Human urocortin II, a new CRF-related peptide, displays selective CRF$_2$-mediated action on gastric transit in rats

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CORTICOTROPIN-RELEASING FACTOR (CRF) and urocortin (Ucn) bind to two distinct seven transmembrane domain G protein-coupled receptors, subtypes CRF$_1$ and CRF$_2$, leading to an increase in intracellular cAMP levels (30). Human/rat CRF (h/rCRF) displays higher affinity to CRF$_1$ than CRF$_2$, whereas rat Ucn (rUcn) exhibits high affinity to both CRF receptor subtypes (29). CRF is best known for its physiological role in mediating the pituitary adrenal activation induced by various stressors, an effect mediated by CRF$_1$ (38).

Convergent studies (4, 7, 36) established that activation of central CRF receptors is also implicated in integrating the behavioral, autonomic, and visceral responses to acute stress, including alterations of gastrointestinal (GI) motor function in various mammalian species.

Recent evidence (10) indicates that peripheral CRF receptors may also be involved in stress-related alterations of gut motor function. h/rCRF and rUcn administered intravenously, intraperitoneally, or subcutaneously inhibit gastric motility and emptying, while stimulating colonic motility and transit, mimicking the pattern of changes induced by stress or central injection of the peptides (1, 6, 14, 18, 21, 26, 27). The specific, nonselective CRF$_1$/CRF$_2$ peptide antagonists, α-helical CRF-(9–41), [D-Phe$^{12}$]CRF-(12–41), and astressin, injected peripherally, blocked peripheral h/rCRF and rUcn actions on GI motor function (1, 14, 18, 21, 26). A few studies also indicate that these CRF antagonists administered peripherally prevented post-operative gastric ileus (21, 26) and reduced restraint- and water avoidance stress-induced stimulation of colonic transit and defecation (5, 18, 39). Although indirect evidence suggested that CRF$_2$ may be involved in intravenous h/rCRF- and rUcn-induced inhibition of gastric emptying of nonnutrient meal (26), direct pharmacological proof was hampered by the lack of selective CRF$_2$ agonists and antagonists.

Recently (16, 32), new mammalian members of the CRF family, Ucn II and Ucn III, were discovered by sequence homology to h/rCRF from the human genome database and the mouse orthologs have been cloned. Mouse Ucn II shares 76% identity with the 38 amino acid putative mature human Ucn (hUcn) II, 42% with rUcn, and 34% with h/rCRF (16, 32). Ucn II and Ucn III from mouse or human origin display selective binding to CRF$_2$ and are postulated to be endogenous ligands for CRF$_2$ (16, 32). So far little is known about the biological actions of Ucn II except for one report (32) showing that mouse Ucn II injected into the lateral...
brain ventricle decreased food intake without altering gross motor activity in rats. Based on the high degree of selectivity to CRF$_{2}$, Ucn II may be of value in dissociating alterations of GI motor functions mediated by CRF receptor subtypes. In addition, the development of selective peptide CRF$_{2}$ antagonists, namely anti-sauvagine-30, and the long-acting analog astressin$_{2}$-B (11, 33, 34) provide new tools to assess the role of CRF$_{2}$ in mediating endogenous CRF ligands and stress-related alterations of gut motor function.

In the present study, we investigated the influence of the novel mammalian member of the CRF family, hUcn II (administered peripherally), on gastric emptying of a solid meal and distal colonic transit monitored simultaneously in conscious rats. We also examined the role of CRF receptor subtypes in mediating hUcn II-, h/rCRF-, and rUcn actions. Partial restraint stress-induced alterations of gastric and colonic motor function (39), using peripheral pretreatment with the selective CRF$_{1}$ antagonist, CP-154,526 (35) and the newly developed (33) selective CRF$_{2}$ antagonist astressin$_{2}$-B.

**MATERIALS AND METHODS**

**Animals**

Adult male Sprague-Dawley rats (Harlan, San Diego, CA), weighing 280–320 g, were housed in group cages with free access to food (Purina rat chow) and tap water. All experiments started between 8 and 9 AM and were performed in 24-h fasted rats, with free access to water. Protocols were approved by the Animal Care Committee (protocol no. 9906-20) of the Veteran Affairs Greater Los Angeles Healthcare System.

**Drugs and Treatments**

The following peptides were used: hUcn II, h/rCRF, rUcn, and astressin$_{2}$-B (Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies). Peptides were synthesized as previously described (32), stored in powder form at −80°C, weighed, and dissolved immediately before their use in saline (hUcn II, h/rCRF, and rUcn) or double distilled water (astressin$_{2}$-B) at pH 7.0. CP-154,526 (CP-154526-01 hydrochloride salt, Pfizer, Groton, CT) was dissolved in vehicle (5% DMSO, 5% Cremaphor EL, and 90% saline) immediately before use. CP-154,526 solution and its respective vehicles were injected subcutaneously at 90 min after the start of the 2-h feeding period. Thirty minutes later, rats were briefly anesthetized with isoflurane for bead insertion into the distal colon and the intravenous injection of saline, h/rCRF (1, 3, or 10 µg/kg), rUcn (1, 3, or 10 µg/kg), or hUcn II (3 or 10 µg/kg) was performed.

**Experimental Protocols**

**Effects of mammalian CRF receptor agonists.** Fasted rats were given preweighed chow for 2 h, and then food and water were removed. All rats were placed under brief anesthesia (2–3 min) with isoflurane for insertion of the bead into the distal colon, and either no treatment or the intravenous injection of saline, h/rCRF (1, 3, or 10 µg/kg), rUcn (1, 3, or 10 µg/kg), or hUcn II (3 or 10 µg/kg) was performed.

**Effects of selective CRF receptor antagonists on h/rCRF and hUcn II actions.** Fasted rats were given preweighed chow for 2 h. Astressin$_{2}$-B (100 or 200 µg/kg), CP-154,526 (20 mg/kg), or their respective vehicles were injected subcutaneously at 90 min after the start of the 2-h feeding period. Thirty minutes later, rats were briefly anesthetized with isoflurane for bead insertion into the distal colon and the intravenous injection of saline, h/rCRF (10 µg/kg), or hUcn II (10 µg/kg) in all pretreated groups.

**Partial Restraint**

Partial restraint was performed as previously described (24) with slight modifications. Under short isoflurane anesthesia, the forelimbs were wrapped together in gauze and secured with tape and the hind limbs were similarly wrapped. A small plastic neck collar was placed around the neck to minimize unwrapping of the gauze and tape. Rats recovered from the anesthesia within 3 min and were placed individually in their home cages.

**Simultaneous Measurement of Gastric Emptying and Distal Colonic Transit**

Fasted rats had free access to preweighed Purina chow for a 2-h period. Then a single 5-mm colored plastic bead was inserted into the distal colon (3 cm past the anus) with a plastic rod while each rat was under brief isoflurane anesthesia. After bead insertion, conscious rats were placed in individual plastic cages without water or food. The time required for expulsion of the bead (in min) was monitored over 4 h. Gastric emptying of the ingested meal was assessed 4 h after food removal as previously described (20). Animals were euthanized by CO$_{2}$ inhalation, the abdominal cavity was rapidly opened, the pylorus and cardia were clamped, and the stomach was removed and its contents weighed. The percentage of gastric emptying in 4 h was calculated using the following formula: [1 − (weight of gastric content/weight of food intake) × 100]. The solid food ingested was determined by the difference between the weight of the Purina chow given to each rat and the weight of the leftover and spill at the end of the 2-h feeding period. All weights were made to the nearest 0.1 g.

**Statistical Analysis**

Values are expressed as means ± SE. Data were analyzed using the Kruskal-Wallis test (for gastric emptying) or one-way ANOVA followed by a Student-Newman-Keuls multiple-comparisons test (for bead expulsion time). P < 0.05 was considered statistically significant.
RESULTS

Differential Actions of Mammalian CRF Agonists

In 24-h fasted rats that received no treatment (n = 6), the gastric emptying at 4 h after the end of an ingested solid meal was 60.0 ± 6.9%, and the distal colonic transit, assessed by the bead expulsion time, was 127.2 ± 12.6 min. Saline injected intravenously (n = 10) did not influence gastric emptying (64.2 ± 5.6%) or distal colonic transit time (141 ± 20.9 min; Fig. 1). hUcn II injected intravenously at 3 (n = 6) and 10 μg/kg (n = 10) significantly reduced gastric emptying of the solid meal to 35.3 ± 6.9% and 28.9 ± 7.9%, respectively, while not altering the distal colonic transit time (Fig. 1). rUcn at 1, 3, and 10 μg/kg iv dose dependently inhibited gastric emptying of the solid meal to 43.6 ± 5.7% (P > 0.05), 32.9 ± 5.4% (P < 0.05), and 29.1 ± 6.7% (P < 0.05), respectively, compared with saline (62.1 ± 7.7%; Fig. 2A) and significantly decreased the bead expulsion time only at 10 μg/kg (44.3 ± 11.3 min, P < 0.05 vs. saline, 116.7 ± 20.6 min; Fig. 2B). h/rCRF injected intravenously significantly reduced gastric emptying of the meal only at 10 μg/kg (34.8 ± 4.6%) while lower doses (1 or 3 μg/kg iv) had no effect (Fig. 2A). In contrast, there was a dose-related shortening in the bead expulsion time with values decreasing to 77.8 ± 16.3% (P > 0.05), 49.5 ± 7.7% (P < 0.05), and 20.5 ± 5.3% (P < 0.05) following intravenous injection of h/rCRF at 1, 3, and 10 μg/kg, respectively (Fig. 2B).

Reversal of CRF and Ucn II Actions by Selective CRF Receptor Antagonists

hUcn II (10 μg/kg iv)-induced inhibition of gastric emptying of a solid meal was dose dependently prevented by the CRF2 antagonist astressin2-B. Fasted rats were given access to food for 2 h and injected intravenously with saline, h/rCRF, or rUcn, and a bead was inserted into the colon. Values are means ± SE of 5–10 rats/group. *P < 0.05 vs. saline (0); #P < 0.05 vs. the corresponding dose (3 μg/kg) of h/rCRF or rUcn.

Reversal of Restraint Stress Effects by Selective CRF Receptor Antagonists

Partial restraint reduced gastric emptying of the solid nutrient meal over 4 h to 38.7 ± 6.2% and short-
ened the distal colonic bead expulsion time to 16.4 ± 1.0 min (P < 0.05 vs. control) compared with 68.3 ± 5.9% and 160.9 ± 15.2 min, respectively, in the nonpretreated and nonrestraint group. The CRF2 antagonist astressin 2-B (200 µg/kg sc) abolished restraint-induced delayed gastric emptying (55.9 ± 4.9%, P < 0.05, compared with 32.9 ± 10.6% in intravenous vehicle + saline; #P < 0.05 vs. h/rCRF + astressin2-B (100 and 200 µg/kg) or h/rCRF + CP-154,526 (20 mg/kg)).

CP-154,526 (20 mg/kg) and astressin2-B (200 µg/kg) injected subcutaneously 30 min before intravenous saline did not modify either gastric emptying of a solid meal or distal colonic transit (Figs. 1, 3, and 4).

DISCUSSION

In the present study, we provide the first evidence that the newly discovered CRF family member hUcn II selectively delayed gastric emptying of a solid meal and that its inhibitory action on gastric motor function is mediated through interaction with CRF2 in conscious rats. Recent in vitro studies (32) established that hUcn II displays a high degree of selectivity for the type 2 CRF receptor and represents an endogenous ligand for this receptor. In the radioreceptor binding assay, hUcn II is equipotent to rUcn for binding to CRF 2 and 1,000-fold less effective at competing for binding to CRF 1 (32). In the cAMP stimulation assay using cells stably transfected with either CRF1 or CRF2, hUcn II showed efficacy comparable to that of rUcn in activating CRF2 in sub- or low-nanomolar concentrations with no preference for the splice variants CRF2α and CRF2β (16, 32). In contrast, hUcn II exhibits a very low potency to activate CRF1 (>100 nM) whereas rUcn has a half-maximal effective dose of 0.15 nM (16, 32). The similar magnitude of gastric emptying inhibition induced by intravenous injection of hUcn II and rUcn (45% and 53% inhibition, respectively) at 3 µg/kg (0.5 nmol/kg) may have a bearing on their comparable efficacy at CRF2 (32). The role of CRF2 in mediating hUcn II’s action was further established by the demonstration that the selective CRF2 antagonist astressin 2-B (33) injected subcutaneously at 100 and 200 µg/kg dose dependently blocked intravenous hUcn II (10 µg/kg)
induced inhibition of gastric emptying by 58% and 100%. Likewise, astressin\(_{2}\)-B (100 \(\mu\)g/kg sc) antagonized h/rCRF (10 \(\mu\)g/kg iv)-induced delayed gastric emptying of a solid meal by 100%. The higher subcutaneous antagonist-to-agonist ratio for hUcn II (20:1) than h/rCRF (10:1) is in line with the greater affinity of hUcn II for CRF2 compared with h/rCRF (11, 32). CRF2 was previously suggested (26) to be involved in peripheral h/rCRF- and rUcn-induced delayed gastric emptying of a nonnutrient solution mainly on the basis of rank order of potency of nonselective nonmammalian CRF\(_{1}\)/CRF\(_{2}\) agonists. The present data provide direct pharmacological proof that CRF2 mediates peripherally administered mammalian CRF receptor ligand-induced delayed gastric emptying of a solid meal in rats by using the recently characterized selective CRF2 agonist hUcn II and antagonist astressin\(_{2}\)-B.

The present study also established that hUcn II injected intravenously displays selective action on gastric emptying while not influencing distal colonic transit as assessed in a new model of simultaneous monitoring of gastric emptying of a solid meal and propulsive function in the distal colon of conscious rats. The hUcn II action contrasts with that of rUcn and h/rCRF, which, when injected intravenously, induced a dose-related acceleration of distal colonic transit and inhibition of gastric emptying. These findings provide functional evidence that hUcn II administered peripherally induced a pattern of GI motor alterations consistent with its in vitro high degree of CRF\(_{2}\) selectivity (32). Indeed, present and previous data strongly support a role of CRF\(_{1}\), unlike CRF\(_{2}\), in mediating the stimulatory action of peripheral CRF on distal colonic transit. h/rCRF and rUcn, which display high affinity for CRF\(_{1}\) (30, 32), administered intraperitoneally or intravenously stimulate colonic motor function in rats. This was shown by the induction of clustered spike burst activity in the cecum and proximal colon, acceleration of large intestinal transit (as monitored by labeled chromium distribution from the cecum to the colon), defecation (5, 6, 15, 18, 39), and shortening of distal colonic transit (present study). In addition, the selective CRF\(_{1}\) antagonist CP-154,526 prevented the intravenous h/rCRF-induced decrease in colonic bead expulsion time whereas the selective CRF\(_{2}\) antagonist astressin\(_{2}\)-B, injected subcutaneously at a dose that prevented the gastric response, did not. Similarly, one previous study (18) reported that intraperitoneal h/rCRF-induced fecal pellet output and clustered spike burst activity in the rat proximal colon were antagonized by the subcutaneous injection of CP-154,526.

These experimental findings in rats may have relevance to human physiology. Ovine CRF is considered to be primarily a CRF\(_{1}\) ligand based on the 180-fold higher affinity to CRF\(_{1}\) than CRF\(_{2}\) (3, 34). In previous studies (23), ovine CRF injected intravenously at a dose (2.8 \(\mu\)g/kg) inducing an almost twofold increase in plasma cortisol levels failed to influence the postprandial motility index in the human antrum. In contrast, h/rCRF (2 \(\mu\)g/kg iv) increased segmental contractions in the descending and sigmoid colon, and the colonic motor response was more pronounced in patients with irritable bowel syndrome compared with healthy volunteers (6). These clinical reports are consistent with CRF\(_{1}\) not being involved in intravenous CRF action on gastric motor function while playing a role in stimulating colonic motor activity in humans, as established in rats.

The pathways through which intravenous hUcn II inhibits gastric emptying via activation of CRF\(_{2}\) will require further investigation to be understood. In longitudinal smooth muscles of rat antrum, h/rCRF dose dependently reduced the motility index of spontaneous activity through TTX-sensitive pathways (31). These data suggest that CRF action does not act directly on smooth muscles but involves neural transmission within the gastric enteric nervous system (31). CRF injected intravenously inhibits Fos expression in the gastric myenteric neurons induced by central vagal activation (37, 40), indicative of a possible modulation of nicotinic excitatory input. Likewise, in vitro studies (8, 18, 19) indicate that CRF stimulatory action in the colon involves activation of the colonic enteric nervous system. This contrasts with the reported direct action of CRF on cecal circular smooth muscle cells inhibiting contractile response (13). Further studies are required to localize the exact sites at which the differential gut motor responses are elicited by peripheral CRF-related peptides.

Very little is known regarding the CRF receptor subtypes mediating stress-related alterations of GI motor function due to the lack of selective CRF\(_{2}\) antagonist. Partial restraint stress for 90 min resulted in a 48% reduction of the gastric emptying of the solid meal whereas distal colonic transit was stimulated by 87% in conscious rats. This stress model reproduced changes identical (in pattern and magnitude) to those of h/rCRF injected intravenously. The selective CRF\(_{2}\) antagonist astressin\(_{2}\)-B injected subcutaneously prevented restraint-induced delayed gastric emptying while not affecting the acceleration of distal colonic transit. Moreover, the CRF\(_{1}\) antagonist CP-154,526 attenuated the restraint stress-induced shortening of distal colonic transit while not influencing the gastric motor response. Peripheral administration of CP-154,526 also reduced water avoidance stress-induced defecation (18) and diarrhea elicited by morphine withdrawal in rats (17). Taken together, the data demonstrate that CRF\(_{2}\) is involved in restraint stress-related delayed gastric transit of a solid meal. These findings also extend to another stressor, the participation of CRF\(_{1}\) in colonic motor action. It is to be noted that peripheral administration of the selective CRF receptor antagonists, at doses abolishing intravenous h/rCRF- or restraint-induced alterations of gut transit, did not influence either the gastric emptying of the solid meal or distal colonic transit in nonstressed rats. These observations indicate that CRF pathways do not modulate postprandial gastrocolonic transit under nonstressed conditions.

The source of endogenous CRF ligands activating CRF receptor subtypes under acute stress could not be
determined in the present study. The possible leakage of CRF from the brain to the periphery during stress is supported by the demonstration of active CRF transport from the brain to the periphery (22). However, CRF and Ucn are expressed in the GI tract (9, 12, 16, 25) and immune cells (2). There is also preliminary evidence from PCR analysis that high levels of selective CRF2 ligands are expressed in the stomach (12), supporting the possibility that CRF-related peptides may act through local nonhormonal mechanisms as reported for other peripheral actions of CRF (2, 28).

In summary, the present data demonstrate that intravenous injection of the newly discovered hUcn II, which in vitro displays selective affinity to CRF2 (32), delayed gastric emptying of a solid meal while not influencing distal colonic motor function monitored simultaneously. The inhibitory action of hUcn was prevented by peripheral administration of the selective CRF2 antagonist astressin2-B. In contrast, rUcn and h/cRF, which have affinity for both CRF1 and CRF2, injected intravenously inhibited gastric emptying and stimulated distal colonic transit, and these responses were mimicked by acute partial restraint. The inhibitory action on gastric emptying induced by intravenous CRF and acute stress was blocked by the selective CRF2 antagonist whereas the stimulation of distal colonic transit was attenuated selectively by the CRF1 vented by peripheral administration of the selective urocortin III, an additional member of the corticotropin-releasing hormone receptor family through CRF receptor subtype selectivity.

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REFERENCES


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