GH-releasing peptide-2 administration prevents liver inflammatory response in endotoxemia

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Submitted 18 May 2007; accepted in final form 1 November 2007

Granado M, Martín AI, López-Menduíña M, López-Calderón A, Villanúa MA. GH-releasing peptide-2 administration prevents liver inflammatory response in endotoxemia. Am J Physiol Endocrinol Metab 294: E131–E141, 2008. First published November 6, 2007; doi:10.1152/ajpendo.00308.2007.—It has been reported that growth hormone (GH)-releasing peptide-2 (GHRP-2), a ghrelin receptor agonist, has an anti-inflammatory effect. We investigated whether this GH secretagogue attenuates liver injury in LPS-treated rats. Wistar rats were simultaneously injected (ip) with LPS (1 mg/kg) and/or GHRP-2 (100 μg/kg). Serum levels of aspartate and alanine transaminases were measured as an index of liver damage. Circulating nitrites/nitrates and hepatic TNF-α and IGF-I were evaluated as possible mediators of GHRP-2 actions. LPS increased serum levels of transaminases and nitrites/nitrates. Moreover, LPS increased hepatic TNF-α and decreased hepatic IGF-1 mRNA. GHRP-2 administration attenuated the effects of LPS on transaminases, nitrites/nitrates, TNF-α, and IGF-1 in vivo. This GHRP-2 effect does not seem to be due to modifications in food intake, since fasting did not modify serum levels of transaminases, serum nitrites/nitrates, and hepatic TNF-α mRNA both in vehicle rats and in LPS-injected rats. To elucidate whether GHRP-2 is acting directly on the liver, cocultures of hepatocytes and nonparenchymal cells and monocultures of isolated hepatocytes were incubated with LPS and GHRP-2. The ghrelin receptor agonist prevented an endotoxin-induced increase in transaminases and nitrite/nitrate release as well as in TNF-α mRNA and increased IGF-1 mRNA from cocultures of hepatocytes and nonparenchymal cells, but not from monocultures. In summary, these data indicate that GHRP-2 has a protective effect on the liver in LPS-injected rats that seems to be mediated by IGF-1, TNF-α, and nitric oxide. Our data also suggest that the anti-inflammatory effect of GHRP-2 in the liver is exerted on nonparenchymal cells.

Transaminases; insulin-like growth factor I; nitrites/nitrates; tumor necrosis factor-α; growth hormone-releasing peptide-2

CATABOLISM AND GROWTH IMPAIRMENT are complications of several situations such as sepsis and inflammatory diseases. Inflammatory response can be induced experimentally by administering lipopolysaccharide (LPS), a component of the wall of gram-negative bacteria. The liver plays a central role in the inflammatory response to LPS, since it clears LPS and also responds to LPS with production of cytokines (38) and reactive oxygen intermediates (1). Most of the toxicities of LPS, both in liver and in systemic circulation, have been related to the release of these inflammatory cytokines and mediators (24, 61). Nitric oxide (NO) and tumor necrosis factor (TNF) are believed to be involved in hepatic dysfunction in sepsis (5, 57). As potent producers of inflammatory cytokines such as TNF-α, Kupffer cells have been implicated in the pathway leading to liver injury (60). In many models of liver injury, elevated TNF-α levels are present and correlate with damage (6, 31). Immunohistochemical studies reveal that TNF-α is exclusively produced by Kupffer cells, whereas hepatocytes are responsible for NO production after LPS stimulation (26). However, it has been reported that LPS not only activates Kupffer cells to release TNF-α and NO (7) but also induces TNF-α and NO production by hepatocytes (20, 66).

Hypercatabolism and liver injury in inflammation are not only due to increased production of inflammatory cytokines. The increase in catabolic hormones together with a decrease in anabolic hormones can contribute to the catabolic state. LPS administration decreases circulating levels of insulin-like growth factor I (IGF-I) both in humans (35) and in experimental animals (10, 54). We have previously observed that the decrease in serum IGF-I is associated with a decrease in IGF-I in the liver (47, 48), which can be independent of pituitary growth hormone (GH) (47). IGF-I plays an important role in the early stages of liver tissue repair (51). Transgenic mice overexpressing IGF-I have accelerated liver regeneration after liver injury (50). In addition, IGF-I treatment resulted in effective prevention of acute liver failure in rats induced by D-galactosamine and LPS (28). This fact suggests a therapeutic potential for IGF-I in the prevention of acute liver failure.

Ghrelin is a circulating hormone mainly produced by the stomach that stimulates GH secretion from pituitary somatotropes (33) and increases food intake and body weight and promotes adiposity (58, 62). Ghrelin exerts these activities through binding of GH secretagogue receptor-1a (GHSR-1a), identified previously as the receptor for the synthetic GH secretagogues (GHSs) (27). Ghrelin, therefore, appears to be a promising candidate to treat hypercatabolic states, and this possibility has already been demonstrated in animal models with cardiac (42) and cancer cachexia (23). Moreover, ghrelin exerts potent anti-inflammatory effects, inhibiting proinflammatory cytokines such as TNF-α, via a GHSR-specific mechanism (9, 13). We have recently reported that, during the active phase of arthritis, administration of GH-releasing peptide-2 (GHRP-2), a synthetic ghrelin receptor agonist, reduced the symptoms of arthritis and nitrite/nitrate and interleukin-6 (IL-6) production by macrophages in response to LPS (17). Ghrelin administration increases body weight in endotoxemic models (25). Furthermore, ghrelin decreased mortality and corrected metabolic abnormalities in rats with septic shock (4) and also protects the hepatic and pancreatic tissues against oxidative injury (32).

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The objective of this study was to investigate whether the administration of the synthetic ghrelin analog GHRP-2 is able to prevent sepsis-induced liver inflammatory response. For this purpose, we studied the effect of GHRP-2 on transaminase release induced by LPS as an index of liver damage as well as on IGF-I, TNF-α, and NO responses to LPS. Moreover, to examine whether GHRP-2 effects can be direct on the liver, hepatocytes and nonparenchymal cells and in cultures of isolated hepatocytes and nonparenchymal cells and in cultures of isolated hepatocytes.

**MATERIAL AND METHODS**

**Animals and experimental design.** Male Wistar rats (250 g; Harlam, Barcelona, Spain) were used for the experiment. The procedures followed the guidelines recommended by the European Union for the care and use of laboratory animals, and the animal protocols were approved by the University Animal Care Committee. Rats were housed three or four per cage with free access to food and water, under constant conditions of temperature (20–22°C) and light (lights on from 0730 to 1930). Rats were randomly assigned to a treatment group after 1-wk adaptation to environment and diet. Rats were divided into four groups: 1) vehicle rats injected with saline, 2) vehicle rats injected with GHRP-2 (100 µg/kg) (Bachem, Bubendorf, Switzerland), 3) rats injected with LPS (1 mg/kg, ip) (serotype O55:B5; Sigma Chemical, St. Louis, MO), and 4) rats injected simultaneously with LPS and GHRP-2. Rats were injected at 1700 and at 0550 h the following day. This LPS administration protocol has been described by Miranda et al. (41). The rats were allowed to resume food and water during the 18 h of the experiment. Trunk blood was collected in cooled tubes, allowed to clot, and centrifuged, and the serum was stored at −80°C until RNA isolation and the mRNA in the liver (47). The GHRP-2 dose was chosen taking into account that this dose was able to decrease serum concentrations of IL-6 and arthritis score (47). The GHRP-2 dose was chosen taking into account that this dose was able to decrease serum concentrations of IL-6 and arthritis score (47). The GHRP-2 dose was chosen taking into account that this dose was able to decrease serum concentrations of IL-6 and arthritis score (47).

Because LPS fed food intake, an experiment with fasting rats was included to elucidate whether the effects of LPS were only due to the increase in eating. LPS (1 mg/kg, ip) or vehicle (250 µl of saline) was injected into two groups of rats: 1) fasted rats during the 18 h of fasting, an experiment with fasting rats was included to elucidate whether the effects of LPS were only due to the decrease in eating. LPS (1 mg/kg, ip) or vehicle (250 µl of saline) was injected into two groups of rats: 1) fasted rats during the 18 h of fasting, an experiment with fasting rats was included to elucidate whether the effects of LPS were only due to the decrease in eating. LPS (1 mg/kg, ip) or vehicle (250 µl of saline) was injected into two groups of rats: 1) fasted rats during the 18 h of fasting, an experiment with fasting rats was included to elucidate whether the effects of LPS were only due to the decrease in eating. LPS (1 mg/kg, ip) or vehicle (250 µl of saline) was injected into two groups of rats: 1) fasted rats during the 18 h of fasting. LPS (1 mg/kg, ip) or vehicle (250 µl of saline) was injected into two groups of rats: 1) fasted rats during the 18 h of fasting. LPS (1 mg/kg, ip) or vehicle (250 µl of saline) was injected into two groups of rats: 1) fasted rats during the 18 h of fasting.

Hepatocytes were isolated by modification of the in situ collagenase perfusion technique as previously described (53). Rats were anesthetized with pentobarbital sodium (Sigma), the portal vein was cannulated after opening of the abdomen and perfused with calcium-free buffer for 7 min, and then the liver was digested with 0.04% collagenase (Roche, Indianapolis, IN) for another 2 min at 37°C. The liver was transferred to a petri dish, and the liver cells were obtained by gentle raking with a comb and filtered through a 100-µm mesh. Hepatocytes were separated from nonparenchymal cells by differential centrifugation at 400 rpm (3 times, 5 min each). Hepatocyte purity was assessed by microscopy and was >90%; viability was measured by Trypan blue exclusion and was >80%.

Hepatocyte isolation. Hepatocytes were isolated by modification of the in situ collagenase perfusion technique as previously described. The objective of this study was to investigate whether the administration of the synthetic ghrelin analog GHRP-2 is able to prevent sepsis-induced liver inflammatory response. For this purpose, we studied the effect of GHRP-2 on transaminase release induced by LPS as an index of liver damage as well as on IGF-I, TNF-α, and NO responses to LPS. Moreover, to examine whether GHRP-2 effects can be direct on the liver, hepatocytes and nonparenchymal cells and in cultures of isolated hepatocytes and nonparenchymal cells and in cultures of isolated hepatocytes.

**Table 1. Primers for real-time PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
<th>Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1</td>
<td>GCCATGCTGCCACGATTCTGG</td>
<td>TCCGGAAGCAACACTGATCC</td>
<td>62</td>
</tr>
<tr>
<td>TNF-α</td>
<td>GCCACAGCTCCCTCTGTTCT</td>
<td>GTCTGGGCGATGGAACTGAT</td>
<td>100</td>
</tr>
<tr>
<td>GHSR</td>
<td>GAAAGCCACACACCACACAG</td>
<td>AGGAACTCATGCGGGACAGC</td>
<td>105</td>
</tr>
<tr>
<td>r18S</td>
<td>AGGGAGGGCACACCCAGAGA</td>
<td>CACCACCCACCGGAACTGCGA</td>
<td>126</td>
</tr>
<tr>
<td>HPRT</td>
<td>CTCTAGGATGATTATGACAGGAC</td>
<td>GCAAGTCTACAGAAGATTAAGGCC</td>
<td>122</td>
</tr>
</tbody>
</table>

GHSR, growth hormone (GH) secretagogue receptor; HPRT, hypoxanthine-guanine phosphoribosyltransferase.
was 20 pg/ml, and the intra-assay coefficient of variation was 8%. All samples were run in the same assay.

RNA extraction and real-time PCR. Total RNA from the liver was extracted by the guanidine thiocyanate method using a commercial reagent (Ultraspec RNA; Biotecx Laboratories, Houston, TX). The extracted RNA was dissolved in diethylpyrocarbonate water with 0.1% SDS and quantified at 260 nm; RNA integrity was confirmed by agarose gel electrophoresis. For RT-PCR analysis, 2 μg of mRNA from hepatic tissue or cells were reverse transcribed using the instructions of the commercial kit Quantitect Reverse Transcription Kit (Quiagen, Valencia, CA). Each RT-PCR reaction consisted of 2.5 μl of cDNA, SYBR Green Premix Ex Taq (Takara Otsu, Shiga, Japan), and 300 nM forward and reverse primers in a reaction volume of 25 μl. Reactions were carried out on a SmartCycler (Cepheid, Sunnyvale, CA). Primers for PCR were obtained from previously published sequences of TNF-α and IGF-I (8), hypoxanthine-guanine phosphoribosyl transferase (HPRT) (45), and r18S (2) or by use of the rat GenBank and the EXIQON ProbeLibrary GHSR (Table 1). Primers were designed to span a single sequence derived from two exons (i.e., separated by an intron in genomic DNA and primary RNA transcripts to minimize amplification). Parameters included an initial activation of hotStar Taq DNA polymerase at 95°C for 10 s, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C, and extension at 72°C for 30 s. Specific amplification was confirmed by the presence of one single peak in the melting curve plots. In addition, the PCR products were analyzed in agarose gel electrophoresis.

Fig. 1. Serum concentrations of aspartate aminotransferase (AST; A), alanine aminotransferase (ALT; B), and nitrites/nitrates (C) in rats treated ip with LPS (1 mg/kg) and growth hormone (GH)-releasing peptide-2 (GHRP-2; 100 μg/kg). Two-way ANOVA revealed that there was an interaction between the effect of LPS and GHRP-2 administration on serum concentrations of AST (F₁,₃⁶ = 4.81, P < 0.05) and on serum concentrations of ALT (F₁,₃₂ = 4.73, P < 0.05), as GHRP-2 decreased serum concentrations of AST and ALT in LPS-injected but not in vehicle rats. There was no interaction between the effect of LPS and GHRP-2 on serum concentrations of nitrites/nitrates (F₁,₃₂ = 1.28, P = 0.26). Values shown represent means ± SE for 9–11 rats/group (AST and ALT) and for 7–9 rats/group (nitrites/nitrates, presented as the percentage of the mean value in vehicle rats treated with saline). *P < 0.05 and **P < 0.01 vs. vehicle-saline. *P < 0.05 and **P < 0.01 vs. LPS-saline. *P < 0.05 vs. vehicle-GHRP-2 (2-way ANOVA and Student’s t-test).

Fig. 2. Effect of GHRP-2 (100 μg/kg) or saline administration on serum concentrations (A) and liver gene expression (B) of IGF-I in vehicle rats and LPS-injected (1 mg/kg) rats. Each bar represents the mean ± SE for 7–10 rats/group. IGF-I mRNA expression was quantified using real-time RT-PCR and is presented as the percentage of the mean value in vehicle rats treated with saline by analyzing the critical threshold (C₇) nos. corrected by CT readings of corresponding internal 18S rRNA as control gene. There was no interaction between the effect of LPS and GHRP-2 on IGF-I mRNA expression (F₁,₃₀ = 0.01, P > 0.91). There was an interaction between the effect of LPS and GHRP-2 on serum concentrations of IGF-I (F₁,₃₃ = 5.38, P < 0.05), as GHRP-2 increased serum concentrations of IGF-I in LPS-injected but not in vehicle rats. *P < 0.05 and **P < 0.01 vs. vehicle-saline. *P < 0.05 vs. LPS saline. #P < 0.01 vs. vehicle-GHRP-2 (2-way ANOVA and Student’s t-test).
Results were calculated as percentage of control rats injected with saline, using the \( \Delta C_T \) method (37) (where \( C_T \) is critical threshold) with HPRT and 18S as control genes.

Statistical analysis. Statistics were computed using the statistics program STATGRAPHICS plus for Windows (Manugistic, Rockville, MD). Data are presented as means ± SE and were analyzed by multifactorial analysis of variance (ANOVA) with LPS and treatment with GHRP-2 or fasting as factors. Post hoc comparisons were made by using the unpaired Student’s \( t \)-test. Data from GHsR were analyzed by chi-square test. Statistical significance was set at \( P < 0.05 \).

RESULTS

Figure 1 shows the effect of LPS and GHRP-2 administration on serum concentrations of AST (Fig. 1A), ALT (Fig. 1B), and nitrites/nitrates (Fig. 1C). LPS induced a significant increase \( (P < 0.01) \) in both serum transaminases activities, and GHRP-2 administration prevented both of these increases, since serum transaminases levels in rats injected with LPS and GHRP-2 were similar to those of the vehicle rats. The serum concentration of nitrites/nitrates was also increased in LPS-injected rats compared with the vehicle group \( (P < 0.05) \). GHRP-2 administration decreased the serum concentration of nitrites/nitrates both in vehicle rats \( (P < 0.01) \) and in LPS-injected \( (P < 0.05) \) rats. The decrease in nitrites/nitrates in rats injected with LPS and GHRP-2 was able to normalize the serum concentration of nitrites/nitrates.

Serum IGF-I concentrations as well as hepatic IGF-I mRNA in vehicle rats and LPS-injected rats treated with GHRP-2 or saline are shown in Fig. 2. There was a significant decrease in serum concentrations \( (P < 0.01) \) and hepatic mRNA \( (P < 0.05) \) of IGF-I in LPS-injected rats (Fig. 2, A and B), and these parameters were increased by GHRP-2 administration \( (P < 0.05) \). However, GHRP-2 did not modify serum concentrations and hepatic gene expression of IGF-I in vehicle rats.

LPS injections increased the TNF-\( \alpha \) mRNA in the liver \( (P < 0.01; \) Fig. 3), and GHRP-2 administration decreased this parameter, although this decrease was not statistically significant in vehicle rats or in LPS-injected rats \( (P = 0.07). \)

The effects of GHRP-2 (Fig. 4A) or fasting (Fig. 4B) on body weight gain in vehicle rats or LPS-injected (1 mg/kg) rats. Two-way ANOVA revealed that there was no interaction between the effect of LPS and GHRP-2 administration on body weight gain \( (F_{1,38} = 2.07, P = 0.15) \). There was an interaction between the effect of LPS and fasting on body weight gain \( (F_{1,31} = 30.13, P = 0.00) \), as fasting decreased body weight gain in vehicle rats but not in LPS-injected rats. Values shown are the means ± SE for 8–11 rats/group. **\( P < 0.01 \) vs. vehicle-saline or vehicle-"ad libitum" fed. "\( P < 0.05 \) vs. LPS-saline. ###\( P < 0.01 \) vs. vehicle-GHRP-2 or vehicle-fasted (2-way ANOVA and Student’s \( t \)-test).

Fig. 3. Hepatic mRNA expression of TNF-\( \alpha \) in vehicle rats and LPS-injected (1 mg/kg) rats treated with saline or GHRP-2 (100 \( \mu \)g/kg). TNF-\( \alpha \) mRNA expression was quantified using real-time RT-PCR and is presented as a percentage of the mean value in vehicle rats treated with saline by analyzing the \( C_T \) nos. corrected by \( C_T \) readings of corresponding internal 18S rRNA as control gene. There was no interaction between the effect of LPS and GHRP-2 on hepatic TNF-\( \alpha \) gene expression \( (F_{1,31} = 0.48, P = 0.49) \). Each bar represents the mean ± SE for 7–9 rats/group. **\( P < 0.01 \) vs. vehicle-saline. \#\( P < 0.05 \) vs. vehicle-GHRP-2 (2-way ANOVA and Student’s \( t \)-test).

Fig. 4. Effect of GHRP-2 (100 \( \mu \)g/kg) administration (A) and fasting (B) on body weight gain in vehicle rats or LPS-injected (1 mg/kg) rats. Two-way ANOVA revealed that there was no interaction between the effect of LPS and GHRP-2 administration on body weight gain \( (F_{1,38} = 2.07, P = 0.15) \). There was an interaction between the effect of LPS and fasting on body weight gain \( (F_{1,31} = 30.13, P = 0.00) \), as fasting decreased body weight gain in vehicle rats but not in LPS-injected rats. Values shown are the means ± SE for 8–11 rats/group. **\( P < 0.01 \) vs. vehicle-saline or vehicle-"ad libitum" fed. "\( P < 0.05 \) vs. LPS-saline. ###\( P < 0.01 \) vs. vehicle-GHRP-2 or vehicle-fasted (2-way ANOVA and Student’s \( t \)-test).
Table 2. Effect of GHRP-2 administration and fasting on food intake and water intake in vehicle or LPS-injected rats

<table>
<thead>
<tr>
<th></th>
<th>Vehicle-Saline</th>
<th>Vehicle-GHRP-2</th>
<th>LPS-Saline</th>
<th>LPS-GHRP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake, g/100 g BW</td>
<td>8.9±0.3</td>
<td>11.6±0.6</td>
<td>0.3±0.1</td>
<td>2.2±0.0</td>
</tr>
<tr>
<td>Water intake, ml/100 g BW</td>
<td>11.0±1.4</td>
<td>12.8±0.0</td>
<td>4.1±1.7</td>
<td>5.9±2.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Vehicle-Ad Libum</th>
<th>Vehicle-Fasted</th>
<th>LPS-Ad Libum</th>
<th>LPS-Fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake, g/100 g BW</td>
<td>6.7±0.1</td>
<td>0</td>
<td>0.6±0.04</td>
<td>0.4±0.01</td>
</tr>
<tr>
<td>Water intake, ml/100 g BW</td>
<td>12.0±1.1</td>
<td>9±0.4</td>
<td>5.9±0.6</td>
<td>3.6±0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE for 2–3 cages/group. Effect of GH-releasing peptide-2 (GHRP-2; 100 μg/kg) administration and fasting on food intake and water intake in vehicle or LPS-injected (1 mg/kg) rats. Food and water intakes per cage were calculated by measuring the difference between the initial and the remaining amount of pellets in the feeder and the volume of water in the drinking bottle and are expressed as grams or milliliters, respectively, per 100 g body wt (BW).

There was no interaction between the effects of LPS and GHRP-2 administration on food intake (F1,32 = 0.85), and water intake (F1,32 = 0.78). LPS induced a significant increase in serum AST (P < 0.01) and ALT (P < 0.05) activities, and fasting did not modify serum transaminases activities in vehicle rats or in LPS-injected rats. The serum concentration of nitrites/nitrates was also increased in LPS-injected rats compared with the vehicle group (P < 0.01). Vehicle rats and LPS-injected rats had a similar serum concentration of nitrites/nitrates in both groups, fed ad libitum and fasted rats.

The effect of fasting on serum IGF-I concentrations as well as on hepatic IGF-I mRNA in rats injected with LPS or vehicle is shown in Fig. 6. There was a significant decrease in serum concentrations (P < 0.01) and hepatic mRNA (P < 0.01) of IGF-I in LPS-injected rats (Fig. 6, A and B), and these parameters were not changed by the fasting. However, fasting decreased serum concentrations (P < 0.05) and hepatic gene expression of IGF-I (P < 0.05) in vehicle rats. The decrease in serum and hepatic IGF-I caused by fasting in vehicle rats was significantly lower than the decrease in serum and hepatic IGF-I caused by LPS injections (P < 0.05 for both parameters).

LPS injections increased the TNF-α mRNA in the liver (P < 0.05; Fig. 7), and fasting had no significant effect on TNF-α mRNA in vehicle rats or in LPS-injected rats.

In vitro experiments. Because GHRP-2 decreased serum concentrations of transaminases and nitrites/nitrates, we examined whether GHRP-2 directly affected cocultures of hepatocytes and nonparenchymal cells or monocultures of hepatocytes in vitro. The addition of LPS to the cocultures of hepatocytes and nonparenchymal cells increased AST and ALT production (P < 0.01; Fig. 8, A and B), and the presence of GHRP-2 in the incubation medium prevented these effects, since transaminase production in cocultures incubated with LPS and GHRP-2 was similar to that of the vehicle cocultures. The addition of LPS to the coculture medium also increased nitrite/nitrate production (P < 0.01; Fig. 8C), and the addition of GHRP-2 attenuated the stimulatory effect of LPS on the nitrite/nitrate production from cocultures. In vehicle cocultures of hepatocytes and nonparenchymal cells, the presence of GHRP-2 did not modify the aminotransferase and nitrite/nitrate production.

The gene expressions of TNF-α and IGF-I in cocultures of hepatocytes with nonparenchymal cells are shown in Fig. 9, A and B, respectively. TNF-α mRNA was increased by LPS in cocultures of hepatocytes and nonparenchymal cells (P < 0.05; Fig. 9A), and the addition of GHRP-2 to the incubation me-
GHRP-2 prevents LPS-induced liver injury

FIG. 6. Effect of fasting on serum concentrations (A) and liver gene expression (B) of IGF-I in vehicle rats and LPS-injected (1 mg/kg) rats. Each bar represents the mean ± SE for 8–9 rats/group. IGF-I mRNA expression was quantified using real-time RT-PCR and is presented as the percentage of the mean value in vehicle-ad libitum-fed rats by analyzing the CT nos. corrected by CT readings of corresponding internal 18S rRNA as control gene. There was an interaction between the effect of fasting and LPS on serum concentrations of IGF-I (F1,34 = 5.73, P < 0.05) and on IGF-I mRNA expression (F1,32 = 4.36, P < 0.05), as fasting decreased serum concentrations of IGF-I and its mRNA expression in vehicle rats but not in LPS-injected rats. *P < 0.05 and **P < 0.01 vs. vehicle-ad libitum fed. #P < 0.05 vs. vehicle-fastened (2-way ANOVA and Student’s t-test).

FIG. 7. Hepatic mRNA expression of TNF-α in vehicle rats and LPS-injected (1 mg/kg) rats fed ad libitum or fasted. TNF-α mRNA expression was quantified using real-time RT-PCR and is presented as a percentage of the mean value in vehicle-ad libitum-fed rats by analyzing the CT nos. corrected by CT readings of corresponding internal 18S rRNA as control gene. There was an interaction between the effect of LPS and fasting on serum concentrations of TNF-α (F1,33 = 0.14, P = 0.70). Each bar represents the mean ± SE for 7–9 rats/group. *P < 0.05 vs. vehicle-ad libitum fed (2-way ANOVA and Student’s t-test).

Discussion

Our data show that GHRP-2 administration prevented liver injury induced by LPS treatment, as demonstrated by the significant reduction in serum and liver cell release of transaminases. This effect seems to be related to the effects of GHRP-2 decreasing hepatic TNF-α and NO and increasing hepatic IGF-I. Since GHRP-2 decreases the LPS-induced increase in TNF-α and NO production by cocultures of hepatocytes and nonparenchymal cells but does not modify the LPS-induced increase in TNF-α and NO production by monocultures of hepatocytes, these data suggest that the protective effect of GHRP-2 on the liver is exerted on nonparenchymal cells.

LPS administration induced an increase in the release of alanine and aspartate transaminases, both in monocultures of hepatocytes and in cocultures of hepatocytes and nonparenchymal cells, which results in an increase in serum concentrations of both transaminases. This effect on serum transaminases has been previously described after LPS administration (29, 39) and in situations in which liver injury was induced by injection of LPS plus a bacillus (21, 67). LPS injections increased serum nitrites/nitrates and TNF-α mRNA in the liver. Increases in serum nitrites/nitrates (15) as well as in hepatic gene expression of IGF-I in monocultures of hepatocytes (P < 0.05; Table 3), and GHRP-2 did not have any effect on the gene expression of IGF-I in vehicle or LPS monocultures.

Table 4 shows the effect of LPS and GHRP-2 administration on GHSR mRNA levels in the whole liver, in cocultures of hepatocytes and nonparenchymal cells, and in hepatocyte monocultures. We could not detect the GHSR mRNA levels in all samples. For this reason, Table 3 shows the frequency of detection of GHSR. In the liver of both vehicle groups, we detected GHSR in only 5 rats of 19 (26.3%). However, in both LPS-injected groups, the frequency was higher than in the vehicle groups: 12 rats of 17 (70.5%, P < 0.05). In cocultures of hepatocytes and nonparenchymal cells, the frequency of samples in which we detected GHSR was similar (~30%). However, in hepatocyte monocultures, we could not detect GHSR mRNA.

Discussion

Our data show that GHRP-2 administration prevented liver injury induced by LPS treatment, as demonstrated by the significant reduction in serum and liver cell release of transaminases. This effect seems to be related to the effects of GHRP-2 decreasing hepatic TNF-α and NO and increasing hepatic IGF-I. Since GHRP-2 decreases the LPS-induced increase in TNF-α and NO production by cocultures of hepatocytes and nonparenchymal cells but does not modify the LPS-induced increase in TNF-α and NO production by monocultures of hepatocytes, these data suggest that the protective effect of GHRP-2 on the liver is exerted on nonparenchymal cells.

LPS administration induced an increase in the release of alanine and aspartate transaminases, both in monocultures of hepatocytes and in cocultures of hepatocytes and nonparenchymal cells, which results in an increase in serum concentrations of both transaminases. This effect on serum transaminases has been previously described after LPS administration (29, 39) and in situations in which liver injury was induced by injection of LPS plus a bacillus (21, 67). LPS injections increased serum nitrites/nitrates and TNF-α mRNA in the liver. Increases in serum nitrites/nitrates (15) as well as in hepatic...
In the liver, LPS increased the nitrite/nitrate levels in the hepatocyte culture medium, as our group (46) and others (20) have previously reported. Moreover, LPS also increased the TNF-α mRNA in hepatocyte cultures in accordance with data on the literature (66). TNF-α mRNA in hepatocytes can be also induced by proinflammatory cytokine IL-1β (3). However, the effects of LPS on TNF-α mRNA are more evident in the cocultures of hepatocytes and nonparenchymal cells than in monocultures of hepatocytes (26, 52, 66).

GHRP-2 administration improved LPS-induced hepatic dysfunction, as shown by the reduction in transaminase levels in serum. This reduction seems to be the result of the GHRP-2 effects on nonparenchymal cells, since this ghrelin agonist decreased transaminase production in cocultures of hepatocytes and nonparenchymal cells, but it had no effect on transaminase production by hepatocyte cultures. In addition, GHRP-2 treatment of LPS-injected animals decreased serum nitrite/nitrate concentrations and TNF-α gene expression in the liver. This last effect was more evident in vitro, since the gene expression of TNF-α significantly decreased in the cocultures.

Fig. 8. Effect of GHRP-2 on AST (A), ALT (B), and nitrite/nitrate (C) production by cocultures of hepatocytes and nonparenchymal cells in response to LPS (100 ng/ml). Cocultures were obtained from adult male Wistar rats. Hepatocytes and nonparenchymal cells (3:2) were incubated with LPS (100 ng/ml) and/or GHRP-2 (10⁻⁷ M), and cell coculture supernatants were collected after 24 h. There was an interaction between the effect of LPS and peptide administration on AST (F₁,₃₅ = 6.69, P < 0.05) and on ALT (F₁,₃₇ = 4.12, P < 0.05), as GHRP-2 decreased AST and ALT release to the culture medium in LPS but not in vehicle cocultures. There was no interaction between the effect of LPS and peptide administration on nitrite/nitrate production by cocultures of hepatocytes and nonparenchymal cells (F₁,₃₇ = 2.44, P = 0.12). Values shown are means ± SE for n = 9–10 petri dishes. Nitrite/nitrate values are presented as the percentage of the mean value in vehicle rats treated with saline. **P < 0.01 vs. vehicle medium. *P < 0.05 vs. LPS medium (2-way ANOVA and Student’s t-test).

Fig. 9. Effect of LPS (100 ng/ml) and GHRP-2 (10⁻⁷ M) on TNF-α mRNA (A) and on IGF-I mRNA (B) levels in hepatocytes and nonparenchymal cell cocultures. Gene expressions of TNF-α and IGF-I were quantified using real-time RT-PCR and are presented as a percentage of the mean value in vehicle rats treated with saline. **P < 0.01 vs. vehicle medium. *P < 0.05 vs. LPS medium (2-way ANOVA and Student’s t-test).
of hepatocytes and nonparenchymal cells. The decrease in serum nitrates/nitrates after GHRP-2 administration in rats injected with LPS may be in part the result of the effect of this peptide on the liver, since the nitrite/nitrate release to the culture medium decreased in cocultures of hepatocytes and nonparenchymal cells. Moreover, GHRP-2 can act on other extrahaepatic immune tissues that exhibit ghrelin receptors (12, 40) and that are contributing to NO production after LPS challenge. In this sense, we have reported that GHRP-2 directly decreased nitrite/nitrate release from peritoneal macrophages in vitro (17).

The data suggest that suppression of TNF-α and NO could be one of the mechanisms by which GHRP-2 attenuated hepatic injury induced by LPS. Consistent with this suggestion, it has been reported that inhibition of NO from iNOS protects against liver injury induced by mycobacterial infection and endotoxins or by LPS and drugs (22, 30). Moreover, the depletion of Kupffer cells by gadolinium chloride (34) or by liposomal clodronate (55) and neutralization of TNF-α with anti-TNF-α antibody (64) are effective in reducing hepatic damage. Our results are also in agreement with the protective role that compounds different from GHRP-2 had in hepatic injuries caused by LPS, through the inhibition of TNF-α or NO (14, 21, 59, 67).

Although it has been reported (65) that ghrelin downregulates TNF-α and IL-6 in sepsis through activation of the vagus nerve, we and others demonstrated that both GHRP-2 and ghrelin directly prevented the LPS-induced release of IL-6 from human monocytes (9) and from rat peritoneal macrophages (17). In the present study, effects of GHRP-2 on TNF-α and nitrates/nitrates in cocultures suggest that GHRP-2 acts directly on nonparenchymal cells, inhibiting TNF-α and NO. In this sense, binding sites for GH secretagogues have been found by radioreceptor assay in the liver (44). On the basis of these observations, we investigated the presence of GHSR in the liver and in cultured cells by RT-PCR. We could not detect GHSR in the liver of all rats. This could be due to the fact that hepatocytes perhaps do not express GHSR. These data are in agreement with those previously reported in humans (12) and mice (56). The fact that the frequency of GHSR expression increased in LPS-induced rats suggests that this expression occurs in macrophages that migrate to the liver. These data are also supported by the presence of GHSR in monocytes (9).

As previously reported, we found that LPS decreased body weight gain (15) and IGF-I in vivo (46). These effects of LPS are not only due to the decrease in food intake, since LPS decreased IGF-I in vitro (Ref. 46 and present study). Moreover, LPS administration to fasted rats was able to decrease body weight gain as well as serum IGF-I and its gene expression in the liver. In agreement with our results, it has been reported that GHRP-2 increased body weight gain in control mice (62) and plasma IGF-I in cachectic conditions (18, 63). The effect of GHRP-2 on IGF-I does not seem to be mainly due to the increase in food intake, as demonstrated by the in vitro studies. Moreover, GHRP-2 increased food intake and body weight gain, but it does not modify circulating IGF-I and its gene

Table 3. Effect of LPS and GHRP-2 on AST, ALT, nitrite/nitrate production, TNF-α mRNA, and IGF-I mRNA levels in hepatocyte monocultures

<table>
<thead>
<tr>
<th></th>
<th>Vehicle-Saline</th>
<th>Vehicle-GHRP-2</th>
<th>LPS-Saline</th>
<th>LPS-GHRP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST, U/l</td>
<td>100.2 ± 20.9</td>
<td>111.5 ± 16.1</td>
<td>144.6 ± 11.5</td>
<td>185.6 ± 15.7</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>21.1 ± 1.8</td>
<td>22.3 ± 1.1</td>
<td>31.0 ± 4.2*</td>
<td>34.6 ± 4.2*</td>
</tr>
<tr>
<td>Nitrite/nitrate, μM</td>
<td>100.0 ± 3.9</td>
<td>101.7 ± 4.7</td>
<td>197.7 ± 9.2*</td>
<td>210.4 ± 7.8*</td>
</tr>
<tr>
<td>TNF-α mRNA⁺</td>
<td>100.0 ± 7.2</td>
<td>111.7 ± 9.5</td>
<td>158.1 ± 16.6*</td>
<td>201.5 ± 26.5*</td>
</tr>
<tr>
<td>IGF-I mRNA⁺</td>
<td>100.0 ± 5.7</td>
<td>114.4 ± 6.3</td>
<td>63.4 ± 13.1*</td>
<td>68.7 ± 13.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE for n = 6–8 petri dishes. Effect of LPS (100 ng/ml) and GHRP-2 (10⁻⁷ M) on aspartate aminotransferase (AST), alanine aminotransferase (ALT), nitrite/nitrate production, TNF-α mRNA, and IGF-I mRNA levels in hepatocyte monocultures. Cultures were obtained from adult male Wistar rats. Hepatocytes (5 × 10⁶) were incubated with LPS and/or GHRP-2, and cell culture supernatants were collected after 24 h. There was no interaction between the effects of LPS and peptide administration on AST (F1,28 = 0.76, P = 0.39), on ALT (F1,28 = 0.13, P = 0.72), and on nitrite/nitrate production by cultures of hepatocytes (F1,28 = 0.64, P = 0.42). *Gene expressions of TNF-α and IGF-I were quantified using real-time RT-PCR and are presented as a percentage of the mean value in vehicle rats treated with saline by analyzing the critical threshold (Ct) nos. corrected by Ct readings of corresponding internal HPRT controls. There was no interaction between the effects of LPS and GHRP-2 on TNF-α mRNA (F1,28 = 0.58, P = 0.45) and on IGF-I mRNA (F1,27 = 0.14, P = 0.71), *P < 0.05 and †P < 0.01 vs. vehicle medium. ’P < 0.05 and ‡P < 0.01 vs. vehicle-GHRP-2 medium (2-way ANOVA and Student’s t-test).

Table 4. Effect of LPS and GHRP-2 on frequency of GHSR expression in the liver in vivo, in cocultures of hepatocytes and nonparenchymal cells, and in hepatocyte monocultures

<table>
<thead>
<tr>
<th></th>
<th>Vehicle-Saline, n/N (%)</th>
<th>Vehicle-GHRP-2, n/N (%)</th>
<th>LPS-Saline, n/N (%)</th>
<th>LPS-GHRP-2, n/N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver in vivo</td>
<td>4/10 (40%)</td>
<td>1/9 (10%)</td>
<td>7/8 (87.5%)*</td>
<td>5/9 (55.5%)‡</td>
</tr>
<tr>
<td>Cocultures of hepatocytes and nonparenchymal cells</td>
<td>2/10 (20%)</td>
<td>5/9 (55.5%)</td>
<td>3/10 (30%)</td>
<td>3/10 (30%)</td>
</tr>
<tr>
<td>Monocultures of hepatocytes</td>
<td>1/8 (12.5%)</td>
<td>0/7 (0%)</td>
<td>0/8 (0%)</td>
<td>0/7 (0%)</td>
</tr>
</tbody>
</table>

Effect of LPS (100 ng/ml) and GHRP-2 (10⁻⁷ M) on frequency of GHSR expression in the liver in vivo, in cocultures of hepatocytes and nonparenchymal cells, and in hepatocyte monocultures. Cultures were obtained from adult male Wistar rats. Cocultures of hepatocytes and nonparenchymal cells (5 × 10⁶ cells in a ratio of 3:2) and hepatocyte monocultures (5 × 10⁶ cells) were incubated with LPS and/or GHRP-2, and cell culture supernatants were collected after 24 h. Gene expression of GHSR was measured using real-time RT-PCR. LPS increased GHSR detection both in saline (χ² = 4.14, P < 0.05) and in GHRP-2 groups (χ² = 4.39, P < 0.05). *P < 0.05 vs. vehicle-saline. ‡P < 0.05 vs. vehicle-GHRP-2 (χ² test).

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expression in the liver in vehicle rats. The increase in hepatic IGF-I after GHRP-2 administration might contribute to the attenuation of the hepatic injury induced by LPS. Recent evidence showed that serum levels of both alanine and aspartate aminotransferases were reduced in transgenic mice expressing IGF-I (50) and in rats treated with IGF-I (28) after liver injury.

The improvement of hepatic IGF-I mRNA and circulating levels of IGF-I after GHRP-2 administration could be the result of the decrease induced by GHRP-2 on hepatic TNF-α mRNA and nitrite/nitrate production. We have reported that TNF-α blockade in arthritic rats prevents the decrease in hepatic IGF-I, suggesting an inhibitory role of TNF-α on IGF-I synthesis (16). Moreover, the inhibition of Kupffer cells by gadolinium chloride administration blocked the inhibitory effect of LPS on serum concentrations of IGF-I and on its gene expression in the liver (15). In addition, the inhibition of NO production by aminoguanidine administration prevented the effect of LPS on circulating IGF-I and its gene expression in the liver (49). All these data suggest that the immunomodulatory effect of GHRP-2 improving IGF-I could be mediated by TNF-α and NO. Although we have not administered GHRP-2 after LPS, but rather simultaneously, in a previous experiment on arthritic rats, we injected GHRP-2 for 1 wk when the illness was already established. In this situation, GHRP-2 treatment had a therapeutic effect, decreasing paw swelling and serum nitrite/nitrate and IL-6 levels (17).

Effects of GHRP-2 on the liver do not seem to be due to the modifications in food intake or water intake, as demonstrated by the in vitro studies. Moreover, fasting did not have any effect on serum concentrations of transaminases, serum nitrate and IL-6 levels (17).

In summary, this is the first time that the effects of GHRP-2, the synthetic ghrelin analog, on LPS-induced liver dysfunction are reported. This study shows that GHRP-2 has a protective effect on LPS-induced liver injury, decreasing the inflammatory response and increasing IGF-I. Moreover, the in vitro experiment supports the direct protective effect of GHRP-2 on the liver, acting on nonparenchymal cells. The present study also suggests that GHRP-2 may be a useful therapeutic tool for endotoxin-induced liver injury.

ACKNOWLEDGMENTS

We thank A. Carmona and M. A. Ramirez for technical assistance and Christina Bickart for proofreading the manuscript. We are also indebted to the National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program for the reagents for IGF-I determinations.

GRANTS

This work was supported by Comunidad Autónoma de Madrid (GR/SAL/0704/2004) and by a Fellowship from Ministerio de Educación y Ciencia to M. Granado (Formación del Profesorado Universitario, AP2003-2564).

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