Exogenous high-mobility group box 1 improves myocardial recovery after acute global ischemia/reperfusion injury

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Background. High-mobility group box 1 (HMGB1) is a mediator of inflammation with dose-dependent effects. In the setting of regional myocardial infarction, a high-dose HMGB1 treatment decreases myocardial function, whereas low-dose HMGB1 improves function; however, it is unknown what role HMGB1 has in the setting of global ischemia/reperfusion (I/R) injury. We hypothesized that a low-dose HMGB1 treatment would improve myocardial functional recovery and decrease infarct size after global I/R injury in association with increased levels of cardioprotective paracrine factors and decreased inflammation.

Methods. Adult rat hearts were isolated and perfused using the Langendorff method and were subjected to global I/R and treatment with either the vehicle, 200-ng HMGB1, or 1-μg HMGB1. The treatment was administered during 1 min at the start of reperfusion, and myocardial function was measured for 60 min of reperfusion. At the end of reperfusion, the hearts were sectioned and incubated in triphenyltetrazolium chloride to assess myocardial infarct size or homogenized to measure levels of inflammatory cytokines and growth factors.

Results. Postischemic treatment with 200-ng HMGB1 significantly improved myocardial functional recovery after global I/R in association with decreased infarct size and decreased interleukin-1 (IL-1), IL-6, IL-10, and vascular endothelial growth factor (VEGF) levels. In addition, 1-μg HMGB1 decreased myocardial inflammation but did not result in subsequent improvement in functional recovery.

Conclusion. In the setting of global I/R, 200-ng posts ischemic HMGB1 treatment improves myocardial function and decreases infarct size in association with suppressed myocardial inflammation. These results suggest a potential role for exogenous HMGB1 therapy in the acute posts ischemic period. (Surgery 2011;149:329-35.)

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The salvage of ischemic myocardium is critical to prevent the adverse effects of postinfarct left ventricular (LV) remodeling (wall fibrosis, eccentric LV dilation, and increased mortality). Acute reperfusion of the injured heart is a key salvage strategy. Reperfusion, however, is associated with an injurious inflammatory cascade and potentially lethal reperfusion injury. Therefore, a substantial amount of cardiovascular research focuses on identifying novel compounds that modulate the inflammatory cascade associated with ischemia/reperfusion (I/R) injury. High-mobility box group 1 (HMGB1) is a potential treatment. HMGB1 is a ubiquitous, DNA-binding nuclear protein that facilitates gene transcription. Recently, HMGB1 has also been identified as an extracellular mediator of inflammation. Damaged or
necrotic cells release HMGB1 passively, whereas activated inflammatory cells such as macrophages release HMGB1 actively.\(^9\) The receptors for HMGB1 include the Toll-like receptors (TLRs) and the receptor for advanced glycated end-products (RAGE), which are associated with the release of proinflammatory cytokines such as tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)), interleukin (IL)-1, and IL-6.\(^9\text{,}^{10}\) Although 1 of the potential deleterious clinical roles of HMGB1 is as a later mediator of sepsis,\(^{11}\) emerging evidence points to 2 different roles for HMGB1 with respect to myocardial injury.

In a mouse model of regional myocardial I/R, Andrassy et al\(^{12}\) noted that preischemic, high-dose HMGB1 treatment decreased myocardial function 48 h after reperfusion. In contrast, Kitahara et al\(^{13}\) reported that hearts overexpressing HMGB1 had improved function and neoangiogenesis after myocardial infarction (MI). Supporting the beneficial role for HMGB1, Linama et al\(^{14}\) reported that low-dose, exogenous HMGB1 improved functional recovery and led to the accumulation of myocytes in the infarct area after MI. All 3 studies point to an integral role for HMGB1 in the setting of regional injury; however, it is not known what effect HMGB1 treatment has in the acute setting of global I/R that is encountered during cardiac surgery and cardiac transplantation. It is also unknown whether HMGB1 treatment after global I/R alters myocardial levels of the proinflammatory cytokines TNF, IL-1, and IL-6, or protective factors such as vascular endothelial growth factor (VEGF) or IL-10.\(^{15\text{,}^{16}}\)

We hypothesized that low-dose HMGB1 treatment in a model of global I/R would (1) increase functional recovery; (2) decrease MI size; (3) increase levels of cardioprotective cytokines; and (4) decrease proinflammatory signaling.

MATERIALS AND METHODS

**Animals.** Normal male (250–300 g, 9–10 weeks old) Sprague-Dawley rats (Harlan, Indianapolis, IN) were fed a standard diet and acclimated in a quiet quarantine room for 1 week before the experiments were conducted. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Indiana University. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, which was published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

**HMGB1 preparation.** Recombinant HMGB1 was obtained from Sigma-Aldrich (St. Louis, MO) and reconstituted per the manufacturer’s instructions. The final concentrations of HMGB1 (200 ng/mL and 1 \(\mu\)g) were made in a modified Krebs-Henseleit (KH) solution (in mmol/L: 11 dextrose, 110 NaCl, 1.2 CaCl\(_2\), 4.7 KCl, 20.8 NaHCO\(_3\), 1.18 KH\(_2\)PO\(_4\), and 1.17 MgSO\(_4\)). The treatment dose of 200 ng (1 mL) was based on previously reported results.\(^{14}\) A dose of 1 \(\mu\)g was chosen as a high dose to investigate for dose response.

**Isolated heart (Langendorff) experiments.** All isolated rat hearts were subjected to the same I/R protocol: 15-min equilibration, 25-min warm global ischemia (37°C), and 60-min reperfusion. The hearts were assigned randomly to 1 of 3 postischemic intracoronary infusion treatments: vehicle (1 mL KH solution, \(n = 9\)), 200-ng HMGB1 (dissolved in 1-mL KH, \(n = 10\)), or 1-\(\mu\)g HMGB1 (dissolved in 1-mL KH, \(n = 10\)). The infusions were performed over 1 min immediately at the start of reperfusion.

The rat hearts were isolated as described previously.\(^{17}\) The rats were anesthetized (60-mg/kg pentobarbital sodium intraperitoneal injection) and heparinized (500 units intraperitoneal injection). The hearts were excised rapidly via median sternotomy and placed in ice-cold KH solution. The aorta was cannulated, and the heart was perfused in the constant pressure, isovolumetric Langendorff mode with 37°C KH solution. The total ischemic time was less than 45 s. The perfusate was bubbled with 95% O\(_2\)-5% CO\(_2\) and filtered continuously through a 0.45-\(\mu\)m filter. A pulmonary arteriotomy and left atrial appendage resection were performed allowing the insertion of a water-filled latex balloon through the left atrium into the left ventricle. The left ventricular preload volume (balloon volume) was held constant during the entire experiment to allow continuous recordings of the left ventricular developed pressure (LVEDP). The balloon was adjusted to a mean left ventricular end-diastolic pressure (EDP) of 8 mm Hg (range, 6–10) during the equilibration period. Pacing wires were fixed to the right atrium and left ventricle, and hearts were paced at 6 Hz, 5 V, 8 ms (approximately 350 beats/min) during equilibrium and reperfusion. A stopcock above the aortic root was used to create global ischemia, during which the heart was placed in a 37°C organ bath. Data were recorded continuously with a PowerLab 8 preamplifier/digitizer (AD Instruments, Milford, MA) and a Mini Mac computer (Apple Computer, Cupertino, CA). The maximum positive and negative values of the first derivative of pressure (+dP/\(dt\) and −dP/\(dt\)) were calculated with PowerLab software (AD Instruments). After 60 min reperfusion, the hearts were removed from the apparatus,
sectioned for MI measurement, or homogenized for cytokine measurement.

**MI area.** At the end of reperfusion, the hearts from each group (n = 3–4/group) were frozen for 30 min at −20°C and cut in 1–2-mm-thick serial cross sections from the apex to the base. The sections were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC; Carolina Biological Supply, Burlington, NC) and fixed in 10% formalin (Fisher Scientific, Hanover Park, IL) similar to protocols described previously.18 Three serial slices at the midpapillary level were scanned using an Epson AL-CX11 flatbed scanner (Epson, Long Beach, CA). The total LV area and the LV infarct area of each slice were measured using the NIH Image J software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/). The percentages of the infarct area/total LV area were then averaged to calculate an overall value for each heart.

**Myocardial cytokine expression.** The remaining hearts (n = 5 in the vehicle group, n = 7 in the 200-ng group, and n = 7 in the 1-µg group) were homogenized for 2 min in a cold buffer solution consisting of 80% RIPA buffer, 10% proteinase inhibitor cocktail, and 10% phosphotase inhibitors (Sigma-Aldrich). The homogenate was then centrifuged at 12,000 rpm for 5 min, and the total protein concentration was determined by the Bradford method using an Eppendorf biophotometer (Eppendorf, Westbury, NY). Myocardial levels of the inflammatory cytokines TNF, IL-1 and IL-6, as well as the myocardial protective cytokines VEGF and IL-10, were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (R&D, Minneapolis, MN). The measured values (pg/mL) were normalized to protein concentration (pg/mg of myocardial protein). All samples and standards were measured in duplicate.

**Presentation of data and statistical analysis.** The reported values represent means ± standard errors of the mean (SEM). All measures of myocardial function were compared using a repeated-measures analysis of variance (ANOVA) with post-hoc Bonferroni correction. Measures of protein expression and infarct size were compared using 1-way ANOVA with post-hoc Bonferroni or Dunnett’s, respectively. A probability value of less than .05 was considered statistically significant.

**RESULTS**

**HMGB1 treatment increases post-ischemic myocardial function.** I/R resulted in markedly decreased LVDP, +dP/dt and −dP/dt in all 3 groups compared with the baseline function (Fig 1). Post-ischemic recovery of LVDP, +dP/dt, and −dP/dt, however, was significantly greater in hearts infused with 200-ng postischemic HMGB1. The postischemic recovery of LVDP was also significantly greater in the 1-µg treated hearts compared with the vehicle group but was not different from the 200-ng treatment. Treatment with 1-µg HMGB1 did not alter significantly the postischemic recovery of +dP/dt and −dP/dt. The difference in the postischemic recovery of these 3 parameters in the 200-ng treatment group was observed as early as 10 min into reperfusion and became statistically significant after 40 min of reperfusion for LVDP and +dP/dt. In the 200-ng treatment group, −dP/dt was significantly different from the vehicle after 30 min of reperfusion.

At the end of reperfusion, the percent recovery of the baseline LVDP was 36% ± 3% in the vehicle group, 53% ± 6% in the 200-ng HMGB1 treatment group, and 50% ± 4% in the 1-µg HMGB1 group (P < .01 vehicle vs 200 ng HMGB1, P < .05 vehicle vs 1-µg HMGB1). The percent recovery of +dP/dt at the end of reperfusion was 39% ± 4% in the vehicle group, 53% ± 5% in the 200-ng treatment group, and 51% ± 5% in the 1-µg HMGB1 group (P < .01, vehicle vs 200-ng HMGB1-treated hearts; P = ns, vehicle vs 1-µg HMGB1). In the vehicle group, the percent recovery of baseline −dP/dt at the end of reperfusion was 47% ± 3% compared with 55% ± 5% in the 200-ng group and 47% ± 3% in the 1-µg group (P < .01, vehicle vs 200 ng HMGB1-treated hearts). The mean EDP at the end of reperfusion did not differ among the groups (74 mm Hg ± 5 mm Hg in the vehicle group, 67.91 mm Hg ± 3.98 mm Hg in the 200-ng group, and 73.22 mm Hg ± 4.72 mm Hg in the 1-µg group).

**MI size.** As expected, I/R resulted in substantial damage to the hearts as evidenced in representative gross sections of the hearts (Fig 2); however, postischemic treatment with HMGB1 decreased the percentage of the LV infarct area to the total LV area in the 200-ng HMGB1 group (38% ± 3%) and the 1-µg treatment group (38% ± 1%) as compared with the vehicle treatment group (49% ± 3%; P < .05 vehicle versus 200 ng and 1 µg).

**Myocardial levels of VEGF and IL-10.** HMGB1 treatment decreased levels of myocardial VEGF significantly in a dose-dependent fashion. Treatment with 200-ng HMGB1 decreased VEGF production to 339 ± 24- pg/mg myocardial protein compared with 420 ± 16-pg/mg myocardial protein in the vehicle group. Then, 1-µg HMGB1 decreased VEGF levels to 269 ± 12-pg/mg of
myocardial protein ($P < .05$, vehicle compared with 200-ng HMGB1, 200 ng compared with 1-$\mu$g HMGB1). Similarly, HMGB1 treatment decreased IL-10 levels. IL-10 decreased from 555 ± 31 pg/mg of myocardial protein to 433 ± 35 pg/mg of myocardial protein in the 200-ng treatment,
and 221 ± 22 pg/mg of myocardial protein in the 1-µg group (P < .05, vehicle compared with 200-ng HMGB1; P < .05, 200-ng compared with 1-µg HMGB1).

**Myocardial expression of inflammatory cytokines.** HMGB1 treatment was also associated with decreased levels of myocardial IL-1 and IL-6. Postischemic infusion of 200-ng HMGB1 decreased myocardial IL-1 from 382 ± 15 pg/mg of myocardial protein in the vehicle group to 259 ± 26 pg/mg of myocardial protein (P < .01). In addition, 1-µg HMGB1 treatment decreased IL-1 to 178 ± 16 pg/mg of myocardial protein (P < .05; 200-ng vs 1-µg HMGB1). The IL-6 levels decreased from 435 ± 15-pg/mg myocardial protein (vehicle) to 392 ± 21-pg/mg myocardial protein with 200-ng HMGB1 treatment to 173 ± 8-pg/mg myocardial protein with 1-µg myocardial protein (P < .01, vehicle compared with 200 ng; P < .05, 1 µg compared with 200 ng). The TNF levels were at the lower limit of the ELISA detection and, therefore, excluded.

**DISCUSSION**

Identification of the mechanisms related to myocardial I/R is critical and may result in the development of targeted therapies that capitalize on the beneficial aspects of I/R-associated inflammation while minimizing adverse effects. HMGB1 is an attractive potential therapy because exogenous administration enhances cardiac stem cell proliferation as early as 24 h after injury. In addition, blockade of HMGB1 increased infarct size and did not suppress TNF and other inflammatory markers linked with myocardial dysfunction in a mouse model of regional I/R. These results imply that HMGB1 may be important in mediating the recovery of the heart after I/R. In light of the previous findings, we hypothesized that HMGB1 would improve myocardial function and protect the myocardium in the setting of global I/R, which is encountered clinically in cardiac surgery and cardiac transplantation.

To test our hypothesis, we used a rat model of global I/R and injected a single, low dose of 200 ng or a greater dose of 1-µg HMGB1 into the coronary circulation in the immediate postischemic period. The low dose was chosen based on a previous report that 200-ng HMGB1 injected into the myocardium of the LV of mice improved function and myocardial regeneration after MI. We use the term “low dose” to differentiate from the greater dose given by Andrassy et al (10 µg/mouse) in their study. Although it should be noted that the serum levels of HMGB1 in humans suffering an acute MI or in control rats are significantly less than 200 ng.

Our results are the first indication that low-dose HMGB1 treatment of the heart after global I/R is associated with improved recovery of baseline LVDP and ±dP/dt early in the time course of reperfusion. These effects became significant after 30–40 min of reperfusion and continued to diverge from the vehicle treatment until the end of the experiment. The increased function associated with exogenous HMGB1 treatment in the hyperacute phase of reperfusion (first minute) was also linked to a significantly smaller LV infarction at the end of reperfusion. These functional and infarct results are encouraging because Piper et al advocated that intervention in the first few minutes of reperfusion is crucial in minimizing cardiomyocyte hypercontracture and subsequent necrosis. Our results support this group’s claim that the early phase of reperfusion may be an important time window for protecting the I/R myocardium. Treatment with 1-µg HMGB1 did not result in improved functional recovery of LVDP and
decreased infarct size when compared with the 200-ng HMGB1 group. In addition, $\frac{\Delta dP}{dt}$ were not different in the 1-μg treated hearts compared with the vehicle hearts. This finding suggests that the functional effects of HMGB1 treatment decrease with increasing doses of HMGB1.

To determine whether the improved function and smaller infarcts observed in the HMGB1-treated hearts were associated with an altered inflammatory reaction, we measured levels of anti- and proinflammatory cytokines in the heart. VEGF, which is a known cardioprotective factor, decreased in response to both HMGB1 treatments. In addition, both HMGB1 treatments were associated with decreased myocardial levels of IL-10. IL-10 has known anti-inflammatory properties in heart disease. Interestingly, the decreased VEGF and IL-10 levels in the HMGB1 group were associated with statistically significant decreases in IL-1 and IL-6. The heart releases TNF in response to injury, and TNF leads to increases in IL-1 and IL-6; however, we could not determine accurately the low levels of TNF in the groups. The functional results and cytokine data suggest that hyperacutre treatment with exogenous HMGB1 is associated with a generalized suppression of proinflammatory and anti-inflammatory cytokine levels produced by the injured heart. Greater levels of exogenous HMGB1, however, do not improve myocardial function recovery beyond what is observed with 200-ng treatment. It is possible that some minimal level of inflammatory signaling is required to counteract reperfusion injury. Our results with the greater dose of 1 μg would support this concept because the lesser levels of myocardial cytokines did not improve myocardial function.

Our results add to the growing body of evidence indicating that HMGB1 is associated with beneficial paracrine actions, improved myocardial function and cardioprotection. It is likely that the timing (preischemic versus postischemic) and the dose of HMGB1 are crucial factors. Initially, Andrassy et al. reported that high-dose intraperitoneal HMGB1 (10 μg/10–12-week-old mouse) 1 h before ischemia was detrimental in a model of regional I/R. More recently, however, Hu et al. reported that 24 h of preconditioning in rats with high doses of intraperitoneal HMGB1 (200 μg/250–300 g rat) resulted in improved function after regional I/R. Although Andrassy et al. did not indicate the weight of the mice, it is likely that these mice weighed no more than 30 g (~0.3-μg HMGB1/g body weight), which would be less than the dose used in the study by Lin et al. These 2 studies suggest that a greater preconditioning period and greater dose may be necessary for the beneficial effects of HMGB1 in the setting of preschismic treatment. Alternatively, it can be argued that Lin et al. did not view any negative effects, because their reperfusion end point was substantially shorter (4 h vs 48 h).

In our study, we chose to investigate a low-dose postconditioning strategy instead of preschismic treatment because several groups reported that post-MI treatment with HMGB1 increased function and decreased LV remodeling. Oozawa et al. are the only authors currently to report on the role of HMGB1 in regional I/R instead of post-MI. They reported that blockade of endogenous HMGB1 30 min prior to reperfusion increased infarct size after regional I/R. Nonetheless, as Taka-hashi recently summarized, there is a gap in our understanding of the role of HMGB1 with respect to ischemic heart disease. Our results provide constitute the initial report that exogenous HMGB1 in the hyperacute period of reperfusion after global I/R is beneficial.

Our study was designed to determine whether exogenous HMGB1 had an effect on the heart in the setting of global I/R and whether that change was associated with alterations in the myocardial cytokine production as a measure of inflammation. One limitation of our study is that it was not designed to determine the precise mechanism. Although our results are encouraging, subsequent studies are needed that will focus on the known receptors for HMGB1 (RAGE, TLR2 and TLR4). RAGE was the first identified receptor for HMGB1; however, HMGB1 has been implicated recently with the activation of TLR2 and TLR4. The 3 receptors have downstream signaling pathways with substantial overlap, which complicates the investigation of the mechanism. Knockout animals (RAGE-deficient and TLR2 and TLR4 knockout mice) will help to define the mechanistic pathway associated with exogenous HMGB1 therapy. Some investigators speculate that HMGB1 may form extracellular complexes that may have adverse physiologic results. Therefore, blocking experiments may also be necessary to prevent the formation of these extracellular complexes that could augment the beneficial effects of exogenous HMGB1.

In conclusion, the role of HMGB1 remains multifaceted in terms of myocardial salvage. Studies have implicated HMGB1 in both beneficial and deleterious roles depending on the model or the disease state being investigated. With respect to the heart and global I/R, postischemic treatment with HMGB1 is associated with improved functional recovery acutely. Whether this effect is durable remains unknown.
involved with exogenous HMGB1 therapy for the heart is likely to involve more than 1 extracellular receptor and merits subsequent investigation.

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