Effect of high-fat diet, surrounding temperature, and enterostatin on uncoupling protein gene expression

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Rippe, Catarina, Karin Berger, Charlotta Böiers, Daniel Ricquier, and Charlotte Erlanson-Albertsson. Effect of high-fat diet, surrounding temperature, and enterostatin on uncoupling protein gene expression. Am J Physiol Endocrinol Metab 279: E293–E300, 2000.—Nonshivering thermogenesis induced in brown adipose tissue (BAT) during high-fat feeding is mediated through uncoupling protein 1 (UCP1). UCP2 is a recently identified homologue found in many tissues. To determine the role of UCP1 and UCP2 in thermoregulation and energy balance, we investigated the long-term effect of high-fat feeding on mRNA levels in mice at two different ambient temperatures. We also treated mice with the anorectic peptide enterostatin and compared mRNA levels in BAT, white adipose tissue (WAT), stomach, and duodenum. Here, we report that high-fat feeding at 23°C increased UCP1 and UCP2 levels in BAT four- and threefold, respectively, and increased UCP2 levels fourfold in WAT. However, at 29°C, UCP1 decreased, whereas UCP2 remained unchanged in BAT and increased twofold in WAT. Enterostatin increased UCP1 and decreased UCP2 mRNA in BAT. In stomach and duodenum, high-fat feeding decreased UCP2 mRNA, whereas enterostatin increased it. Our results suggest that the regulation of uncoupling protein mRNA levels by high-fat feeding is dependent on ambient temperature and that enterostatin is able to modulate uncoupling protein 1; uncoupling protein 2; colipase; thermogenesis; thermoneutrality

Diets rich in fat are generally associated with the development of obesity. Studies in rodents show that some strains will become obese on a high-fat diet, whereas others are relatively resistant to obesity (31, 33, 38). Various explanations have been presented to understand the difference in susceptibility to hyperlipidic regimens.

One explanation for the development of obesity is reduced thermogenic activity in brown adipose tissue (BAT) (15, 32). Nonshivering thermogenesis in BAT serves to regulate body temperature, as well as body weight. It is stimulated both by exposure to cold (cold-induced thermogenesis) and by high-fat feeding (diet-induced thermogenesis) (28). Heat is generated in BAT through the presence of uncoupling protein 1 (UCP1) (25). UCP1 is found in the mitochondrial inner membrane of BAT and is able to uncouple the oxidative phosphorylation, producing heat (26).

Recently, proteins related to UCP1 have been discovered and named UCP2 (10, 22, 37), UCP3 (2, 22, 37), UCP4 (21), and brain mitochondrial carrier protein 1 (BMCP1) (30). UCP2 is expressed in most tissues, whereas UCP3 has been found mainly in BAT and muscle (2, 10, 22, 37). UCP4 and BMCP1 have both been found in the central nervous system (21, 30) and have weak sequence homologies to UCP1. High-fat feeding has been shown to increase UCP2 in white adipose tissue (WAT) (10, 13, 22, 36) and UCP3 in muscle (13, 22, 36). It has been speculated that UCP2 and UCP3 upregulation could be of importance in regulating diet-induced thermogenesis.

Another reason for the development of obesity during high-fat feeding is increased energy intake (9, 14). Food intake is controlled by a number of peptides released from the gut in response to a meal. One such peptide, enterostatin, has been found to decrease food intake and body weight during high-fat feeding (8). Enterostatin has been shown to specifically decrease the macronutrient intake of fat (7) and to increase the sympathetic activation of BAT (24). Because the precursor molecule for enterostatin, procolipase, has been found to increase during high-fat feeding (23, 39), it has been hypothesized that enterostatin may act as a signal to regulate the appetite for fat and to be an important determinant for the susceptibility to develop obesity on high-fat diets. The influence of enterostatin on uncoupling protein expression is not known.

The aim of the present work was to investigate the effects of high-fat feeding on the expression of UCP1 and two mRNA levels in mice housed at room temperature and at thermoneutrality. Because obesity was much greater at thermoneutrality, we chose to examine the effects of enterostatin on food intake and body weight, as well as on the expression of UCP1 and UCP2 mRNA, at this temperature. We also studied the ex-
pression of UCP2 mRNA in the stomach and duodenum in response to high-fat feeding and after enterostatin treatment.

METHODS

Animals and Diets

Seven-week-old male NMRI mice were obtained from Bornholt, Denmark. The animals were kept in separate cages and housed either at room temperature (23 ± 1°C) or at thermoneutral temperature (29 ± 1°C), both with a 12:12-h light-dark cycle. Food and water were available ad libitum. The low-fat (LF) and high-fat (HF) diets were made according to Table 1, the LF diet consisting of 10% fat, 24% protein, and 66% carbohydrate (% of total calories) and the HF diet containing 63% fat, 24% protein, and 13% carbohydrate. In addition, a HF + enterostatin diet was made. This was achieved by mixing mouse enterostatin (27) (APGPR, Ferring, Malmö, Sweden), dissolved in the lipid phase, into the HF diet. The HF + enterostatin diet contained 166 μg enterostatin/g food.

Control Experiment at 23°C

Thirty mice were divided into two groups. One group was fed the LF diet, and the other group was fed the HF diet. The mice were adapted to their respective diets for 1 wk before the experiment (20). The mice weighed 36.9 ± 0.3 g (LF) and 37.8 ± 0.7 g (HF) at the start of the experiment. Food intake and body weight were measured every 3rd day. After 25 days, the mice were fasted overnight (8 h) and anesthetized with pentobarbital sodium (20 mg/kg ip), and a glucose tolerance test was performed. The mice were then killed by exsanguination.

Experiment at 29°C with Enterostatin Treatment

Thirty-one mice were first divided into two groups. Eleven mice were fed the LF diet, and the other group was fed the HF diet. The mice were adapted to the diets 1 wk before the experiments. At the start of the experiment, the mice weighed 36.3 ± 0.6 g (LF) and 37.7 ± 0.6 g (HF). The HF-fed mice were divided into two subgroups, one control and one receiving enterostatin. Food intake and body weight were measured every 3rd day. After 25 days, the mice were fasted for 5 h (starting between 0700 and 0900) and anesthetized with pentobarbital sodium (20 mg/kg ip), and an intraperitoneal glucose tolerance test was performed before the mice were killed by exsanguination.

Blood and Tissue Sampling

During the glucose tolerance test, blood samples were collected from the tail vein. Glucose was immediately measured with Accutrend glucose gauge (Boehringer Mannheim). The epididymal fat pads and BAT were dissected, weighed, and frozen in liquid nitrogen. Pancreas, stomach, and duodenum were rapidly collected and immediately frozen in liquid nitrogen.

Isolation of RNA and Northern Blot

Total cellular RNA was extracted from BAT, WAT, stomach, and duodenum using TRIzol reagent (GIBCO-BRL). The quality and concentration of the RNA were determined by measuring the absorbance at 260 and 280 nm. Twenty micrograms of total RNA were separated on 1% agarose gel containing 2% formaldehyde and transferred to a nylon membrane (Zeta-Probe, Bio-Rad). Prehybridization and hybridization were carried out overnight either at 60°C (for colipase and UCP probes) or at 37°C (for the 18S probe). The UCP and colipase probes were labeled using [α-32P]deoxyctydine triphosphate (Amersham) according to a Nick translation kit (Boehringer Mannheim), whereas the 18S probe was end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP. The filters were washed twice for 60 min in 0.20 mM Na2HPO4 and 5% SDS, pH 7.2, and twice more in 0.20 mM Na2HPO4 and 1% SDS, pH 7.2. The washes were carried out at the hybridization temperature. The filters were then analyzed with a phosphomager (Fujix Bas 2000), and the radioactivity was quantified. Rehybridization with the 18S probe was made as above at 37°C overnight after stripping the blot in 0.1 × standard sodium citrate, 0.5% SDS for 2 × 30 min at 95°C. The UCP and colipase levels were compared with the 18S control and are expressed in arbitrary units.

Probes

Plasmids containing rat UCP1 and mouse UCP2 cDNA have previously been described (10). These were transferred into Escherichia coli Top 10 cells (Invitrogen, Groningen, Netherlands). One colony was picked from the plate and incubated overnight in LB (Luria-Bertani), (Tryptone (10g/l), yeast extract (5 g/l), NaCl (10 g/l), and medium containing ampicillin and then purified with a Maxi plasmid purification kit (Qiagen). The UCP1 insert was released by use of BamH I and the UCP2 insert by use of EcoRI and Sac I (GIBCO-BRL). The inserts were separated from the vectors on a 1% agarose gel and purified with a DNA purification kit (Promega). The colipase cDNA probe was kindly provided by Professor Mark Lowe (Washington University School of Medicine, St. Louis, MO). As a control, an 18S probe (DNA Technology, Aarhus, Denmark) was used, according to the RNA sequence determined by Chan et al. (3).

Assays

pH-stat titration. The lipolytic activity of lipase and colipase was measured with a pH-stat titrator (PHM 290, Boehringer Mannheim) with tributyrin as substrate, as described earlier by Borgstrom and Erlanson (1). Lipase activity was determined by the addition of procolipase, and colipase activity was measured by the addition of lipase. The released fatty acids were titrated with 0.1 M NaOH, and the activity was calculated in units per milliliter where one unit

Table 1. Composition and energy content of the high-fat and low-fat diets

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<th>High-Fat Diet</th>
<th>Low-Fat Diet</th>
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<tr>
<td></td>
<td>Weight, g/100 g</td>
<td>%kcal</td>
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<td>Casein</td>
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<td>13</td>
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<tr>
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<td>Coconut fat</td>
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<tr>
<td>Vitamin mix</td>
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<tr>
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<tr>
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corresponds to one micromole of released fatty acids per minute.

Protein assay. The protein content of the pancreas was measured with a DC protein assay kit (Bio-Rad).

Statistical Analyses

Data are presented as means ± SE. Statistical significance was calculated by means of one-way ANOVA and unpaired t-test, with the significance levels set at * P < 0.05, ** P < 0.01, and *** P < 0.005.

RESULTS

Effects of HF Feeding at Room Temperature (23°C) and at Thermoneutrality (29°C)

Body weight and food intake. The caloric intake and the body weight gain at the different temperatures are shown in Fig. 1. At both temperatures, HF feeding increased the daily energy intake compared with LF feeding (*P < 0.005; Fig. 1A). The HF-fed mice gained approximately twice as much body weight when housed at 29°C compared with 23°C (**P < 0.01; Fig. 1B). However, the LF-fed mice decreased their food intake at 29°C (*P < 0.005) compared with 23°C and gained approximately the same weight at the two temperatures. The daily food intake was constant over the experimental period in the individual groups (data not shown).

UCP1. The effect of HF feeding on the mRNA expression of UCP1 at 23°C and 29°C is illustrated in Fig. 2. As shown, HF feeding caused a fourfold increase in UCP1 expression in BAT at 23°C (*P < 0.05), whereas at 29°C, the mice instead slightly decreased their UCP1 expression (*P < 0.05). The mice fed the LF diet significantly decreased their UCP1 mRNA levels at 29°C compared with 23°C (*P < 0.005).
The expression of UCP2 in BAT and WAT after HF feeding is shown in Fig. 3. UCP2 expression in BAT increased threefold at 23°C ($P < 0.05$), whereas there was no difference between LF and HF feeding at 29°C (Fig. 3A). However, housing the mice at thermoneutrality during LF feeding decreased the UCP2 expression significantly ($P < 0.005$). When measuring UCP2 expression in WAT after HF feeding, we found increased expression at both temperatures, fourfold at 23°C ($P < 0.005$) and twofold at 29°C ($P < 0.005$; Fig. 3B). We were not able to measure the relative amount of UCP2 mRNA in WAT at the two temperatures.

Colipase activity in pancreas. To be able to estimate the amount of endogenous enterostatin produced, we measured the activity of pancreatic colipase in homogenates from pancreas. As shown in Fig. 4, housing the mice at 29°C decreased the pancreatic colipase activity at both HF and LF feeding ($P < 0.05$).

Body weight and food intake. Figure 5 shows the body weight gain and the daily food intake in mice fed the HF diet with and without enterostatin. The mice receiving enterostatin significantly decreased their

\[ UCP2. \] The expression of UCP2 in BAT and WAT after HF feeding is shown in Fig. 3. UCP2 expression in BAT increased threefold at 23°C ($P < 0.05$), whereas there was no difference between LF and HF feeding at 29°C (Fig. 3A). However, housing the mice at thermoneutrality during LF feeding decreased the UCP2 expression significantly ($P < 0.005$). When measuring UCP2 expression in WAT after HF feeding, we found increased expression at both temperatures, fourfold at 23°C ($P < 0.005$) and twofold at 29°C ($P < 0.005$; Fig. 3B). We were not able to measure the relative amount of UCP2 mRNA in WAT at the two temperatures.

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\[ \text{Effect of Enterostatin on HF Feeding at Thermoneutrality (29°C)} \]

\[ \text{Body weight and food intake. Figure 5 shows the body weight gain and the daily food intake in mice fed the HF diet with and without enterostatin. The mice receiving enterostatin significantly decreased their} \]

Fig. 3. Expression of UCP2 in interscapular BAT and epididymal white adipose tissue (WAT) after HF and LF feeding at 23 and 29°C. Total cellular RNA was prepared, and Northern blot hybridizations were made with a UCP2 probe. The UCP2 levels are expressed in relation to 18S. Representative autoradiographs are shown (top). A: HF feeding increased UCP2 in BAT at 23°C ($^*P < 0.05$) but had no effect at 29°C. Housing the mice at 29°C significantly decreased UCP2 levels during LF feeding ($###P < 0.005$). B: UCP2 in WAT increased by HF feeding at both 23°C ($###P < 0.005$) and 29°C ($###P < 0.005$).

Fig. 4. Colipase activity in pancreas was measured in mice fed HF or LF diet at 23°C and 29°C. Pancreas was homogenized, and colipase activity was determined by pH-stat titration using tributyrin as substrate. Protein concentration was measured in the homogenates, and the activity is expressed as U/mg protein. Housing the mice at 29°C significantly decreased the colipase activity in both LF-fed ($P_{ac} < 0.05$) and HF-fed animals ($P_{bd} < 0.05$).

Fig. 5. Body weight gain and food intake after 25 days of HF feeding and enterostatin treatment. A mean of the daily caloric intake during the whole period was calculated. Both the daily food intake ($^*P < 0.05$) and the body weight gain ($^*P < 0.05$) significantly decreased during enterostatin treatment.
food intake ($P < 0.05$), and, as shown, these mice also gained less weight ($P < 0.05$) compared with the control mice. There was no significant effect of enterostatin treatment on the weight of the epididymal fat pads or interscapular BAT (Table 2).

**UCP1 and UCP2 in BAT and WAT.** We also investigated the effect of enterostatin on the expression of UCP1 in BAT and UCP2 in BAT and WAT. Enterostatin treatment caused a significant increase in UCP1 mRNA expression in BAT during HF feeding ($P < 0.05$; Fig. 6). In BAT, the UCP2 mRNA levels were significantly decreased ($P < 0.05$) by enterostatin, whereas there was no change in WAT (Fig. 6).

**UCP2 mRNA in duodenum and stomach.** We found UCP2 to be present in both duodenum and stomach. As shown in Fig. 7A, HF feeding caused a decrease in UCP2 mRNA expression in duodenum ($P < 0.005$) compared with LF feeding, whereas enterostatin treatment increased UCP2 mRNA levels ($P < 0.01$). In stomach (Fig. 7B), HF feeding decreased the UCP2 mRNA expression ($P < 0.005$), and enterostatin increased UCP2 mRNA to a level similar to that with LF feeding ($P < 0.005$).

**Expression of colipase mRNA in stomach and lipase/colipase activity in pancreas.** Recently, expression of colipase has been found in stomach (34). Therefore, we measured the mRNA levels of colipase in stomach after HF feeding and enterostatin treatment. HF feeding at 29°C did not influence the expression of colipase compared with that at LF feeding, but enterostatin treatment significantly decreased the expression of colipase in stomach (Fig. 7C). Enterostatin treatment did not affect the activity of lipase or colipase in pancreas after HF feeding (Table 2).

**Effect of HF Feeding and Enterostatin Treatment on Glucose Tolerance at Thermoneutrality**

A glucose tolerance test was performed on day 25 after 5 h of fasting. There was no significant difference in fasting glucose levels between LF- and HF-fed mice. However, 30 min after intraperitoneal glucose administration, the HF-fed mice had significantly higher blood glucose concentrations, which lasted until 120 min (Fig. 8). There was no significant difference in glucose levels between the control and enterostatin-treated mice (Fig. 8), even though the mice treated with enterostatin seemed to reach basal glucose values somewhat faster.

**DISCUSSION**

The data presented in this paper show that NMRI mice fed a high-fat diet gain twice as much body weight when housed at 29°C compared with 23°C. This gain in body weight is most likely caused by a suppressed thermogenesis, because the mice consumed similar amounts of calories at both temperatures (Fig. 1). Previous investigators have found that mice kept at room temperature have a high thermogenic activity, 30% of the calories ingested being used for facultative thermogenesis (18). Also, a thermoneutral environment degenerates BAT, decreasing the total amount of uncoupling protein (11). This is consistent with our finding of a significantly reduced expression of UCP1 mRNA in mice housed at 29°C. We found that mice housed at...
room temperature indeed increased their UCP1 mRNA expression approximately fourfold in response to high-fat feeding, whereas mice kept at 29°C instead slightly decreased their UCP1 mRNA expression (Fig. 2). The difference in UCP1 mRNA expression during high-fat feeding at the two temperatures may thus explain the difference in body weight gain (Fig. 1). However, the low-fat-fed mice did not gain additional weight at 29°C despite a reduction in UCP1. This can be explained by the reduced food intake at thermoneutrality compared with that at room temperature (Fig. 1A). Taken together, these findings indicate that thermogenesis plays an important role in defense against hyperlipidic diets. In contrast, during low-fat feeding, the animals are able to regulate their appetite, resulting in a maintained body weight balance.

The increased UCP1 mRNA expression in BAT at room temperature is in agreement with previous studies (36), supporting a role for BAT thermogenesis during high-fat feeding. In contrast, Enerbäck et al. (6) have shown that mice lacking UCP1 do not become obese on a high-fat diet, but these mice were not hyperphagic, and they also compensated for the loss of UCP1 with increased expression of UCP2 mRNA, which may explain the lack of obesity (6). Commins et al. (4) have shown that ambient temperature influences the ability of ob/ob mice to respond to exogenous leptin, which is known to upregulate UCP1 mRNA in BAT. The lack of upregulation of UCP1 in our experiment at 29°C could be caused by a blunted effect of leptin.

We found the expression of UCP2 mRNA to be regulated in a tissue-specific manner. In BAT, UCP2 mRNA expression increased during high-fat feeding at 23°C but did not change at 29°C, a pattern similar to the expression of UCP1 (Fig. 3A). Previous investigators failed to demonstrate any upregulation of UCP2 in BAT in response to high-fat feeding (22, 36), which may be explained by an elevated ambient temperature. The upregulation of UCP1 and UCP2 in BAT at 23°C but not at 29°C could be explained by a higher sympathetic innervation at 23°C, as shown by Denjean et al. (5).

In WAT, UCP2 mRNA increased during high-fat feeding at both temperatures, the upregulation being

Fig. 7. Northern blot analysis of UCP2 expression in duodenum and stomach and colipase expression in stomach. Mice were fed LF, HF, or HF + enterostatin diet. A: in duodenum, HF feeding decreased UCP2 expression (###P < 0.005), whereas enterostatin treatment increased the expression of UCP2 (**P < 0.01). A representative autoradiograph is shown. B: in stomach, levels of UCP2 decreased significantly during HF vs. LF feeding (###P < 0.005). Enterostatin treatment significantly increased UCP2 in HF-fed mice (***P < 0.005). C: representative autoradiograms of UCP2, 18S, and colipase are shown. HF feeding did not change the expression of colipase in stomach, whereas enterostatin treatment decreased colipase expression.

Fig. 8. An intraperitoneal glucose tolerance test was performed on day 25, between 0700 and 1000. HF feeding significantly increased blood glucose levels from 30 to 120 min vs. LF feeding. Mice treated with enterostatin during HF feeding reached basal glucose levels somewhat faster, but not significantly.
more extensive at room temperature than at thermoneutrality (Fig. 3B). Increased expression of UCP2 in WAT by high-fat feeding is consistent with earlier reports (10, 22). Surwit et al. (36) showed that UCP2 expression in WAT was increased by high-fat feeding in obesity-resistant but not in obesity-prone mice, and they suggested that upregulation of UCP2 in WAT was a defense mechanism against diet-induced obesity. However, in our experiments conducted at 29°C, the animals became obese and insulin resistant despite increased UCP2 expression in WAT (Fig. 8). Recent data linking UCP2 gene expression in muscle to fatty acid metabolism support other roles of UCP2 (29). We did not, however, study the expression of uncoupling proteins in skeletal muscle.

We also found that high-fat feeding caused a decrease in UCP2 gene expression in the stomach, as well as in the intestine (Fig. 7). The importance of this finding is not known. It is clear that the intestine should provide energy rather than dissipating it; hence, downregulation of an uncoupling mechanism seems advantageous for uptake and storage of energy. That digestion and absorption of fat is specifically dependent on ATP production in the epithelial cells is evident from the fact that, after being liberated from dietary fat through hydrolysis, fatty acids need ATP for further transport and transformation into triacylglycerol and chylomicrons.

Because we found that the obesity attained by high-fat feeding at 29°C was caused by hyperphagia in combination with reduced thermogenesis (Figs. 1 and 2), we were interested in studying the effect of enterostatin, a peptide that has been shown to reduce fat intake and increase sympathetic activity to BAT (8, 24). With orally administered enterostatin, we found a minor but significant reduction in food intake and body weight gain (Fig. 5). The importance of the slight reduction in food intake for the observed decrease in body mass is not known. One reason for overeating at 29°C could be the lower amount of colipase, and therefore of enterostatin, at this temperature compared with that at 23°C (Fig. 4). Earlier studies have shown that colipase, but not lipase, is temperature sensitive (12). The significance of the temperature-dependent regulation of colipase is not known. Colipase is also produced in the stomach (35), and we did not find any change of colipase mRNA expression in the stomach during high-fat feeding (Fig. 7A), in contrast to what previous investigators have found (19, 34). However, treatment with enterostatin decreased the gastric colipase mRNA levels, in agreement with Lin et al. (19) (Fig. 7A).

According to the thermostatic regulation of feeding (16, 17), a rise in body temperature occurs during a meal and is terminated when the body reaches a certain critical temperature. In our study, we observed that, during low-fat feeding, the food intake was lower at the higher ambient temperature (Fig. 1A), supporting a role for the principle of thermostatic regulation during this regimen. However, during high-fat feeding, the animals failed to suppress their food intake at the higher surrounding temperature (Fig. 1A). This phenomenon could be explained by a downregulation of UCP1 in BAT (Fig. 2) and possibly also of UCP2 in the gut (Fig. 7) observed during high-fat feeding. This downregulation of uncoupling protein expression may prolong the time for reaching critical body temperature associated with meal termination. In fact, upon enterostatin treatment, an increased expression of UCP1 in BAT and UCP2 in the gut (Figs. 6 and 7) was observed, which may explain the lower food intake under these conditions. The mechanism for increased UCP1 expression by enterostatin may be through stimulated sympathetic innervation (24) or caused by increased corticosterone levels (8).

In conclusion, we found that high-fat feeding caused obesity in mice when thermogenic mechanisms could not be utilized. In contrast, low fat-fed mice were able to suppress appetite, remaining in weight balance during the same conditions. The expression of UCP1 and UCP2 was regulated in a similar way in BAT during high-fat feeding, whereas the UCP2 expression was differently regulated in the various tissues after high-fat feeding. The hyperphagia induced by high-fat feeding was partly suppressed by enterostatin, which at the same time affected the expression of UCP1 and UCP2.

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