CCK inhibits the orexigenic effect of peripheral ghrelin

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Various peptide hormones have been proposed to play an important role in the short-term regulation of food intake and satiety by influencing initiation and termination of meals (52). Compelling evidence for the existence of “satiety factors” came from initial observations in the 1970s demonstrating that peripheral CCK administered before food exposure causes a dose-dependent decrease in meal size (21). Since then, the satiating effect of CCK has been confirmed and extended by numerous reports (reviewed in Refs. 44 and 40). More recently, a 28-amino acid peptide hormone named ghrelin (27) has been identified as the first gut-brain peptide stimulating food intake in rats (33, 47, 55, 56), mice (4, 50), and humans (54).

CCK and ghrelin exert antagonistic effects on ingestive behavior. The potent orexigenic effect of peripheral injection of ghrelin (33, 47, 55, 56), the rise in plasma ghrelin levels induced by food deprivation or weight loss (15, 47) and the reduction of feeding in response to central administration of growth hormone secretagogue receptor (GHS-R) antagonist (3) in rodents implicate that endogenous ghrelin is involved in the initiation of food intake (14). On the other hand, several lines of evidence (reviewed in Ref. 40) have shown that endogenous CCK, released in the small intestine (36) in response to nutrients (28, 29), elicits potent anorexigenic effects and is involved in meal termination.

Recent reports indicate that both ghrelin and CCK effects on food intake are mediated at least partly by distinct brain nuclei, as demonstrated by changes in neuronal activity after peripheral administration of each peptide. Brain pathways activated by intraperitoneal injection of these peptides have been revealed by c-Fos-like immunoactivity (c-FLI) (43). Sulfated CCK octapeptide (CCK-8S) induces c-FI in the paraventricular nucleus of the hypothalamus (PVN) and selective medullary nuclei, namely, the nucleus of the solitary tract (NTS) and the area postrema (10, 17, 31, 34, 39). In contrast, ghrelin injected intraperitoneally induces c-Fos expression, primarily in the arcuate nucleus of the hypothalamus (ARC) (24, 50), in the PVN (42), but not in the dorsal vagal complex (50) in rats and mice.

The satiation effect of CCK depends on signaling via the vagus nerve, as demonstrated by the significant attenuation of the feeding inhibitory properties of CCK by either surgical or chemical deafferentation of the vagal nerve (41, 45). Likewise, a recent study indicates that vagal afferent-dependent mechanisms may be involved in peripheral ghrelin-induced increase in food intake and c-FLI in the ARC (16).

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Collectively, these findings provide the conceptual framework for an antagonistic interaction between ghrelin and CCK to regulate food intake. Therefore, the initial purpose of the present study was to determine whether CCK modulates the orexigenic response to ghrelin. We investigated first the changes in food intake after simultaneous peripheral injection of ghrelin and increasing doses of CCK-8S in rats. Furthermore, recent electrophysiological studies (16), which demonstrated opposing effects of ghrelin and CCK on the afferent discharge activity of a vagal nerve filament, suggest that the dual influence of CCK and ghrelin may be associated with distinct neuronal activation patterns in the brain. Consequently, we also examined the influence of simultaneous intraperitoneal injection of ghrelin and CCK on the pattern of Fos expression in the PVN, ARC, and NTS. Brain nuclei were selected on the basis of the presence of GHS-R for ghrelin (ARC, PVN) and input from vagal afferent terminals after activation by peripheral CCK (NTS) (38, 51).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan-Winkelmann, Böchen, Germany) weighing 300–350 g were housed in groups of four rats per cage under conditions of controlled illumination (12:12-h light-dark cycle, lights on/off: 6:30 AM/6:30 PM), humidity, and temperature (22 ± 2°C). Animals were fed with a standard rat diet (Altromin, Lage, Germany) and tap water ad libitum. All animals were trained daily for 14 days before starting the experiments to accustom them to the behavioral studies. During this handling phase, holding them on their backs was practiced to prepare the animals for receiving an intraperitoneal injection. Similarly, every day, the animals were removed from their social group for 2 h and set in single cages. This period of social isolation exactly mimicked the time schedule of the experiments. Animals used for neuronal mapping (c-Fos) were handled daily for 5 days before the start of the experiment. They were also trained to be held in the back position for intraperitoneal injection and to be isolated in single cages for 2 h.

Animal care and experimental procedures followed institutional ethical guidelines and conformed to the requirements of the state authority for animal research conduct.

Peptide Preparation

Rat ghrelin [GSS(octanoyl)FLSPEHQKAQQRE5KKPPAKL-QPR] (Bachem, Heidelberg, Germany) and CCK-8S [DTI(SO,H)MGWMDF] (Bachem) were dissolved in distilled water (1 mg/ml) and stored at −20°C. Immediately before starting the experiments, peptides were diluted in vehicle solution consisting of sterile 0.15 M NaCl solution (Braun, Melsungen, Germany) to reach the final concentration of 13 μg/kg body wt (4 nmol/kg body wt) for ghrelin and 2 or 25 μg/kg body wt for CCK-8S. Peptide solutions were kept on ice for the duration of the experiments.

Experiment 1: Effects of Peripheral Ghrelin and CCK-8S on Food Intake

All experiments were started at the same time of day (between 10:00 and 10:30), at 3.5 to 4 h after the start of the light cycle to achieve maximum consistency.

On the day of the experiment, freely fed animals were injected intraperitoneally simultaneously (final volume: 0.5 ml) with vehicle plus vehicle (0.15 M NaCl + 0.15 M NaCl, n = 15), ghrelin plus vehicle (13 μg/kg body wt + 0.15 M NaCl, n = 15), vehicle plus CCK-8S [0.15 M NaCl + 2 (n = 14) or 25 (n = 15) μg/kg body wt], or ghrelin plus CCK-8S (13 μg/kg body wt + 2 (n = 12) or 25 (n = 15) μg/kg body wt) and returned to single housing cages immediately after the injection. Thereafter, preweighed rat chow was made available to the animals. Food intake was determined by measuring the difference between the preweighed standard chow and the weight of chow at the end of the first 30 min, 1 h, and 2 h of food exposure. Selected doses of peptides were based on previous studies (31, 42, 57).

Experiment 2: Effects of Peripheral Ghrelin and CCK-8S on c-Fos-like Immunoreactivity in the Hypothalamus and Brain Stem

Freely fed rats were injected intraperitoneally (final volume 0.5 ml) with vehicle plus vehicle (0.15 M NaCl + 0.15 M NaCl, n = 8), ghrelin plus vehicle (13 μg/kg body wt + 0.15 M NaCl, n = 8), vehicle plus CCK-8S [0.15 M NaCl + 2 (n = 4) or 25 (n = 4) μg/kg body wt], or ghrelin plus CCK-8S [13 μg/kg body wt +2 (n = 4) or 25 (n = 4) μg/kg body wt]. Immediately after injection, animals were deprived of food to avoid an influence of ghrelin-induced increase in food intake on c-Fos expression in the brain but had ad libitum access to water (50). At 90 min after the intraperitoneal injection, animals were deeply anesthetized with intraperitoneal injections of 100 mg/kg body wt ketamine (Ketanest, Curared, Karlsruhe, Germany) and 10 mg/kg body wt xylazine (Rompun 2%, Bayer, Leverkusen, Germany) and heparinized with 2,500 U heparin intraperitoneally (Liquemin, Hoffmann-La Roche, Grenzach-Whylen, Germany). Transcardial perfusion was performed as described before (20) and consisted of a 10-s flush of a plasma substitute (Longasteril 70; Fresenius, Bad Homburg, Germany) followed by a mixture of 4% (wt/vol) paraformaldehyde, 0.05% (vol/vol) glutaraldehyde, and 0.2% (vol/vol) picric acid in 0.1 M phosphate buffer, pH 7.4, for 30 min and ended with a 5% (wt/vol) sucrose solution for 5 min. After dissection, brains were kept in a 5% (wt/vol) sucrose solution overnight and then cut into 1.0- to 4.5-mm coronal blocks, enclosing the hypothalamic and brain stem regions, respectively, using a Plexiglas brain matrix. For cryoprotection, blocks were moved through a sucrose gradient [15% (wt/vol) and 27.3% (wt/vol)], then shock-frozen in hexane at −70°C, and stored at −80°C until further processing.

Immunohistochemistry

Staining for c-Fos-like immunoreactivity. First, 25-μm, free-floating sections were pretreated with 1% (wt/vol) sodium borohydride in PBS for 15 min. Subsequently, sections were incubated in a solution containing 10% (wt/vol) BSA and 0.3% (vol/vol) Triton X-100 in PBS for 60 min for blockade of nonspecific antibody binding. Thereafter, the diluted primary antibody solution [rabbit anti-rat c-Fos protein; Oncogene Research Products, Boston, MA; 1:10,000 in a solution of 10% (wt/vol) BSA, 0.3% (vol/vol) Triton X-100, and 0.1% (wt/vol) sodium azide in PBS] was applied for 24 h at room temperature.

After rinsing sections in PBS three times and incubation in a solution containing 10% (wt/vol) BSA and 0.3% (vol/vol) Triton X-100 for 60 min, FITC-labeled goat anti-rabbit IgG (Sigma, St. Louis, MO) was applied for 12 h at room temperature in an appropriate dilution [1:800 in 10% (wt/vol) BSA in PBS]. Sections were rinsed in PBS three times again and stained with propidium iodide (2.5 μg/ml in 4 nmol/kg body wt) for ghrelin and 2 or 25 μg/kg body wt for CCK-8S. Peptide solutions were kept on ice for the duration of the experiments.

Immunohistochemistry. Semiquantitative assessment of c-Fos-like immunoreactivity (c-FLI) was achieved by counting the number of c-FLI-positive cells. Cells with green nuclear staining were considered c-FLI positive. Every second of all consecutive coronal 25-μm
sections was counted for c-FLI-positive staining bilaterally in the ARC, PVN, and NTS throughout their rostrocaudal extent. Anatomical correlations were made according to landmarks given in a stereotaxic atlas (35). c-FLI-positive cells were counted in 10 sections of the PVN per rat and 15 sections of the ARC and NTS per rat. The investigator counting the number of c-Fos-positive cells was blinded to treatments received by the animals.

The average number of c-FLI-positive cells per section for the brain nuclei mentioned above was calculated for each rat. Data are expressed as median and interquartile ranges of the average number of cells per section. Differences between groups were evaluated by the nonparametric Kruskal-Wallis and Mann-Whitney U-tests; \( P < 0.05 \) was considered significant.

Feeding experiments. The cumulative food intake monitored at the end of the first 30-min, 1-h, and 2-h periods after peptide or vehicle injection was expressed as food intake (g) per body weight (kg). Data are expressed as means ± SE and analyzed by ANOVA. Differences between groups were evaluated by the least significant difference test; \( P < 0.05 \) was considered significant.

RESULTS

Experiment 1: Effects of Ghrelin and CCK-8S Injected Intraperitoneally on Food Intake

Ghrelin (13 \( \mu g/kg \) body wt ip) significantly increased the food intake within the first half hour after intraperitoneal injection of vehicle plus peptide (2.89 ± 1.04 g/kg body wt) compared with the intraperitoneal vehicle plus vehicle group (1.23 ± 0.52 g/kg body wt, \( P < 0.028 \); Fig. 1). CCK-8S injected intraperitoneally at either 2 or 25 \( \mu g/kg \) body wt) significantly increased food intake compared with the vehicle plus vehicle group, CCK injected intraperitoneally alone at both doses (1.96 ± 0.82 g/kg body wt, \( P < 0.048 \); Fig. 1)). Interestingly, despite the lack of a significant influence of CCK alone at both doses on food intake, we observed a decrease in food intake in comparison with the vehicle group when 2 \( \mu g \) CCK-8S/kg body wt was administered together with ghrelin (4.1 ± 1.2 g/kg body wt vs. vehicle 4.1 ± 1.2 g/kg body wt, \( P = 0.49 \); Fig. 1). Further, a significant difference in reduction of food intake was observed between the vehicle plus 2 \( \mu g \) CCK-8S/kg body wt group and the ghrelin plus 2 \( \mu g \) CCK-8S/kg body wt group (4.36 ± 1.39 g/kg body wt vs. 0.83 ± 0.72 g/kg body wt, \( P < 0.036 \); Fig. 1).

Experiment 2: Effects of Ghrelin and CCK-8S Injected Intraperitoneally on c-fos Expression in Hypothalamic and Medullary Nuclei

Ghrelin (13 \( \mu g/kg \) body wt ip) induced a robust increase in the density of c-FLI-positive neurons in the ARC, predominantly in the ventromedial part (Fig. 2), compared with vehicle (median number of c-FLI-positive neurons per section: 31.35 vs. 9.86, \( P < 0.0001 \); Fig. 3). The effect of peripheral ghrelin (13 \( \mu g \) ghrelin/kg body wt) on neuronal activity in the ARC was significantly diminished by simultaneous treatment with CCK-8S injected intraperitoneally at a dose of 2 \( \mu g/kg \) body wt (13.33 neurons per section, \( P < 0.008 \) vs. ghrelin dose; Fig. 3) or 25 \( \mu g/kg \) body wt (12.86 neurons per section, \( P < 0.004 \) vs. ghrelin dose; Fig. 3), as shown by the few c-FLI-positive nuclei found in the ARC (Fig. 2). CCK-8S alone had no effect on neuronal activity in the ARC, as assessed by c-FLI, when the peptide was injected at doses of 2 or 25 \( \mu g/kg \) body wt (5.33 and 11.21 cells per section, respectively; Fig. 3).

The density of c-FLI-positive neurons in the PVN and NTS (data shown in Figs. 4 and 5) was dose dependently increased after treatment with CCK-8S (2 or 25 \( \mu g/kg \) body wt ip). Simultaneous treatment with ghrelin (13 \( \mu g/kg \) body wt + 2 or 25 \( \mu g/kg \) body wt) did not affect the CCK-induced
Fig. 2. Effects of ghrelin, CCK, and ghrelin + CCK injected intraperitoneally on c-Fos-like immunoreactivity (c-FLI) in the arcuate nucleus (ARC) of the hypothalamus. At treatment with 13 μg ghrelin/kg body wt (A), we observed c-FLI (green staining) at 90 min after intraperitoneal injection of the peptides in freely fed rats (n = 8/group). At intraperitoneal injection of ghrelin (13 μg/kg body wt) + 2 (G) or 25 (H) μg CCK-8S/kg body wt (n = 4/group), 2 μg CCK-8S/kg body wt (n = 4/group) (B) and 25 μg CCK-8S/kg body wt (n = 4/group) (C) alone, or saline injection (n = 8/group) (I), none or only a few c-FLI-positive neurons were found in the ARC. Cell nuclei are stained red as a result of the counterstaining with propidium iodide in the same slice from animals treated with ghrelin (D), ghrelin + 2 (J) or 25 (K) μg CCK-8S/kg body wt, 2 (E) or 25 (F) μg CCK-8S/kg body wt alone, and saline (L). The white line delineates the area of the hypothalamic ARC in accordance with landmarks from the Paxinos and Watson rat brain atlas (35). White scale bar represents 100 μm. 3V, third ventricle; ME, median eminence.
increase in c-fos expression in the PVN and NTS (Figs. 4 and 5).

In the present study, we could also show that ghrelin administered intraperitoneally caused a significant increase in the number of c-FLI-positive neurons in the PVN compared with the vehicle group (median number of c-FLI-positive cells per section: 50.56 vs. 20.25, \(P < 0.05\); Fig. 4).

**DISCUSSION**

Short-term control of ingestive behavior is regulated by various gut-derived peptides such as CCK and ghrelin (21). In the present study, we provide evidence for an inhibition of the stimulatory effect of peripheral ghrelin on food intake and neuronal activation in the hypothalamic ARC nucleus by peripheral CCK-8S in rats.

Ghrelin injected intraperitoneally exerted an orexigenic effect in freely fed rats, which is in agreement with earlier studies (33, 47, 55, 56). The time course study indicates that CCK-8S at doses of 2 or 25 \(\mu\)g/kg body wt induced a suppression of ghrelin stimulatory action on food intake that was similarly rapid in onset (within 30 min) and duration (over 2 h). Interestingly, the reduction in food intake in animals treated with ghrelin plus CCK-8S at both doses was more pronounced and...
longer lasting than with treatment with CCK-8S alone. It may be speculated that this effect of a combined application of both peptides is mediated via hypothalamic mechanisms, since peripheral injection of ghrelin and CCK-8S affects neuronal activity in different hypothalamic nuclei, as shown by changes in c-fos expression. However, the mechanisms and pathways still have to be elucidated in detail. CCK still displays a similar potency to reduce the 1-h accumulated food intake increase when injected intraperitoneally at 2 or 25 μg/kg body wt, resulting in 94% and 83% reduction, respectively. Thus both doses of CCK in our experiments act to suppress ghrelin-induced food intake in rats. It has been shown that doses of 8 μg CCK-8S/kg body wt ip or higher might induce unspecific effects in laboratory animals (e.g., nausea, taste aversion) (32). However, doses less than 8 μg CCK-8S/kg body wt do not induce unspecific effects on behavior in rats (32), suggesting that the observed effect of CCK on ghrelin-induced increase in food intake is specific to the peptide.

Various medullary and hypothalamic brain nuclei have been suggested to convey nutrient-related signals from the gastrointestinal tract to higher brain centers (18, 19, 52). In particular, several studies established that both CCK and ghrelin are gut peptides that regulate ingestive behavior by modulating the activity of distinct brain nuclei (22, 24, 49, 50, 57), as shown by a specific pattern of c-FLI in the brain (10, 17, 24, 31, 34, 39, 50). It therefore seemed reasonable to assume that the interaction between the two peptides to regulate food intake may be accompanied by changes in the specific patterns of neuronal activation in the hypothalamus and/or the brain stem. The intraperitoneal injection of ghrelin did not influence intraperitoneal CCK-induced Fos expression in the PVN and NTS. By contrast, CCK-8S at both doses of 2 and 25 μg/kg body wt significantly reduced the intraperitoneal ghrelin-induced increase of c-Fos density in the ARC monitored at 90 min postinjection by 57% and 59%, respectively.

The effect of ghrelin on ARC neurons has been recently characterized. First, peripheral administration of ghrelin induces a significant increase of c-Fos-positive neurons in the ARC in rats and mice (24). Furthermore, it has been found that a large population of c-Fos-positive neurons contains the orexigenic peptide neuropeptide Y (NPY) (50). Thus one could speculate that such brain areas are involved in the mechanisms that regulate meal initiation. Consequently, the inhibition of NPY/agouti-related protein neurons in the ARC by CCK could be involved in the suppressive effects of CCK on ghrelin-induced stimulation of food intake. The pathways by which intraperitoneal ghrelin signals to the ARC are impaired by intraperitoneal CCK could not conclusively be identified from the present study.

In our experiments, both doses of CCK-8S (2 and 25 μg/kg body wt) caused a significant reduction of the ghrelin-induced increase of c-Fos density in the ARC and blocked the ghrelin-induced stimulation of food intake. The control of meal size by CCK has been reported to involve brain stem mechanisms (23). However, it has also been shown that bilateral midbrain transection rostral to the NTS blocks peripheral injection of CCK-induced reduction of feeding (12). Furthermore, the destruction of the PVN (11) or the dorsomedial nucleus of the hypothalamus (6, 7) inhibits the suppression of feeding induced by peripheral CCK. Consistent with these functional studies, CCK induced a significant increase in c-fos expression in the NTS and PVN at 90 min after intraperitoneal injection, as previously reported (10, 17, 31, 34, 39). The PVN and NTS might exert an inhibitory influence on the neuronal activity in the ARC because both nuclei send direct projections to the ARC (26, 37). Therefore, it seems possible that the inhibition of ghrelin-induced activation of ARC neurons by CCK is mediated by afferent projections from the PVN and/or NTS.

Other recent studies support a role of the vagus in ghrelin signaling pathways to the ARC. Date and colleagues (16) have shown that both subdiaphragmatic or gastric vagotomy and perivagal capsaicin treatment attenuate the increase in the density of c-Fos in the ARC after intravenous injection of ghrelin. Also, in such vagally impaired animals, the change in food intake after ghrelin administration decreased significantly. Moreover, afferent fibers of the vagal nerve carry the GHS-R (16). Ghrelin is an endogenous ligand of the GHS-R (27). It is also well known that vagal afferent fibers play a primary role in the satiety effect of a low dose of CCK (41, 45). Therefore, one could speculate that the inhibition of the ghrelin effect by CCK is mediated by mechanisms involving afferent fibers of the vagal nerve. Recent electrophysiological studies showed opposing effects of ghrelin and CCK on the multiunit afferent discharge activity of gastric vagal nerve filaments (16). However, more recent electrophysiological studies looking at single gastric vagal afferent units in response to ghrelin and CCK injected intravenously showed that ghrelin has both inhibitory and excitatory action on vagal afferent activity, while application of CCK results in a constant stimulatory effect (11). In the present study the NTS, which receives vagal afferent input (38), was activated similarly by CCK injected alone or with ghrelin, suggesting that additional mechanisms unrelated to vagal afferent signaling pathways may also play a role. Recently, in vitro CCK studies of mouse brain slices demonstrated that a subset of neurons in the ARC responds to CCK by slowing the firing of ARC type-C neurons (9). Thus it cannot be ruled out that peripheral CCK reaching neurons in the ARC through the “leaky” blood-brain barrier at the median eminence could affect the responsiveness of ARC neurons to peripheral ghrelin (9). Further electrophysiological studies will be needed to support the hypothesis of a direct central effect of peripheral CCK on ARC neurons sensitive to peripheral ghrelin. Therefore, the present observations that peripheral CCK inhibits peripheral ghrelin-induced activation of ARC neurons, as assessed by c-FLI, and abolishes ghrelin-induced food intake in rats could be also explained by such a local hypothalamic mechanism.

In studies related to c-fos expression in the brain induced by peripheral injection of CCK, and ghrelin injected alone or in combination, fed animals were subsequently deprived of solid food to avoid confounding effects of food intake. Acute stress (swimming stress) in rats showed increased c-fos expression distinctly in the lateral part of the ARC (13). However, it is unlikely that the food deprivation may induce stress in ghrelin-treated rats and possibly change the c-fos expression in the ARC. We observed c-Fos signals in the medial part of the ARC after intraperitoneal injection of ghrelin, as previously reported (24, 50), and only a few signals of c-Fos in the lateral part of the ARC.

It has been well established that CCK suppresses food intake after intraperitoneal injection in rats (2, 8), although only certain experimental conditions reveal the anorexigenic effect.
of CCK (44, 48). The present studies were performed in nonfasted rats to detect meal initiation and its blockade. During the first half hour after CCK injection, we observed a trend toward a reduction in food intake, although this effect did not reach statistical significance. Most studies showing an inhibitory effect of peripheral CCK on food intake were conducted in fasted animals to assess the termination of hyperphagia induced by a fast in rats or mice (5, 30). Thus the nonfasting state of the animals is very likely the reason for the lack of a significant reduction of food intake at treatment with both doses of CCK in the present study.

Evidence supports that peripheral ghrelin interferes not only with the CCK-related mechanism in the regulation of satiety and food intake but also with other neuropeptides involved in homeostatic regulation of energy intake and expenditure. Hewson et al. (25) have shown an increase of c-Fos-positive neurons in the ARC, induced by intravenous administration of growth hormone-releasing peptide (GHRP-6), a synthetic growth hormone secretagogue (25). This effect of GHRP-6 on ARC neurons was diminished by central administration of ghrelin (25). Further, it has been shown that neurons in the ARC that are c-Fos positive, as a result of peripheral administration of ghrelin, carry the leptin receptor (Ob-R) (46) and that there is an interaction between ghrelin and leptin in the regulation of food intake (33).

In summary, the present data show that the orexigenic effect of peripheral ghrelin is modulated by peripheral CCK in freely fed rats. The stimulation of food intake and neuronal activity in the ARC induced by ghrelin administered intraperitoneally is abolished by intraperitoneal CCK. In contrast, the activation of neurons in the PVN and NTS induced by peripheral CCK is not influenced by ghrelin. These observations suggest that ARC neurons play a role in the mediation of ghrelin-induced stimulation of food intake and also in the mechanisms involved in the inhibition of the ghrelin-induced increase in food intake by peripheral CCK. However, it still has to be determined whether the influence of peripheral CCK on ghrelin-induced changes in food intake and neuronal activity in the hypothalamus occurs primarily at the level of peripheral afferents, like the vagus nerve, or at the central level.

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