Interaction of apolipoprotein AIV with cholecystokinin on the control of food intake

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Submitted 10 May 2007; accepted in final form 17 July 2007

Obesity increases the risk of type II diabetes mellitus, hypertension, coronary heart disease, osteoarthritis, respiratory problems, and cancer of the breast, endometrium, prostate, and bowel (20). Obesity has become an epidemic in developed countries, and the prevalence of obesity continues to increase drastically in the United States (2). Obesity develops when caloric intake exceeds energy expenditure over time with the excess energy eventually being stored as fat (39). Gastrointestinal hormones and peptides contribute to energy homeostasis in a variety of ways. In particular, cholecystokinin (CCK) and apolipoprotein AIV (apo AIV) are examples of gastrointestinal satiation signals whose synthesis and secretion by the small intestine are induced by consumption of fatty meals. In addition to what occurs in the small intestine, fasting and lipid feeding also regulate hypothalamic apo AIV mRNA in the rat (21). Peripheral injection of the octapeptide of CCK (CCK-8) increases neuronal activation (c-Fos) in the nucleus of the solitary tract and in the dorsomedial hypothalamic nucleus (19, 41). Thus, circulating levels of these two peptides can also influence the brain to control food intake (36).

Apo AIV is a protein found in many species; the human form has a molecular weight of 46 kDa (1, 16). After meals, circulating levels of apo AIV are increased by >50% above fasting levels in serum, which are typically between 80–100 μg/ml (11). The jejunum is the major site of apo AIV synthesis, but it is also produced in duodenum and ileum, and apo AIV secretion is stimulated by lipid absorption (7, 18). Apo AIV synthesis and secretion into the lymph are associated with chylomicron formation as digested lipid is being processed in mucosal cells; when chylomicron formation is blocked by infusion of a hydrophobic surfactant known as Pluronic L-81 during lipid infusion, both lymphatic lipid and lymphatic apo AIV output are completely attenuated (17). Once chylomicrons enter the circulation from the lymph, apo AIV readily dissociates from the chylomicron remnants, the majority of apo AIV in the circulation existing as the free protein and the remainder associated with circulating high-density lipoproteins (12).

Apo AIV was implicated in food intake when it was observed that intravenous administration of chylous lymph significantly reduced intake in rats and that when apo AIV was removed from the chylous lymph before administration, food intake was not suppressed (9). Consistent with this, the administration of purified apo AIV inhibits food intake (9, 22). Collectively, these observations suggest that endogenous apo AIV induced by consumption of fatty meals plays an important role in the control of food intake and the overall maintenance of energy homeostasis. Subsequent studies determined that at doses that reduce food intake, exogenous apo AIV does not cause malaise (22) and that comparable doses of a comparable apolipoprotein, apo AI, are without effect (9).

CCK is secreted by intestinal I cells after lipid and protein consumption. CCK is involved in many functions, such as triggering gallbladder contraction, stimulating pancreatic enzyme secretion, modulating intestinal motility, and controlling food intake (4, 40). Experimental rats administered exogenous CCK-8 display short-term satiation by reducing meal size, an effect that can be attenuated by vagotomy or deactivation of vagal afferents with capsaicin (13, 27, 28, 30, 31).

Secretion of CCK and apo AIV by the small intestine is induced by fat absorption, and this fat-induced stimulation is dependent on the formation and secretion of chylomicrons (17, 30). CCK and apo AIV both act peripherally (after either

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intraperitoneal or intravenous administration) as well as centrally to reduce food intake (6, 10, 14, 18, 24–26, 37). On the basis of all of this evidence, we hypothesized that systemic CCK and apo AIV interact to reduce food intake. The aim of the present experiments therefore was to determine the interaction between these peptides by comparing the effects of the administration of apo AIV alone, CCK-8 alone, or both, on food intake.

**MATERIALS AND METHODS**

Male Sprague-Dawley rats and a pelleted standard low-fat diet (LM-485) were obtained from Harlan Sprague Dawley, (Indianapolis, IN). *Escherichia coli* (DE3) was purchased from Invitrogen (Rockville, MD). AffinityPak Detoxi-Gel endotoxin removing gel was obtained from Pierce Biotech (Rockford, IL). His-bind columns were obtained from Novagen (Darmstadt, Germany). Sulfated CCK-8 and lorglumide sodium salt were obtained from Sigma (St. Louis, MO).

Adult rats weighing 350–400 g were individually housed in tub cages in a facility approved by the American Association for the Accreditation of Laboratory Animal Care. Corn cob bedding was used, and animals were kept in a room that was temperature controlled and on a 12:12-h light-dark cycle (illuminated from 0600 to 1800). They had free access to pelleted chow and water. All animals were transferred to clean cages and deprived of food for 24 h (0930–0930) prior to each experiment. All animal protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Recombinant apo AIV was produced by a bacterial expression system, and its final concentration after the removal of endotoxin was 1.5 mg/ml in physiological saline (16). Briefly, rat apo AIV cDNA was contained in a pSP65 maintenance vector, and an AffinityPak Detoxi-Gel endotoxin removing gel was obtained from Pierce Biotech (Rockford, IL). His-bind columns were purchased from Novagen (Darmstadt, Germany). Sulfated CCK-8 and lorglumide sodium salt were obtained from Sigma (St. Louis, MO).

After induction of apo AIV protein synthesis in the cells, the cells were harvested and sonicated. Apo AIV protein from the lysate was purified by standard procedures, such as His-bind affinity column chromatography and dialysis. Finally, endotoxin was removed by passing the protein through an AffinityPak Detoxi-Gel endotoxin removing gel, and the resultant apo AIV protein was diluted to a final concentration of 1.5 mg/ml in saline.

**Experiment 1. Effect of recombinant apo AIV on food intake.** To determine the effect of intraperitoneal apo AIV on food intake, 5–10 fasted rats per group received 0.5 ml of either recombinant apo AIV (50 or 100 μg/kg) or vehicle (saline) at 0930. Twenty minutes later, food was returned, and intake was assessed after 15, 30, 60, and 120 min. A dose that proved ineffective in suppressing food intake was determined to be subthreshold and was used in the study examining effects of coadministration of exogenous apo AIV and CCK-8.

**Experiment 2. Effect of CCK-8 on food intake.** Groups of rats (n = 7–10) received 0.5 ml of either saline or sulfated CCK-8 (0.06, 0.125, 0.25, 0.5 μg/kg in 0.9% saline) intraperitoneally 5 min prior to the return of food. Intake was measured 15, 30, 60, and 120 min after the injection.

**Experiment 3. Interaction of apo AIV and CCK-8 in the inhibition of food intake.** Groups of rats (10 per group) were administered two injections on the test day. The injections contained either saline (0.5 ml) + saline (0.5 ml) or CCK-8 (0.5 ml) + apo AIV (0.5 ml). Two different combinations of CCK and apo AIV were studied (50 μg/kg apo AIV + 0.06 μg/kg CCK-8; 100 μg/kg apo AIV + 0.06 μg/kg CCK-8). The first injection (saline or apo AIV) occurred 15 min prior to the return of food and the second (saline or CCK-8) occurred 5 min prior to food return. Food intake was measured at 15, 30, 60, 120, and 240 min.

**Experiment 4. Effect of a CCK1 receptor antagonist on food intake suppression caused by the coadministration of apo AIV and CCK-8.** Three groups of rats (7 per group) were administered two intraperitoneal injections on the test day. The rats were used in prior experiments. Lorglumide was used as a CCK1 receptor antagonist (34, 35). The injections contained either saline (0.5 ml) + saline (0.5 ml), CCK-8 (0.5 ml) + apo AIV (0.5 ml), or CCK-8 (0.5 ml) + apo AIV + lorglumide (0.5 ml). The combination doses of CCK + apo AIV were 200 μg/kg apo AIV + 0.6 μg/kg CCK-8, and the lorglumide 300 μg/kg lorglumide. The first injection (saline, apo AIV, or apo AIV + lorglumide) occurred 15 min prior to return of food and the second injection (saline or CCK-8) occurred 5 min prior to food return. Food intake was measured at 30, 60, 120, and 240 min.

**Statistical analysis.** All values are presented as means ± SE. Parametric statistical analyses and two-way repeated-measures ANOVA were performed using GraphPad Prism (version 3.0; GraphPad, San Diego, CA), and differences were considered significant if the probability of P values were <0.05.

**RESULTS**

**Experiment 1. Effect of recombinant apo AIV on food intake.** Rats injected with 50 μg/kg ip apo AIV consumed similar amounts of chow as the control animals with no significant differences found at any interval (Fig. 1). When the dose of recombinant apo AIV was increased to 100 μg/kg, food intake was significantly reduced relative to saline after 15 min (P < 0.004), 30 min (P < 0.0116), and 60 min (P < 0.0382). The efficacy was short-lived, with no difference detected at 2 h after apo AIV administration. These results suggest that apo AIV at 50 μg/kg is not effective at suppressing food intake (i.e., is subthreshold) and that a dose of 100 μg/kg is minimally effective for up to 1 h. They also demonstrate for the first time that intraperitoneal administration of recombinant apo AIV reduces food intake.

**Experiment 2. Effect of CCK-8 on food intake.** Reduced food consumption was observed at doses of CCK-8 at or above 0.125 μg/kg (Fig. 2), and the effect lasted about 1 h. For example, CCK-8 at 0.125 μg/kg ip significantly inhibited food intake during the first hour (~34% reduction at 30 min, P < 0.0001); at 0.25 μg/kg CCK, food intake was reduced by 38%...
during the first 30 min (P < 0.0001); and at 0.5 μg/kg, it was reduced by 50% (P < 0.0001). CCK-8 at 0.06 μg/kg had no effect on food intake during the first hour or at any time point studied. This study demonstrates that 0.06 μg/kg is subthreshold for inhibiting food intake under these conditions and that the smallest effective dose was 0.125 μg/kg. These results are consistent with the findings of many others (3, 28).

Experiment 3. Interaction of apo AIV and CCK-8 in the inhibition of food intake. When the combined subthreshold doses of apo AIV (50 μg/kg) and CCK (0.06 μg/kg) were administered, they significantly inhibited food intake at 15 min (P < 0.004), 30 min (P < 0.0001), and 60 min (P < 0.0011) (Fig. 3). When the dose of apo AIV was increased to 100 μg/kg, the effect lasted longer, and the combination reduced food intake for 4 h compared with the control condition. Experimental rats consumed 40% less food after 30 min, and 23% less over 4 h compared with the saline controls. These observations suggest that these two gut peptides produce an additional additive or possibly a synergistic effect on the suppression of food intake when given in combination compared with what happens when given alone.

**DISCUSSION**

We compared the intraperitoneal administration of recombinant apo AIV and CCK-8 either alone or in combination on short-term food intake in fasted rats. Recombinant apo AIV at a dose of 100 μg/kg significantly reduced food intake over the first hour of refeeding after a 24-h fast, whereas a lower dose was without effect. These results indicate that intraperitoneal recombinant apo AIV is comparably effective in inhibiting food intake as intravenously administered native apo AIV or recombinant apo AIV administered into the third cerebral ventricle (9, 10, 25). In this study, we found that the effective dose of recombinant apo AIV (100 μg/kg) required to inhibit food intake is comparable to the requisite dose of native rat apo AIV purified from plasma (135 μg) (9). Obtaining native apo
AIV requires extraction and purification of the protein from plasma or lymph; this process is time consuming and labor intensive (17). In contrast, recombinant apo AIV produced in a bacterial expression system is more time efficient and can thus be modified for larger-scale preparations (22). We previously observed that when recombinant apo AIV was infused into the third cerebral ventricle at 4 μg per rat, food intake was significantly inhibited (22). In the present experiments, intraperitoneal administration of recombinant apo AIV elicited a relatively rapid satiation effect. The mechanism by which this effect is mediated is not clear at the moment and will require further investigation. It is possible that the recombinant apo AIV acts locally by activating the vagus nerve, and it may also interact with other peripheral satiety signals, such as CCK in this regard.

Many reports have demonstrated that peripheral administration of CCK-8 decreases food intake (e.g., see Refs. 3 and 4). The smallest effective dose of CCK-8 we observed to effectively inhibit food intake was 0.125 μg/kg; this is comparable to the smallest effective dose of 0.25 μg/kg reported in other studies (3, 4). The 24-h deprivation of food prior to the start of the experiment, the age and strain of the animals, and other factors likely contribute to these minor differences. We also found in the present study that doses of exogenous CCK-8 above 0.06 μg/kg resulted in inhibition of short-term food intake that lasted for about 30 min, and this finding is in agreement with other investigators (3, 4). Previous studies have demonstrated that CCK binds with CCK1 receptors on local vagal afferent nerve terminals and activates ascending vagal fibers for relaying information to the brain (34, 35).

In the present study, we identified subthreshold doses for both CCK-8 (0.06 μg/kg) and apo AIV (50 μg/kg) that were then used to address whether they become effective in inhibiting food intake when administered in combination. We found that the anorectic effect of apo AIV was enhanced by the coadministration of a subthreshold concentration of CCK-8. This is the first demonstration that coadministration of apo AIV and CCK-8 at physiological doses suppresses food intake, and it clearly implies a close interaction between apo AIV and CCK in regulating food intake. The conclusion that CCK-8 enhances the anorectic effect of apo AIV is further illustrated by the observation that the inhibitory effect was prolonged to 4 h when CCK-8 was coadministered together with 100 μg/kg apo AIV, since the satiating effect normally lasts for only 1 h. We do not currently know the exact mechanism of the interaction between apo AIV and CCK that leads to additional suppression of food intake over the administration of either peptide alone. From earlier studies, it has been reported that active fat absorption results in increased secretion of CCK and apo AIV and that this stimulation is dependent on the formation and secretion of chylomicrons. The satiation effect of both CCK and apo AIV, when administered alone, has been demonstrated to be vagally mediated through interaction with the CCK1 receptors on the vagal afferent fibers as well as potentially through the release of factor or factors into the circulation (10, 11, 15, 17, 31, 35).

In the present study, rats administered lorglumide (300 μg/kg) had attenuated suppression of food intake by CCK + apo AIV over the 4-h observation period. The dose of lorglumide used (300 μg/kg) did not completely reverse the inhibition of food intake produced by CCK-8 and apo AIV, suggesting either that some additional (non-CCK1 receptor-mediated) pathway is involved in the interaction causing satiation, or that a higher dose of lorglumide will be required. One previous study indicated that lorglumide was effective at a dose of 200 μg/kg to almost completely reverse CCK-8-induced inhibition of food intake (33). Early reports demonstrated that central insulin or central leptin increases the sensitivity to CCK-induced food suppression (8, 23, 32). We therefore speculate that some additional factor, such as insulin or leptin that is stimulated by the combination of CCK + apo AIV, may be involved in enhancing the satiating effect of the combination of CCK + apo AIV via circulation to brain. In conclusion, we hypothesize that apo AIV and CCK-8 work together to stimulate vagal afferent nerves via both a CCK1 receptor as one route (15) and via additional, as yet unknown, pathways to inhibit food intake.

CONCLUSION

We have demonstrated in these experiments that intraperitoneal administration of recombinant apo AIV alone and CCK-8 alone significantly reduce short-term food intake, the effect lasting for 1 h or less in 24-h fasted rats. An anorectic effect of a subthreshold dose of apo AIV was apparent only with the coadministration of a subthreshold dose of CCK-8. The CCK-8 enhanced the satiation effect of apo A-IV and also prolonged its satiating effect. The satiation effect produced by CCK-8 + apo AIV was mediated in part via CCK1 receptor.

GRANTS

This work was supported in part by National Institutes of Health Grants DK-38180, DK-17844, DK-56910, ES-14464, DK-76928, DK-63907, and HL-82734 and by a postdoctoral fellowship from American Heart Association, Ohio Valley Affiliate.

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