Chronic inflammation modulates ghrelin levels in humans and rats

M. Otero, R. Nogueiras, F. Lago, C. Dieguez, J. J. Gomez-Reino and O. Gualillo

Objectives. The aim of this work was to investigate whether changes in plasma ghrelin, the recently discovered 28-amino acid gastric hormone that regulates growth hormone (GH) secretion and energy homeostasis, occur during inflammation in adjuvant-induced arthritis (AA) in rats. For completeness, ghrelin plasma levels were measured in rheumatoid arthritis (RA) patients.

Methods. AA was induced in male Lewis rats using Freund's complete adjuvant. Animals were monitored for weight and food intake, every 2 or 3 days, along all time-course experiments. Plasma ghrelin concentrations in 31 RA patients and 18 healthy controls, as well as in rats, were determined by a specific double-antibody radioimmunoassay. Gastric ghrelin mRNA expression was evaluated by northern blot analysis. Human GH and insulin-like growth factor (IGF)-1 were determined by quantitative chemiluminescence assay.

Results. Compared with controls, arthritic rats gained significantly (P < 0.01) less body weight than controls until the end of the study, when a partial recovery occurred. Ghrelin plasma levels were significantly lower at day 7 after arthritis induction than in controls (AA 7 = 91.2 ± 5.6 pg/ml vs controls = 124.75 ± 5.9 pg/ml), but they recovered to control levels by day 15. RA patients had ghrelin plasma levels significantly lower than healthy controls (RA = 24.54 ± 2.57 pg/ml vs 39.01 ± 4.47 pg/ml of healthy controls; P = 0.0041).

Conclusion. In AA, there is a compensatory variation of ghrelin levels that relates to body weight adjustments. Recovery of ghrelin levels in the latter stage suggests an adaptive response and may represent a compensatory mechanism under catabolic conditions. In RA patients, chronic imbalance in ghrelin levels suggests that this gastric hormone may participate, together with other factors, in alterations of metabolic status during inflammatory stress.

Key words: Ghrelin, Rheumatoid arthritis, Inflammation experimental models.

Chronic inflammation is associated with a significant loss of body mass. Patients with rheumatoid arthritis (RA) have approximately 15% less cell mass than their age-, sex-, weight- and race-matched controls. Inflammatory cachexia develops in the absence of malabsorption and it is accompanied by hypermetabolism and accelerated protein breakdown [1]. These metabolic abnormalities are associated with increased production of inflammatory cytokines [2] and also mediated by disturbances of the endocrine system [3], particularly those related to the adrenal axis [4] and pituitary [5].

Ghrelin is a novel growth hormone (GH) releasing peptide, isolated from the stomach, that is identified as the endogenous ligand for GH secretagogue receptor [6]. Recent studies show that ghrelin produces a positive energy balance by stimulating food intake [7] and decreasing fat utilization through a GH-independent mechanism. It also antagonizes leptin through the activation of the hypothalamic neuropeptide Y/Y1 receptor pathway [8]. Interestingly, leptin may promote an inflammatory response via nitric oxide production [9]. These findings raise the possibility that ghrelin may play an important role in the regulation of metabolic balance in inflammatory diseases such as RA. Nevertheless, the role of ghrelin in the metabolic imbalance associated with these conditions and its relationship with disease activity remain unknown.

Freund's complete adjuvant (FCA)-induced arthritis is a useful model for the understanding of the mechanisms by which humoral mediators of inflammation, including cytokines and hormones, cause cachexia. Hence, in this animal model we investigated ghrelin plasma levels during the inflammatory process in connection with body weight variation. For completeness, we have determined ghrelin plasma levels in patients affected by RA in comparison with healthy controls.

Subjects and methods

Freund's complete adjuvant-induced arthritis

Male Lewis rats weighing 160–180 g, obtained from Charles River (Barcelona, Spain), were used for this study. Animals were kept in standard conditions and were given an ad libitum diet with free access to food and water. The animals were housed under standard conditions and were sacrificed after 7 days of adjuvant-induced arthritis and 15 days of recovery. The rats were divided into two groups: control (C) and arthritic (AA). The arthritic group was induced by FCA injection (250 μg) and the control group received an equivalent volume of saline.

Results

Compared with controls, arthritic rats gained significantly less body weight than controls until the end of the study, when a partial recovery occurred. Ghrelin plasma levels were significantly lower at day 7 after arthritis induction than in controls (AA 7 = 91.2 ± 5.6 pg/ml vs controls = 124.75 ± 5.9 pg/ml), but they recovered to control levels by day 15. RA patients had ghrelin plasma levels significantly lower than healthy controls (RA = 24.54 ± 2.57 pg/ml vs 39.01 ± 4.47 pg/ml of healthy controls; P = 0.0041).

Conclusion

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access to water. All animals were acclimatized to a 12-h day/night cycle and were kept under clean but not germ-free conditions. The study protocols were approved by the animal care and use committee of Santiago de Compostela University. Animals were randomly divided into four groups; control 7 (n=12), AA 7 (n=18), control 15 (n=12), and AA 15 (n=18). On day 0, experimental arthritis was induced in animals of group AA 7 and AA 15 by injecting once with 200 μl of FCA [a suspension of Mycobacterium tuberculosis (1 mg/ml) in sterile mineral oil; Sigma-Aldrich, St Louis, MO] in the right paw, while control groups received 200 μl of vehicle alone. Food consumption was determined by weighing the food consumed by each animal daily. Animals were weighed every 3 days and examined daily for evidence of joint swelling. Swelling of the ankle, incapacity to bend the ankle and the presence of nodules at the base of the tail and ears were used as parameters of arthritis evolution.

AA 7 and AA 15 animals were killed on day 7 and 15 after experimental arthritis induction, together with their respective controls, and blood samples were harvested for ghrelin determination. Stomachs were dissected and immediately snap-frozen on dry ice and stored at −80°C until use.

**Northern blot analysis**

Ghrelin mRNA was isolated and analysed as previously described [10]. Briefly, after electrophoresis and transfer to a nylon membrane, total RNA was hybridized to a 32P-labelled cDNA probe (501 bp; a kind gift of Dr M. Kojima, National Cardiovascular Center Research Institute, Osaka, Japan) to rat ghrelin. A rat 18s ribosomal RNA probe, 5'-ACGGTATCTGATCGTCTTCG AACC-3' (Invitrogen, Barcelona, Spain), end-labelled with 32P, was used to assess the amount and integrity of total RNA loaded in each gel. The filters were then successively washed and processed as described above.

**Rat ghrelin radioimmunoassay**

Blood was collected into Vacutainer® (BD Biosciences, Madrid, Spain) tubes, which contained EDTA, between 9.00 and 10.00 am on day 0. After centrifugation at 1600 g (0.6 IU/ml) was added and tubes were gently rocked to inhibit the activity of proteinases. Blood was centrifuged at 1600 g (0.6 IU/ml) was added and tubes were gently rocked to inhibit the activity of proteinases. Blood was centrifuged at 1600 g (0.6 IU/ml) was added and tubes were gently rocked to inhibit the activity of proteinases. Blood was centrifuged at 1600 g (0.6 IU/ml) was added and tubes were gently rocked to inhibit the activity of proteinases. Blood was centrifuged at 1600 g (0.6 IU/ml) was added and tubes were gently rocked to inhibit the activity of proteinases. Blood was centrifuged at 1600 g (0.6 IU/ml) was added and tubes were gently rocked to inhibit the activity of proteinases. Blood was centrifuged at 1600 g (0.6 IU/ml) was added and tubes were gently rocked to inhibit the activity of proteinases.

Ghrelin determination in patients and controls

Thirty-one patients with a diagnosis of RA (mean age ± s.e.m. = 46.1 ± 14.1; body mass index, BMI, = 25.88 ± 0.63) according to the classification criteria of the American College of Rheumatology were enrolled in the study. Twenty-two patients had more than eight inflamed joints along with an elevated erythrocyte sedimentation rate. Twenty-eight (81%) were being treated with methotrexate, 23 (62%) with low doses of prednisone (< 10 mg daily) and nine (26%) with tumour necrosis factor (TNF) antagonists.

Eighteen healthy controls of similar gender, age (mean age ± s.e.m. = 48.3 ± 16.1) and BMI (24.36 ± 0.83) were used as controls. Informed consent was obtained from patients and controls according to the declaration of Helsinki; the design of the work was approved by the Galician Ethical Committee of Clinical Investigation. In all participants blood was collected into Vacutainer tubes containing EDTA between 9.00 and 11.00 am after overnight fasting. Blood samples were processed and used for human ghrelin and human GH determination. Plasma ghrelin was determined by a specific radioimmunoassay as described above. Human GH and insulin-like growth factor (IGF)-1 were determined by a chemiluminescence immunoassay for the quantitative determination of the hormones using materials and protocols provided by the supplier (Nichols Institute Diagnostics, S. Juan Capistrano, CA 92675, USA).

**Statistical analysis**

Data are expressed as means ± s.e.m. and were analysed with a computer package for statistical analysis. Statistically significant differences were determined by ANOVA followed by a post-hoc multiple comparison test; a two-tailed P value < 0.05 was considered as significant.

**Results**

**Experimental arthritis in rats**

More than 90% of the rats developed signs of inflammation 5–7 days after adjuvant injection (rats that were non-responders were not included in the study analysis), reaching the maximum level at day 13–14.

Animals were monitored, during the 15 days of the experiments, to study body weight profile. As shown in Fig. 1A, body weight gain in the arthritic rats did not differ from controls until day 5. At this time, arthritic rats began to gain significantly less body weight than controls (P < 0.01), yet at the end of the study (day 13–15) a partial recovery of body weight occurred.

As shown in Fig. 1B, a significant (P < 0.01) decrease of ghrelin plasma levels was observed in arthritic rats killed 7 days after FCA injection in comparison with controls (AA 7 = 91.2 ± 5.6 pg/ml vs controls = 124.75 ± 5.9 pg/ml). A significant (P < 0.001) recovery of plasma levels, slightly higher than controls (125.32 ± 4.21), occurred on day 15 (136.5 ± 5.96 pg/ml). Northern blot analysis of gastric ghrelin mRNA in the day 7 and day 15 groups showed no significant differences in comparison with control animals (Figs 1C and 1D).

**Rheumatoid arthritis**

Blood ghrelin levels in RA patients (24.54 ± 2.57 pg/ml) were significantly (P = 0.0041) lower than controls (39.01 ± 4.47 pg/ml) (Fig. 2). For completeness, we determined GH and IGF-1 plasma concentration in RA patients and in healthy controls. There were no significant differences in GH basal concentrations between patients with RA and healthy control subjects (control patients: 0.699 ± 0.222 ng/ml; RA patients: 0.641 ± 0.156 ng/ml). In addition, mean IGF-1 levels tended to be lower in patients with RA than in controls, but the difference was not significant (control patients: 116.02 ± 11.5 ng/ml; RA patients: 94.9 ± 5.45 ng/ml).

**Discussion**

In the present study we have investigated the putative role of ghrelin in the changes of body weight that occur during inflammation. Our results suggest that decrease of plasma levels of this orexigenic peptide could contribute in part to weight loss in animal models and RA patients.

Tissue damage, induced by inflammation, triggers body energy adjustments that include elevated energy expenditure, fat mobilization, enhanced gluconeogenesis, protein catabolism, negative nitrogen balance and marked body weight loss. This happens in

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**Ghrelin in arthritis**

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animal models of inflammation and infection as well as in human chronic inflammatory diseases such as RA [11]. Thus, rheumatoid cachexia is believed to accelerate morbidity and mortality in RA. Nevertheless, some authors consider that post-inflammation anorexia and tissue catabolism could be beneficial during acute inflammatory diseases [12], in the same way as an adaptive metabolic response to external injuries. Currently, there is no established mechanism for rheumatoid cachexia, but it is likely to be mediated by multifactorial components such as hormones and cytokines. The increased production of pro-inflammatory cytokines such as TNF-α is probably one of the principal factors involved in rheumatoid cachexia. This cytokine acts synergistically with interleukin 1β to promote wasting syndrome in RA. In addition, bi-directional neuroendocrine-immune relationships are accountable for some of the homeostatic perturbations. In fact, defective hypothalamic–pituitary–adrenal axis functions are found in RA [13]. Ghrelin, the recently cloned endogenous GH-releasing hormone, was first characterized as a powerful inducer of GH secretion, interplaying with GHRH and somatostatin [14]. It behaves as an orexigenic signal from the gut to the brain, regulating metabolic homeostasis in physiological and/or pathological conditions [15].

In this work, we have analysed plasma ghrelin variations in a well-known experimental model of inflammatory cachexia in rats. Our results show that ghrelin plasma levels significantly decrease during the first week after induction of experimental

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**FIG. 1.** (A) Weight changes in rats with adjuvant arthritis (○) and control rats (□). Data are expressed as mean ± S.E.M., **P < 0.01** vs control rats injected with vehicle. (B) Changes in rat plasma concentration of ghrelin, 7 and 15 days after FCA injection. Data represent the mean ± S.E.M., **P < 0.01** vs control, ###P < 0.001 vs FCA 7 days. (C) Upper panel. Northern blot analysis of ghrelin mRNA in rat stomach. Controls (lanes 1–4), AA 7 (lanes 5–7), AA 15 (lanes 8–11). Quantity and quality control of RNA was confirmed by hybridizing the blot with an oligonucleotide probe for 18S RNA (lower panel). (D) Densitometric analysis of gastric ghrelin mRNA expression in control animals and arthritic rats. Data are normalized with 18S and expressed in arbitrary units.
Ghrelin in arthritis

309

Fig. 2. Ghrelin plasma levels in RA patients (n = 31) and in healthy controls (n = 18). **P < 0.01.

arthritis, while a significant recovery of ghrelin plasma levels occurs in the latter part of the process. This biphasic profile indicates that ghrelin could be regulated in chronic inflammatory processes, perhaps by pro-inflammatory factors such as leptin, interleukin 1β and TNF-α [2]. This hypothesis is consistent with observations of other authors showing that (i) leptin is able to negatively regulate ghrelin, and (ii) increases of ghrelin induced by weight loss arise because of diminished inhibitory input from leptin [16, 17]. The belated increase of ghrelin levels observed in our experimental model, up to values comparable with controls, was accompanied by a significant recovery of body weight, suggestive of a compensatory mechanism under catabolic–anabolic imbalance. It is conceivable that in rat experimental arthritis the magnitude of weight loss achieved in arthritic animals is sufficient to trigger an increase in ghrelin. Thus, ghrelin recovery might represent an attempt to antagonize the anorexigenic and well-known pro-inflammatory effects of leptin suggesting that these two hormones might act in parallel as opposing metabolic counterparts, although at present this is far from being elucidated since the nature of the relationships between ghrelin and leptin remain controversial [18].

Of note were our results obtained by northern blot analysis showing a marginal or non-existent modulation of ghrelin mRNA at gastric level, indicating that blood level changes pertained to extra-gastric sites of expression that are generally responsible for 30–35% of circulating hormone [19]. Thus, we hypothesize that non-gastric sources of ghrelin may be reduced in arthritic animals, but it is equally possible that ghrelin catabolism is increased or visceral blood flow is altered, reducing the availability of ghrelin to the circulation.

In agreement with the results obtained in experimental arthritis, ghrelin plasma levels in RA patients were significantly lower than those present in healthy controls. Low ghrelin concentrations could reflect the presence of an inhibitory anorexigenic signal/s. Whether this anorexigenic signal is evoked by leptin or other factors remains to be established. Thus, the chronic decrease of ghrelin plasma levels in RA patients could be one of the drivers of rheumatoid cachexia. For completeness, we determined plasma GH and IGF-1 concentrations in the two groups. In RA patients the GH and IGF-1 concentrations were similar to those of the controls and no significant statistical difference between the two basal values was observed. Thus, ghrelin decrease is unlikely to be related to alterations of GH secretory pattern since other authors have reported that GH kinetics are unaltered in RA patients [20]. All in all, our results confirm a main role for ghrelin in the maintenance of body orexigenic signals, independently from its action as a GH secretagogue, which might have a significant impact on metabolism.

In conclusion, data presented in this work suggest that ghrelin could switch the body to a more efficient use of the energy reservoir in wasting syndromes. The potential of ghrelin-substitutive therapy, in association with other anti-inflammatory treatments, as an important therapeutic countermeasure against inflammatory cachexia needs to be explored further.

Acknowledgements

This work was supported by grant 01/3137 and PI 02–0431 from the Spanish Ministry of Health (Fondo de Investigación Sanitaria). OG and FL are recipients of a research contract from the Instituto de Salud Carlos III and Complexo Hospitalario Universitario de Santiago.

The authors especially acknowledge Prof. Felipe F. Casanueva (Department of Medicine, University of Santiago de Compostela) for his helpful advice and valuable discussions during the course of these investigations. The authors are very grateful to Dr Manuel Paz and Jesús Devesa for their collaboration and help.

References


