaus and Nawroth), and the Netzwerk Altersforschung (SFB405 to Dr Nawroth), the Juvenile Diabetes Research Stiftung (to Dr Andrassy), the Deutsche Forschungsgemeinschaft. This work was in part supported by grants from the Carl Baresel Foundation. Various studies also have documented increased RAGE expression in I/R injury in the heart, and thereby imply that the clinical situation is most likely a situation of excessive HMGB1-RAGE interaction. Because blocking ligamation of HMGB1 to RAGE by HMGB1 box A effectively reduces I/R injury in RAGE-bearing WT mice, approaches in which excessive HMGB1-RAGE interactions are targeted but that allow for RAGE-independent cardioprotective actions of HMGB1 might offer attractive novel pathogenetic-oriented treatment options.

Conclusions

HMGB1 plays a major role in early I/R injury by binding to RAGE, which results in the activation of proinflammatory pathways and enhanced myocardial injury. Therefore, blockage of HMGB1 might represent a novel therapeutic strategy to reduce I/R injury.

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Disclosures

Dr Bianchi is part owner of HMGBiotech, a company specializing in HMGB proteins, and holds a patent application on the use of HMGB1 in tissue damage. The other authors report no conflicts.

References


**CLINICAL PERSPECTIVE**

One of the major therapeutic goals of modern cardiology is to design strategies aimed at reducing myocardial tissue damage and optimizing cardiac repair after myocardial infarction. It has become apparent in recent years that high-mobility group box-1 (HMGB1) is instrumental in mediating response to tissue damage and infection. HMGB1, a nuclear factor released by necrotic cells and activated immune cells, was recently rediscovered to be a potent innate “danger signal” for the initiation of host defense or tissue repair. Here, we demonstrate that HMGB1 acts as an early mediator of inflammation and organ damage in ischemia/reperfusion injury of the heart. We further provide evidence that HMGB1-induced cell responses are mediated at least in part by the receptor for advanced glycation end products (RAGE), a multifunctional receptor of the immunoglobulin superfamily inductively expressed in most tissues and present on a wide range of cells where it plays a key role in inflammatory processes, especially at sites where its ligands accumulate. Treatment of mice with recombinant HMGB1 worsened ischemia/reperfusion injury, whereas treatment with HMGB1 box A, a functional antagonist of extracellular HMGB1 cytokine activity, and HMGB1 interaction with the receptor for advanced glycation end products significantly reduced infarct size and markers of tissue damage.
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