HMGB1 in renal ischemic injury

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Rabadi MM, Ghaly T, Goligorksy MS, Ratliff BB. HMGB1 in renal ischemic injury. Am J Physiol Renal Physiol 303: F873–F885, 2012. First published July 3, 2012; doi:10.1152/ajprenal.00092.2012.—Factors that initiate cellular damage and trigger the inflammatory response cascade and renal injury are not completely understood after renal ischemia-reperfusion injury (IRI). High-mobility group box-1 protein (HMGB1) is a damage-associated molecular pattern molecule that binds to chromatin, but upon signaling undergoes nuclear-cytoplasmic translocation and release from cells. Immunohistochemical and Western blot analysis identified HMGB1 nuclear-cytoplasmic translocation and release from renal cells (particularly vascular and tubular cells) into the venous circulation after IRI. Time course analysis indicated HMGB1 release into the venous circulation progressively increased parallel to increased renal ischemic duration. Ethyl pyruvate (EP) treatment blocked H2O2 (oxidative stress)-induced HMGB1 release from human umbilical vein endothelial cells in vitro, and in vivo resulted in nuclear retention and significant blunting of HMGB1 release into the circulation after IRI. EP treatment before IRI improved short-term serum creatinine and albuminuria, proinflammatory cyto-/chemokine release, and long-term albuminuria and fibrosis. The renoprotective effect of EP was abolished when exogenous HMGB1 was injected, suggesting EP’s therapeutic efficacy is mediated by blocking HMGB1 translocation and release. To determine the independent effects of circulating HMGB1 after injury, exogenous HMGB1 was administered to healthy animals at pathophysiologic dose. HMGB1 administration induced a rapid surge in systemic circulating cyto-/chemokines (including TNF-α, etoxatin, G-CSF, IFN-γ, IL-10, IL-1α, IL-6, IP-10, and KC) and led to mobilization of bone marrow CD34+/Flk1+ cells into the circulation. Our results indicate that increased ischemic duration causes progressively enhanced HMGB1 release into the circulation triggering damage/repair signaling, an effect inhibited by EP because of its ability to block HMGB1 nuclear-cytoplasmic translocation.

renal damage; ischemia-reperfusion injury; ethyl pyruvate treatment

The proinflammatory effects of HMGB1 have been recently targeted to determine the beneficial efficacy of blocking HMGB1 release in the circulation. In endotoxemia studies, neutralizing antibodies against HMGB1 prevented endotoxemic-induced lethality (1, 76). In a model of IRI, neutralizing antibodies against HMGB1 offered significant protection against renal IRI damage, as was demonstrated with a reduction in tubular apoptosis, serum creatinine, blood urea nitrogen (BUN), TNF-α expression, and pathologic injury (37, 78). However, not all of HMGB1’s downstream effects are detrimental as various injury models have strongly implicated HMGB1 in regeneration and repair after tissue damage (2, 10, 35, 38, 43, 48, 54, 55, 60, 75, 83). Once released into the circulation, HMGB1 induces migration, proliferation, and homing of vascular-associated stem cells to sites of injury (10, 46) and regeneration of epithelia by mobilization of platelet-derived growth factor receptor-α-positive (Lin-/PDGFRα+) bone marrow cells (65). In addition, HMGB1 has been shown to be essential for mitochondria quality control (67) in eukaryotic cells.

While generalized HMGB1 protein release has been shown to occur during IRI, the dynamics of its translocation and release in response to various intensities of injury have not been examined. Here, we analyzed the time course and degree to which HMGB1 is released from the kidney into the circulation during IRI and whether its release is proportional to the duration of ischemic episode. Using immunohistochemistry, we aimed to unmask which renal cells may be responsible for release of HMGB1. Other studies showed neutralizing HMGB1 antibodies in vivo prevents renal IRI injury (37).

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with antibodies after its release following IRI improves renal damage function (37, 78). We attempted to explore the efficacy of ethyl pyruvate (EP) in preventing HMGB1 release. It has been shown that EP can improve renal function after IRI, but its therapeutic mechanism and ability to prevent nuclear-cytoplasmic translocation and release from renal and endothelial cells remain unclear. Furthermore, while previous studies suggested that circulating HMGB1 promotes both proinflammatory and regenerative effects, the effects circulating HMGB1 has on the cyto-/chemokine response and its ability to stimulate mobilization of bone marrow progenitor cells, such as endothelial progenitor cells (EPC), following IRI remain to be fully examined. For example, Li et al. (37) and Wu et al. (78) showed neutralizing HMGB1 during IRI reduces TNF-α, IL-6, and MCP-1 release; however, these studies did not fully examine the effects HMGB1 blockade has on secretion of various other important cyto-/chemokines such as IL-1, IL-8, IL-10, etc. during IRI. Therefore, we aimed to better understand the effects of HMGB1 once it is released into the systemic circulation during IRI and whether the therapeutic potential of EP during IRI is directly related to inhibition of HMGB1 translocation and release from stressed cells.

MATERIALS AND METHODS

IRI model. The animal study protocol was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Male FVB/NJ mice (Jackson Laboratory, Bar Harbor, ME), ages 10–12 wk, were anesthetized with intraperitoneal injection of 60 mg/kg ketamine and 6.6 mg/kg xylazine before the induction of renal IRI and placed on a heated surgical pad to maintain a constant body temperature. All animals received 500 U heparin (APP Pharmaceuticals, Schaumburg, IL) by intraperitoneal injection 15 min before surgery. After a 1.5-cm midlateralotomy, both kidneys were exposed and clamping of the renal pedicles was performed (bilaterally) with microserrefines.

For time trial experiments aimed at measuring the extent of HMGB1 translocation and release during increased periods of ischemia, renal pedicles were clamped for 25, 40, and 55 min. Following ischemia, clamps were removed and venous and arterial blood samples were taken immediately during reperfusion. For blood collection, the renal vein was cannulated to obtain venous blood exiting the kidney. To obtain systemic arterial blood, samples were drawn from the left ventricle. Upon death, kidneys were removed and subject to lysis and nuclear-cytoplasmic fractionation.

For IRI (excluding time trials) experiments, renal arteries were clamped for 40 min. After removal of the clamp, the abdominal incision was closed with 4–0 sutures. Mice were killed at various times after IRI (1 h, 36 h, and 2 mo later). Blood (collected by cardiac puncture), urine, and kidneys were collected for further analysis.

For animal experiments using EP, animals received a one-time intraperitoneal bolus injection of 40 mg/kg body wt of EP (Sigma, St. Louis, MO) (26, 31, 41, 73, 82) 15 min before IRI surgery. Due to the acidic nature of EP, 15 mM HEPES was added and the resulting

![Image](http://ajprenal.physiology.org/)

**Fig. 1.** Renal high-mobility group box-1 protein (HMGB1) immunohistochemistry staining after ischemia-reperfusion injury (IRI). Immunohistochemistry on kidney sections stained for HMGB1 (red) and nuclei (blue) immediately and 1 h after IRI (40-min ischemia). In control kidneys, HMGB1 demonstrates colocalization with nuclei in vascular endothelial cells, tubular cells, and peritubular capillary cells. Immediately and 1 h after IRI, HMGB1 localization becomes more diffused. All images are ×100. Arrows indicate HMGB1.
solution was set to a pH of 7.2. To assess the effects of EP on kidney function and cyto-/chemokine release, animals were killed 36 h after EP treatment and IRI surgery. Serum was obtained by cardiac puncture. Serum samples were analyzed for creatinine content using a creatinine assay kit (Abcam, Cambridge, MA).

For animal experiments using recombinant HMGB1 (rHMGB1), animals received an intravenous bolus (tail vein) injection of 100 ng/ml of rHMGB1 (R&D Systems, Minneapolis, MN), a dose representative of the pathophysiological concentration found in patient serum (3, 9, 73). Animals received rHMGB1 injection 1 h after IRI surgery.

Long-term effects. Two months after IRI surgery, 40-min ischemia with and without EP treatment, mice were killed and analyzed for long-term renal effects of blocking translocation and secretion of HMGB1 with EP. Kidneys were removed, fixed with 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA), paraffin embedded, sectioned, stained with Masson’s trichrome, and visualized by microscopy for fibrosis analysis. Serum was obtained from animals by cardiac puncture and analyzed for creatinine content. Before death, urine was obtained from the bladder using a 26-gauge needle (Becton Dickinson, San Jose, CA). Urine was analyzed for creatinine and albumin content to determine urine albumin:urine creatinine ratio (albuminuria). Urine albumin was measured using a murine microalbuminuria Elisa kit (Exocell, Philadelphia, PA). For short-term analysis, albuminuria was measured using the same kit from Exocell.

Immunohistochemical staining for HMGB1. Upon death, ischemic kidneys (40-min ischemia) were fixed by perfusion with 4% PFA. After excision, kidneys were dehydrated with 30% sucrose, frozen in O.C.T. (Tissue-Tek, Torrance, CA), and cryosectioned (10-μm-thick sections). Sections were stained for HMGB1 as previously described (6). Briefly, sections were permeabilized with 0.25% Triton X-100 in PBS-BSA (1%; Sigma), blocked with PBS-BSA (1%), and stained using rabbit polyclonal antibody to HMGB1 (Abcam) followed by AlexaFluor 594-conjugated donkey anti-rabbit (Invitrogen, Carlsbad, CA) secondary antibody, according to the manufacturer’s recommendation. Nuclei were stained by adding 10 μg/ml of DAPI for 1 min followed by washing and addition of antifade reagent in PBS (Invitrogen). Sections were imaged on an Axiosvert fluorescence microscope at ×100 with an Axioscan MRm camera using the Axiovision 4.6.3SP1 Software (Zeiss, Thornwood, NY).

Isolation of cytoplasmic and nuclear proteins. To obtain cytoplasmic and nuclear fractions from kidney tissue, Abcam’s subcellular and nuclear fractionation protocols with modifications (60) were utilized (53). In brief, samples were incubated on ice for 10 min in cytoplasmic lysis buffer [10 mM Tris·HCl (pH 8.0), 60 mM KCl, 1 mM EDTA, 1 mM DTT, protease inhibitor and 0.5% NP-40] and gently
Fig. 3. HMGB1 in kidney nuclear and cytoplasmic fractions following ischemic insult of increasing duration. HMGB1 was measured by Western blot analysis in kidney nuclear and cytoplasmic fractions after renal ischemia of 25, 40, and 55 min of IRI with (A) and without (B) EP treatment. Representative gel images and quantified data indicate HMGB1 decreased in the nucleus during ischemia with a dip in reduction occurring after 25 min of ischemia, meanwhile, HGMB1 remained elevated in the cytoplasm during all ischemic durations (A). EP treatment attenuated the nuclear reduction and cytoplasmic elevation of HMGB1 during increased ischemia length. EP treatment did not significantly reduce basal HMGB1 levels in cytoplasmic fractions of nonischemic kidneys (C). *P < 0.05 vs. control. #P < 0.05 vs. IRI + EP (same IRI duration); n = 6.
Fig. 4. Human umbilical vein endothelial cells (HUVEC) transfected with HMGB1-green fluorescent protein (GFP) plasmids were treated with H$_2$O$_2$ with/without EP. In image panels (A, C, E) of HUVEC, duration after treatment with H$_2$O$_2$ and EP is indicated in minutes. HUVEC treated with H$_2$O$_2$ demonstrated HMGB1 translocation, as indicated by increased GFP fluorescence in the cytoplasm (A). H$_2$O$_2$-induced HMGB1 translocation was inhibited when cells were treated with EP (C). HMGB1 translocation was not observed in untreated control cells (E). Resulting fluorescence intensities of images were quantified and graphed (B, D, F). Each data point within the graphs represents an average of 5 separate experiments. Trend lines demonstrate the nuclear-to-cytoplasm translocation rate of HMGB1-GFP when cells were treated with H$_2$O$_2$ (B), an effect inhibited by EP (D). *P < 0.05 vs. baseline; n = 5.
triturated using a 26-gauge needle (Becton Dickinson). After centrifugation at 2,300 rpm for 5 min at 4°C, the supernatant fraction was removed and the pellet was resuspended in nuclear lysis buffer [20 mM Tris·HCl (pH 8.0), 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 1.5 mM MgCl₂, Ultra pure water, protease inhibitor, 1 mM DTT, and 1% Triton X-100], incubated on ice for 30 min, repeatedly triturated, centrifuged at 14,000 rpm for 5 min at 4°C, and supernatant containing nuclear fraction was retained. Three Kunitz units of DNase (Qiagen, Valencia, CA) were added to nuclear fractions.

Western blot analysis. HMGB1 was examined in tissue and cell samples by Western blotting (27, 53). In brief, samples were dissolved in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA), boiled at 80°C for 10 min, and separated on 4–20% polyacrylamide Mini-Protean TGX gels (Bio-Rad Laboratories). Proteins were electrotransferred to a polyvinylidene difluoride membrane (Millipore, Medfors, MA). After being blocked with 5% wt/vol nonfat milk, membranes were incubated at 4°C for 24 h with primary antibody, followed by incubation with secondary antibody. Polyclonal rabbit antibody to HMGB1 (Abcam), monoclonal mouse antibody to β-tubulin (Sigma), and polyclonal rabbit antibody to histone (Calbiochem, Gibbstown, NJ) were used as primary antibodies, according to the manufacturer’s recommendation (including dilutions). Anti-rabbit and anti-mouse antibodies (GE Healthcare Lifesciences, Piscataway, NJ) conjugated to horseradish peroxidase were used as secondary antibodies, according to the manufacturer’s recommendation. Detection was performed using enhanced chemiluminescence (Thermo Scientific, Rockford, IL) and exposure to X-ray.

Relative protein levels were calculated as densitometric ratios to histone or β-tubulin. To ensure the purity of cytoplasmic fractions, histone antibodies were used in Western blotting of cytoplasmic fraction samples. To ensure the purity of nuclear fractions, cytoplasmic tubulin antibodies were used in Western blotting of nuclear fraction samples. Furthermore, for Western blotting of plasma samples, equal amounts of total protein (as determined by Bradford assay) of each sample were loaded onto Western blot gels and utilized as a loading control.

HMGB1-GFP plasmid transfection. Immortalized human umbilical vein endothelial cells (HUVEC; ATCC, Manassas, VA) were transfected with HMGB1-green fluorescent protein (GFP) plasmid using the Amaxa HUVEC Nucleofector Kit (Lonza, Allendale, NJ) and Amixa Biosystems Nucleofector II Transfection Unit (Lonza). In brief, cells were cultured in EB2 medium (Lonza) supplemented with 2% FBS, hydrocortisone, human basic fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor-1, ascorbic acid, human epidermal growth factor, GA-1000, heparin. HUVEC were maintained at 37°C/5% CO₂. Using the Amixa Biosystems Nucleofector II Transfection Unit (Lonza), HUVEC were transfected with HMGB1-GFP plasmid (kindly provided by Dr. Samuel H. Wilson at NIH) following the Amaxa manufacturer’s protocol. After transfections, cell suspensions were plated in glass-bottom petri dishes and cultured in EB2 medium. Following 48 h of incubation at 37°C/5% CO₂, cells were washed and treated with (a nonlethal dose of) 500 μM H₂O₂ with/without 25 mM EP (13, 14, 41, 53, 73, 74) in Krebs buffer supplemented with 15 mM HEPES and imaged using a Nikon Y-FL epifluorescence intravital microscope (Nikon, Melville, NY) equipped with an intensified CoolSNAP HQ tube camera (Photometrics, Tucson, AZ) and imaged at magnification ×600. Fluorescence.
cence intensity was quantified using MetaVue software (Universal Imaging, Downingtown, PA). Cells were maintained at 37°C using a thermal plate (Tokai Hit, Japan) during videomicroscopy imaging.

Flow cytometry analysis. Excised kidneys were homogenized with collagenase type 1 (Invitrogen) at 37°C. Kidney homogenates along with bone marrow (obtained from flushed femurs) and blood samples were filtered through a 40-μm nylon filter (Fisher Scientific, Pittsburgh, PA). Resulting cell suspensions were subject to ficoll histopaque-1077 (Sigma) density gradient centrifugation to obtain mononuclear cells. Cells were costained with rat anti-mouse CD34 (Abcam; dilution 1:100) and rabbit anti-mouse Flk-1 (Abcam; dilution 1:50), followed by FITC-conjugated goat anti-rat or R-phycoerythrin conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA; dilution 1:200). After being stained, cells were fixed with 4% PFA and subjected to FACS analysis as previously described (80). Data were acquired using a FACSscan cytometer equipped with a 488-nm argon laser and a 620-nm red diode laser and analyzed using CellQuest software (Becton Dickinson). FACSscan parameters were determined using unstained and secondary antibody-stained cells.

Cytokine/chemokine release. At designated time points, serum (collected by cardiac puncture) was evaluated for cyto-/chemokine levels using the multiplex Luminex 100 system (Luminex, Austin, TX) and an anti-mouse cyto-/chemokine panel I assay (Millipore), according to the manufacturer’s protocol. Resulting assay data were analyzed by Luminex CellQuest software (Becton Dickinson).

Statistical analysis. Data are presented as means ± SE. For multiple comparisons between groups, either a one-way ANOVA with Tukey’s posttest was performed or a two-way ANOVA with a Bonferroni posttest using NCSS 2007 statistical package (NCSS, Kaysville, UT). Differences were considered significant at $P < 0.05$.

RESULTS

We initially performed immunohistochemical studies of HMGB1 in renal tissues following ischemia (Fig. 1). In control kidneys, HMGB1 was observed in the nucleus of vascular and tubular cells. After IRI, nuclear HMGB1 was diminished as staining appeared diffused out of the nuclear compartments (while concomitantly increasing in perivascular areas), suggesting the translocation of HMGB1 out of the nucleus of renal cells during IRI injury (Fig. 1).

After observing HMGB1 and its potential nuclear-to-cytoplasmic translocation in IRI kidneys, we sought to examine 1) the release of HMGB1 into the renal venous circulation after IRI and 2) whether increasing the duration of ischemic insult is accompanied by the increased release of HMGB1 from the kidneys. After 25 min of renal ischemia, immediately after release of the vascular clamps from the renal artery/vein, HMGB1 was observed in the venous circulation exiting the kidneys while arterial levels of HMGB1 remained minimal, thus indicating the renal, and not systemic, origin of HMGB1 during IRI (Fig. 2). Increasing the duration of ischemic insult augmented the renal release of HMGB1 into the venous circulation, particularly when ischemic insult duration lasted 55 min (Fig. 2). While venous levels of HMGB1 were significantly higher than systemic arterial levels, at longer ischemic times, arterial HMGB1 increased suggesting a possibility of systemic release (Fig. 2).

EP has been reported to inhibit the release of HMGB1 in various in vivo models (13, 14, 74). When animals were pretreated immediately before renal ischemic insult, the release of HMGB1 in the renal venous circulation was significantly abrogated while arterial HMGB1 was almost completely abolished (Fig. 2).

In further analysis, IRI kidneys were homogenized, separated into nuclear-cytoplasmic fractions, and examined by
Western blot. HMGB1, initially low, increased significantly in the cytoplasmic fraction during ischemic insult, indicative of nuclear-cytoplasmic translocation (Fig. 3). HMGB1 was predominantly nuclear before injury, but already after the first 25 min of ischemic insult and at longer durations of ischemia (40 and 55 min), it was reduced drastically in the nucleus (Fig. 3A). Pretreatment of animals with EP before IRI significantly decreased HMGB1 nuclear-cytoplasmic translocation (Fig. 3B), as observed by retention of HMGB1 in the nucleus and absence of a significant increase of cytoplasmic HMGB1 (Fig. 3B). However, at 55 min of ischemia, EP blockade of HMGB1 translocation was diminished. EP pretreatment slightly decreased HMGB1 in the cytoplasm of renal cells before IRI; however, this reduction was statistically insignificant (Fig. 3C).

To confirm EP’s ability to inhibit HMGB1 nuclear-cytoplasmic translocation during IRI, we conducted in vitro studies. HUVEC were transfected with HMGB1-GFP plasmids, which allowed for visualization of HMGB1 in the nucleus of transfected endothelial cells. When these cells were subject to H2O2 (representative of oxidative stress during the reperfusion period), HMGB1 translocated from the nucleus to the cytoplasm (Fig. 4), as indicated by increased GFP in the cytoplasm. However, treatment with EP prevented the H2O2-induced translocation (Fig. 4). While HMGB1 translocation occurred more rapidly in the IRI in vivo model, the slower translocation observed in the in vitro oxidative stress HMGB1-GFP-transfected cell model may be attributed to the complex dynamics of multiple signaling events that are present in vivo during IRI, but absent in isolated in vitro experiments.

EP’s ability to block cellular HMGB1 translocation and release prompted us to examine the therapeutic effect of inhibiting HMGB1 release during IRI. At 36 h post-IRI, renal function was less impaired, as indicated by decreased serum creatinine and albuminuria (urine albumin:creatinine ratio) in animals pretreated with EP (Fig. 5). When rHMGB1 was administered at pathophysiological dose (100 ng/ml) intravenously (3, 9, 73), the renoprotective effect of EP was abrogated indicating EP’s therapeutical benefits were due to inhibition of HMGB1 release from renal cells during IRI. Interestingly, when rHMGB1 was administered to healthy animals without IRI, serum creatinine remained unchanged but albuminuria increased. While increased HMGB1 in the circulation leads to renal function impairment, its detrimental effects appear to be enhanced synergistically with other factors that are released during IRI.

The improvement in renal function afforded by EP could be mediated through attenuation of HMGB1-induced release of proinflammatory cyto-/chemokines during IRI. At 36 h post-IRI, EP treatment resulted in the reduction of circulating proinflammatory cytokines TNF-α, IL-6, and IL-1β, and proinflammatory chemokines MCP-1, KC, Rantes, and IP-10 (Fig. 6). Leukocyte-stimulating factors GM-CSF and G-CSF were also reduced in the circulation by EP (Fig. 6). In long-term studies (animals examined 2 mo after IRI), pretreatment with EP attenuated renal fibrosis (Fig. 7), serum creatinine (Fig. 8A), and albuminuria (Fig. 8B). The long-term therapeutic advantages of EP are not necessarily a reflection of EP’s effect on the underlying pathophysiology of fibrosis and progressive renal damage, but rather highlight its ability to rescue the kidney from initial damage first sustained early on during initial IRI insult preventing the progression of kidney damage and alleviating long-term impairment.

To further define the specific effects elicited by HMGB1 once it is released by injured renal cells following IRI, we introduced exogenous HMGB1 into the circulation of healthy animals. rHMGB1 was intravenously injected into mice and cyto-/chemokine release into the circulation was examined. After intravenous injection of rHMGB1, GM-CSF, IFN-γ, KC, IP-10, MIP-1α, TNF-α, and the anti-inflammatory IL-10 were all released within 1 h (Fig. 9). Three hours postinjection, eotaxin, G-CSF, and IL-12 increased in the plasma (Fig. 9). The increase in cyto-/chemokines and their effects on the kidney may contribute to the albuminuria we observed in healthy mice 36 h after rHMGB1 intravenous injection (Fig. 5). Following rHMGB1 injection, the mobilization of CD34+/Flk-1, presumably EPC (4, 24, 30, 57, 81), from the bone marrow into the circulation was also examined by FASC analysis. rHMGB1 injection resulted in upregulated mobilization of EPC from the bone marrow 1 h after injection (Fig. 10). EPC were significantly elevated in the peripheral blood, depleted from the bone marrow, and progressively accumulated in the spleen up to 6 h postinjection (Fig. 10). This would suggest once HMGB1 is released from renal cells during IRI, circulating HMGB1 signals the bone marrow to mobilize EPC into the circulation for possible regenerative potential.

**DISCUSSION**

The pathophysiological process of IRI is mediated by hypoxic organ damage that is accentuated following the return of
blood flow and oxygen delivery by inflammation and oxidative damage (68). The initiators that lead to propagation of the inflammatory response and ensuing damage are the focus of intense investigation. Recent reports have identified the DAMP alarmin HMGB1 as one of these propagation initiators that exacerbate the pursuing signal cascade thus promoting further tissue damage after ischemic insult (12, 13, 37, 40, 53, 78). Originally, HMGB1 was shown to be released by monocytes/macrophages, but more recently, other cell types have been shown to release HMGB1 upon stimulation including endothelial cells (15, 21, 22, 45, 53) and perhaps smooth muscle cells (52). Immunohistochemical analysis here indicated a redistribution of HMGB1 in vascular and tubular renal cells upon IRI preceding its release into the venous circulation (Fig. 1). However, the extent of HMGB1 release from renal cells during IRI and its ability to promote damage/repair after release remain to be fully examined.

It is known that HMGB1 can be released from cells by either an active regulated process or passively during cell necrosis. Hypoxia alone leads to minimal renal cell death during the first few hours of ischemia-reperfusion (61), therefore suggesting that release of HMGB1 from renal cells occurs actively from live cells. Relevant to our study here on renal IRI, Tsung et al. (72) showed that oxidative stress leads to HMGB1 release by hepatocytes in the absence of cell death and that production of reactive oxygen species (ROS) and subsequent HMGB1 release by hypoxic hepatocytes required intact TLR4 signaling, downstream calcium/calmodulin-dependent kinases, with release of HMGB1 parallel to the extent of oxidant production. Similarly, our previous studies indicated that the rise in circu-

Fig. 9. Effects of circulating HMGB1 on chemokine/cytokine release. rHMGB1 was intravenously injected into animals and chemokine/cytokine release into the circulation was measured at varying time points after injection by a Luminex technology. Within 1 h after HMGB1 administration, GM-CSF, IFN-γ, IL-8, IP-10, MIP-1α, and IL-10 increased in the circulation. After 3 h, eotaxin, G-CSF, and IL-12 were also elevated in the circulation. *P < 0.05 vs. all groups. †P < 0.05 vs. control. #P < 0.05 vs. control and 1 h. ‡P < 0.05 vs. control and 6 h; n = 6.

AJP-Renal Physiol • doi:10.1152/ajprenal.00092.2012 • www.ajprenal.org
lating uric acid that accompanies IRI leads to HMGB1 translocation in endothelial cells, a process that requires an increase in intracellular calcium and the MEK/Erk pathway (53). The importance of calcium is further evident by studies from other labs that showed HMGB1 active release to involve calcium/calmodulin interaction and calcium-dependent secretory pathways involving CRM1, an interaction further stimulated by ROS (15, 23, 28, 29, 32, 51, 53, 64, 72, 85). In addition, the active release of HMGB1 during IRI appears to involve other signaling cascades in nonnecrotic cells including acetylation and possible phosphorylation of HMGB1 (9, 27, 53), a process that seemingly requires a reduction in deacetylase activity in the nucleus (17).

While HMGB1 is released by cells during periods of hypoxia and oxidative stress (71), particularly the kidney (12, 13, 37, 40, 78), the detailed time course of its release remains unexamined. Results here demonstrate that the duration of the ischemic episode applied to the kidneys determines the extent of HMGB1 release (Fig. 2), and therefore suggest that HMGB1 release is linear with the time of ischemia, rather than “yes-or-no” response. Furthermore, during IRI the kidney (as opposed to systemic sources) is the primary source of HMGB1 release into the circulation. Moreover, the ischemic kidney does not require a substantial reperfusion period for significant HMGB1 release to occur from renal cells (Figs. 2 and 3).

EP is a pharmacological inhibitor of HMGB1 secretion (13, 14, 18, 31, 41, 53, 59, 62, 73, 74, 82); however, the precise mechanism(s) that mediate its inhibitory effects have not been identified as of yet. Treatment with EP has been shown to improve survival and alleviate organ dysfunction in a wide variety of preclinical models of critical illness (19, 20) including the amelioration of IRI in organs (13, 34, 77, 84) including the kidney (13). For instance, Chung et al. (13) demonstrated that EP improved serum creatinine after IRI in rats, although HMGB1 mRNA expression remained unchanged. Here, we build on the findings by Chung et al. and show in both renal in vivo (Fig. 3) and endothelial cell in vitro (Fig. 4) models that EP specifically prevents HMGB1 nuclear-cytoplasmic translocation during insult. Moreover, we confirm that therapeutic efficacy of EP is due to its ability to prevent HMGB1 release during IRI, because renoprotective effects of EP treatment can be overcome by intravenous injection of rHMGB1 during IRI (Fig. 5).

EP’s initial prevention of HMGB1 release during IRI resulted in improved short-term (Fig. 7) and subsequent long-term renal function (Fig. 8). The improvement in long-term renal function and fibrosis is most likely attributed to the reduction in the initial renal damage sustained immediately after IRI. Another key contributor to renal damage after IRI is the proinflammatory response of cytotoxic/chemokine release. Other laboratories showed that pretreatment with EP significantly attenuates the release of TNF-α, IL-6, and MCP-1 after IRI (13, 78). Our study confirms and expands these findings as we observed EP treatment reduces the secretion of proinflammatory cytokines TNF-α, IL-6, IL-1β, and chemokines including leukocyte colony-stimulating GM-CSF, G-CSF, and chemotactic MCP-1, KC, Rantes, and IP-10. Reducing the release of these cytokines leads to a reduction of infiltrating leukocytes in the IRI kidney, thus potentially improving short- and long-term renal function. The improvement in renal outcome associated with EP administration is similar to the beneficial effects observed when HMGB1 neutralizing antibody is administered during IRI, as demonstrated by Li et al. (37) when proinflammatory TNF-α release, serum creatinine, BUN, neutrophil infiltration, and tubular injury were reduced. However, the use of EP to alleviate renal damage is much more attractive because it can be safely used in the clinical setting.

During IRI, a plethora of both long- and short-term damage-signaling molecules released by the injured kidney may mask HMGB1’s ability to independently promote the release of cytotoxic/chemokines into the circulation. To address this, rHMGB1 at a concentration (100 ng/ml) commensurate with plasma levels during insult (3, 9, 76) was intravenously injected into healthy animals and the cytotoxic/chemokine response was examined (Fig. 9). While other labs observed an increase in secretion of TNF-α, IL-8, and G-CSF in in vitro cell models in response to rHMGB1 treatment (41, 45), we expand on these findings and show that in vivo administration of rHMGB1 increases within hours the secretion into the circulation of eotaxin, C-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-6, IL-10, IP-10, KC, MIP-1α, Rantes, and INF-α. Results from experiments here provide evidence of HMGB1’s ability to directly stimulate release of an array of proinflammatory molecules.

HMGB1’s systemic effects are not limited to proinflammation, but also appear to mediate repair/regenerative responses. Reports have identified HMGB1 to be a strong chemoattractant and a proliferative molecule for vessel-associated stem cells (mesangioblasts) (46). For instance, Chavakis et al. (10) reported that HMGB1 stimulates EPC homing to ischemic tissues. In a study by Limana et al. (38), after myocardial infarction, direct delivery of HMGB1 to the peri-infarcted tissues. In a study by Limana et al. (38), after myocardial infarction, direct delivery of HMGB1 to the peri-infarcted tissues resulted in the formation of new monocytes within the infarcted portion of the wall. The regeneration of heart tissue in the Limana et al. study involved the proliferation and differentiation of endogenous cardiac c-kit+ progenitor cells (38). Kohno et al. (35) supported Limana and colleagues’ reports in which blockade of HMGB1 after postmyocardial infarction resulted in impairment of the infarct healing process and...
marked hypertrophy of the noninfarcted surrounding areas. HMGB1 has also been found to be involved in the healing of other injured tissues, such as skin wounds (54, 55, 65). Tamai et al. (65) demonstrated HMGB1 has the ability to mobilize PDGF-Rα-positive (and lineage negative) cells from the bone marrow to regenerate injured skin epithelial cells. In addition, HMGB1 has been reported to have proangiogenic activity (8, 42, 58, 75).

The ability of HMGB1 to promote proliferation, migration, and differentiation of several cell types, particularly those involved in angiogenesis, prompted us to examine the ability of HMGB1 to mobilize CD34+/Flk-1+ (presumably EPC) (4, 24, 30, 50, 57, 81) from their bone marrow storage pools. We observed significant release of bone marrow-derived CD34+/Flk-1+ into the circulation within hours after HMGB1 was infused into the circulation. However, without a homing signal for circulating CD34+/Flk-1+, they are sequestered by the spleen. Our finding supports the idea that HMGB1 has activity that can mobilize stem cells to potentially aid in tissue repair and regeneration after injury. However, in the presence of IRI, the deleterious proinflammatory effects of HMGB1 dominate its preregenerative properties.

In summary, the present study shows that HMGB1 translocates from the nucleus to the cytoplasm in renal cells and is then released into the venous circulation by the kidneys during IRI. Importantly, the extent of HMGB1 release is proportional to the duration of ischemic insult with longer ischemic episodes causing greater amounts of HMGB1 to be released into the venous circulation. While the therapeutic benefit of EP administration during renal IRI has been previously reported, we demonstrate that its therapeutic efficacy is abolished by rHMGB1 administration indicating that EP’s ability to prevent HMGB1 translocation and release into the circulation during IRI mediates its renoprotective mechanism. This finding was supported in vitro when EP treatment prevented HMGB1 translocation from endothelial cells subject to oxidative stress. Moreover, blocking HMGB1 release with EP during IRI improves short- and subsequently long-term renal function, a finding comparable to that observed by other laboratories using neutralizing antibody to HMGB1 but with more clinical potential. Furthermore, we were able to show the details of the cellular effect of HMGB1 once it is released in the circulation. HMGB1 induces various cyto-/chemokines secretion and also leads to the mobilization of CD34+/Flk-1+ from the bone marrow.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

F884
HMGB1 DURING RENAL ISCHEMIA


63. Stros M, Bernues J, Querol E. Calcium modulates the binding of high-mobility-group protein 1 to DNA. Biochem Int 21: 891–899, 1990.


