Different metabolic responses to central and peripheral injection of enterostatin

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Lin, Ling, MieJung Park, Matt Hulver, and David A. York. Different metabolic responses to central and peripheral injection of enterostatin. Am J Physiol Regul Integr Comp Physiol 290: R909–R915, 2006. First published December 8, 2005; doi:10.1152/ajpregu.00045.2005.—Enterostatin, a pentapeptide cleaved from procolipase, suppresses fat intake after peripheral and central administration. Chronic treatment of rats with enterostatin decreases body weight and body fat. The effect was greater than could be accounted by the reduction in food intake alone. Hence, we have investigated the effect of enterostatin on energy metabolism. Male Sprague-Dawley rats adapted to a high-fat diet were implanted with lateral cerebral ventricular or amygdala cannulas. The metabolic effects were determined by indirect calorimetry. After habituation to the test cages, fasted rats were injected with either saline vehicle or enterostatin given either intraperitoneally (100 nmol) or intracerebroventricularly (1 nmol) or into specific brain regions [amygdala (0.01 nmol) or paraventricular nucleus (PVN) (0.1 nmol)]. Respiratory quotient (RQ) and energy expenditure were monitored over 2 h. Intraperitoneal enterostatin reduced RQ (saline: 0.81 ± 0.02 vs. enterostatin: 0.76 ± 0.01) and increased energy expenditure by 44%. Intracerebroventricular enterostatin increased the energy expenditure without any effects on RQ, whereas PVN enterostatin increased metabolic rate, while preventing the increase in RQ observed in the control animals. In contrast, neither RQ nor energy expenditure was altered after enterostatin was injected into the amygdala. Enterostatin activated AMP-activated protein kinase in primary cultures of human myocytes in a dose- and time-dependent manner and increased the rate of fatty acid β-oxidation. These findings suggest that enterostatin regulates energy expenditure and substrate partitioning through both peripheral and central effects.

amygdala; indirect calorimetric system; respiratory quotient; adenosine 5'-diphosphate-active protein kinase; myocytes; β-oxidation

ENTEROSTATIN, a PENTAPEPTIDE, is a cleavage product of pancreatic procolipase released during activation of the cofactor in the intestine after the ingestion of dietary fat (8). Procolipase is also expressed in the stomach, duodenal mucosa, and in specific brain regions (20, 36, 47, 52). Enterostatin acts as an early satiety factor to regulate energy intake, specifically the intake of dietary fat (7, 9, 22). Exogenous administration of enterostatin selectively suppressed fat intake in rats after both peripheral and central administration (18, 19, 21, 22, 24, 27, 29, 46). The peripheral site of action is in the gastroduodenal region, and this response is mediated through the afferent vagus (18). Central action sites include the paraventricular nucleus (PVN) in the hypothalamus and the amygdala region of the limbic system, with maximum inhibitory effect being observed in the central nucleus of amygdala (26, 27). The central pathway activated by enterostatin includes opioid, serotonin, and CCK components (23, 25, 38).

Maintenance of energy balance requires a balance between energy intake and expenditure. The regulation of these two limbs of energy balance is physiologically linked, through hypothalamic and other central nervous system (CNS) circuits (10, 48). Most of the peptides that regulate energy intake also affect energy expenditure (EE), such as neuropeptide Y (NPY), leptin, melanin-concentrating hormone (MCH), corticotropin-releasing hormone (CRH) (4, 5, 12, 30, 31, 49). Obesity may result from either increased feeding and/or decreased EE, as exemplified by the obesity that results from defects in either leptin signaling or the melanocortin pathway (3, 11).

Although there are numerous reports to support the effect of enterostatin on feeding, little is known about the metabolic effects of enterostatin. The possible effects on EE or energy substrate utilization are suggested by several lines of evidence. First, chronic infusion of enterostatin intraperitoneally or intracerebroventricularly caused weight and body fat loss that seemed to be greater than could be accounted for the reduction of food intake (19, 35); second, enterostatin treatment increases sympathetic outflow to brown adipose tissue (34) and enhances uncoupling protein 1 mRNA levels and heat production (42); third, acute and chronic administration of enterostatin increases the serum corticosterone levels (19, 35), which is critical to the EE (12). Moreover, an in vitro study showed that enterostatin stimulated oxygen consumption in INS-1 cells (2). The present study was designed to characterize the effects of acute peripheral and central injection of enterostatin on EE in rats by using indirect calorimetry. In addition, we have demonstrated a direct effect of enterostatin on muscle to activate AMP-activated protein kinase (AMPK) and β-oxidation of fatty acids. AMPK is now recognized to have a central role in regulating the balance between anabolic and catabolic pathways in peripheral tissues (13, 43).

MATERIALS AND METHODS

Peptides and antibodies. Enterostatin was synthesized by solid-phase chemistry, purified by HPLC, and estimated to be of greater than 90% purity by the Core Laboratory of Louisiana State University Medical Center (New Orleans, LA). Antibodies against phospho-AMPK and AMPK were purchased from Upstate Biotechnology (Lake Placid, NY). β-Actin antibody was obtained from Abcam (Cambridge, UK).

Animals and diet. Forty-eight male Harlan Sprague-Dawley rats (300–400 g body wt, Harlan Sprague-Dawley, Indianapolis, IN) were used in the current study. They were housed individually in acrylic cages in a temperature-controlled room (22–23°C) with a 12:12-h light-dark cycle (lights on at 0700) and with free access to an automatic watering system. The rats were adapted to a high-fat diet...
(4.78 kcal/g, 56% of energy as fat) ad libitum for a minimum of 2 wk. The composition of the diet has been described elsewhere (19). The experimental procedures and protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Surgery. Animals were anesthetized with pentobarbital sodium (Nembutal; 0.1 ml/100 g body wt ip). Each rat was stereotaxically implanted with one unilateral cannula. A stainless steel cannula was aimed to the lateral ventricle, PVN, or central nucleus of the amygdala. The coordinates (AP/L/DV to bregma) were lateral ventricle: −1.4/−0.8/−4.0 mm; PVN: −1.9/−0.4/6.0 mm; amygdala: −2.4/−3.8/−6.0 mm (40). Each cannula was secured in place with three anchor screws and dental acrylic and occluded with a stylet. The injectors for PVN and amygdala were designed to project 2 mm beyond the guide cannula tip. The animals were returned to the home cages after recovery from the anesthesia and maintained on the diet. Experiments began after rats had regained their preoperative weight (about 10 days).

Peptide injections. Enterostatin was dissolved in saline (0.9% w/v NaCl) and given as a single injection at doses of 120, 1, 0.1 or 0.01 nmol ip, icv, PVN, or amygdala, respectively. All of the above doses were experimentally shown previously to have maximum inhibitory effect on feeding (22, 27, 37). The rats assigned as a vehicle control group were given saline in a volume of 0.1 ml for ip, 5 μl for icv, 0.1 μl for PVN or amygdala.

Apparatus. An open circuit calorimeter (Oxymax, Columbus Instruments, Columbus, OH) was used to monitor oxygen and carbon dioxide gas fractions at both the inlet and output ports to the test chamber. The RQ was allowed to reach equilibrium again (~21 min) before measurements were taken. Food and water were not available during measurement of EE (5, 12). At the conclusion of the experiments, rats were killed, and brain cannula placements were determined by histology and comparison to the rat brain atlas of Paxinos and Watson (40).

Immunoblotting. Whole cell lysates were made by sonication in lysis buffer (1% Triton X-100, 50 mM KCl, 25 mM HEPES, pH 7.8, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 125 μM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). Samples (100 μg of total protein) in 50 μl of reducing sample buffer were boiled for 5 min and resolved on 10% mini-SDS-PAGE for 90 min at 100 V. The contents of the gel were transferred onto polyvinylidene difluoride membrane (Roche, Indianapolis, IN) at 50 v for 120 min. The membrane was preblotted in milk buffer [5% nonfat milk in Tris-buffered saline-Tween (TBST); 10 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20] for 120 min and then immunoblotted with primary antibody for 18 h followed by secondary antibody for 30 min after washing with TBST. Horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used in conjunction with chemiluminescence reagent (ECL detection kit, Amersham Pharmacia, Buckinghamshire, UK, or visualizer from Upstate Biotechnology).

Fatty acid oxidation in human primary muscle cell cultures. Human skeletal muscle cells were cultured as previously described (32, 33). Briefly, muscle samples were obtained from the vastus lateralis muscle by needle biopsy, and satellite cells were isolated by trypsin digestion (14). Cells were grown to confluency on type I collagen-coated T25 flask at 37°C in a humidified atmosphere of 5% CO2 in DMEM supplemented with 10% FBS, 0.5 mg/ml BSA, 0.5 mg/ml fedtin, 20 ng/ml human epidermal growth factor, 0.39 μg/ml dexamethasone, and 50 μg/ml gentamicin/ampicillin B. After reaching ~90% confluence, myoblasts were subcultured onto a six-well plate, type I collagen-coated plates at densities of 96 × 103 cells per well. When cells reached 90% confluence, differentiation was induced by changing to low-serum differentiation media consisting of 2% heat-inactivated horse serum, 0.5 mg/ml BSA, 0.5 mg/ml fedtin, and 50 μg/ml gentamicin/ampicillin B.

Fatty acid oxidation was measured on the 7th day of differentiation under the following conditions: control, 0.01 μM enterostatin, and 1.0 μM enterostatin. Briefly, cells were incubated at 37°C in sealed six-well plates, containing 2.0 ml of serum-free differentiation media plus 12.5 mmol/l HEPES, 0.2% BSA, 1.0 mmol/l carnitine, 100 μmol/l oleate, 50 μg/ml gentamicin, and 1.0 μCi/ml[14C]oleate.

Fig. 1. Photography of the coronal sections of rat brain showing the cannula tract in the paraventricular nucleus (PVN; A) and central nucleus of amygdala (CeA) (B). The inlets (A and B) are the pictures taken under a microscope with ×10 magnification. Schematic illustrations of those sites (C: PVN; D: central nucleus of the amygdala) are adapted from Paxinos and Watson (40). Open arrows show PVN, and solid arrows show the amygdala.
After 3 h, incubation media were sampled and assayed for 14C-labeled CO2 and acid-soluble metabolites (ASM), as previously described (15, 17). The cells were placed on ice, washed twice with ice-cold PBS, and scraped into a 1.5-ml Eppendorf tube in two additions of 0.30 ml 0.05% SDS lysis buffer. Lysates were stored at −80°C for determination of protein content. Total fatty acid oxidation is denoted as CO2/ASM production in nmol/mg protein/h.

Data analysis. RQ and EE data are expressed as means ± SE with six rats in each treatment group. EE was derived from the computer-generated data and normalized by body weight across subjects as J·h−1/100 g body wt−1 (1, 28). The difference between the treatments was analyzed by ANOVA with repeated measures, and post hoc tests were performed by using the Bonferroni correction.

RESULTS

Verification of the cannula placements. Figure 1 shows an example of histological verification of the positions of the cannula. The tract of the unilateral cannula in the rat coronal sections terminates directly above either the PVN (Fig. 1A) or the central nucleus of the amygdala (Fig. 1B), compared with the schematic illustrations of rat brain (Fig. 1, C and D).

Changes of RQ after the injection of enterostatin. The time course of the effects of enterostatin on RQ after injection into intraperitoneal, intracerebroventricular, PVN, or amygdala sites is shown in Fig. 2. Rats receiving a peripheral injection of enterostatin (120 nmol) (Fig. 2A) had a decreased RQ compared with vehicle controls after 35 min (saline 0.80 vs. enterostatin 0.75). ANOVA showed a significant difference over the observed period with intraperitoneal enterostatin treatment [main treatment effects: F(1,10) = 19.13, P < 0.001]. However, this resulted mainly from an increase in RQ in the control animals rather than a decrease in RQ in the enterostatin-treated rats. A similar effect was also seen after injection of enterostatin (0.1 nmol) into the PVN (saline 0.88 vs. enterostatin 0.82 at 35 min). This effect was statistically significant [main treatment effects: F(1,10) = 5.28, P < 0.05]. No effects of treatments were observed after intracerebroventricular [main treatment effects: F(1,10) = 0.759, P = 0.404] or amygdala [main treatment effects: F(1,10) = 1.908, P = 0.197] injections (Fig. 2, B and D).

In Fig. 2E, RQ is expressed as the average over the 2-h testing period. There was a significant decrease in RQ after intraperitoneal enterostatin treatment (saline: 0.790 ± 0.004 vs. enterostatin: 0.761 ± 0.003, P < 0.001) and after PVN treatment (saline: 0.862 ± 0.003 vs. enterostatin: 0.841 ± 0.002, P < 0.001). No effects were observed with enterostatin injected intracerebroventricularly or into the amygdala.

Effects of enterostatin on EE. As shown in Fig. 3, A and E, intraperitoneal enterostatin increased EE by 32%, the effect being observed within 14 min (saline 2,141.7 ± 51.5 vs. enterostatin 2,837.9 ± 56.1 J·h−1/100 g body wt−1) and lasting throughout the whole test period (126 min) [main treatment effects: F(1,10) = 38.36, P < 0.0001]. Intracerebro-
ventricular injections reduced metabolic rates, but enterostatin-treated rats maintained a higher EE (20% at 21 min) than saline vehicle-treated rats (Fig. 3, B and E). The main treatment effect of intracerebroventricular enterostatin was significant \([F_{1,10} = 6.584, P < 0.05]\). PVN injection of enterostatin (Fig. 3, C and E) increased EE by ~25% overall with a maximum 35% increase at 56 min [saline: 2,604.3 ± 105.9 vs. PVN enterostatin: 3,527.7 ± 211.5 J h\(^{-1}\) 100 g body wt\(^{-1}\), \(F_{1,10} = 5.537, P < 0.05\)]. Injection of enterostatin into the amygdala had no effect on EE \([F_{1,10} = 0.035, P = 0.856]\).

**Effect of enterostatin on AMPK and \(\beta\)-oxidation in primary myocyte cultures.** Enterostatin-activated AMPK in primary myocytes in a time- and dose-dependent manner (Fig. 4, A and B). Maximum levels of phospho-AMPK were observed at 15-min incubation time with enterostatin, and the strongest activation was observed with 10 nM enterostatin but was lower at higher concentrations of enterostatin. Likewise, the fourfold increase in fatty acid oxidation (Fig. 4 C) was observed at 10 nM enterostatin but not at the higher dose of 1 \(\mu\)M.

**DISCUSSION**

This study reports new observations that the anorectic peptide enterostatin regulates EE and substrate oxidation after both peripheral and central administration. Injection of enterostatin intraperitoneally and into the PVN reduced RQ, indicating an increased fat utilization, and also increased EE. In contrast, the finding that enterostatin injections into the central nucleus of amygdala did not affect RQ or EE suggests that the regulation of EE and food intake by enterostatin involves different neural circuits. Further, the studies with primary myocyte cultures suggest that enterostatin has a direct effect on muscle to enhance fatty acid oxidation.

Energy balance is determined by food intake and EE. Increasing the food intake and/or decreasing the EE will lead to obesity (41). Enterostatin, like many other gut peptides, appears to have both peripheral and central effects (22, 37). The central actions probably do not relate to uptake of circulating enterostatin into the CNS, as recent data, using immunohistochemistry and RT PCR, have provided evidence for the synthesis of procolipase and release of enterostatin in specific brain regions (20). Most of the peptides that regulate food intake have also been shown to have an impact on EE. These dual effects have been well demonstrated for NPY, CRH, leptin, \(\alpha\)-MSH, and galanin (4, 5, 12, 30, 31, 49, 53). In this light, it is not surprising that enterostatin, an early satiety factor, also has effects on EE, as found in this study.

Another characteristic of most neuropeptides that affect feeding is the opposing effects on food intake and EE. Thus the anorectic peptides (for example, NPY, agouti-related protein) provoke food intake and reduce EE, whereas the catabolic peptides (for example, \(\alpha\)-MSH, CCK) decrease food intake, while enhancing sympathetically mediated EE. Enterostatin, likewise, affects both sides of the energy balance equation, suppressing dietary fat intake, while positively increasing fat oxidation.
utilization (decrease of RQ) and EE. These observations are consistent with our previous findings that chronic administration of enterostatin, both peripherally and centrally, decreases fat pad weight and body weight (19).

The hypothalamus plays an important role in the regulation of feeding and EE. Multiple subregions are involved in EE, including the arcuate PVN, ventromedial hypothalamus, and lateral hypothalamus (5, 6, 32, 44). The circuitry regulating food intake has been described in detail in recent years (45), but there is less insight into the circuits regulating EE. However, studies with NPY suggest a separation of the two control circuits. For example, the perifornical hypothalamus appears to mediate only the feeding-stimulatory actions of NPY, whereas PVN regulates metabolic processes (5). The current study had a similar observation that enterostatin into the PVN changes both RQ and EE, but neither RQ nor EE was affected by injection of enterostatin into the amygdala. We previously demonstrated that the amygdala is the most sensitive site for induction of enterostatin feeding response within the brain (26, 27) and have described the circuits that are activated in response to amygdala enterostatin. Together with other recent reports that the amygdala is critical for ingestion of dietary fat (16, 50), the evidence suggests that the amygdala may be the center for the regulation of fat intake by enterostatin but that enterostatin regulation of EE may be initiated by a circuit activated in the PVN.

In this study, we only used a single dose of enterostatin for each route of administration. It may be argued that a dose-response curve might identify effects of enterostatin at higher doses. However, the doses we chose have been previously demonstrated to have maximal effects on inhibition of feeding (22, 27, 37). The absence of any effects on EE, using the maximal amygdala dose, and the presence of effects in the PVN provide us with confidence in our conclusion that the amygdala is a major site for enterostatin regulation of dietary fat intake, whereas the PVN is a major site for enterostatin regulation of EE. It is unlikely that the effects of central enterostatin on EE relate to the induction of illness, as previous studies on feeding behavior have shown that enterostatin does not induce a conditioned taste aversion (26).

AMPK is thought to play a central role in the regulation of nutrient partitioning and the control of anabolic and catabolic pathways. Activation of AMPK by phosphorylation has been associated with an increase in fatty acid oxidation and glucose transport in muscle and a decrease in fatty acid synthesis and gluconeogenesis in liver (43, 51). In the current studies, using primary human myocyte cultures, we showed that enterostatin had a direct effect on myocytes to activate AMPK and increase fatty acid oxidation. This occurred in a dose-dependent manner with maximum activation around 10 nM enterostatin. Previous studies with enterostatin have also shown a U-shaped dose-response curve on food intake (18) and on receptor binding (39). The amino acid sequence of enterostatin is identical in rat and man, Ala-Pro-Gly-Pro-Arg, so the effect of enterostatin to increase fatty acid oxidation in human myocytes is suggestive that a similar response may be evident in rodent muscle. Certainly, these in vitro culture data are consistent with the in vivo data reported here that intraperitoneal enterostatin increased EE and decreased RQ, that is, increased fatty acid oxidation. Our results suggest that this stimulation of fatty acid oxidation in muscle was, at least, partly responsible for this effect. Whether enterostatin also increases fatty oxidation in other tissues such as liver, is not known at this time.

In conclusion, the experiments reported here provide convincing data that enterostatin has metabolic effects in addition to its effects on feeding behavior. The interpretation of the data on RQ is difficult. The RQ of enterostatin-treated rats was significantly reduced compared with vehicle-treated rats after intraperitoneal and PVN administration. However, in both cases, the difference mainly resulted from an increase in RQ in the control vehicle-treated rats. Because these measurements were made in the absence of food, it is possible that the handling of rats was associated with a sympathetically mediated increase in glycogenolysis and subsequent increase in
carbohydrate availability and oxidation that was not observed in enterostatin-treated rats or was counteracted by the promotion of lipolysis and fat oxidation. Likewise, it is not clear why the RQ levels of all rats with cannulas directed at the PVN was higher, around 0.85, despite the sole access to a high-fat diet. It is possible again that the indwelling cannulas themselves caused an activation of the autonomic nervous system that led to enhanced availability of carbohydrates for oxidation.

The mechanism through which enterostatin increases EE is probably twofold. First, it increases sympathetic drive to brown adipose tissue, uncoupling protein 1 transcription, and thermogenesis (42) and would be expected to increase lipolysis and fatty acid oxidation. Second, the demonstration that membrane-located β-subunit of F1,F-ATP synthase is the enterostatin receptor (39) and that enterostatin reduces cell ATP levels suggests that all cells that have enterostatin receptors will also increase ATP turnover and heat production. Such a change in ATP levels is likely responsible for the activation of AMPK and the stimulation of muscle fatty acid oxidation that we have reported in this study.

REFERENCES


