Anti-inflammatory effect of the ghrelin agonist growth hormone-releasing peptide-2 (GHRP-2) in arthritic rats

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Ghrelin is a gastrointestinal hormone that has been proposed as a treatment to prevent cachexia. Ghrelin is a well-established experimental model of rheumatoid arthritis, and GHRP-2 treatment decreased the serum IL-6 levels (P < 0.01). To elucidate whether GHRP-2 is able to modulate IL-6 release in arthritic rats. Male Wistar rats were injected with Freund’s adjuvant, and 15 days later arthritic and control rats were daily injected with GHRP-2 (100 µg/kg) or with saline for 8 days. Arthritis induced an increase in serum ghrelin (P < 0.01) and a decrease in serum concentrations of leptin (P < 0.01), whereas GHRP-2 administration increased serum concentrations of leptin. GHRP-2 increased food intake in control rats but not in arthritic rats. However, in arthritic rats GHRP-2 administration ameliorated the external symptoms of arthritis, as it decreased the arthritis score (10.4 ± 0.8 vs. 13.42 ± 0.47, P < 0.01) and the paw volume. In addition, circulating IL-6 and nitrites/nitrates were increased by arthritis, and GHRP-2 treatment decreased the serum IL-6 levels (P < 0.01). To elucidate whether GHRP-2 is able to modulate IL-6 release directly on immune cells, peritoneal macrophages cultures were incubated with GHRP-2 or ghrelin, the endogenous ligand of the growth hormone (GH) secretagogue receptor. Both GHRP-2 (10−7 M) and ghrelin (10−7 M) prevented endotoxin-induced IL-6 and decreased nitrate/nitrate release from peritoneal macrophages in vitro. These data suggest that GHRP-2 administration has an anti-inflammatory effect in arthritic rats that seems to be mediated by ghrelin receptors directly on immune cells.

 interleukin-6; inflammation

RHEUMATOID ARTHRITIS IS A CHRONIC DISEASE associated with multiple inflammatory mediators that lead to joint damage, cachexia, and disability. Adjuvant-induced arthritis in rats is a well-established experimental model of rheumatoid arthritis and chronic inflammatory illnesses (43). Adjuvant arthritis can be induced in rats by an intradermal injection of Freund’s adjuvant. On days 14–15 after adjuvant injection, rats develop chronic inflammation and polyarthritis, which lead to a marked decrease in body weight and cachexia (23, 32). Similarly, rheumatoid arthritis patients have cachexia, hypermetabolism, and accelerated protein breakdown (33). Chronic arthritis induced a decrease in food intake, but it was not the only factor responsible for the decrease in body weight gain (32).

Growth hormone (GH) secretagogues (GHS) are a group of synthetic compounds that induce GH secretion through the activation of the GHS receptor (GHS-R). Ghrelin, a recently discovered peptide hormone secreted mainly by the stomach, has been identified as the endogenous ligand of the GHS-R and has a potent GH-releasing effect (22). In addition, ghrelin stimulates food intake and promotes adiposity by a GH-independent action (40). These data suggest that ghrelin plays an important role in the regulation of metabolic balance. Peripheral administration of ghrelin attenuated body weight loss in a rat model of cachexia with chronic heart failure (29) or in mice with cancer cachexia (15). For this reason, ghrelin has been suggested as a treatment to prevent cachexia resulting from different illnesses (20). Ghrelin administration increases body weight in rats with endotoxin-induced wasting syndrome (17). However, there are no data on changes in plasma ghrelin in rats with chronic arthritis or about the effects of exogenous ghrelin administration in chronic inflammation.

Because ghrelin has a short half-life (40), we used the synthetic analog growth hormone-releasing peptide-2 (GHRP-2) to study GHS-R-mediated effects on chronic arthritis induced by adjuvant injection. GHRP-2 is a hexapeptide and a potent ghrelin receptor agonist of the GHRP family (8). Chronic GHRP-2 administration increases body weight and bone mass in mice (41). In addition, administration of the ghrelin receptor agonist GHRP-2 to critically ill patients is able to improve protein catabolism (42).

Thus the objectives of this study were to examine whether plasma ghrelin is affected by chronic arthritis and to investigate whether the administration of the GHRP-2 to arthritic rats improves the illness. As all GHS stimulate the release of ACTH and glucocorticoids (38) and this hormone has anti-inflammatory effects, serum concentrations of ACTH and corticosterone were determined. Finally, the effect of GHRP-2 and ghrelin directly on the immune cells in culture was also studied. We observed that administration of GHRP-2 to arthritic rats, during the chronic phase of the illness, ameliorates the inflammation and decreases IL-6 levels. Moreover, GHRP-2 and rat ghrelin also have an anti-inflammatory effect in vitro, since they are able to decrease bacterial lipopolysaccharide (LPS)-induced IL-6 and nitrate/nitrate release from peritoneal macrophages. These results may have clinical impact, as they show the possible usefulness of GHS for immunotherapy. To our knowledge, this is the first report on an anti-inflammatory effect of the ghrelin agonists in experimental arthritis.
MATERIALS AND METHODS

Animals. Arthritic and control male Wistar rats were purchased from Charles River (Barcelona, Spain) and weighed 150–175 g (6 wk) at the beginning of the experiment. Arthritis was induced in the rats by an intradermal injection of 1 mg of heat-inactivated Mycobacterium butyricum in incomplete Freund’s adjuvant in the right paw. Control animals were injected with mineral oil. The procedures followed the guidelines recommended by the European Union for the care and use of laboratory animals and were approved by the Animal Care Committee of the Faculty of Medicine, Universidad Complutense, Madrid.

Rats were housed three to four per cage under controlled conditions of light (lights on from 0730 to 1930) and temperature (22 ± 2°C). Food and water were available ad libitum. The arthritis index of each animal was scored by grading each paw from 0 to 4, determined as 0, no erythema or swelling; 1, slight erythema or swelling of one or more digits; 2, entire paw swollen; 3, erythema and swelling of the ankle; and 4, ankylosis, incapacity to bend the ankle. The severity score was the sum of the clinical scores of each limb, the maximum value being 16. Rats having an arthritis score on day 15 below 9 (6 of 30 rats) were excluded from the experiment. On day 15 after adjuvant injection, 24 arthritic and 20 control rats were randomly divided into two groups; one group was subcutaneously injected daily with 100 μg/kg GHRP-2 (Bachem, Bubendorf, Switzerland) during the following 16 days and the second group received 250 μl of saline. The GHRP-2 dose was chosen taking into account that chronic administration of ghrelin (100 μg/kg sc) was able to increase body weight in rats with heart failure (15). All rats were weighed daily, and the arthritis score was taken. Food intake per cage was calculated daily by measuring the difference between the initial and the remaining amount of pellets in the feeder, and expressed as grams per rat per 100 grams body weight.

Control and arthritic rats were killed by decapitation 22 days after adjuvant or vehicle injection and after 8 days of GHRP-2 treatment, 2.5 h after the last injection. Trunk blood was collected in cooled tubes, allowed to clot, and centrifuged, and the serum was stored at −20°C until nitrites and leptin assays were performed and at −80°C until ghrelin and IL-6 analyses were performed. The left hindpaw was amputated, and volume was measured by water displacement.

Peritoneal macrophage cultures. Male Wistar rats (250 g, 8 wk) were killed by decapitation. Cells were obtained by peritoneal lavages. Ten milliliters of sterile saline were injected in the abdominal cavity, and after massaging, the solution was extracted again, collected in a sterile tube, and kept on ice. Cell suspension was washed and centrifuged twice at 1,000 rpm at 4°C and then suspended in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Paisley, UK) supplemented with glutamine (2 mM), gentamicin sulfate (50 μg/ml), fetal bovine serum (10%), and mercaptoethanol (1 mM) (all from Sigma, Madrid, Spain). Cellular viability was estimated by the Trypan blue test, and cellular recount was done. Cells (5 × 10⁶/well in 500 μl of culture medium) were distributed in 24-well culture plates (Nunc, Roskilde, Denmark) and grown at 37°C with 5% CO₂. After 2 h of preincubation, wells were washed to remove nonadherent cells. The macrophages, still adhering to the bottom of the wells, were incubated for 24 h in 500 μl of supplemented DMEM with the different stimuli. LPS (1 or 100 ng/ml, serotype 055:B5, Sigma), GHRP-2 (10⁻⁶ M) and rat ghrelin (10⁻⁹ M, Sigma) were dissolved in the assay medium. At this concentration, both compounds are able to increase rat pituitary GH secretion in vitro (31, 39). The culture medium was collected and stored at −20°C until nitrite/nitrate analysis and at −80°C until IL-6 determination. The experiments were performed twice.

Leptin, ghrelin, corticosterone, ACTH, and IL-6 measurements. Serum concentrations of rat leptin and rat ghrelin (active) were determined by radioimmunoassay (RIA) using commercial kits from Linco Research (St. Charles, MO; www.lincoresearch.com) following the manufacturer’s instructions. Serum concentrations of corticosterone were determined by a competitive protein-binding assay (26). ACTH levels were measured by RIA with a commercial kit from Diagnostic System Laboratories (Webster, TX; www.dslabs.com). Rat IL-6 levels in serum and culture medium were measured by rat Biotrak enzyme-linked immunosorbent assay (ELISA) system kit (Amersham Biosciences, Little Chalfont, UK; www.amershambiosciences.com).

Nitrite determination. Nitrite and nitrate concentrations in serum and culture medium were measured by a modified method of the Griess assay, described by Miranda et al. (27). Serum was deproteinized to reduce turbidity by centrifugation through a 30-kDa molecular mass filter by use of a Centrifree Micropartition Device with a YM-30 ultrafiltration membrane (Amicon Division, Millipore, Bedford, TX), at 15,000 rpm for 1 h at 37°C for 300-μl samples. One hundred microliters of filtrated serum or 1:10-diluted culture medium was mixed with 100 μl of vanadium chloride, which was quickly followed by the addition of the Griess reagents. The determination was performed after incubation at 37°C for 30 min. Absorbance was measured at 540 nm. Nitrite and nitrate concentrations were calculated using a NaNO₂ standard curve and expressed as micromoles per liter. Statistical analysis. Statistics were computed using the statistics program Statgraphics plus for Windows. Statistical significance was calculated by multifactorial analysis of variance (ANOVA), with arthritis and GHRP-2 administration as factors. Post hoc comparisons were made using the unpaired Student’s t-test. A P value of <0.05 was considered significant.

RESULTS

As shown in Fig. 1, there was a decrease in cumulative body weight gain in arthritic rats (P < 0.01). GHRP-2 administration
increased body weight gain during the 8 days of treatment (P < 0.05). Arthritis induced a decrease in cumulative food intake (P < 0.01), although this decrease (10% over control rats treated with saline) was not as big as the decrease in body weight gain induced by arthritis (74% over control rats injected with saline). GHRP-2 administration increased food intake in control but not in arthritic rats (Fig. 1).

Serum concentrations of ghrelin and leptin are shown in Fig. 2. The effect of arthritis on serum ghrelin was the opposite of the effect that it had on serum concentrations of leptin, as arthritic rats had higher serum ghrelin (P < 0.01) and lower serum leptin levels (P < 0.01) than control rats. GHRP-2 administration increased the serum concentration of leptin (P < 0.01) in both control and arthritic rats.

As shown in Fig. 3, the arthritic rats injected with saline had higher serum concentrations of corticosterone and ACTH (P < 0.01) than the control rats treated with saline. GHRP-2 administration had a different effect in control than in arthritic rats. In control rats, GHRP-2 induced an increase in serum concentrations of ACTH (P < 0.05) and in corticosterone, although this increase was not statistically significant. However, in the arthritic rats, GHRP-2 administration decreased the serum concentrations of ACTH (P < 0.05) and corticosterone (P < 0.01).

Arthritis induced a significant increase in paw volume (P < 0.01). GHRP-2 treatment reduced paw volume in arthritic rats (P < 0.01), whereas GHRP-2 administration had no effect on paw volume in control rats (Fig. 4). The anti-inflammatory effect of GHRP-2 administration was also evident in the evolution of the arthritis score. In the arthritic rats injected with GHRP-2, the arthritis score was lower than in the rats injected with saline. This difference was significant starting at the 6th day of treatment (Fig. 4).

The serum concentrations of nitrites/nitrates was increased by 10.22 ± 0.32 in October 29, 2017 http://ajpendo.physiology.org/ Downloaded from arthritis-saline. **P < 0.01; Fig. 5). In the arthritic rats injected with saline, this difference was significant starting at the 6th day of treatment (Fig. 4).

In vitro experiment. Because GHRP-2 administration decreased IL-6 in serum, we examined whether GHRP-2 and the natural ligand of the GH secretagogue receptor ghrelin directly affected macrophage mediator production in vitro. The addition of LPS (1 or 100 ng/ml) to the culture medium increased macrophage nitrite/nitrate production (P < 0.01; Fig. 6). In the peritoneal macrophages incubated with GHRP-2 or ghrelin,
administration of LPS (1 and 100 ng/ml) also increased nitrite/nitrate production \((P < 0.01)\). However, the amount of nitrite/nitrate released after LPS stimulation was lower in the macrophages incubated with GHRP-2 or ghrelin than in the control cultures (Fig. 6).

IL-6 production was also increased in macrophages by 1 ng/ml \((P < 0.05)\) and 100 ng/ml \((P < 0.01)\) of LPS (Fig. 7). GHRP-2 and ghrelin did not modify the basal IL-6 release by macrophages. However, both GHRP-2 and ghrelin totally prevented the LPS-induced IL-6 release from macrophages (Fig. 7).

**DISCUSSION**

In the present study, we have shown that daily GHRP-2 administration to arthritic rats decreases the external signs of inflammation, as evidenced by a decrease in paw edema and serum IL-6 levels. This anti-inflammatory effect seems to be mediated by ghrelin receptors, as GHRP-2 and ghrelin are able to prevent LPS-induced IL-6 release from peritoneal macrophages in vitro.

Adjuvant-induced arthritis is associated with an increase in serum concentrations of ghrelin and a decrease in leptin. Because chronic arthritis dramatically decreases body weight, the reduction in serum concentration of leptin and the increase in serum ghrelin were not unexpected. An increase in serum ghrelin in humans has been reported in several catabolic
Circulating leptin also has a biphasic response to inflammatory stimuli but completely opposite to that of ghrelin. Acute LPS administration induces an increase in serum concentration of leptin (13), whereas a reduction in leptin in chronic inflammation, such as Staphylococcus aureus-induced arthritis, has previously been described (18). The GHRP-2-induced increase in serum concentration of leptin both in control and in arthritic rats can be secondary to the increase in body weight gain induced by the treatment. However, the increase in body weight gain after GHRP-2 administration is higher in control rats than in arthritic rats, but the increase in serum leptin is similar or higher in arthritic rats than in control rats. Similarly, serum concentration of leptin is also decreased, and it increases after ghrelin administration in rats with cancer (28) or in LPS-injected rats (17).

In our data, the orexigenic effect of GHRP-2 is smaller than its effect on body weight gain. It has previously been described that the effect of GHRP-2 administration on body weight is higher than its effect on food intake (41). This difference has been explained by the fact that GHRP-2 may increase the efficiency of assimilating calories from food.

The results indicate that GHRP-2 administration decreases edema evolution in the hindpaw of arthritic rats and serum IL-6 levels. Our data suggest that the anti-inflammatory effect of GHRP-2 in arthritic rats is not mediated by the increase in serum concentrations of glucocorticoids, since GHRP-2 administration has different effects in control and arthritic rats. Furthermore, it decreased both ACTH and corticosterone levels in arthritic rats. The stimulatory effect of GHS on ACTH and glucocorticoids is well documented (2, 38). Taking into account that adjuvant arthritis increases ACTH and corticosterone secretion (35), the decrease in both hormones after GHRP-2 treatment could be the result of the anti-inflammatory effect of the treatment. Although the in vitro data suggest a direct effect on immune cells, we cannot exclude other factors that may be involved in the in vivo anti-inflammatory effect of GHRP-2 administration.

Because ghrelin receptors have been found in peripheral tissues such as bone marrow, spleen, and lymphocytes (12, 25), the anti-inflammatory effect of GHRP-2 can also be mediated by ghrelin receptors directly on immune cells. To explore this possibility, we analyzed IL-6 and nitrite/nitrate release in LPS-stimulated macrophages. Taking into account that GHRP-2 is able to decrease LPS-induced IL-6 release in vitro, the anti-inflammatory effect of this compound may be independent of pituitary GH secretion. This hypothesis is in accord with our previous observations that chronic GH administration to arthritic rats did not ameliorate the arthritis score or the increase in paw volume, although it increased body weight gain (19). A exhaustive study has recently been published that confirms our data (11). It shows that GHS-R are expressed in human T lymphocytes and monocytes and that ghrelin acting via GHS-R inhibits the expression of cytokines such as IL-1β, IL-6, and TNF-α (11).

Similarly to GHRP-2, leptin administration to arthritic rats has been reported to decrease the severity of the disease (18). However, the anti-inflammatory effect of leptin is not well known, as both pro- and anti-inflammatory effects have been described (24, 44), and leptin-deficient mice develop less severe arthritis than control mice (6). Nevertheless, GHRP-2 administration increased serum leptin levels, but the effect of...
GHRP-2 administration in the arthritic rats does not seem to be due to the increase in leptin, since both ghrelin and GHRP-2 are able to decrease nitrite/nitrate and IL-6 release after LPS stimulation in peritoneal macrophages in vitro. Therapeutic effects of ghrelin in other inflammatory conditions, such as endotoxic shock in rats, have recently been described (7, 11). These effects include reduced mortality and improved glycemia and cardiovascular disturbances.

We observed that GHRP-2 ameliorated adjuvant-induced arthritis during the chronic stage of the disease. In adjuvant-induced arthritis, the acute response to antigens is correlated with the presence of IL-1 and TNF, whereas IL-6 is involved in the systemic and local events underlying adjuvant arthritis, especially in the later phase (36). IL-6 has been reported to be a good marker of arthritis, including adjuvant-induced arthritis (37) and rheumatoid arthritis (4). A high correlation between IL-6 in serum and in synovial fluid and the severity of chronic arthritis in rats has been reported (5). IL-6, produced mainly by monocytes and macrophages, is one of the main mediators of tissue destruction and participates in the development and clinical manifestations of arthritis (1). Thus decreased production of this cytokine can be the mechanism by which GHRP-2 decreases inflammation in arthritic rats. Our in vitro data are in agreement with the in vivo results, in that GHRP-2 and ghrelin prevent the increased release of IL-6 from LPS-stimulated macrophages. Although GHRP-2 and ghrelin are able to decrease both IL-6 and nitrite/nitrate release from stimulated macrophages, their inhibitory effect is higher on IL-6 than on nitrite/nitrate release. Similarly, in vivo GHRP-2 administration decreased serum concentrations of IL-6 but not nitrite/nitrate levels. Suppression of adjuvant arthritis in rats by bromocriptine treatment did not alter nitric oxide production despite the total prevention of paw swelling and core temperature (21). Moreover, treatment with intraperitoneal Mycobacterium inhibited the arthritis development as well as the increase in plasma IL-6 levels, but not the nitrite/nitrate plasma levels (9). All of these data suggest that the anti-inflammatory effect of GHRP-2 in the arthritic rats can be due to a GHRP-2 direct influence on IL-6 production. Considering the positive anti-inflammatory effect of ghrelin, increased ghrelin levels in arthritic rats may represent a compensatory mechanism in this catabolic condition.

In summary, we observed that GHRP-2 administration during the active phase of arthritis reduced the symptoms of arthritis as well as the serum concentration of IL-6. This anti-inflammatory effect of GHRP-2 seems to be a direct effect on the immune cells mediated by ghrelin receptors, since both GHRP-2 and ghrelin are able to decrease IL-6 release in vitro.

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