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Ghrelin Protects against Experimental Sepsis by Inhibiting High-Mobility Group Box 1 Release and by Killing Bacteria[1,2]

Alejo Chorny, Per Anderson, Elena Gonzalez-Rey, and Mario Delgado[3]

Sepsis, a life-threatening complication of infections and the most common cause of death in intensive care units, is characterized by a hyperactive and out-of-balance network of endogenous proinflammatory cytokines. None of the current therapies are entirely effective, illustrating the need for novel therapeutic approaches. Ghrelin (GHR) is an orexigenic peptide that has emerged as a potential endogenous anti-inflammatory factor. In this study, we show that the delayed administration of GHR protects against the mortality in various models of established endotoxemia and sepsis. The therapeutic effect of GHR is mainly mediated by decreasing the secretion of the high mobility box 1 (HMGB1), a DNA-binding factor that acts as a late inflammatory factor critical for sepsis progression. Macrophages seem to be the major cell targets in the inhibition of HMGB1 secretion, in which GHR blocked its cytoplasmic translocation. Interestingly, we also report that GHR shows a potent antibacterial activity in septic mice and in vitro. Remarkably, GHR also reduces the severity of experimental arthritis and the release of HMGB1 to serum. Therefore, by regulating crucial processes of sepsis, such as the production of early and late inflammatory mediators by macrophages and the microbial load, GHR represents a feasible therapeutic agent for this disease and other inflammatory disorders. ***The Journal of Immunology,*** **2008, 180: 8369–8377.**

Sepsis is the third leading cause of death in developed societies and the most common cause of death in many intensive care units. Despite extensive research on the pathophysiology of sepsis and the technical advances, sepsis incidence is constantly rising (1.5–8% per year), and the increase in survival statistics of septic patients are merely due to the improvements in supportive treatment (1). The pathogenesis of sepsis is characterized by overwhelmed inflammatory and immune responses that can lead to tissue damage, multiple organ failure, and death. Most therapeutic strategies have targeted proinflammatory mediators, but they did not improve survival of patients when studied in large multicenter clinical trials (2). Therapies designed to block one single cytokine, such as TNF-α and IL-1β, have shown limited efficacy probably due to the early and transient kinetic of these inflammatory cytokines. In the last years, different evidences indicate that the high mobility group box 1 (HMGB1)[4] is a necessary and sufficient late mediator of severe sepsis and, therefore, its targeting provides a wider window for clinical intervention (3).

Institute of Parasitology and Biomedicine, Consejo Superior de Investigaciones Cientificas, Granada, Spain

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[2] A.C. and M.D. designed research, performed research, and wrote the paper. E.G.-R. and P.A. performed research.

[3] Address correspondence and reprint requests to Dr. Mario Delgado, Instituto de Parasitologia y Biomedicina, Consejo Superior de Investigaciones Cientificas, Avenida Conocimiento, PT Ciencias de la Salud, Granada 18100, Spain. E-mail address: mdelgado@ipb.csic.es

[4] Abbreviations used in this paper: HMGB1, high mobility group box 1; GHR, ghrelin; CLP, cecal ligation and puncture; CIA, collagen-induced arthritis; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid) trimethine oxonol; FAM-GHR, carboxyfluorescein-labeled GHR.

Originally described as a nuclear protein that bends DNA, HMGB1 was recently found to be secreted by activated monocytes and macrophages (4), acting as a late proinflammatory factor (5) and promoting epithelial cell dysfunction (6). High levels of systemic HMGB1 are present in humans and animals with sepsis or endotoxemia (4, 7). Administration of recombinant HMGB1 to mice causes gut barrier dysfunction and lethal multiple organ damage (4, 6). In addition, passive immunization with neutralizing Abs against HMGB1 improves survival and prevents organ failure in septic mice (8, 9). Although the secretion of HMGB1 might be critical for the successful elimination of pathogens, it needs to be limited, because its excessive release results in severe inflammation and collateral tissue damage. In general, inflammatory responses are self-controlled by anti-inflammatory mediators secreted by host innate immune system during the ongoing process, but the endogenous factors involved in the control of HMGB1 secretion are poorly known.

Classically considered as neuroendocrine mediators, certain neuropeptides and hormones are also produced by immune cells and have recently emerged as potent endogenous anti-inflammatory factors involved in the control of the immune homeostasis (10). Ghrelin (GHR) is a 28-aa acylated polypeptide mainly produced by stomach cells, initially identified as an endogenous circulating ligand for the growth hormone secretagogue receptor, has potent orexigenic actions and is involved in the control of growth hormone secretion, energy expenditure, and adiposity (11). GHR and its receptors were recently detected in immune cells (12), indicating that this peptide may play a role in the immune system. Indeed, GHR has been described as a potential anti-inflammatory peptide that inhibits the production of proinflammatory cytokines by activated human T cells, monocytes, endothelial cells, and by animals with septic shock (12–14). In addition, GHR delays the onset, decreases the frequency and reduces the disease severity and mortality in experimental colitis, down-regulating both inflammatory and Th1-driven autoimmune responses (15).

In this study, we demonstrate that GHR is an endogenous inhibitor of HMGB1 release. Consistently, delayed administration of GHR attenuates the circulating levels of HMGB1, rescues from lethal experimental sepsis and attenuates the severity in arthritis. Notably, administration of recombinant HMGB1 to GHR treated mice reversed the therapeutic effect of the neuropeptide. Interestingly, this anti-inflammatory effect is accompanied by a potent bactericidal effect of GHR. In vitro and ex vivo studies indicate that macrophages are the major cell targets of GHR in the inhibition of HMGB1 secretion.

Materials and Methods

Animal models

Animal experimental protocols were reviewed and approved by the Ethical Committee of the Spanish Council of Scientific Research. To induce endotoxemia, BALB/c mice (6–8 wk old; The Jackson Laboratory) were injected i.p. with different amounts (25–600 μg/mouse; 400 μg/mouse unless otherwise indicated) of LPS (Sigma-Aldrich), or with a bacterial suspension containing 10^8 live *E. coli* (DH5α). To induce sepsis, cecum of anesthetized BALB/c mice was ligated 5.0 mm from the cecal tip and punctured once with a 22-gauge needle, and the stool was then extruded (1 mm). Vehicle (controls) or GHR (American Peptides) were administered i.p. starting at 12 or 24 h (at 12, 18, 24, 36, 48, and 72 h) after cecal ligation and puncture (CLP), 2 h (a single administration) after *E. coli* injection or at different times (a single administration) after LPS infusion. The effective concentrations of GHR used in the study were chosen based in previous experiments performed in our laboratory. In some experiments, recombinant HMGB1 (100 μg/mouse; HMGBiotech) was administered i.p. in GHR-treated animals 18 h after CLP. Animals were monitored daily for survival and clinical signs. Sera were obtained at different time points by cardiac puncture.

To induce collagen-induced arthritis (CIA) DBA/1J mice (7–10-wk-old; The Jackson Laboratory) were injected s.c. with 200 μg of type II collagen (Sigma-Aldrich) emulsified in CFA containing 200 μg of *M. tuberculosis* H37 RA (Difco). At day 21 after primary immunization, mice were boosted s.c. with 100 μg of type II collagen in CFA. GHR treatment consisted in the administration i.p. of GHR (1 nmol) on five consecutive days starting at day 24 when all mice showed established arthritis (clinical score >2). Control group was injected i.p. with PBS (untreated). Mice were analyzed by two blinded examiners every other day and monitored for signs of arthritis onset measuring the clinical score as described (16). Serum samples were collected at day 35 postimmunization for HMGB1 determination.

Cell culture

BALB/c peritoneal macrophages or RAW264.7 cells were cultured at 10^6 cells/ml in RPMI medium 1640 (with 10% heat-inactivated FBS, 2 mM glutamine, and antibiotic-antimycotic mixture) for 2 h, washed with Opti-MEM (Invitrogen) 2 h later, and stimulated for 24 h with LPS in the presence or absence of GHR in Opti-MEM. Supernatants were assayed for cytokine contents and precipitated with trichloroacetic acid for HMGB1 determination.

Cytokine and NO determination

Cytokine contents in sera and culture supernatants were determined by Multiplex assay (Bio-Rad) or by a specific sandwich ELISA by using capture/biotinylated detection Abs (BD Pharmingen) following the manufacturer's recommendations. NO amounts were estimated from the accumulation of the stable NO metabolite nitrite by the Griess assay. Serum amyloid A levels in sera were determined by ELISA (Tridelta).

HMGB1 Western blot analysis

Serum was filtered and concentrated through Centricon YM-100 and YM-10 (Millipore), respectively. Proteins in concentrated sera and cell culture supernatants were separated on 12% SDS-polyacrylamide gels and transferred to Immuno-blot membranes. Blots were blocked with 5% dry milk in PBS-Tween 20, incubated with a rabbit anti-HMGB1 Ab (1/5000; BD Pharmingen) and with a peroxidase-conjugated anti-rabbit Ab (Dako-Cytomation), and developed with ECL plus substrate (Amersham). HMGB1 expression was expressed as densitometric units relative to CLP, LPS, or CIA control samples on the same blot.

Antimicrobial assays

To determine the antimicrobial activity of GHR in vivo, peritoneal lavage fluid recovered from mice 24 h post-CLP was analyzed for bacterial CFU/ml by dilution plating onto trypticase soy agar with 5% sheep blood (BD Biosciences) and colony counting following overnight incubation.

To determine the bactericidal activity of GHR in vitro, 10^4 *E. coli* (DH5α, mid-exponential growth phase) were incubated with different concentrations of GHR (added at time 0 and 3h) for 6 h at 37°C with shaking. After stopping the reaction with PBS at 4°C (dilution 1/100), the samples were plated, incubated overnight at 37°C, and the number of CFU/ml calculated.

To analyze membrane potential and permeability, 5×10^5 *E. coli* were incubated with 5 μM of GHR (added twice in an interval of 30 min) for 1 h at 37°C with shaking and then stained either with propidium iodide (PI, 10 μg/ml; Sigma-Aldrich) or bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($(DiBAC_4(3))$, 1 μg/ml; Molecular Probes) for 10 min at room temperature. After incubation, cells were washed and analyzed on a FACSCalibur flow cytometer (BD Biosciences). To determine the binding of GHR to the bacteria, 5×10^5 *E. coli* cells were incubated with GHR (4 μM or 9 μM) plus carboxyfluorescein-labeled GHR ((FAM-GHR), 1 μM; Phoenix Pharmaceuticals) for 30 min at 37°C with shaking. After incubation, cells were washed, immobilized on poly-L-lysine-covered glass slides for 1 h at 4°C, mounted in Vectashield medium (Vector Laboratories) and visualized by confocal microscopy (LEICA TCS SP5). Alternatively, FAM-GHR treated cells were analyzed by flow cytometry.

Immunofluorescence

Macrophages adhered to coverslides were fixed in 4% paraformaldehyde for 20 min at room temperature and then incubated with glycine 30 mM for 5 min. After washing three times with PBS, cells were permeabilized with 0.2% Triton X-100 for 15 min, and blocked with 2% BSA for 1 h. Cells were incubated with rabbit anti-HMGB1 Ab (dilution 1/2000 in PBS/2% BSA/0.1% Tween 20) for 12 h at 4°C. Slides were then washed and incubated with FITC-labeled goat anti-rabbit Ab (1/500; Molecular Probes). After extensive washing, samples were mounted in DAPI-containing Vectashield medium and acquisition was performed with a microscope system (Cell R IX81; Olympus).

Results

Late administration of GHR rescues from sepsis lethality and inhibits HMGB1 release

Different evidences have suggested the role of GHR in inflammation (12, 13, 15). Wu and coworkers (14, 17) have recently shown that GHR protects rats from experimental sepsis by down-regulating the production of some inflammatory cytokines. We first confirmed the therapeutic action of GHR in systemic inflammation and septic shock syndrome by using a model of endotoxemia induced by high-dose of endotoxin and of bacterial peritonitis by injection of *E. coli*. Early administration of GHR protected against septic death caused by a wide range of bacterial endotoxin LPS doses and 10^8 live *E. coli* (Fig. 1, *A* and *B*). The GHR effect on LPS-induced endotoxemia was dose-dependent, showing maximal protection at doses between 1 and 5 nmol (survival for 5, 1, 0.5, and 0.1 nmol were 80, 80, 50, and 10%, respectively). The protective effect of GHR on endotoxemia seems to be mediated through the regulation of the secretion of a wide array of inflammatory mediators, including several cytokines and chemokines, NO, and an acute phase protein (Fig. 1*C*). Because the production of inflammatory cytokines occurs in a rapid sequence starting with TNF-α, which reaches a maximum 2 h after LPS infusion, we next asked whether delayed administration of GHR could still protect in conditions of already established endotoxemia. Kinetic studies show that GHR exerts a full protective effect when administered 4 h after LPS injection (Fig. 2*A*). Even when the treatment was delayed until 12 h following the endotoxemia induction, GHR retained significant protective action (Fig. 2*A*). Notably, most of the inflammatory mediators returned to baseline at the time of GHR administration (Fig. 1*C*). Thus, the inhibitory effect of GHR on the release of the panel of early inflammatory mediators could only

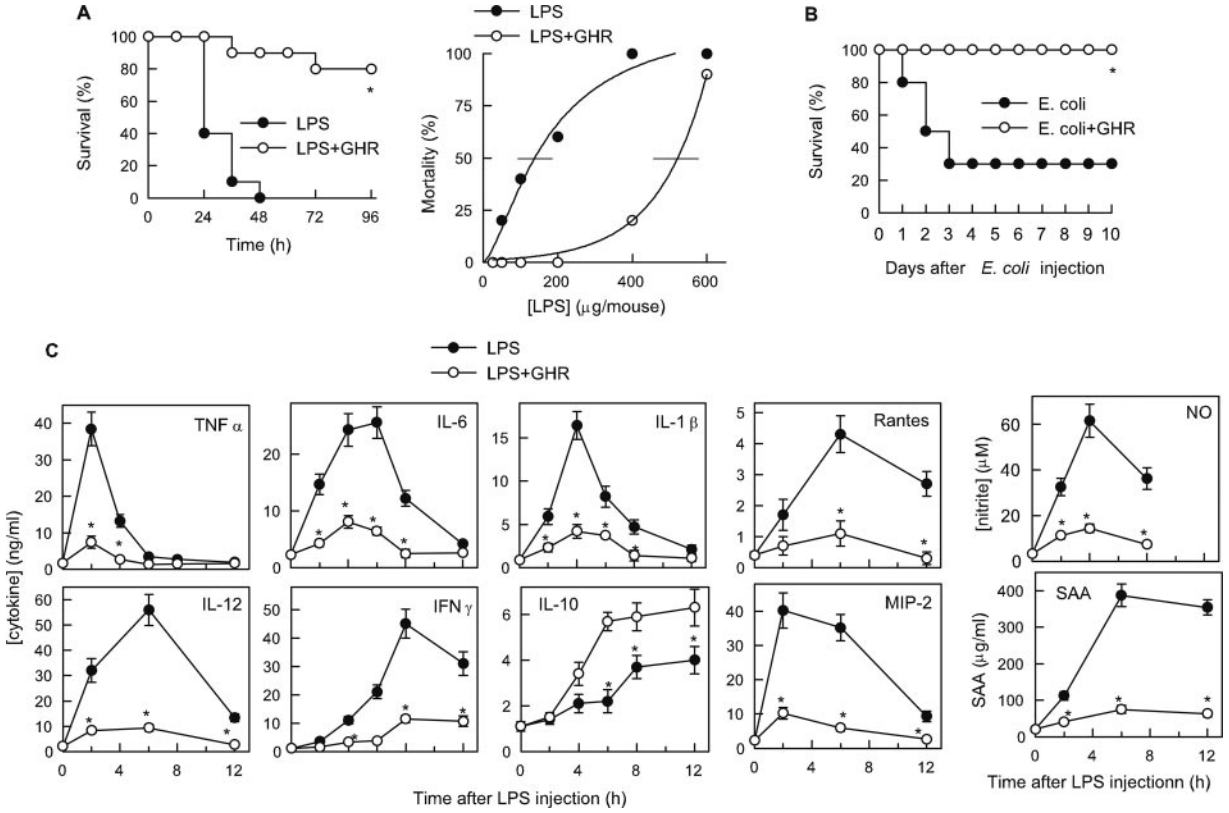

FIGURE 1. GHR protects against endotoxemia and *E. coli* induced lethality. *A*, Mice were injected i.p. with different doses of LPS (400 μg/mouse *in left panel*). GHR (1 nmol/mouse) was injected i.p. 30 min after LPS administration. Survival was monitored during the next 96 h. *Right panel*, Mortality curves were used to calculate LD_{50}, and horizontal bars indicate the 95% confidence limits of LD_{50} determinations. GHR treatment shifted the LD_{50} from 150 to 450 μg LPS. *B*, GHR protects against lethality induced by i.p. injection of 10^8 live *E. coli*. GHR (1 nmol/mouse) was injected 2 h after bacterial injection. $n = 10$–20 mice/group. *, $p < 0.001$ vs untreated control mice (two-tailed Fisher's exact test). *C*, GHR treatment reduces the systemic levels of early inflammatory mediators in endotoxemic mice. Endotoxemia was induced by i.p. injection of LPS (400 μg/mouse). Mice were treated 30 min later with medium (controls) or with GHR (1 nmol/mouse). Serum was collected at various time points after endotoxin injection. Serum cytokine and chemokine contents were determined by ELISA and NO levels were estimated from the accumulation of the stable NO metabolite nitrite by the Griess assay. Serum amyloid A (SAA) contents were determined by ELISA. $n = 5$–8 mice/group. Data are mean ± SD. *, $p < 0.001$ vs untreated control mice (Mann-Whitney *U* test).

explain the therapeutic effect of GHR in a short therapeutic window. However, the significant therapeutic effect shown by GHR in established endotoxemia could only be sustained by a potential effect on late mediators. Therefore, we next investigated whether GHR could attenuate circulating HMGB1 levels during endotoxemia. Consistent with previous observations (4), endotoxin administration resulted in increased systemic levels of HMGB1 (Fig. 2*B*). Treatment of endotoxemic mice with GHR reduced the secretion of HMGB1 into the circulation (Fig. 2*B*). Taken together, these data indicate that early administration of GHR prevents endotoxin-induced lethality by attenuating the release of both early and late inflammatory mediators.

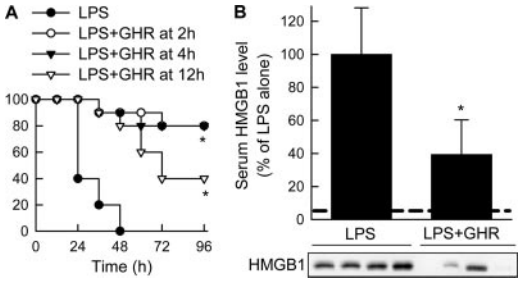

FIGURE 2. Delayed GHR treatment protects against established endotoxemia and reduces the systemic levels of HMGB1 levels. *A*, Endotoxemia was induced by i.p. injection of LPS (400 μg/mouse). GHR (1 nmol/mouse) was injected i.p. at 2, 4, or 12 h after LPS challenge. Survival was monitored during the next 96 h. $n = 10$ mice/group. *, $p < 0.001$ vs untreated control mice (two-tailed Fisher's exact test). *B*, Mice were injected with LPS (100 μg/mouse) and 30 min later with medium or with GHR (1 nmol/mouse). Serum was collected 24 h after endotoxin administration and circulating HMGB1 levels were determined by Western blot, and expressed as band densities relative to control samples (LPS alone) on the same blot. Dashed line represents basal serum HMGB1 levels in normal mice. Data are mean ± S.E.M. $n = 7$–10/group *, $p < 0.05$ vs LPS alone (Mann-Whitney *U* test).

There is general agreement among investigators that endotoxin administration may represent a model of systemic inflammation or endotoxic shock but not sepsis (2). On the contrary, the CLP model of peritonitis is considered for many researchers to be the most reliable animal model for human sepsis and a critical preclinical test for any new treatment of severe sepsis (18, 19). For example, TNF-α-blockade therapies were efficient in lethal endotoxemia and *E. coli* injection models (20–22), but failed in septic humans and CLP mice (23, 24). Sepsis induction by CLP results in increased circulating HMGB1 levels, with a peak at ~18 h after surgery (8). By contrast, other proinflammatory cytokines, such as TNF-α and IL-1β, reach their peak in the first hours of disease progression. Because GHR inhibits HMGB1 secretion in endotoxemia, we investigated whether GHR could improve sepsis survival. We induced sepsis by CLP and started GHR treatment 12 or 24 h after CLP procedure, i.e., after the early mediators' peak. GHR administration to mice

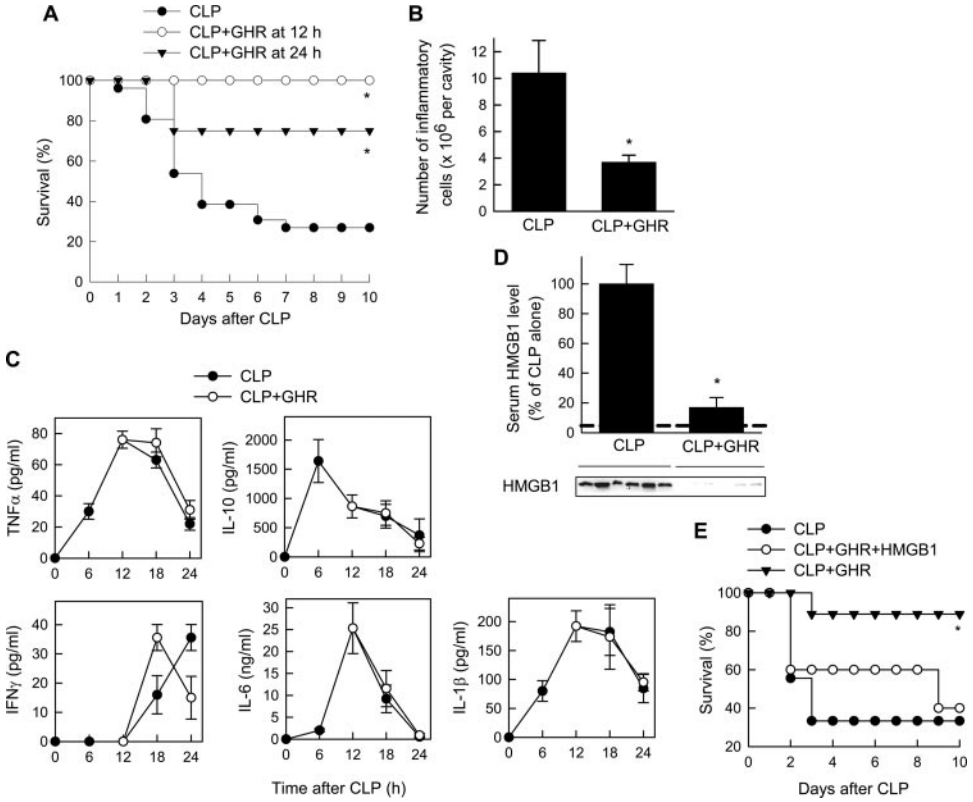

FIGURE 3. GHR improves survival and inhibits HMGB1 release in sepsis. *A*, Sepsis was induced by CLP. GHR treatment (1 nmol GHR/mouse at 12, 18, 24, 36, 48, and 72 h) was started 12 or 24 h later and survival was monitored for 10 d. $n = 20$/group. *, $p < 0.01$ vs CLP (two-tailed Fisher's exact test). *B*, GHR reduces leukocyte recruitment to the peritoneal cavity. Mice were subjected to CLP and treated i.p. with GHR (1 nmol/mouse) 12 h later. Peritoneal lavage was obtained 18 h after sepsis induction the number of inflammatory cells determined. Data are mean ± SEM. $n = 10$/group. *, $p < 0.001$ vs CLP alone (Mann-Whitney *U* test). *C*, Mice were subjected to CLP and treated with vehicle or with GHR (1 nmol) at 12 and 18 h after sepsis induction. Serum was collected at different times after CLP and the levels of different cytokines determined as described in *Materials and Methods*. Data are mean ± SEM. No significant differences were found between untreated or GHR-treated mice. $n = 5$–10 mice/point. *D*, Mice were subjected to CLP and GHR treatment was started 12 h later. Serum was collected 24 h after sepsis induction and the HMGB1 levels were determined by western blot and expressed as band densities relative to control samples (CLP alone) on the same blot. Dashed line represents basal serum HMGB1 levels in normal mice. Data are mean ± SEM. $n = 16$/group. *, $p < 0.001$ vs CLP alone (Mann-Whitney *U* test). *E*, HMGB1 reversed the therapeutic effect of GHR in sepsis. Sepsis was induced by CLP and mice were treated with vehicle (CLP) or with GHR (1 nmol/mouse) at 12, 18, 24, and 36 h after sepsis induction. Recombinant HMGB1 (100 μg/mouse) was added to GHR-treated animals at 18 h after CLP. Survival was monitored for 10 days. *, $p < 0.05$ vs CLP alone and vs HMGB1-treated animals (two-tailed Fisher's exact test). $n = 5$–10 mice/group.

with severe sepsis completely prevented the mortality caused by the cecal perforation (Fig. 3*A*). GHR treatment showed a wide therapeutic window, because even when the initiation of the treatment was delayed to 24 h after the induction of sepsis, GHR increased the survival from 27% to 75% (Fig. 3*A*). The delayed administration of GHR also attenuated the clinical signs of sepsis, including lethargy, diarrhea, huddling, and piloerection, and reduced the histopathological signs of the disease, such as massive accumulation of leukocytes in the peritoneum (Fig. 3*B*) and intestinal tract and in the gut and liver serosas, as well as segmental ischemia of the bowel with regions of hemorrhage, loss of intestinal crypts and intestinal wall perforation (data not shown). Previous studies and the present work (Fig. 1) showed that early administration of GHR prevent lethal endotoxemia by down-regulating a wide spectrum of inflammatory mediators. However, delayed administration of the neuropeptide in animals with severe sepsis did not significantly affect the systemic levels of proinflammatory (TNF-α, IL-1β, and IL-6) and anti-inflammatory (IL-10) cytokines (Fig. 3*C*). These data suggest that the therapeutic effect of GHR in established sepsis is mediated by the control of a late mediator other than the early inflammatory cytokines. Western blot analysis of the serum proteins showed increased HMGB1 levels in mice with sepsis (Fig. 3*D*). GHR treatment significantly reduced the circulating levels of HMGB1 (Fig. 3*D*). Moreover, administration of recombinant HMGB1 partially reversed the therapeutic effect GHR in CLP-induced sepsis (Fig. 3*E*). Taken together these results indicate that GHR rescues mice from septic death by down-regulating the release of HMGB1.

GHR improves bacterial clearance in vivo and shows bactericidal properties in vitro

CLP leads to a focal infection that subsequently becomes systemic and persistence of the local bacterial infection plays a critical role in sepsis mortality. Indeed, antibiotics can improve survival in murine sepsis induced by CLP (25). To evaluate whether GHR treatment influences the natural response against infection, we determine the bacterial levels at the primary peritoneal site of infection of septic mice. Contrary to the expected results, septic mice treated with GHR had lower peritoneal bacterial counts than septic mice treated with vehicle (Fig. 4*A*). This result suggests that GHR promotes bactericidal activities or that GHR is itself an antimicrobial peptide.

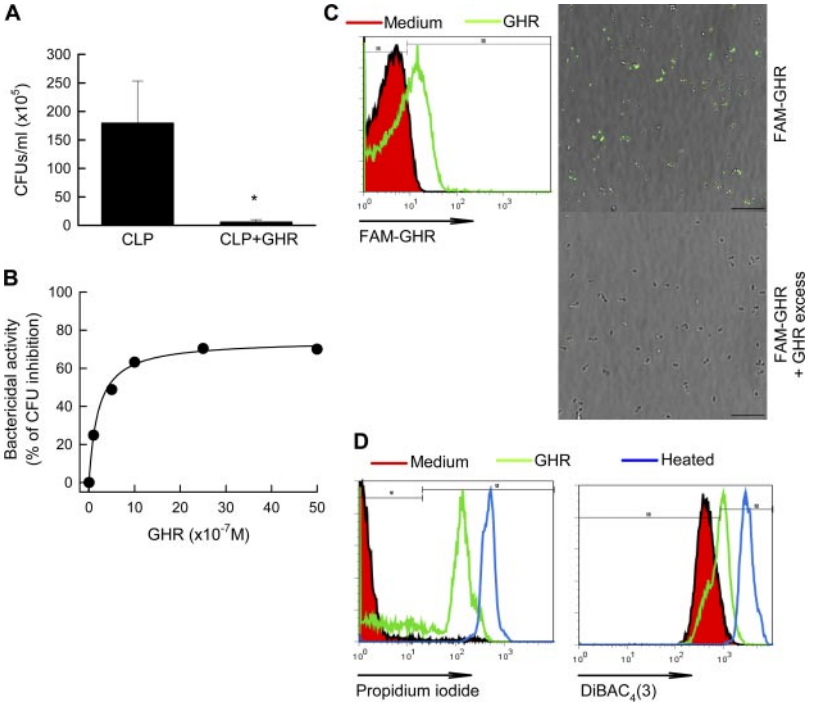

FIGURE 4. GHR improves bacterial clearance in vivo and shows bactericidal properties in vitro. *A*, Sepsis was induced by CLP, and mice were treated with GHR (1 nmol GHR/mouse at 12 and 18 h after CLP). Peritoneal lavage was obtained 24 h after surgical procedure, plated and incubated at 37°C, and the number of CFU/ml was counted. Data are mean ± SEM. $n = 16$/group. *, $p < 0.0005$ vs CLP alone (Mann-Whitney *U* test). *B*, Bactericidal activities of GHR against *E. coli* (DH5α). Each point represents the mean of triplicate viable counts determined as described in *Materials and Methods*, and expressed as 100 - the percentage of number of CFU/ml in control samples without neuropeptide. *, $p < 0.001$ (Student's *t* test). *C*, GHR binding to *E. coli*. Bacteria were incubated with medium (red histogram) or FAM-GHR (1 μM, green histogram) for 30 min and analyzed by flow cytometry and visualized by confocal microscopy. In binding competition assays, the addition of an excess of unlabeled GHR (9 μM) significantly reduced the number of FAM-GHR$^+$ cells while it increased the fluorescence background in the medium (bottom micrograph). Results are representative of three experiments. Bars in microscopy correspond to 10 μm. *D*, GHR treatment results in disruption of bacterial membrane. *E. coli* were cultured with medium (red histogram) or GHR (5 μM, green histogram) for 1 h. The membrane integrity and potential were indirectly determined by flow cytometric analysis of the incorporation of propidium iodide and DiBAC$_4$(3), respectively. *E. coli* heated at 80°C for 5 min (blue histogram) were used as controls of bactericidal activity (99.5% cells were positive for propidium iodide and DiBAC$_4$(3) staining). Results are representative of three experiments.

Antimicrobial peptides are integral components of the innate immune system and have a central role in host defense against infections. It has been recently described that some neuropeptides may participate in the immune defense against infections by directly killing bacteria (26). Because septic mice treated with GHR have decreased number of bacteria in the peritoneal fluid, we assessed the direct bactericidal activity of GHR in vitro, by incubating Gram-negative bacteria (*E. coli*) with different concentrations of GHR. GHR showed potent bactericidal properties against *E. coli* (Fig. 4*B*). This effect was dose dependent with a 75% of bacteria killed at 2.5 μM. The lack of recovery of bacterial viability after removal of GHR would indicate that the neuropeptide is bactericidal as opposed to bacteriostatic. We next investigated potential mechanisms involved in the bactericidal effect of GHR. It has been proposed that cationic antimicrobial peptides target the bacteria through electrostatic interactions with the microbial membrane, resulting in most cases in transmembrane pore formation and loss of membrane integrity that causes cell death. By using FAM-GHR, we observed that GHR was able to target the bacterial membrane (Fig. 4*C*). After binding, GHR seems to disrupt the membrane of the bacteria because a significant proportion of *E. coli* (69% of cells) treated with GHR incorporated propidium iodide, a small cationic molecule frequently used to monitor damage of microbial membrane integrity (Fig. 4*D*, *left panel*). This finding was confirmed by the fact that bacteria (54%) cultured with GHR showed significant increases in the uptake of DiBAC$_4$(3), a lipophilic anion sensitive to changes in membrane potential that enter into membranes only if their membrane potential has collapsed (Fig. 4*D*, *right panel*). These data collectively demonstrate that GHR acts as a bactericidal neuropeptide in vivo and in vitro, probably by disrupting the integrity of the cell membrane.

GHR inhibits the translocation and secretion of HMGB1 by activated macrophages

Macrophages are a major source of proinflammatory cytokines and secrete HMGB1 upon stimulation with LPS, and the HMGB1 release by macrophages is in agreement with that observed in serum of mice with sepsis (4, 8). To examine whether macrophages are direct cell targets of the inhibitory effect of GHR, we stimulate these cells with LPS in the presence or absence of the neuropeptide. GHR dose-dependently inhibited the release of several LPS-induced proinflammatory cytokines and chemokines and of NO (Fig. 5*A*). Thus, we examined the direct effect of GHR on the secretion of HMGB1 by LPS-stimulated macrophages. Unstimulated macrophages secreted very low levels of HMGB1, and LPS stimulation resulted in an increased secretion of HMGB1 (Fig. 5*B*). GHR significantly inhibited the release of HMGB1 induced by LPS. This effect was dose-dependent with a maximal effect at 10^{-7} M (Fig. 5*B*), a concentration that is within the physiological range (11). Although unstimulated macrophages displayed a strong staining for HMGB1 mostly restricted to the nucleus, LPS stimulation induced HMGB1 translocation from the nucleus to the cytoplasm before its secretion in 60% of the cells. GHR prevented the LPS-induced HMGB1 cytoplasmic translocation and retained

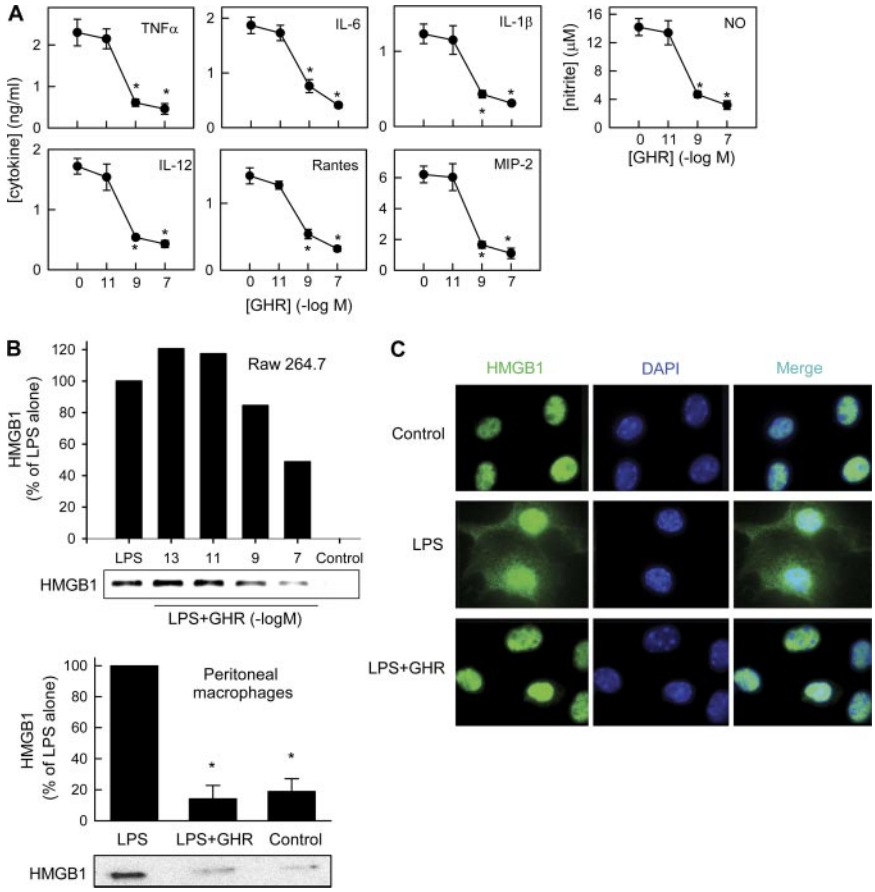

FIGURE 5. GHR inhibits the cytoplasmic translocation and secretion of HMGB1 in activated macrophages in vitro. *A*, GHR down-regulates the production of inflammatory factors by activated macrophages. Peritoneal macrophages were stimulated with LPS (1 μg/ml) in the absence or presence of different concentrations of GHR. At different time points (6 h for TNF-α and 24 h for IL-6, IL-12, IL-1β, RANTES, MIP-2, and NO), the levels of proinflammatory mediators in the culture supernatants were determined as described in the legend of the Fig. 1*C* ($n = 5$). $*$, $p < 0.001$, vs untreated control mice (Student's *t* test). *B*, GHR decreases HMGB1 secretion by activated macrophages. RAW 264.7 (*upper panel*) or peritoneal macrophages (*lower panel*) were stimulated with LPS (1 μg/ml) and different concentrations of GHR (10^{-7} M for peritoneal macrophages) for 24 h. HMGB1 content in culture supernatants was assayed by Western blotting and expressed as densitometric units relative to the LPS-treated condition on the same blot. Data are representative of three independent experiments. *C*, GHR inhibits cytoplasmic translocation of HMGB1 in macrophages. Peritoneal macrophages were cultured with medium (control), or stimulated with LPS (100 ng/ml) or with LPS plus GHR (10^{-7} M). After 12 h, macrophages were fixed-permeabilized, stained with DAPI (blue channel) and anti-HMGB1 Ab (green channel) and analyzed by fluorescent microscopy.

HMGB1 in the nucleus (only 20% of the cells showed translocation; Fig. 5*C*). A single administration of GHR was enough to signal macrophages and exert a long-term inhibition of HMGB1 release in vitro. Thus, GHR still down-regulated HMGB1 secretion when it was added 4 h after LPS stimulation (data not shown). These in vitro findings were relevant to the secretion of HMGB1 by macrophages during sepsis, because whereas peritoneal macrophages isolated from septic animals spontaneously produced high amounts of HMGB1 ex vivo, macrophages isolated from GHR-treated animals secreted much lower levels of HMGB1 (Fig. 6*A*). Furthermore, GHR efficiently blocked the in vivo translocation of HMGB1 from the nucleus to the cytoplasm (Fig. 6*B*). These results collectively demonstrate that GHR inhibits HMGB1 secretion by macrophages during sepsis.

GHR reduces serum HMGB1 during arthritis

HMGB1 has been proven to be a successful therapeutic target not only in severe sepsis, but also in experimental models of diverse infectious and inflammatory diseases. Among them, HMGB1 is elevated in serum and synovial tissue of patients with rheumatoid arthritis as well as in the joints of animals with arthritis. Indeed, in the mouse model of CIA, treatment with Abs to HMGB1 attenuates the severity of the disease. In addition, it has been described a protective effect of the GHR agonist growth hormone-releasing peptide 2 in arthritic rats (27). Thus, to further investigate the ability of GHR to down-regulate the release of HMGB1 during the course of an immune disorder, we induced CIA, treated the animals with GHR, and analyzed the levels of HMGB1 in the serum. GHR treatment of mice with established clinical signs of arthritis progressively decreased incidence and severity of CIA as compared with untreated mice, as assessed by clinical score (Fig. 7*A*).

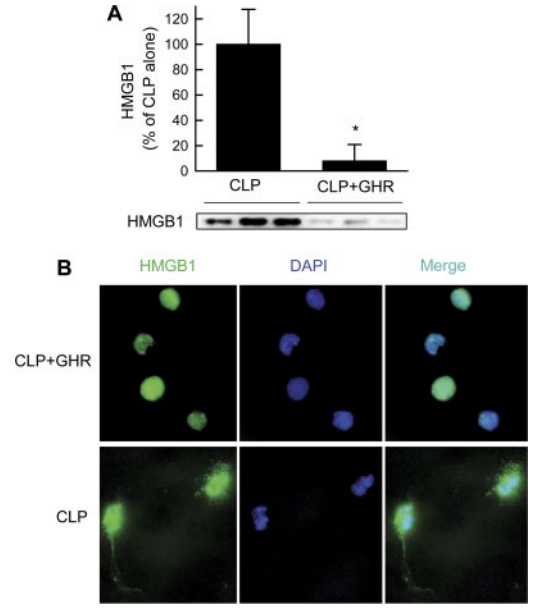

FIGURE 6. GHR deactivates peritoneal macrophages during sepsis. Mice were subjected to CLP and treated i.p. with GHR (1 nmol/mouse) 12 h later. Peritoneal lavage was obtained 18 h after sepsis induction. *A*, Peritoneal macrophages were isolated, cultured with medium at 10^6 cells/ml for 36 h, and the concentration of HMGB1 in the culture supernatants was determined by Western blotting and expressed as band densities relative to control samples (CLP alone) on the same blot. Data are mean $\pm$ SEM. $n = 8$/group. $*$, $p < 0.001$ vs CLP alone. *B*, Peritoneal macrophages were allowed to adhere for 2 h, fixed-permeabilized, stained with 4′,6-diamidino-2-phenylindole (blue channel) and anti-HMGB1 Ab (green channel) and analyzed by fluorescent microscopy.

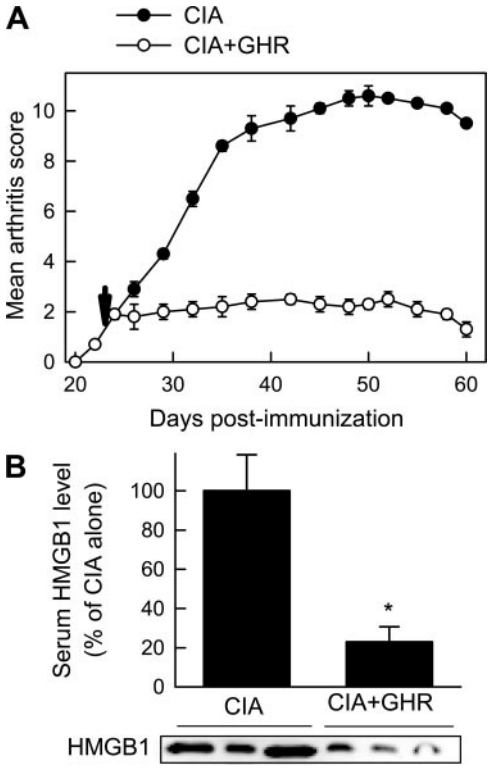

FIGURE 7. GHR treatment reduces the systemic levels of HMGB1 levels in arthritic mice. *A*, Severity of arthritis, assessed by clinical scoring in mice with established CIA injected i.p. (arrow) either with PBS (control) or with GHR (1 nmol/mouse) daily for 5 days. $n = 8–12$ mice/group. $p < 0.001$ vs control after day 28. *B*, Serum samples were obtained on the day 35 post immunization and assessed for the presence of HMGB1 by western blot and expressed as band densities relative to control samples (CIA alone) on the same blot. Data are mean ± SEM. $n = 5$/group. *, $p < 0.001$ vs CIA mice (Mann-Whitney *U* test).

Whereas untreated CIA mice show high levels of circulating HMGB1, GHR reduced the concentration of serum HMGB1 (Fig. 7*B*). These findings support the concept that GHR is an endogenous inhibitor of HMGB1 release and suggest that inhibition of HMGB1 could be a general mechanism of action of the neuropeptide.

Discussion

This work provides the evidence that GHR might represent a feasible therapeutic agent for the treatment of sepsis. GHR functions at several levels to regulate many crucial aspects of sepsis, such as bacterial clearance and the inflammatory response to infection. Thus, GHR has the potential to provide therapeutic advantage over agents that are directed against only one component of the disease.

Our data show that GHR is a potent immunomodulatory factor that directly inhibits the secretion of early and late inflammatory mediators of sepsis, with a special relevancy of down-regulation of HMGB1. Consistent with our results, during the past few years, evidence has been accumulated regarding the role of GHR in inflammation. GHR down-regulates the production of proinflammatory cytokines and chemokines by activated human PBMCs and coadministration of GHR with LPS to rats attenuated the plasma levels of TNF, IL-8, and MCP-1 (12, 13). Recently, Wu et al. (14, 17) have found that GHR down-regulates TNF and IL-6 and improves acute lung injury in CLP rats. These effects have been partially related to stimulation of the vagus nerve and to inhibition of the sympathetic nervous activity by GHR (14, 28). In addition, GHR improves the hemodynamic changes and glucose and lactate abnormalities (29) and tissue perfusion in septic rats (30). Although some of these mechanisms could contribute to the therapeutic action of this neuropeptide in sepsis, the evidence presented in this study strengthens the hypothesis of inhibition of a late mediator. We show that GHR is still protective against endotoxin lethality even if administered 12 h after a LD_{100} injection of LPS, and that GHR treatment can be delayed until 24 h after sepsis induction, several hours after the peak of early mediators. Indeed, administration of GHR 12 h after CLP does not modulate the serum levels of proinflammatory cytokines, but down-regulates the systemic levels of HMGB1. Furthermore, administration of recombinant HMGB1 to GHR-treated mice abolished the therapeutic effect of the neuropeptide on sepsis. In this sense, our study demonstrates that GHR is the most potent physiological inhibitor of HMGB1 release described to date in experimental sepsis. HMGB1 is a nuclear protein with cytokine activity that is involved in the pathogenesis of a wide range of immune-mediated diseases. Remarkably, numerous works has shown that HMGB1 is a necessary and sufficient late mediator of sepsis (reviewed in Ref. 3) and, therefore, it has been established as one of the major targets for its treatment. Because in many clinical cases hours pass before sepsis is diagnosed and specific treatment is implemented, targeting HMGB1 would expand the therapeutic window to a clinical relevant time. In any case, sepsis is a complex disease with different aetiologies and the physiology of HMGB1 is starting to be unraveled. In a recent work, Angus et al. (31) found that HMGB1 was elevated in both uncomplicated pneumonia and pneumonia with severe sepsis, although higher HMGB1 concentrations were associated with mortality.

The inhibitory effect of GHR on the release of HMGB1 in vitro and ex vivo by peritoneal macrophages resembled closely the profile observed in mice treated with GHR. Therefore, it is likely to be deactivation of resident and infiltrating macrophages the major mechanism in the therapeutic effect of the neuropeptide. Although activated macrophages release HMGB1 with a similar kinetics observed in serum of septic mice (8), other cells, including NK, pituicytes, enterocytes, and endothelial and dendritic cells also secrete HMGB1 upon stimulation (32–35). Although the contribution of these cells in the increase of circulating HMGB1 levels during sepsis have not yet been addressed, we cannot rule out the possibility that GHR exerts some effects on them in septic mice. In fact, GHR inhibits endothelial cell activation by TNF-α (13).

The ability of delayed administration of GHR to ameliorate ongoing disease fulfills an essential prerequisite for an anti-septic agent, as treatment starts after the onset of sepsis. In contrast, lysophosphatidylcholine and Abs to macrophage inhibitory factor loose their therapeutic effects if administration starts at 8–10 h after infection induction (36, 37). Similar to GHR, therapies based in neutralizing Abs against HMGB1 improve survival when started 24 h after CLP (8, 9, 38, 39). However, Ab-blocking strategies increase the formation of Ab-Ag complexes and clinical studies designed to block cytokines are disappointing. In this regard, inhibition of HMGB1 secretion might represent a therapeutic advantage compared with anti-HMGB1 Abs. Indeed, the therapeutic window shown by GHR in experimental sepsis has been uniquely achieved by specific inhibitors of HMGB1 (7, 8, 40). Thus, ethyl pyruvate and nicotine (7, 36) inhibit the secretion of HMGB1 and showed a similar therapeutic effect as GHR in severe sepsis. However, GHR is the first endogenous inhibitor of HMGB1 secretion shown to improve sepsis survival in a clinical relevant time frame.

Interestingly, patients with sepsis have also features consistent with immunosuppression, characterized by loss of lymphocytes

and dendritic cells, and manifested by an inability to clear the primary infection and by the development of secondary infections. In this regard, treatment strategies for severe sepsis are directed, at least in part, to eradicate the infection (41, 42). In the present study, we describe that GHR significantly diminishes peritoneal bacterial counts of septic animals and directly kills bacteria in vitro. Remarkably, the GHR concentrations found effective in both in vivo and in vitro antimicrobial studies are in the same range. The antimicrobial activity of GHR (with a LD_{50} of 0.2 μM) against *E. coli* is similar to that described for other neuropeptides, and it corresponds to the concentration of bactericidal neuropeptides found in certain microenvironments, such as in the proximity of nerve endings (26). Interestingly, GHR shares some important properties with natural antimicrobial peptides, including small size ($<$10 kDa), high positive charge, and amphipathic α-helix structures adopted upon interaction with membranes. Our data show that GHR initially binds to bacteria and alter the permeability and potential of the membrane. Therefore, GHR could interact with the negatively charged outer leaflet of the plasma membranes of bacteria and insert into the cell membrane. This would lead to a rapid loss of the cell homeostasis and eventually death of the pathogen by membrane disruption.

The lower bacterial load in the peritoneum of GHR-treated mice might mean lower stimulation for HMGB1 secretion and, therefore, contribute to the GHR-induced inhibition of circulating HMGB1. However, the fact that GHR improves survival and attenuates systemic HMGB1 levels in mice injected with LPS, and that GHR inhibits the secretion of HMGB1 by LPS-activated peritoneal macrophages in vitro, strongly supports that the neuropeptide directly inhibits HMGB1 release in our model. However, GHR could also modulate HMGB1-induced lethality through other indirect mechanisms. Endotoxin and HMGB1 seem to act synergistically in septic death, because coadministration of non-lethal doses of HMGB1 and LPS results in a mortality of 90% (4), and HMGB1 can bind CpG DNA leading to an augmentation of TLR9-dependent secretion of proinflammatory cytokines (43, 44), suggesting that some bacterial products could act as potential cofactors with HMGB1. Moreover, a recent study (45) has demonstrated that purified HMGB1 fails to induce the production of inflammatory cytokines by activated macrophages and that HGMB1 acquires proinflammatory activities through the binding to inflammatory mediators, such as IL-1β. In this context, the decreased bacterial levels observed at the peritoneal cavity of GHR-treated mice may also influence the HMGB1-induced lethality.

From a therapeutic point of view, it is important to consider that HMGB1 has been involved in the progression of various inflammatory and autoimmune diseases. In addition to sepsis, HMGB1 acts as a mediator of inflammation and organ damage in hepatic ischemia/reperfusion injury (3). Moreover, epithelial barrier disruption and bacterial infection are related to the pathogenesis of some inflammatory bowel disorders, and HMGB1 impairs intestinal barrier function and is a mediator of necrotizing enterocolitis (6, 46). In addition, HMGB1 administration to the joints causes arthritis and its blockade confers protection in this disorder (3). In this context, the GHR attenuation of lesions evoked by ischemia/reperfusion, improvement of the outcome in inflammatory bowel disease, and the reduction of arthritic signs (15, 26, 47, 48 and Fig. 7) could be explained, at least partially, by the inhibition of HMGB1 and the antibacterial activity of the neuropeptide. In fact, here we show that GHR attenuates the secretion of HMGB1 into the circulation of arthritic mice.

Of physiologic relevance is the observation that the secretion of GHR dramatically drops in certain pathologic inflammatory conditions, such as CLP, endotoxemia, rheumatoid arthritis, and *H. pylori* infection (17, 49–52). Furthermore, decreased endogenous GHR contribute to the increased incidence of atherosclerosis in patients with obesity (53). Therefore, it is tempting to speculate that during inflammatory processes, a reduction of the levels of GHR might exacerbate the ongoing inflammatory insult. Thus, GHR emerges as a natural antimicrobial and anti-inflammatory peptide, widely distributed in all body tissues and especially abundant in the proximity to physical barriers such as stomach, gut, and skin, where one of its primary functions could be related to the control of innate immunity and response against infections.

In summary, the present study provides the first evidence that GHR is an endogenous inhibitor of HMGB1 release and a bactericidal neuropeptide. In this way, our findings provide a new concept in the role of GHR in host defense and highlight this neuropeptide as a potential multistep therapeutic agent for human sepsis and other immune disorders.

Disclosures

The authors have no financial conflict of interest.

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