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Intravenous injection of urocortin 1 induces a CRF_2 mediated increase in circulating ghrelin and glucose levels through distinct mechanisms in rats

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Abstract

Urocortins (Ucns) injected peripherally decrease food intake and gastric emptying through peripheral CRF_2 receptors in rodents. However, whether Ucns influence circulating levels of the orexigenic and prokinetic hormone, ghrelin has been little investigated. We examined plasma levels of ghrelin and blood glucose after intravenous (iv) injection of Ucn 1, the CRF receptor subtype involved and underlying mechanisms in *ad libitum* fed rats equipped with a chronic iv cannula. Ucn 1 (10 μg/kg, iv) induced a rapid onset and long lasting increase in ghrelin levels reaching 68% and 219% at 0.5 and 3 h post injection respectively and a 5-h hyperglycemic response. The selective CRF_2 agonist, Ucn 2 (3 μg/kg, iv) increased fasting acyl (3 h: 49%) and des-acyl ghrelin levels (3 h: 30%) compared to vehicle while the preferential CRF_1 agonist, CRF (3 μg/kg, iv) had no effect. Ucn 1's stimulatory actions were blocked by the selective CRF_2 antagonist, $astressin_2$-B (100 μg/kg, iv). Hexamethonium (10 mg/kg, sc) prevented Ucn 1-induced rise in total ghrelin levels while not altering the hyperglycemic response. These data indicate that systemic injection of Ucns induces a CRF_2-mediated increase in circulating ghrelin levels likely *via* indirect actions on gastric ghrelin cells that involves a nicotinic pathway independently from the hyperglycemic response.

Keywords

acyl ghrelin; $astressin_2$-B; CRF; des-acyl ghrelin; radioimmunoassay; rat; urocortin

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Disclosure
The authors have nothing to disclose. No conflicts of interest exist.

1. Introduction

The CRF signaling systems in mammals encompass CRF and three related peptides, urocortin (Ucn) 1, Ucn 2, and Ucn 3, as well as two main receptor subtypes, CRF_1 and CRF_2 [15]. CRF ligands interact with CRF receptors with distinct affinity, with CRF showing a 40-fold higher affinity to CRF_1 than CRF_2 receptors [15]. Ucn 1 displays a high affinity to both CRF_1 and CRF_2 receptors while Ucn 2 and Ucn 3 bind selectively to CRF_2 [15]. While the CRF_1 signaling system in the brain is well established to orchestrate the endocrine, behavioral, and visceral responses to stress [27,43,48], growing anatomical and physiological evidence also supports an important role of the peripheral CRF_2 signaling system within the viscera, namely the gut and heart [12,28]. In particular, in the rat and human stomach CRF_2 receptors are prominently expressed at the gene and protein levels although the cellular identification of CRF_2 positive is still to be further investigated [4,5,55]. Convergent functional studies also showed that injection of Ucn 1 or 2 either intraperitoneally (ip) or intravenously (iv) inhibits gastric emptying, antral motility, and food intake in rodents [13,20,33,52]. We also reported that iv Ucn 1 induces a sustained hyperglycemia in fasted rats [50]. The demonstration that the selective antagonist, astressin$_2$-B [37] injected peripherally prevents the ip or iv injected Ucn 1-induced inhibition of gastric emptying and food intake in mice and rats [13,31,37] while peripheral injection of CRF_1 antagonists has no effect [29, 31] supports a mediation through CRF_2 receptors.

Conversely, acyl ghrelin is a peptide hormone mainly released from gastric X/A-like cells, [8] stimulating food intake, gastric emptying and motility [6,18]. Acyl ghrelin release is increased by fasting or before a meal while being reduced postprandially in rodents and humans, indicative of a physiological role in meal initiation [7,44,46]. Previous reports indicate that visceral (abdominal surgery) or immunological (lipopolysaccharide, LPS, injected intraperitoneally at a low dose) stressors induced suppression of food intake and gastric emptying which was associated with the reduction of plasma ghrelin in rats [38,41,50]. In addition, LPS under these conditions up-regulated Ucns mRNA expression in the rat gastric corpus mucosa [55]. These findings indicate a potential influence of peripheral Ucns on circulating ghrelin which so far has been little investigated. One clinical study showed that Ucn 1 infused iv for one hour reduces the fasted ghrelin plasma levels starting at 2-h post infusion in healthy subjects [10] while an iv bolus of Ucn 1 in fasted rats did not change the plasma levels of total ghrelin and induced a hyperglycemia over the 5-h experimental period [50].

In the present study, we investigated the influence of Ucn 1, injected iv at a dose known to inhibit gastric emptying in rats [31,33], on plasma levels of ghrelin and blood glucose in *ad libitum* fed rats, linked with lower circulating levels of ghrelin compared with fasted conditions [42]. Next, we characterized the CRF receptor mediating the iv Ucn 1 action, using the selective CRF_2 antagonist, astressin$_2$-B [37]. We also examined whether the selective CRF_2 agonist Ucn 2 injected iv would influence the fasting ghrelin levels including the acylated and the most abundant form, non-acylated (des-acyl) ghrelin that does not bind to the ghrelin receptor [22,23]. Lastly, in light of previous evidence that iv Ucn 1 activates brain nuclei regulating sympathetic outflow to the viscera as shown by Fos expression [51] and that ghrelin release is regulated by the autonomic nervous system [17], we also investigated the influence of ganglionic blockade by hexamethonium on ghrelin and glucose alterations induced by the iv injection of Ucn 1.

2. Materials and Methods

2.1. Animals

Adult male Sprague-Dawley rats (Harlan, San Diego, CA, USA, 280–320 g) were housed 4 animals/cage under conditions of controlled illumination (12:12 h light/dark cycle, lights on/off: 6.00 h/18.00 h) and temperature (22±2 °C) unless otherwise stated. Animals were fed a standard rodent diet (Prolab RMH 2500; LabDiet, PMI Nutrition, Brentwood, MO, USA) and tap water *ad libitum*. Animal care and experimental procedures followed institutional ethic guidelines and conformed to the requirements of the federal authority for animal research conduct. All procedures were approved by the Animal Research Committee at the Veterans Affairs Greater Los Angeles Healthcare System (animal protocol # 05058-02).

2.2. Compounds

Rat CRF, rat Ucn 1, human Ucn 2 and astressin$_2$-B were synthesized as described before [37] at the Clayton Foundation Laboratories (Peptide Biology Laboratories, Salk Institute, La Jolla, CA). Peptides, stored in powder form at −80 °C and hexamethonium (Sigma-Aldrich, San Louis, MO) stored at room temperature, were dissolved in vehicle immediately before use.

2.3. Blood collection and assays

2.3.1. Intravenous catheterization—Intravenous catheterization was performed as described in our previous studies [50]. Briefly, rats were anesthetized with a mixture of ketamine (75 mg/kg ip; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (5 mg/kg ip; Mobay, Shawnee, KS) and a sterile PE-50 catheter was inserted into the right external jugular vein. The catheter, filled with saline-heparin (200 units/ml) to maintain lumen patency, was exteriorized between the scapulae *via* subcutaneous tunneling, then secured to the skin and closed using a wire. Rats were singly housed after surgery and allowed to recover for three days during which they were accustomed to the experimental procedures including light hand restraint for blood withdrawal. Body weight was monitored before the iv catheterization and three days after the surgery.

2.3.2. Blood withdrawal and processing—Blood (0.5 ml) was withdrawn into a syringe while rats were lightly hand restrained. The first blood sampling time started between 9.00 h and 10.00 h for each batch of rats. Blood samples were processed according to the RAPID method for the measurement of acyl and total ghrelin as previously described [42]. Briefly, immediately after withdrawal, blood was diluted 1:10 in an ice-cold buffer (pH 3.6) containing 0.1M ammonium acetate, 0.5 M NaCl, and enzyme inhibitors (diprotin A, E-64-d, antipain, leupeptin and chymostatin, 1 μg/ml; Peptides International, Louisville, KY), and immediately centrifuged at 3000 rpm for 10 min at 4 °C. Sep-Pak C18 cartridges (360 mg, 55–105 μm, product no. WAT051910, Waters Corporation, Milford, MA) were charged with 5 ml 100% acetonitrile and equilibrated with 10 ml 0.1% trifluoroacetate (TFA). The equilibrated cartridges were loaded with sample, rinsed with 3 ml 0.1% TFA and eluted with 2 ml 70% acetonitrile in 0.1% TFA. The eluted samples were dried by vacuum centrifugation and stored at −80°C until further processing.

For measurement of total ghrelin alone, blood was collected in ice-cooled tubes containing EDTA (7.5%, 10 μl/0.5 ml blood; Sigma-Aldrich) and aprotinin (0.6 trypsin Inhibitory Unit per 0.5 ml blood; ICN Pharmaceuticals, Costa Mesa, CA) as previously described [50]. Samples were kept on ice until centrifugation at 3000 rpm for 10 min at 4°C. Plasma was collected and stored at −80°C.

2.3.3. Determination of acyl ghrelin, des-acyl ghrelin and total ghrelin plasma levels—Radioimmunoassay was performed using a commercial RIA kit for rat/mouse total ghrelin (Phoenix Pharmaceuticals, Belmont, CA). The limit of the assay sensitivity was 54 pg/ml and the intra- and inter-assay variations were less than 5% and 14% respectively.

For acyl and des-acyl ghrelin determinations, samples were re-suspended in double distilled H_2O according to the original volume of plasma and thereafter, acyl and total ghrelin were measured using specific radioimmunoassay kits according to the manufacturer's instructions (# GHRT-89HK and GHRA-88HK, respectively, Millipore, Billerica, MA). Des-acyl ghrelin was calculated as the difference of total minus acyl ghrelin for each individual sample. Intra-assay variability was < 5% and all samples were processed in one batch.

2.3.4. Blood glucose levels—Blood glucose levels were determined using a glucometer (One-Touch Ultra; LifeScan, Milpitas, CA).

2.4. Experimental protocols

All experiments were performed between 9.00 h and 14.00 h. Rats had similar body weight before and three days after the surgery for iv catheter (277.3 ± 11.1 vs 277.3 ± 12.2 g; n=39).

2.4.1. Influence of iv Ucn 1 on plasma total ghrelin and blood glucose levels: Time course study—Freely fed rats implanted with a chronic intra-jugular catheter were injected iv (0.2 ml) with vehicle (pyrogen-free water) or Ucn 1 (10 μg/kg dissolved in vehicle) and returned to their home cages with access to water but not food to avoid potential confounding factors linked with differential influence of treatment on food intake. Blood (0.5 ml) was withdrawn before and at 0.5, 1, 3 and 5 h post injection and processed for plasma levels of total ghrelin and blood glucose measurements were performed as described above. The iv dose of Ucn 1 was selected based on our previous dose response studies in rats showing maximal suppression of gastric emptying in rats [33].

2.4.2. Influence of CRF_2 receptor antagonist on total ghrelin and glucose responses to iv Ucn 1—Freely fed rats implanted with a chronic jugular catheter were injected iv (0.1 ml) with vehicle (pyrogen-free water) or astressin$_2$-B (100 μg/kg dissolved in vehicle) and 15 min later with vehicle (pyrogen-free water) or Ucn 1 (10 μg/kg in vehicle). The regimen of astressin$_2$-B administration was based on previous studies showing the blockade of gastric transit induced by iv Ucn 1 in rats [31]. Blood (0.5 ml) was withdrawn before iv injection of astressin$_2$-B and 1 h post injection of iv Ucn 1 for determination of total ghrelin plasma and blood glucose levels.

2.4.3. Influence of Ucn 2 or CRF on fasted plasma acyl and des-acyl ghrelin levels—Rats implanted with a chronic intra-jugular catheter were deprived of food but not water overnight and injected iv (0.2 ml) with vehicle (saline or pyrogen-free water containing 0.1% bovine serum albumin, BSA, Sigma-Aldrich), Ucn 2 (3 μg/kg in pyrogen-free water containing 0.1% BSA), or CRF (3 μg/kg in sterile saline containing 0.1% BSA) and returned to their home cages without access to food but with water *ad libitum*. Blood (0.5 ml) was withdrawn at 3 and 5 h post injection of Ucn 2, CRF or vehicle and processed for total and acyl ghrelin measurements and the determination of des-acyl ghrelin from these individual values. Doses of peptides were selected based on their inhibitory effects on gastric emptying upon peripheral injection in rats [31,33].

2.4.4. Influence of hexamethonium on iv Ucn 1-induced rise in plasma total ghrelin and hyperglycemia—Rats fed *ad libitum* were injected subcutaneously (sc, 0.3

ml/rat) with vehicle (saline) or hexamethonium (10 mg/kg, dissolved in vehicle), 30 min before the iv injection (0.2 ml/rat) of vehicle (pyrogen-free water) or Ucn 1 (10 μg/kg, dissolved in pyrogen-free water). The dose of hexamethonium was modified from our previous studies showing that 15 mg/kg prevented the iv CRF induced decrease in intraluminal gastric pressure in anesthetized rats [36]. Blood (0.5 ml) was withdrawn at 1 h post injection and processed for total ghrelin and glucose determinations.

2.5. Statistical analysis

Data are expressed as mean ± SEM and analyzed by one-way or two-way analysis of variance (ANOVA) followed by Tukey *post hoc* test. Data obtained in the CRF_2 antagonist and ganglionic blocker experiments were expressed as percentage of change calculated from basal level for each value in the same rat. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Intravenous Ucn 1 increases plasma total ghrelin and blood glucose levels: time course study

In freely fed rats, pre-injection levels of total ghrelin were similar in both groups (Fig. 1A). In the control group injected iv with vehicle, plasma total ghrelin levels increased during the 5-h observation period compared with pre-injection levels, reaching significance at 3 h post injection, which represented a 74% increase at this time point that was maintained at 5 h (0 h *vs.* 3 h: 0.77 ± 0.09 *vs.* 1.36 ± 0.19, $p < 0.05$ and *vs.* 5h: 1.39 ± 0.13 ng/ml; $p < 0.05$; n = 6; Fig. 1A). By contrast, blood glucose levels were stable throughout the 5-h experimental period in iv vehicle injected rats (0 h: 117.2 ± 2.2 *vs.* 5 h: 124.5 ± 3.5 mg/dl, n = 6; $p > 0.05$; Fig. 1B). Injection of Ucn 1 (10 μg/kg, iv) increased total plasma ghrelin levels compared to the vehicle-injected group at 0.5 h (1.41 ± 0.18 *vs.* 0.85 ± 0.11 ng/ml, n = 7; $p < 0.05$), 1 h (1.69 ± 0.18 *vs.* 1.19 ± 0.15 ng/ml; $p < 0.05$), and 3 h (2.61 ± 0.25 *vs.* 1.36 ± 0.19 ng/ml; $p < 0.05$) post injection, whereas at 5 h, no significant difference was observed compared to vehicle (1.81 ± 0.32 *vs.* 1.39 ± 0.13 ng/ml; $p > 0.05$; Fig. 1A). Likewise, blood glucose was significantly increased after iv Ucn 1 compared to vehicle at 0.5 h (155.7 ± 3.9 *vs.* 119.2 ± 2.7 mg/dl, $p < 0.05$) with a plateau increase at 1 h (164.4 ± 6.0 *vs.* 124.0 ± 4.7 mg/dl, $p < 0.05$) and 3 h (163.4 ±3.1 *vs.* 126.3 ± 1.7 mg/dl, $p < 0.05$), and a slow decrease at 5 h post injection (148.7 ± 3.2 *vs.* 124.5 ± 3.5 mg/dl, $p < 0.05$; Fig. 1B). Two way ANOVA showed that total ghrelin levels were significantly influenced by iv Ucn 1 treatment ($F_{(1,55)} = 22.6$, $p < 0.001$) and time ($F_{(4,55)} = 11.4$, $p < 0.001$), but not by treatment × time ($F_{(4,55)} = 2.5$, $p = 0.05$). The increase in blood glucose levels was also significantly influenced by treatment ($F_{(1,55)} = 127.3$, $p < 0.001$) and time ($F_{(4,55)} = 21.6$, $p < 0.001$), as well as treatment × time ($F_{(4,55)} = 12.6$, $p < 0.001$).

3.2. Intravenous Ucn 1-induced elevations of plasma ghrelin and blood glucose levels are CRF_2 receptor mediated

In freely fed rats pretreated with iv vehicle, iv Ucn 1 (10 μg/kg) induced a significant 58.0 ± 9.7% increase in plasma ghrelin (Fig. 2A) and 53.8 ± 9.8% blood glucose levels compared with iv vehicle + iv vehicle as measured at 1 h after peptide injection (Fig. 2B). The selective peptide CRF_2 antagonist astressin$_2$-B (100 μg/kg, iv) completely blocked the iv Ucn 1-induced rise in ghrelin and glucose circulating levels (Fig. 2). When injected alone the antagonist had no effect on either total ghrelin or glucose levels compared to vehicle ($p > 0.05$; Fig. 2).

3.3. Intravenous Ucn 2, but not CRF, increases plasma acyl and des-acyl ghrelin

In overnight fasted rats, Ucn 2 (3 μg/kg, iv) significantly increased plasma acyl ghrelin levels compared with the iv vehicle group by 48.8% at 3 h (1.37 ± 0.13 *vs.* 0.92 ± 0.19 ng/ml, $p < 0.05$) and 66.8 % at 5 h post injection (1.52 ± 0.16 *vs.* 0.91 ± 0.13 ng/ml, $p < 0.05$; Fig. 3A). Two-way ANOVA showed a significant influence of treatment ($F_{(1,16)} = 11.9$, $p < 0.01$) but not time ($F_{(1,16)} = 0.2$, $p > 0.05$). Likewise, plasma des-acyl ghrelin levels were significantly elevated after iv Ucn 2 compared to vehicle injected animals by 30.4% at 3 h (3.30 ± 1.15 *vs.* 2.53 ± 0.19 ng/ml) and 37.7% at 5 h (3.25 ± 0.23 *vs.* 2.36 ± 0.30 ng/ml; Fig. 3B). Two-way ANOVA indicated a significant influence of treatment ($F_{(1,16)} = 14.5$, $p < 0.01$) but not time ($F_{(1,16)} = 0.3$, $p > 0.05$). By contrast, plasma levels of acyl and des-acyl ghrelin did not change significantly over the experimental period following the iv injection of the preferential CRF_1 agonist, CRF (3 μg/kg, iv) compared to iv vehicle as monitored at 0.5, 1, 3 and 5 h post injection in fasted rats ($p > 0.05$; Fig. 3C, D).

3.4. Intravenous Ucn 1-induced CRF_2 receptor mediated increase in plasma total ghrelin levels is blocked by hexamethonium, unlike the hyperglycemia

Hexamethonium (10 mg/kg) injected sc 30 min before the iv injection of Ucn 1 (10 μg/kg, iv) completely blocked the increase of total ghrelin as measured at 1 h post iv injection in freely fed rats (hexamethonium/Ucn 1 *vs.* vehicle/Ucn 1: 42.3 ± 14.6% *vs.* 138.1 ± 21.0%, n = 7–8; $p < 0.05$; Fig. 4A). By contrast, the concomitant elevation of blood glucose was blunted without reaching statistical significance (hexamethonium/Ucn 1 *vs.* vehicle/Ucn 1, 30.1 ± 5.5% *vs.* 41.5 ± 5.5%, $p > 0.05$; Fig. 4B). Hexamethonium alone under these conditions did not influence levels of plasma ghrelin or blood glucose (Fig. 4).

4. Discussion

The present data established that Ucn 1 injected intravenously increases ghrelin plasma levels compared with iv vehicle in freely fed conscious rats. Time course studies showed that the Ucn 1 response was rapid in onset and long lasting as shown by a 68% elevation at 30 min post iv Ucn 1 injection that reached 216% at 3 h compared to pre-injection levels with a return to vehicle levels at 5 h. The sustained response may be linked with pharmacokinetic properties of Ucn 1 that has a long half life in the circulation [34]. However, the underlying mechanisms contributing the peak response observed at 3 h post injection are not clear at the present time. It may be speculated that there is synergistic interaction between Ucn 1's ghrelin-releasing action and additional mechanisms recruited at 3 h. Indeed, in the vehicle-injected group, we observed a gradual increase in ghrelin levels reaching significance at 3 h (76%) and maintained at 5 h (84%) post injection compared with pre-injection values which may be linked with known diurnal changes in circulating ghrelin with a peak occurring at 5 h after onset of the light phase in *ad libitum* fed rats [3]. Consistent with such a possibility is the drop to vehicle values at 5 h post urocortin 1 injection when the peptide (which has a half life of 2.9 h) is cleared [34].

We also showed that the response is not specific to Ucn 1, since the related peptide, Ucn 2 [15] injected iv also induced a sustained ghrelin elevation reaching 49% and 57% at 3 and 5 h post injection respectively compared with vehicle. As the Ucn 2 experiment was performed in fasted rats, a metabolic state well established to increase circulating levels of ghrelin compared to the fed state [42,46], these data provide the first evidence in rats that a systemic treatment with Ucns induces a consistent elevation of circulating ghrelin levels irrespective of the feeding status, overnight fasted or freely fed associated with low or already elevated endogenous ghrelin levels. However, the ghrelin response to iv Ucn 1 is still equivocal as in our previous studies in fasted rats, iv Ucn 1 at a similar dose did not influence plasma levels of total ghrelin [50], indicative that fasting may influence the effect

of Ucn 1. In one clinical study, Ucn 1 (50 μg/healthy subject/h) or vehicle was infused iv for 1 h starting at 1 h after breakfast. There was a time-related elevation of ghrelin levels with a peak response at 3 h in vehicle group and Ucn 1 reduced the elevated plasma total ghrelin levels at 3 and 5 h post initiation of the infusion while not influencing the rise for the first 2 h [10]. Whether these differential patterns reflect species or dose differences remains to be investigated.

Total ghrelin encompasses two major circulating forms, acyl ghrelin, the only form binding to the ghrelin receptor, and des-acyl ghrelin, the predominant form in the blood [9,32]. Recent evidence indicates that the ratio of acyl and des-acyl ghrelin can be altered in experimental animals and humans under stress conditions [45]. We previously determined that the ratio of plasma acyl:des-acyl ghrelin was 1:3 (acyl:total ghrelin 1:5) in rats using the RAPID method based on blood processing to improve the recovery of acyl ghrelin compared with previous methods that yield a lower acyl:des-acyl ratio [16,38,42]. Likewise, in the present study, the acyl:des-acyl ghrelin ratio at 3 and 5 h after iv vehicle was 1:3.1 and 1:2.7, respectively ($p > 0.05$) in fasted rats. Ucn 2 injected iv did not significantly modify this ratio (1:2.5 and 1:2.2 at 3 and 5 h post injection, respectively $p > 0.05$ *vs.* vehicle), although there was a trend to enhance acyl ghrelin *vs.* des-acyl ghrelin. Ghrelin-*O* acyltransferase (GOAT) which is expressed in rat gastric corpus ghrelin cells [40], is the unique enzyme highly conserved across vertebrates able to catalyze the octanoylation of ghrelin, leading to the active form of the peptide [54]. The present data indicate that the rise in ghrelin after iv Ucn 2 may not reflect changes in GOAT expression or activity.

Next, we used a pharmacological approach to assess the receptor subtype(s) involved in the action of the CRF_1/CRF_2 agonist, Ucn 1 [15,47]. We demonstrated that the selective CRF_2 antagonist, astressin$_2$-B injected iv before Ucn 1 completely blocked the rise in ghrelin elicited by Ucn 1. Moreover, the rise in plasma ghrelin can be reproduced by the iv injection of the selective CRF_2 agonist, Ucn 2 [15] while the preferential CRF_1 agonist, CRF [15] had no effect throughout the 5-h experimental period including the 0.5 h sampling period as well as in the first hour and every two hours thereafter. Consistent with the lack of CRF action, a previous study showed that CRF microinfused through a probe inserted into the submucosal layer of the rat gastric corpus did not significantly influence the release of ghrelin monitored in the microdialysis perfusate while noradrenaline had a stimulatory effect [11]. Collectively, these data provide convergent pharmacological evidence that systemic injection of Ucns leads to a CRF_2 receptor-mediated rapid onset and prolonged elevation of circulating acyl and des-acyl ghrelin under basal or fasting-stimulated conditions in rats. This contrasts with the intracerebroventricular injection of Ucn 1 in rats which was recently reported to decrease plasma ghrelin levels during the first 2 h post injection with a longer lasting inhibitory effect on des-acyl ghrelin than acyl ghrelin [53]. The opposite effects of Ucn 1 injected into the lateral brain ventricle *vs.* systemic circulation is indicative of distinct centrally *vs.* peripherally initiated Ucn 1 sites of action to influence circulating levels of ghrelin. In addition, the low basal entry of circulating Ucn 1 across the blood brain barrier compared with other peripheral peptides [19] does not support a direct action of iv Ucn 1 into the brain except in areas outside of the blood-brain barrier.

Then, we investigated the pathways involved in the CRF_2-mediated Ucn 1 action. The majority of circulating forms of ghrelin is produced by ghrelin positive X/A-like cells that are located in both gastric oxyntic and, to a lesser extent, antral mucosa of rats [2]. Previously, we obtained pharmacologic and anatomic evidence indicative that the iv somatostatin (sst)-induced sst_2 receptor mediated inhibition of circulating ghrelin may involve a direct action on sst_2 receptors expressed on gastric corpus ghrelin cells [41]. However, there is no report that CRF_2 immunoreactivity is present in endocrine cells of the rat gastric mucosa [5,35]. In addition, CRF_2 mRNA is not expressed in a purified

preparation of mouse gastric ghrelin cells carrying a reporter, M-cherry, analyzed by microarray compared to whole gastric mucosal scrape (Dr. N. Lambrecht, private communication). Studies available to date are indicative that the CRF_2 mediated action of iv Ucn 1 is unlikely to be exerted directly on ghrelin producing cells in the gastric corpus. However, this contention will need to be ascertained using preparations of pure and not-immortalized rat ghrelin cells when available.

An indirect action is further supported by the complete blockade of the hyperghrelinemic response to iv Ucn 1 by the ganglionic blocker, hexamethonium. Of note, as reported previously [50], iv Ucn 1 induced a sustained rise in blood glucose measured concomitantly with ghrelin. However, while the hyperglycemia was also blocked by the CRF_2 antagonist, hexamethonium had no effect. Previous reports established that the iv Ucn 3-induced hyperglycemia involves a CRF_2 mediated action exerted directly on α-cells in rat pancreatic islets [26]. These data are consistent with the present demonstration that the CRF_2 receptor mediated hyperglycemic responses to iv Ucn 1 are initiated by distinct independent mechanisms. It is well established that the activation of the vagal and sympathetic nervous system increases circulating ghrelin levels [17]. It may be speculated that CRF_2 receptors expressed at peripheral sites such as the nodose ganglia or in brain areas outside the blood-brain barrier, namely the area postrema [25,30,49], can initiate reflex control over efferent autonomic mechanisms [14,21]. In line with this contention, we previously reported that the iv injection of Ucn 1 at a similar dose induced the expression of Fos, a marker of neuronal activation, in the nucleus tractus solitarius, area postrema and other hypothalamic brain nuclei involved in autonomic regulation [51]. In particular, iv Ucn 1 activates C1/A1 catecholaminergic neurons [51] unlike the dorsal motor nucleus of the vagus, favoring an involvement of the sympathetic nervous system. The implication of these neural mechanisms needs to be substantiated in future investigations.

In conclusion, systemic injection of Ucns induces a rapid onset and sustained CRF_2 mediated increase in circulating ghrelin levels unlikely to be related to a direct action on gastric ghrelin cells but requiring the integrity of ganglionic nicotinic transmission in conscious rats. Circulating ghrelin is known to play a physiological orexigenic and gastric prokinetic role to regulate food intake and gastric motility [1,39]. The hyperghrelinemia in response to iv Ucn 1 and Ucn 2, rules out the role of ghrelin in mechanisms underlying peripheral Ucns-CRF_2 mediated inhibition of food intake, gastric emptying and motility that is well established under similar conditions of peripheral administration [13,20,33,52]. Whether such a rise in ghrelin plays a role to dampen the Ucn 1 inhibitory action needs to be assessed using ghrelin antagonists. In addition, the CRF_2 mediated hexamethonium-independent concomitant hyperglycemia induced by iv Ucn 1 strengthens the emerging role of Ucns-CRF_2 signaling as novel peripheral modulators of glucose homeostasis and metabolic functions [24,26].

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Highlights

- Intravenous urocortin 1 increases plasma total ghrelin and blood glucose in fed rats
- Intravenous CRF_2 receptor agonist, urocortin 2 increases fasted plasma acyl ghrelin levels.
- The increase in plasma ghrelin and glucose by urocortin 1 is CRF_2 receptor mediated.
- Ghrelin but not glucose rise by urocortin 1 requires the integrity of autonomic transmission

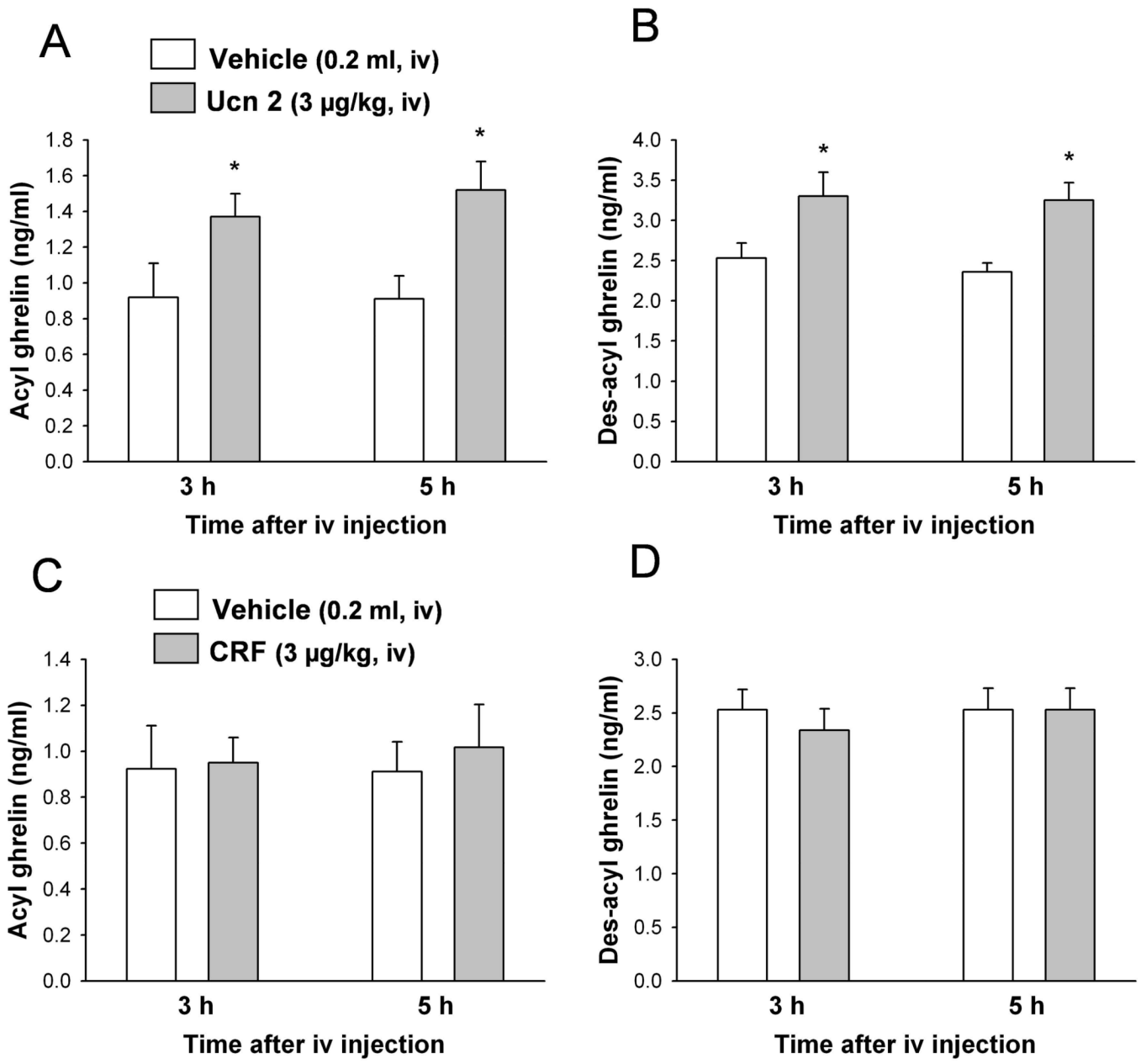

Fig. 1.

Time course of rise in plasma total ghrelin (A) and blood glucose (B) levels induced by iv urocortin 1 in freely moving rats. Freely fed rats implanted with a chronic iv catheter were injected iv with Ucn 1 or vehicle and returned to their home cages with access to water but not food. Blood was withdrawn at various time intervals. Blood glucose and plasma total ghrelin were measured. Data are expressed as mean ± SEM, n = 6–7/group, *: $p < 0.05$ *vs.* time 0 and #: *vs.* vehicle at the same time point.

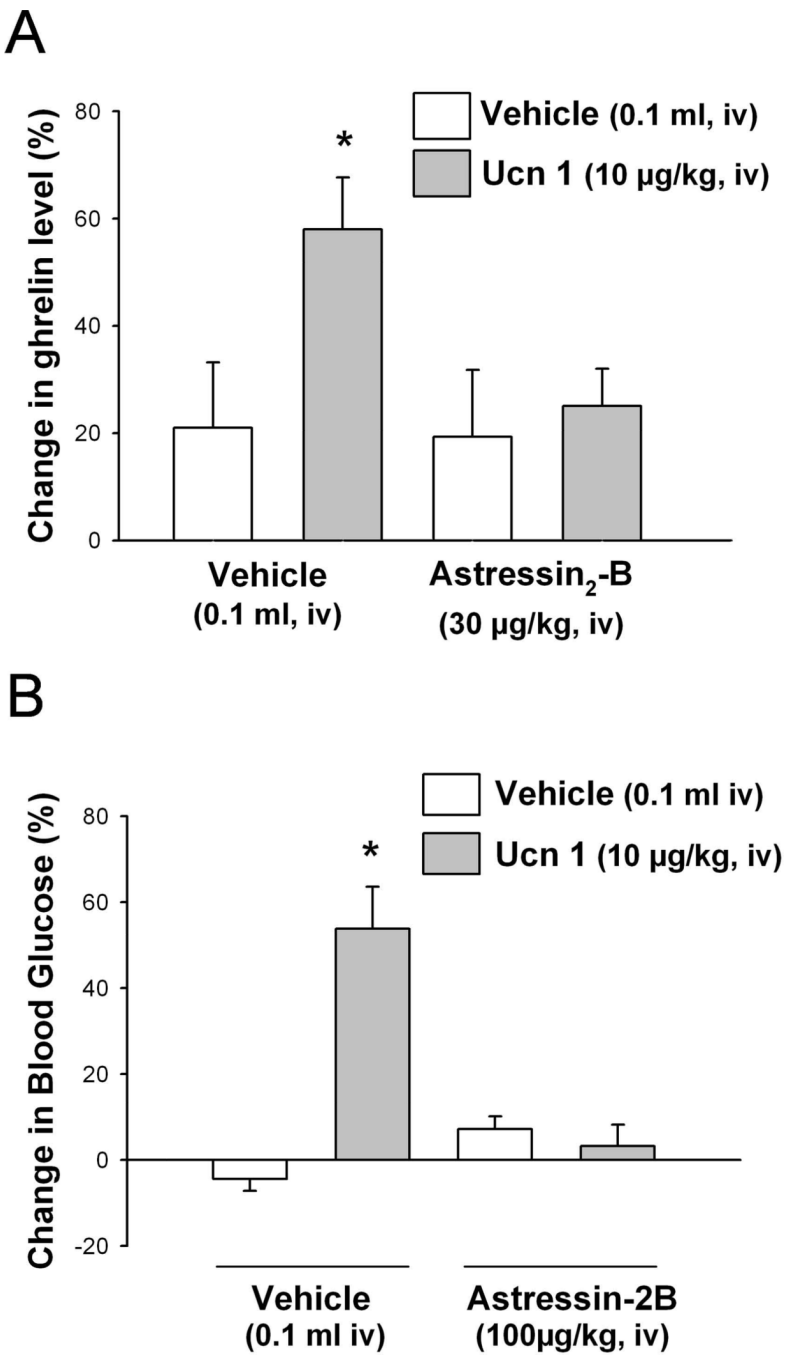

Fig. 2.

The CRF_2 receptor antagonist, astressin$_2$-B blocks the iv urocortin 1-induced increase in plasma total ghrelin (A) and blood glucose (B) levels in freely moving rats. Freely fed rats implanted with a chronic iv catheter were injected iv with astressin$_2$-B or vehicle 15 min before iv injection of Ucn 1 or vehicle, and returned to their home cages with access to water but not food. Blood was withdrawn before injection of astressin$_2$-B and at 1 h post injection of Ucn 1. Blood glucose and plasma total ghrelin were measured. Data are expressed as mean of percent changes ± SEM, n= 5–7/group. * $p < 0.05$ *vs.* vehicle/vehicle.

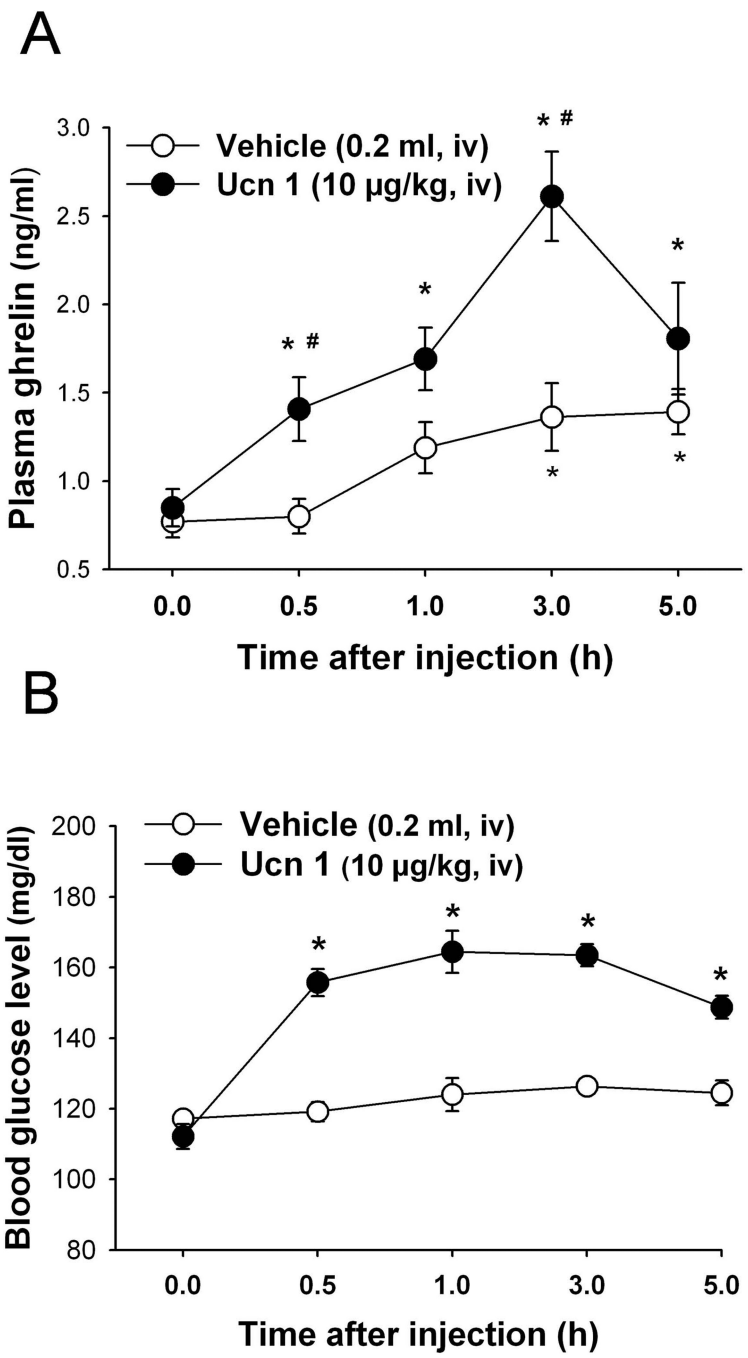

Fig. 3.

Urocortin 2, unlike CRF, increases fasted levels of acyl and des-acyl ghrelin in freely moving rats. Overnight fasted rats implanted with chronic iv catheter were injected iv with Ucn 2, CRF or vehicle and returned in their home cages with access to water but not food. Blood was withdrawn at 3 and 5 h post injection of Ucn 2 (A, B) and CRF (C, D) and processed for acyl and total ghrelin measurements. Des-acyl ghrelin was determined as the difference between total and acyl ghrelin for each measurement. Data are expressed as mean of percent changes ± SEM, of n=5/group. * $p < 0.05$ and *vs.* vehicle.

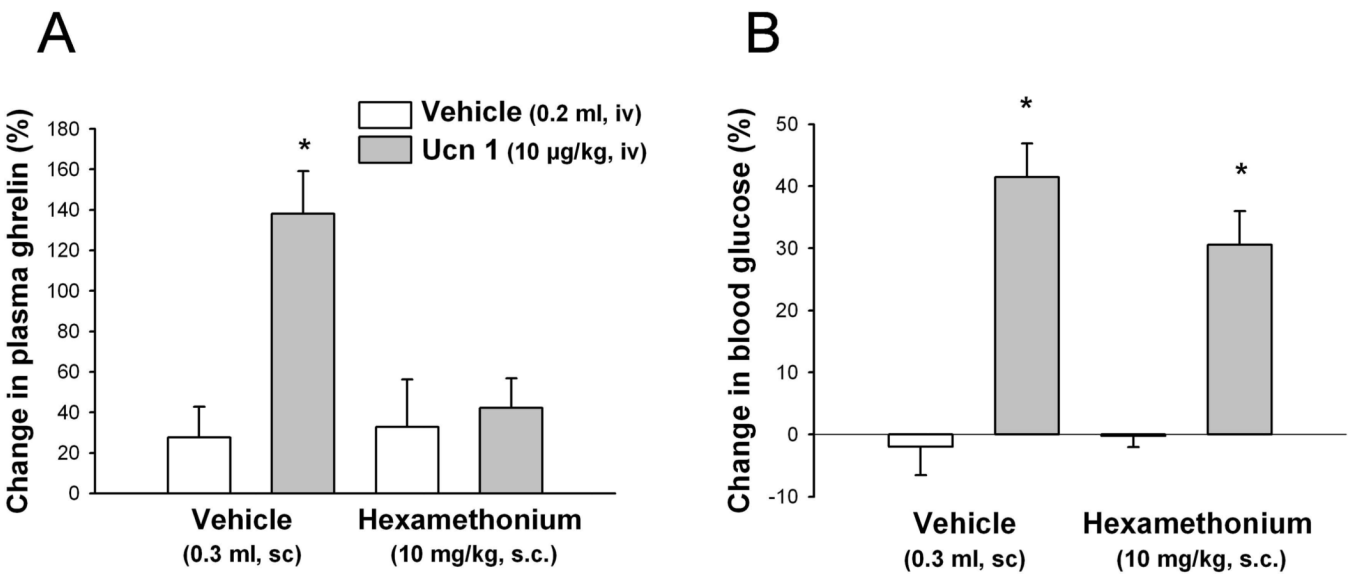

Fig. 4.

Hexamethonium blocks the iv urocortin 1-induced increase in plasma total ghrelin but not blood glucose levels. Rats fed *ad libitum* implanted with a chronic iv catheter were injected sc with hexamethonium or vehicle and 30 min later with Ucn 1 or vehicle iv and blood was withdrawn at 1 h post injection and processed for total ghrelin (A) and glucose (B) measurements. Data are expressed as mean ± SEM, n= 5–6/group. * $p < 0.05$ *vs.* all other groups.