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Exogenous ghrelin attenuates endotoxin fever in rats

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ABSTRACT

Ghrelin is a gut-derived peptide that plays a role in energy homeostasis. Recent studies have implicated ghrelin in systemic inflammation, showing increased plasma ghrelin levels after endotoxin (lipopolysaccharide, LPS) administration. The aims of this study were (1) to test the hypothesis that ghrelin administration affects LPS-induced fever; and (2) to assess the putative effects of ghrelin on plasma corticosterone secretion and preoptic region prostaglandin (PG) E_2 levels in euthermic and febrile rats. Rats were implanted with a temperature datalogger capsule in the peritoneal cavity to record body core temperature. One week later, they were challenged with LPS (50 μg/kg, intraperitoneal, i.p.) alone or combined with ghrelin (0.1 mg/kg, i.p.). In another group of rats, plasma corticosterone and preoptic region PGE_2 levels were measured 2 h after injections. In euthermic animals, systemic administration of ghrelin failed to elicit any thermoregulatory effect, and caused no significant changes in basal plasma corticosterone and preoptic region PGE_2 levels. LPS caused a typical febrile response, accompanied by increased plasma corticosterone and preoptic region PGE_2 levels. When LPS administration was combined with ghrelin fever was attenuated, corticosterone secretion further increased, and the elevated preoptic region PGE_2 levels were relatively reduced, but a correlation between these two variables (corticosterone and PGE_2) failed to exist. The present data add ghrelin to the neurochemical milieu controlling the immune/thermoregulatory system acting as an antipyretic molecule. Moreover, our findings also support the notion that ghrelin attenuates fever by means of a direct effect of the peptide reducing PGE_2 production in the preoptic region.

1. Introduction

Ghrelin is a recently discovered 28-amino acid peptide that has been recognized as an orexigenic gut/brain molecule with a number of physiological effects. Its role on food intake and lipogenesis/obesity are well established [21]. In essence, plasma ghrelin levels are increased in anticipation of a meal, and decreased after food intake [7].

Recent studies have implicated ghrelin in systemic inflammation as well (*cf.* [11,19]). In agreement with this notion, Wang et al. [32] reported increased serum ghrelin levels during endotoxemia. Additionally, the authors observed that ghrelin administration attenuates LPS-induced serum cytokine levels (TNF-α, IL-1β, and IL-6) as well as nitric oxide (NO) production. Moreover, recent studies have shown an association of ghrelin with markers of inflammation in endotoxemic dogs and rats (cf. [19]).

The development of febrile response when animals are submitted to inflammatory stimuli, such as LPS, is under the influence of several modulators [3]. In the present study, we tested the hypothesis that ghrelin modulates LPS-induced fever. Furthermore, we evaluated the mechanisms of action altering the febrile response by assessing the putative influence of ghrelin on plasma glucocorticoid secretion and PGE_2 levels in the preoptic/anteroventral third ventricular region (AV3V), where PGE_2 acts as the terminal mediator of fever [8,17,23].

2. Materials and methods

2.1. Animals

Experiments were performed on 59 male Wistar rats (180–260 g) obtained from the vivarium of the University of São Paulo, campus of Ribeirão Preto. The animals were kept in a room at controlled temperature (23–24 °C) and exposed to a daily 12:12-h light–dark cycle (lights on at 06:00 AM). They had free access to tap water and regular rat chow. To eliminate possible effects of circadian variations, all experiments started between 08:00 and 09:00 AM. Experimental protocols were carried out according to

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the Brazilian Society of Neuroscience and Behavior Guidelines for Care and Use of Laboratory Animals, and with the approval from the local Animal Care and Use Committee.

2.2. Drugs

Endotoxin (lipopolysaccharide, LPS; serotype 0111:B4) and rat ghrelin were purchased from Sigma (St Louis, MO, USA), and they both were dissolved in pyrogen-free saline (0.90% (w/v) of NaCl).

2.3. Surgery

Surgical procedure was performed under ketamine–xylazine anesthesia (100 and 10 mg/kg, respectively; 1 ml/kg, intraperitoneal, i.p.). Rats were submitted to a median laparotomy for the insertion of a temperature datalogger capsule (SubCue, Calgary, Alberta, Canada) into the peritoneal cavity. At the end of the surgical procedure, antibiotic solution (160,000 U/kg benzylpenicilin, 33.3 mg/kg streptomycin, and 33.3 mg/kg dihydrostreptomycin; 1 ml/kg, intramuscular) and analgesic medication (Flunixine; 2.5 mg/kg, 1 ml/kg, subcutaneous) were administered, and the animals were kept in individual cages.

2.4. Body core temperature (T_b) measurements

T_b was recorded by means of the temperature datalogger capsule (4.2 g/2 cc, 1.5 cm diameter × 0.5 cm thick; SubCue Dataloggers, Calgary, Alberta, Canada). Fully conscious, freely moving rats were housed in individual cages and placed in the experiment room at controlled temperature (23 °C) 24 h prior to the experiment in order to get used to the experimental room and conditions. T_b of the animals was recorded at 10-min intervals throughout the experiments.

2.5. Experimental protocols

Experiment 1: This experiment was performed to evaluate the effect of ghrelin administration on LPS-induced fever. At an ambient temperature of 23 °C, rats were bolus-injected with LPS (50 μg/kg, 1 ml/kg, i.p.), ghrelin alone (0.1 mg/kg, 1 ml/kg, i.p.) or ghrelin combined with LPS. Control rats were treated with pyrogen-free saline (1 ml/kg, i.p.). The doses of LPS [22] and ghrelin [34] were chosen on the basis of previous studies and pilot experiments.

Experiment 2: The second set of experiments was aimed at evaluating whether ghrelin affects the febrigenic signaling in the brain as well as the modulation of febrile response by the endogenous glucocorticoids. The levels of PGE_2 (the terminal mediator of fever) in its presumed site of action – the preoptic/anteroventral third ventricular region [4,17,23] – was used as an index of febrigenic signaling, and plasma corticosterone to assess the hypothalamic-pituitary-adrenal axis activation. Animals were bolus-injected with LPS (50 μg/kg, 1 ml/kg; or saline, 1 ml/kg, i.p.), combined or not with ghrelin (0.1 mg/kg, 1 ml/kg, i.p.), and decapitated 2 h post-injection. The brains were then immediately excised from the skull and promptly frozen by immersion into isopentane cooled with dry ice, and blood was collected for corticosterone measurements.

2.6. Measurement of prostaglandin E_2

This experiment was aimed at evaluating PGE_2 production (cyclooxygenase, COX, activity) in the preoptic/AV3V region, as previously described [1,26]. Briefly, 2 h after injections rats were decapitated, their brains immediately excised, and the AV3V, which includes the preoptic region, was dissected. The AV3V region was cut at its borders with the vertical limb of the diagonal band of Broca (anterior), the posterior end of the optic chiasm (posterior), the ventral thalamus (dorsal), and the lateral hypothalamus (lateral); the ventral limit of the AV3V region was the optic chiasm. The samples were frozen by immersion in liquid nitrogen and stored at −70 °C until assayed. They were homogenized on ice in methanol (150 μl) containing indomethacin (1 M). The homogenates were centrifuged at 10,000 × *g* for 10 min at 2 °C. The resulting supernatants and pellets were used for PGE_2 and protein determination, respectively. The samples were reconstituted in the assay buffer provided in the kit (Cayman, 500141 Prostaglandin E_2 EIA Kit), and PGE_2 levels were measured using enzyme immunoassay according to the manufacturer's instructions.

2.7. Sampling procedure

To assess hypothalamic-pituitary-adrenal axis activation trunk blood was collected (~2 ml). Animals were sampled without anesthesia. Control and experimental animals were removed from their cages and decapitated within 10 s [31].

2.8. Corticosterone assay

Blood was allowed to coagulate at 4 °C overnight. Samples were centrifuged at 1500 × *g* for 10 min; plasma was sampled and stored at −70 °C until assay. Total plasma corticosterone (free and bound) was determined by a commercially available ELISA kit, according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA).

2.9. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Thermal indexes, expressed as °C min, were calculated from area under curve, from 120 to 300 min. Scatterplot of the log of plasma corticosterone levels versus the log of preoptic PGE_2 levels from rats treated with LPS combined with ghrelin is defined by its Pearson correlation coefficient (r), which represents direction and strength of the correlation between these two physiological variables [38]. Statistical differences (Statistica™, version 8.0, StatSoft 2008; Tulsa, OK, USA) among groups were assessed by Linear Mixed Model [10] followed by Fisher LSD post hoc test (T_b time courses) or one-way ANOVA followed by the Tukey post hoc test (thermal index, plasma corticosterone and preoptic region PGE_2 levels). Values of $P < 0.05$ were considered statistically significant.

3. Results

The putative role of ghrelin in modulating LPS-induced fever was studied by evaluating the effect of ghrelin on T_b of euthermic (saline-treated) and febrile (LPS-injected) animals. Pyrogen-free saline (1 ml/kg, i.p.) or ghrelin (0.1 mg/kg, 1 ml/kg, i.p.) caused no significant change in T_b of euthermic animals ($P > 0.05$, Fig. 1A). Conversely, injection of LPS (50 μg/kg, i.p.) elicited the well characterized LPS-induced febrile response (for review see [22]). Interestingly, when LPS administration was associated with ghrelin the febrile response was attenuated at the third phase of fever ($P < 0.05$, Fig. 1A). Thermal indexes (TIs) (area under curve; indicated by the horizontal bar in Fig. 1A) were calculated to emphasize the effect of ghrelin on LPS-induced fever (Fig. 1B). As shown in Fig. 1B, injection of LPS induced the highest TI (252.9 ± 12.6 °C min). TI of ghrelin + LPS (169.5 ± 34.3 °C min) was lower than that of LPS alone ($P < 0.05$), and it was significantly higher than both pyrogen-free saline (86.9 ± 21.8 °C min, $P < 0.05$) and ghrelin (68.1 ± 24.5 °C min, $P < 0.05$).

Plasma corticosterone levels were assessed to evaluate the putative influence of ghrelin administration on the LPS-induced hypothalamic-pituitary-adrenal axis activation. As shown in Fig. 2,

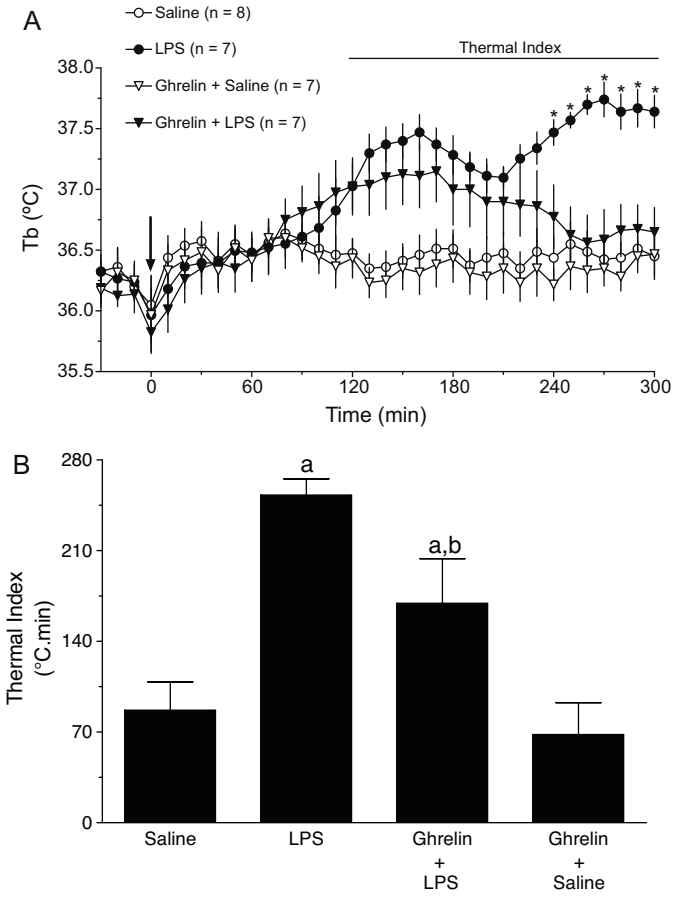

Fig. 1. Body core temperature (T_b) time courses in rats bolus-injected with LPS (50 μg/kg, i.p.) or pyrogen-free saline (1 ml/kg, i.p.) combined or not with ghrelin administration (0.1 mg/kg, i.p.). (A) LPS injection induced a typical febrile response (regulated increase in T_b), which was significantly attenuated by ghrelin. * indicates $P < 0.05$, LPS vs ghrelin + LPS, by Linear Mixed Model followed by Fisher LSD post hoc test. Number of animals is shown in parenthesis. Arrow indicates the moment of injections. (B) Thermal index calculated from area under curve, from 120 to 300 min (as indicated by the horizontal bar in A). (a) $P < 0.05$, vs saline-treated groups; (b) $P < 0.05$, LPS vs ghrelin + LPS, by one-way ANOVA followed by the Tukey post hoc test.

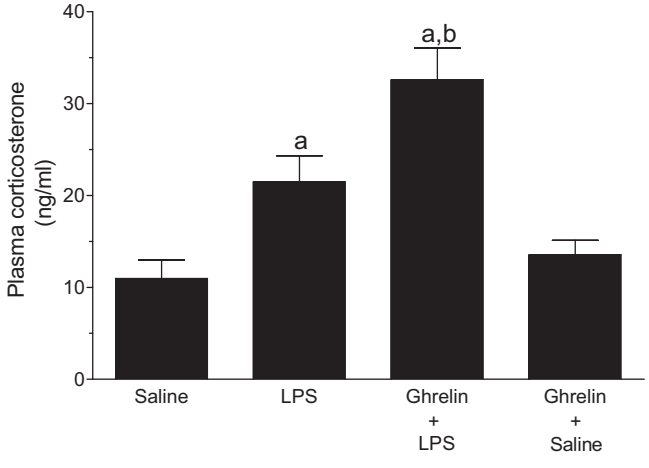

Fig. 2. Plasma corticosterone levels in rats bolus-injected with LPS (50 μg/kg, i.p.) or pyrogen-free saline (1 ml/kg, i.p.) combined or not with ghrelin administration (0.1 mg/kg, i.p.). LPS induced a significant increase in corticosterone levels, which was significantly potentiated by ghrelin. Number of animals: 7–8 per group. (a) $P < 0.05$, vs saline-treated groups; (b) $P < 0.05$, LPS vs Ghrelin + LPS, by one-way ANOVA followed by the Tukey post hoc test.

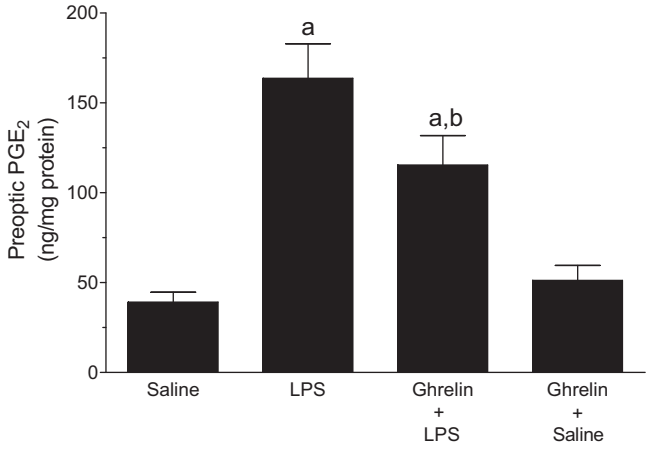

Fig. 3. Prostaglandin E_2 (PGE_2) levels in the preoptic region in rats bolus-injected with LPS (50 μg/kg, i.p.) or pyrogen-free saline (1 ml/kg, i.p.) combined or not with ghrelin (0.1 mg/kg, i.p.). Administration of LPS evoked a marked augmentation of preoptic PGE_2 levels, and such an increase was significantly blunted by ghrelin. Number of animals: 7–8 per group. (a) $P < 0.05$, vs saline-treated groups; (b) $P < 0.05$, LPS vs ghrelin + LPS, by one-way ANOVA followed by the Tukey post hoc test.

injection of LPS alone induced a significant increase in plasma corticosterone levels (from 11.0 ± 2.0 to 21.5 ± 2.8 ng/ml; $P < 0.05$). Administration of ghrelin combined with LPS potentiated the increased secretion of corticosterone induced by LPS (from 11.0 ± 2.0 to 32.6 ± 3.4 ng/ml; $P < 0.05$), whereas ghrelin alone did not alter the basal levels of plasma corticosterone (from 11.0 ± 2.0 to 13.5 ± 1.6 ng/ml).

To address whether the attenuated LPS-induced fever in ghrelin-treated rats resulted from inhibited central COX activity, we measured the levels of PGE_2 in the preoptic region of the hypothalamus. Even though PGE_2 production tended to be higher in ghrelin-treated rats (51.2 ± 8.4 ng/mg of protein) compared to saline-treated controls (39.1 ± 5.5 ng/mg of protein) no significant difference between these groups was found ($P > 0.05$). Administration of LPS, on the contrary, evoked a marked increase in preoptic PGE_2 levels (from 39.1 ± 5.5 to 163.7 ± 19.2 ng/mg of protein, $P < 0.05$), which was significantly reduced by ghrelin (from 39.1 ± 5.5 to 115.4 ± 16.4 ng/mg of protein, $P < 0.05$). These data are depicted in Fig. 3.

In order to assess whether ghrelin affects PGE_2 production directly or whether its action is mediated through increased corticosterone secretion, a scatterplot of the log of plasma corticosterone levels versus the log of preoptic PGE_2 levels from rats treated with LPS combined with ghrelin is shown (Fig. 4). The calculated correlation coefficient (r) is −0.19.

4. Discussion

In 1999 ghrelin was first identified as a gastric peptide hormone in the rat stomach acting as a mediator of growth hormone (GH) release [15]. This peptide, besides being involved in the appetite regulation, has been recently demonstrated to be required for the normal integration of sleep [28]. Recent studies now indicate that ghrelin affects a number of other systems and has diverse effects (*cf.* [27]), including a role in modulating immune cell response [9,19]. This notion is based on the fact that ghrelin and its target receptors have been found in neutrophils, lymphocytes, and macrophages [33]. Moreover, studies have shown that ghrelin inhibits various pro-inflammatory cytokine production, including TNF-α, IL-1β, IL-6, and IL-8 [9,18]. Conversely, ghrelin was initially reported as an immune enhancing factor (*cf.* [20]). The causes of such discrepancies between initial studies showing the

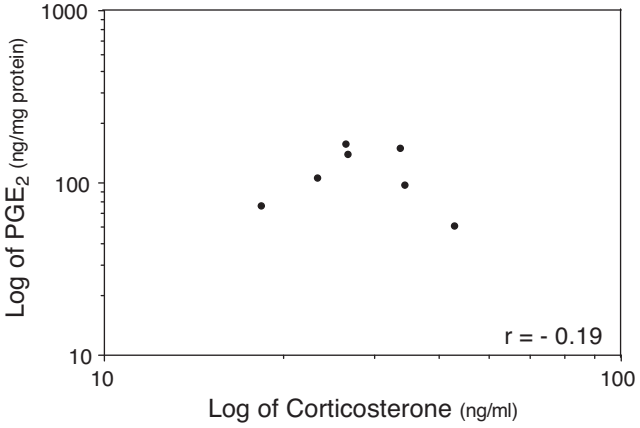

Fig. 4. Scatterplot of the log of corticosterone plasma levels vs the log of preoptic PGE_2 levels of eight sets of paired data. Pearson correlation coefficient: $r = -0.19$.

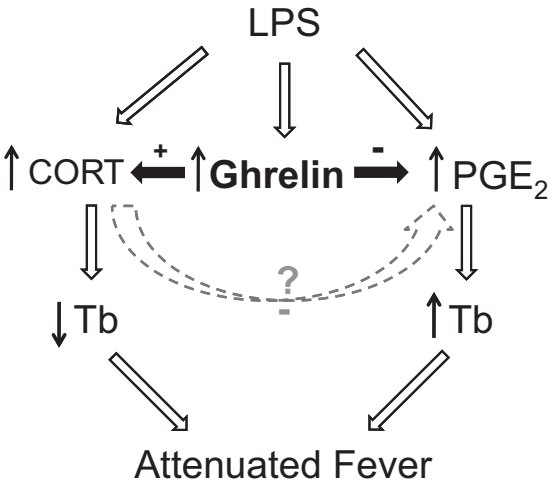

Fig. 5. Possible mechanism for ghrelin-induced antipyresis. LPS stimulates: (1) hypothalamic-pituitary-adrenal axis and thus corticosterone (CORT) secretion, (2) ghrelin secretion, and (3) preoptic PGE_2 production. Ghrelin, in turn, further stimulates CORT secretion, which is an antipyretic molecule and counteracts excessive increases in T_b, whereas inhibits PGE_2 production, which is the terminal mediator of fever in its presumed site of action – the preoptic/anteroventral third ventricular region. Both ghrelin effects favor an attenuated febrile response. The effect of ghrelin inhibiting PGE_2 production seems to be directly mediated rather than a CORT-dependent mechanism (indicated by "?" and dashed arrow).

immune-enhancing effects of ghrelin and recent studies suggesting anti-inflammatory functions of this peptide still remains to be clarified [20,33]. Taken together, available data indicate that ghrelin may play a key role in improving immune cell responses and pathologic inflammatory states. It is interesting to note that the effects of ghrelin on the immune system seem to be beneficial, as recently demonstrated in pathophysiology of cachectic diseases such as cancer [29], and suppression of excessive immune reactions such as sepsis [14]. Therefore, ghrelin may play a protective role, enhancing or inhibiting immunity depending on specific situations. The present data add ghrelin to a neurochemical milieu controlling the immune/thermoregulatory system acting as an antipyretic molecule.

It is worth mentioning that ghrelin plasma levels have been reported to be increased in rats treated with LPS [5,32,36], and that increased ghrelin secretion causes a decrease in mortality rate in rats with endotoxic shock [5]. Perhaps, the increased plasma ghrelin levels observed after treatment with LPS result from the release of adrenergic agents by sympathetic neurons acting directly on β1 receptors on the ghrelin-secreting cells of the stomach [37].

The aims of the present study were to characterize the role of ghrelin in LPS-induced fever and to assess putative mechanisms of action of this peptide. Our results indicate that ghrelin may have therapeutic value for systemic inflammation, as ghrelin reduced LPS-induced fever. We further investigated the antipyretic role of ghrelin in LPS-induced fever. To this end we tested the hypothesis that ghrelin attenuates fever by reducing the LPS-induced PGE_2 production in the preoptic region. To address this possibility, we evaluated whether the increased production of PGE_2 induced by LPS, which in the preoptic region activates febrigenic thermoeffector pathways [8,17,23], was altered in ghrelin-treated rats. We found that the increased preoptic PGE_2 levels in LPS-treated rats were significantly reduced when ghrelin was administered (Fig. 3). PGE_2 was measured 2 h after LPS administration when T_b of rats treated with LPS alone or LPS combined with ghrelin started to differ, whose effect was fully observed at the end of the experimental period.

In general, the present finding about an antipyretic effect of ghrelin is not only in agreement with several previous articles showing that starvation decreases the LPS-induced fever in rats [12,13] but also with a fairly recent study that reported that food deprivation reduces T_b responses to LPS by enhancing inflammatory signaling that decreases T_b rather than by suppressing inflammatory signaling that increases T_b [16]. Further studies are needed to evaluate the hypothesis that ghrelin increases such inflammatory signaling that decreases T_b, *i.e.*, favoring the cryogenic inflammatory signaling via prostaglandin D2, as recently suggested for food deprived rats [16]. Any ways, it may be beneficial to suppress immune/thermoregulatory responses to LPS when animals are under food deprivation, since such responses have a high energy cost, and the present data are consistent with the notion that ghrelin acts as mediator of such down-modulation.

How does ghrelin reduce preoptic PGE_2 production in LPS-treated rats? First of all, it is well established that corticosterone plays a key role as an antipyretic molecule during both LPS- [6] and stress-induced fever [27]. Interestingly, ghrelin did not alter either basal plasma corticosterone levels or T_b of euthermic animals, but was accompanied by a more pronounced increase in plasma corticosterone levels in response to LPS (Fig. 2), which may have contributed to the reduction in the PGE_2 production in the preoptic region. However, this possibility is unlikely because the correlation coefficient value calculated from the scatterplot (Fig. 4) between corticosterone plasma levels and PGE_2 is −0.19 (weakly negative), *i.e.*, in the expected direction (since corticosterone is inversely proportional to PGE_2 production) but lacking strength of correlation (see Ref. [38] for further details). This lack of correlation favors the hypothesis of a direct effect of ghrelin on PGE_2 production. This is in agreement with the notion that ghrelin reduces PGE_2 production and COX expression, as recently reported [25]. As to the kinetics of the effect of ghrelin, it is worth mentioning that ghrelin effect occurred in the late phase of the febrile response, which has been suggested to be related to an increase in the permeability of the blood–brain barrier [24].

In agreement with our findings (Fig. 1), the lack of an effect of ghrelin on basal maintenance of T_b has been observed before [35]. Even though ghrelin-treated rats showed no change in basal PGE_2 production, it seems that these animals are likely to produce relatively less PGE_2 in their brains in response to LPS (Figs. 3 and 5). Still in relation to the combined effects of LPS and ghrelin the present data are consistent with the notion that an enhanced hypothalamic-pituitary-adrenal axis response to LPS occurs when ghrelin is administered (Figs. 2 and 5). Albeit this enhanced axis activation has been suggested to be linked to a suppressed COX activation/PGE_2 production by means of the well known

anti-inflammatory effect of corticosterone [6,30], this is unlikely to be the mechanism of action of ghrelin modulating LPS-induced fever because of the already mentioned lack of correlation (Figs. 4 and 5).

5. Conclusions

Neurochemical mechanisms modulating immune challenge events have become a topic of immense interest over recent years. It is worth noting that recent reports have described the intimate interaction between cells of the nervous and immune systems that takes place in the gut, and may have a role in diverse inflammatory disorders [2,19]. The present study reports the effect of the gut-derived peptide ghrelin on the mechanisms underlying immune-inflammatory modulation of the febrile response. Our results shed light on the new role of ghrelin in the regulation of inflammation, indicating an anti-inflammatory effect (at least, predominantly), which corroborates a recent study [18]. More specifically, we observed an immunosuppressive effect of ghrelin during endotoxemia. As described in Fig. 5, alterations to hypothalamic-pituitary-adrenal axis following LPS exposure appear to be up-modulated by ghrelin, whereas preoptic PGE_2 production seems to be down-modulated by ghrelin. Both the effects of ghrelin favor a reduced T_b (Fig. 5). Moreover, the effect of ghrelin on PGE_2 production seems not to be mediated by the increased glucocorticoids plasma levels (Fig. 4) but rather due to a direct effect of the peptide.

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