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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.

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 receptor 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

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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 1 h 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 3 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 3 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 0.1 M 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 3 days after the surgery for iv catheter in the jugular vein (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 which as described above. The iv