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

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Granado M, Martín AI, López-Menduña 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 IGF-I and 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 N-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 nitrate/nitrite 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).
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, and taking into account that the interactions between hepatocytes and nonparenchymal cells are critical for the development of a hepatic inflammatory response (26, 52, 66), we compared the effects of LPS and GHRP-2 in cocultures of 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 0800 the following day. This LPS administration protocol has been shown to decrease levels of serum IGF-I and its 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 in arthritic rats (17). Rats were killed by decapitation 4 h after the second injection.

Because LPS food intake decreased, 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 the experiment, from 1700 until 1200 the following day, but with free access to drinking water; and 2) “ad libitum” rats with free access to food and water during the 18 h of the following day. Trunk blood was collected in cooled tubes, allowed to clot, and centrifuged, and the serum was stored at −20°C until aspartate aminotransferase (AST), alanine aminotransferase (ALT), nitrites/nitrates, and IGF-I analyses were performed. Immediately after decapitation, the liver was removed, frozen in liquid nitrogen, and stored at −80°C until RNA extraction was performed. Body weight 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 expressed as grams or milliliters, respectively, per 100 gms of body weight.

Hepatocyte isolation. 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%.

Nonparenchymal cell isolation. The supernatants from the differential centrifugations were collected and centrifuged at 2,200 rpm for 6 min at 4°C. The pellet was resuspended in culture medium. Light microscopic observation revealed a purity >90% for nonparenchymal cells; viability was >80% as assessed by Trypan blue exclusion.

Cell cultures. Hepatocytes (5 × 10⁶ in 5 ml of medium) or hepatocytes and nonparenchymal cells (in a ratio of 3 × 10⁶ hepatocytes to 2 × 10⁶ nonparenchymal cells, in 5 ml of medium) were cultured on 100-mm gelatin-coated petri dishes. Medium consisted of Williams’ medium E (Gibco, Cedex, France) with L-glutamine (2 mM), insulin (1 μM), HEPES (15 mM), penicillin-streptomycin (100 units/ml + 100 μg/ml), and 10% low endotoxin calf serum. After 24-h incubation (37°C in 95% air-5% CO₂), the medium was changed to include the pulses (LPS at 100 ng/ml, GHRP-2 at 10⁻⁷ M, or both) in serum-free medium. At these concentrations, LPS is able to increase nitrite/nitrate production by hepatocyte cultures and by peritoneal macrophages (46), and GHRP-2 is able to decrease nitrite/nitrate production by peritoneal macrophages (17). Cells were incubated with the different stimuli for another 24 h. At the end of the incubation period, the medium was removed and stored at −80°C for nitrite/nitrate determination. Total RNA from cells was isolated to measure TNF-α, IGF-I, and GHSR mRNAs by real-time PCR.

Determination of transaminases and nitrite and nitrate concentration. Serum and culture medium AST and ALT were measured following the instructions of a commercial kit from Spinreact (Barcelona, Spain). Serum and culture medium nitrite and nitrate concentrations were measured by a modified method of the Griess assay described by Miranda et al. (41). The serum was deproteinized to reduce turbidity by centrifugation through a 30-kDa molecular mass filter using a Centrifree Micropartition Device with a YM-30 ultrafiltration membrane (Amicon Division, Millipore, Bedford) at 1,500 rpm for 1 h at 37°C for 300-μl samples. One hundred microliters of filtered serum or culture medium were mixed with 100 μl of vanadium chloride, rapidly followed by the addition of the Griess reagents. Thirty minutes later, the determination was performed at 37°C. The absorbance was measured at 540 nm. Nitrite and nitrate concentrations were calculated using a sodium nitrite standard curve.

IGF-I determination. Serum IGF-I was removed from IGF-binding proteins by an acid ethanol extraction and was measured by a double-antibody RIA (54). The IGF-I antiserum (UB2-495) was a gift from Drs. L. E. Underwood and J. J. Van Wyk and was distributed by the Hormone Distribution Program of National Institute of Diabetes and Digestive and Kidney Diseases through the National Hormone and Pituitary Program. Levels of IGF-I were expressed in terms of IGF-I from Gropep (Adelaide, Australia). The sensitivity of the assay

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
<th>Product, bp</th>
</tr>
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<tr>
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<td>TCCGGAGAAGAACAGCTACCC</td>
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<tr>
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<tr>
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<td>GCAAGTCCAGAAGACATGTAACGCC</td>
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GHSR, growth hormone (GH) secretagogue receptor; HPRT, hypoxanthine-guanine phosphoribosyl transferase.

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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.

**Figs. 1 and 2.** 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 \((F_{1,39} = 4.81, P < 0.05)\) and on serum concentrations of ALT \((F_{1,32} = 4.73, P < 0.05)\), 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 \((F_{1,32} = 1.28, P = 0.26)\). 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. 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 \((F_{1,39} = 4.81, P < 0.05)\) and on serum concentrations of ALT \((F_{1,32} = 4.73, P < 0.05)\), 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 \((F_{1,32} = 1.28, P = 0.26)\). 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 (CT) 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 \((F_{1,30} = 0.01, P = 0.91)\). There was an interaction between the effect of LPS and GHRP-2 on serum concentrations of IGF-I \((F_{1,33} = 5.38, P < 0.05)\), 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 ΔΔC_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-α 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 (F1,33 = 2.07, P = 0.15). There was an interaction between the effect of LPS and fasting on body weight gain (F1,33 = 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 7–11 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).

The effects of GHRP-2 (Fig. 4A) or fasting (Fig. 4B) on body weight gain, food intake, and water intake response to LPS administration are shown in Fig. 4 and Table 2. LPS induced a significant decrease in body weight gain (P < 0.01; Fig. 4A) and food intake (P < 0.01; Table 2) compared with vehicle. GHRP-2 administration resulted in a significant increase in body weight gain and food intake both in vehicle rats and in LPS-injected rats (Fig. 4A and Table 2). Water intake was not modified by GHRP-2 administration. As expected, fasting decreased body weight gain in vehicle rats (P < 0.01; Fig. 4B). However, LPS administration was able to induce a significant decrease in body weight gain in fasted rats (−11.9 ± 0.9 vs. −20.1 ± 1.1, P < 0.01; Fig. 4B). Both fasting and LPS decreased water intake, but this decrease was only statistically significant in fasted LPS-injected rats (P < 0.05; Table 2).

Figure 5 shows the effect of LPS administration and fasting on serum concentrations of AST (Fig. 5A), ALT (Fig. 5B), and
There was no interaction between the effects of LPS and GHRP-2 administration on food intake (F1,7, P = 0.86). There was no interaction between the effects of LPS and fasting on food intake (F1,7, P = 0.99). There was no interaction between the effects of LPS and fasting on water intake (F1,7, P = 0.31) and water intake (F1,9, P = 0.03) and on serum concentrations of nitrites/nitrates (F1,32, P = 0.01). 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-

Table 2. Effect of GHRP-2 administration and fasting on food intake and water intake in vehicle or LPS-injected rats

<table>
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<th>Vehicle-Saline</th>
<th>Vehicle-GHRP-2</th>
<th>LPS-Saline</th>
<th>LPS-GHRP-2</th>
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</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±0.1b</td>
<td>2.2±0.0a</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>
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</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 amounts 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, P = 0.31) and water intake (F1,9, P = 0.03). The addition of LPS to the coculture medium also increased transaminase concentrations (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-

Fig. 5. Serum concentrations of AST (A), ALT (B), and nitrites/nitrates (C) in fasted rats treated ip with LPS (1 mg/kg). Two-way ANOVA revealed that there was no interaction between the effect of LPS and fasting on serum concentrations of AST (F1,32, P = 0.78, P = 0.38), on serum concentrations of ALT (F1,31, P = 0.03, P = 0.85), and on serum concentrations of nitrites/nitrates (F1,33, P = 0.02, P = 0.89). Values shown represent means ± SE for 6–9 rats/group. Nitrites/nitrates are presented as the percentage of the mean value in vehicle-ad libitum-fed rats. *P < 0.05 and **P < 0.01 vs. vehicle-ad libitum fed. #P < 0.05 and ###P < 0.01 vs. vehicle-fasted (2-way ANOVA and Student’s t-test).
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dium prevented this effect, since TNF-α mRNA in cocultures incubated with LPS and GHRP-2 was similar to that of the vehicle cocultures. Moreover, GHRP-2 decreased TNF-α mRNA in vehicle cocultures of hepatocytes with nonparenchymal cells (P < 0.01; Fig. 9A). The gene expression of IGF-I was decreased by LPS in the incubation medium (P < 0.01; Fig. 9B), and GHRP-2 increased the IGF-I mRNA from cocultures of hepatocytes and nonparenchymal cells with LPS (P < 0.05; Fig. 9B).

As for cocultures of hepatocytes and nonparenchymal cells, the addition of LPS to the monolayer of hepatocytes increased transaminases (P < 0.05 for ALT; Table 3) and nitrite/nitrate production (P < 0.01; Table 3). However, the presence of GHRP-2 in the incubation medium did not prevent these effects. TNF-α mRNA was increased by LPS in monocultures of hepatocytes (P < 0.05; Table 3), and the addition of GHRP-2 to the incubation medium did not modify TNF-α mRNA in vehicle or LPS monocultures of hepatocytes. Incubation of cells with LPS decreased the gene expression of IGF-I in monolayers 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 monolayers. 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 monolayers, 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

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 no interaction between the effect of LPS and fasting on hepatic TNF-α gene expression (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).
inducible NO synthase (iNOS) and TNF-α mRNAs after LPS have been previously reported (39, 43). The increased TNF-α and NO can contribute to liver damage, as previously described (22, 36).

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 (F_{1,35} = 6.69, P < 0.05) and on ALT (F_{1,37} = 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 (F_{1,37} = 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).**
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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 ± 18.6</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 x 10^5) 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 (F1,26 = 0.76, P = 0.39), on ALT (F1,28 = 0.13, P = 0.72), and on nitrite/nitrate production by cultures of hepatocytes (F1,26 = 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 (F1,26 = 0.58, P = 0.45) and on IGF-I mRNA (F1,27 = 0.14, P = 0.71). *P < 0.05 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%)‡</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 x 10^6 cells in a ratio of 3:2) and hepatocyte monocultures (5 x 10^5 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).
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 an anti-inflammatory 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 nitrites/nitrates, and TNF-α gene expression in the liver, both in vehicle rats and in LPS-injected rats, although it decreased water intake in LPS-injected rats.

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.

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