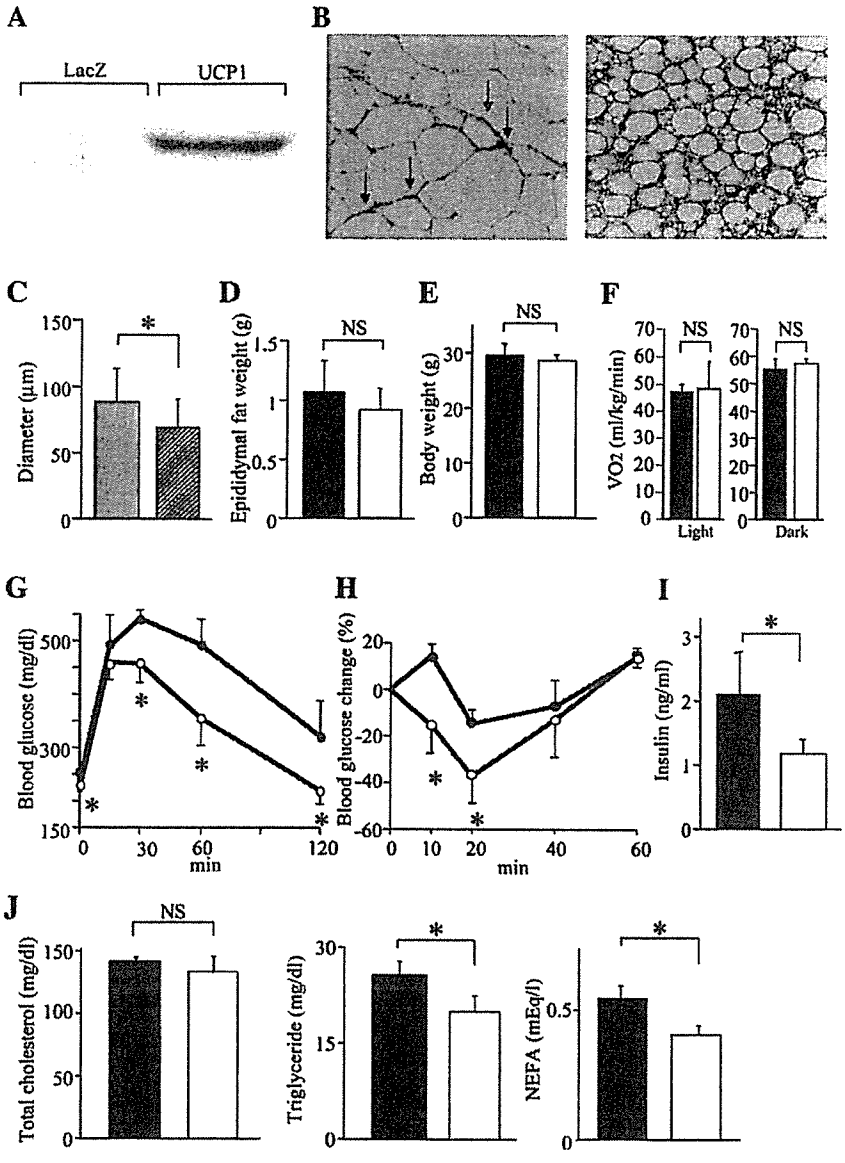

Figure 1. UCP1 expression in Epi improved glucose tolerance and insulin sensitivity

A) Immunoblotting, with anti-UCP1 antibody, of Epi extracts from LacZ and UCP1 mice on day 3 after adenoviral administration.

B) Immunohistochemistry, with anti-UCP1 antibody, of Epi (left panel) and BAT (right panel) sections from a UCP1 mouse on day 3 after adenoviral administration. These two samples were immunostained under the same conditions.

C) Diameters of UCP1-nonexpressing (gray bar) and UCP1-expressing (hatched bar) adipocytes in Epi from UCP1 mice on day 3 after adenoviral administration.

D–J) Epididymal fat weights (**D**), body weights (**E**), resting oxygen consumption during light and dark phase (**F**), and metabolic parameters (**G–J**) of LacZ mice (black bars) and UCP1 mice (white bars) on day 3 after adenoviral administration. Glucose-tolerance (**G**) and insulin-tolerance tests (**H**) were performed on day 3. Data in (**H**) are expressed as percentages of the blood glucose levels immediately before intraperitoneal insulin loading. Serum insulin levels (**I**) and serum lipid parameters ([**J**]; left: total cholesterol, middle: triglyceride, right: free fatty acids) were measured after a 10 hr fast (n = 6 per group). Data are presented as means ± SD (n = 6 per group). *p < 0.05 by unpaired t test.

We further confirmed enhanced metabolism by adenoviral UCP1 expression using 3T3-L1 adipocytes. UCP1 expression decreased intracellular ATP concentrations (Figure S1C) and increased levels of peroxisome proliferator-activated receptor γ coactivator (PGC) 1α and cytochrome c expression (Figure S1D). Thus, exogenous UCP1 was functionally active, resulting in increased mitochondrial biosynthesis in adipocytes.

However, neither total Epi weights nor body weights differed between LacZ and UCP1 mice on day 3 after adenoviral administration (Figures 1D and 1E). Oxygen consumption was not affected by UCP1 expression in Epi during either the light or the dark phase (Figure 1F), also reflecting the very limited UCP1 expression. Therefore, to avoid the secondary effects of body-weight change, we analyzed metabolic parameters on day 3. To our surprise, however, even very limited UCP1 expression in Epi resulted in marked changes in metabolic phenotype.

Glucose- and insulin-tolerance tests indicated marked improvements in glucose tolerance and insulin sensitivity (Figures 1G and 1H). Fasting blood glucose (Figure 1G) and insulin (Figure 1I) levels were significantly lower in UCP1 mice, further confirming improved insulin sensitivity. In addition, serum lipid parameters, including triglycerides and free fatty acids (Figure 1J), were also improved with UCP1 expression in Epi. Thus, limited regional expression of UCP1 in Epi markedly improved systemic insulin resistance, resulting in improvement of diabetes and dyslipidemia.

Next, we measured serum adipocytokine levels (Figure 2A). Adiponectin and tumor necrosis factor α levels were not significantly altered. In contrast, serum leptin was markedly decreased, by 46%, with UCP1 expression in Epi. Although intra-abdominal fat-tissue weights were unaltered or only very slightly decreased in UCP1 mice (Figure 1D and Figure S1E),

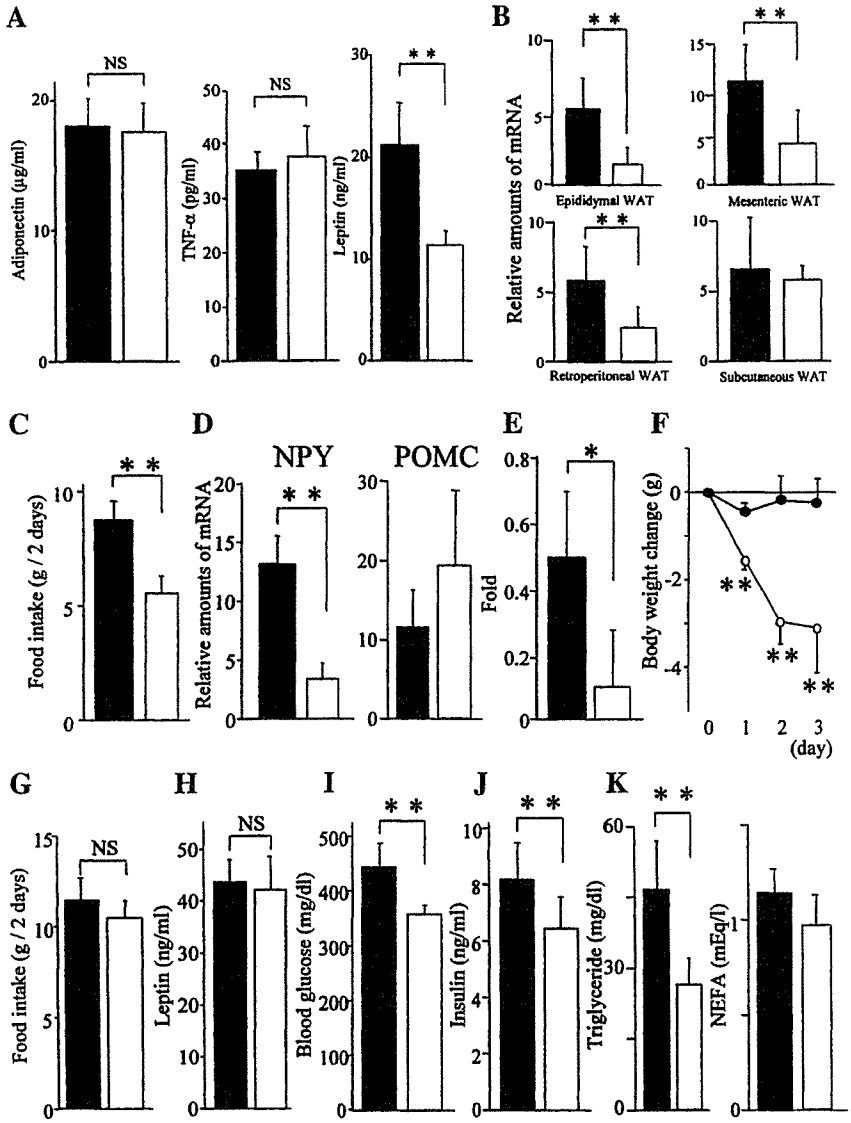

Figure 2. UCP1 expression in Epi improved leptin sensitivity

A–F) LacZ (black bars) or UCP1 (white bars) adenovirus was injected into Epi of mice with dietary obesity.

A) Serum adipocytokine levels (left: adiponectin, middle: TNFα, right: leptin) in LacZ mice and UCP1 mice after a 10 hr fast on day 3 after adenoviral administration.

B) Relative amounts of leptin mRNA in adipose tissues.

C) Total food intakes on days 2 and 3 after adenoviral administration.

D) Relative amounts of neuropeptide Y (left) and proopiomelanocortin (right) mRNA were measured by quantitative RT-PCR using total RNA obtained from the hypothalamus on day 2 after adenoviral administration. Data were corrected with β-actin as the standard (**B** and **D**).

E and F) Leptin-tolerance tests were performed on day 3 after adenoviral administration. Data were expressed as ratios to the food intakes of vehicle-treated mice (**E**). Mice were weighed at 12 hr after each daily injection of leptin or vehicle (**F**).

G–K) LacZ (black bars) or UCP1 (white bars) adenovirus was injected into Epi of db/db mice.

G) Total food intakes on days 2 and 3 after adenoviral administration are presented.

H–K) Blood leptin (**H**), glucose (**I**), and insulin (**J**) levels and serum lipid parameters ([**K**]; left: triglyceride, right: free fatty acids) of db/db mice were measured after a 10 hr fast. Data are presented as means ± SD ($n = 8$ per group). $^{*}p < 0.05$; $^{**}p < 0.01$ by unpaired t test.

leptin mRNA expression was markedly decreased in intra-abdominal fat tissues (Figure 2B). Thus, the effects of UCP1 expression in Epi are also exerted in fat tissues other than those injected with the adenovirus. Food intake was significantly suppressed (Figure 2C), indicating that hypothalamic leptin sensitivity was markedly improved despite the lack of significant changes in body weights. Decreased leptin expression in several adipose tissues suggests efferent sympathetic nerve activation, which also supports leptin signal enhancement.

Administration of green fluorescent protein-adenovirus exerted minimal metabolic effects (Figures S1F–S1J). On day 7, when adenoviral UCP1 expression was markedly decreased (Figure S1B), blood glucose, insulin, and leptin levels did not differ between the UCP1 and LacZ mice (Figure S2). In addition, we confirmed the metabolic effects of UCP1 expression in Epi using three other obese models: AKR mice on high-fat chow and KK mice and KK-Ay mice on normal chow. In these three models, similar metabolic impacts were observed with UCP1 adenovirus administration into Epi (Figure S3). Thus, UCP1 expression in Epi exerts acute, beneficial metabolic effects in both diet-induced and genetically obese models.

Increased leptin signals in the hypothalamus induced by UCP1 expression in Epi were further confirmed by changed levels of hypothalamic neuropeptide expression in UCP1 mice on day 3 after adenoviral administration. Real-time RT-PCR revealed adipose UCP1 expression to significantly decrease expression of neuropeptide Y, an orexigenic neuropeptide, while tending to increase that of proopiomelanocortin, a precursor of an anorexigenic neuropeptide, in the hypothalamus (Figure 2D).

To directly test whether leptin sensitivity was improved, we performed leptin-tolerance tests. When leptin was injected intraperitoneally into fasting mice on day 3, leptin-induced food-intake inhibition was far more profound in UCP1 mice than in LacZ mice (Figure 2E). In addition, when leptin was given daily, body weights were significantly decreased (Figure 2F). Thus,

even very limited UCP1 expression in Epi exerts a remote therapeutic effect on hypothalamic leptin resistance, which had already developed in response to preloading with high-fat chow. Transgenic overexpression of UCP1 (Kopecky et al., 1995) and rather minor induction of UCP1 in white adipose tissue (Cederberg et al., 2001; Leonardsson et al., 2004; Tsukiyama-Kohara et al., 2001; Um et al., 2004) result in resistance to high-fat-diet-induced obesity but do not reportedly cause hyperphagia. In this study, however, we expressed UCP1 after the development of obesity and leptin resistance and were thus able to observe acute, beneficial effects, i.e., improved leptin sensitivity, which would be difficult to detect using congenitally UCP1-overexpressing mice.

Increased leptin sensitivity is likely to be involved in the phenotype of UCP1 mice. If this is the case, at least some of the phenotypic features of UCP1 mice would presumably be absent in mice lacking the hypothalamic leptin signal. To test this, UCP1 or LacZ adenovirus was injected into Epi of db/db mice, leptin-receptor Ob-Rb mutants. Food intake (Figure 2G) and serum leptin (Figure 2H) did not differ between LacZ-expressing and UCP1-expressing db/db mice. These findings confirm that the effect of UCP1 expression in Epi on food intake is leptin-signal dependent. On the other hand, UCP1 expression in Epi of db/db mice caused small but significant decreases in blood glucose (Figure 2I), insulin (Figure 2J), and triglyceride (Figure 2K) levels, as well as tending to decrease serum free-fatty-acid levels (Figure 2K). These findings demonstrate that UCP1 expression in Epi improves insulin sensitivity, in part, independently of leptin signaling.

To eliminate the secondary effects of reduced food intake, pair-feeding experiments were performed using C57BL/6 wild-type mice (Figure S4). Pair feeding did not significantly alter the body weights of LacZ mice. Fasting blood glucose did not differ between UCP1 mice and pair-fed LacZ mice, but after glucose loading, blood glucose levels were significantly lower in UCP1 mice. In addition, serum insulin and leptin levels were significantly lower in UCP1 mice than in pair-fed LacZ mice. Taken together with the results obtained using db/db mice, the improved insulin sensitivity induced by UCP1 expression in Epi appears not to be mediated solely by decreased food intake.

The same amounts of recombinant adenovirus encoding UCP1 were directly injected into subcutaneous fat tissues in the flank of C57BL/6 mice with dietary obesity and diabetes. UCP1 expression levels were similar to those obtained by injection into Epi (data not shown). Food intake was significantly decreased by UCP1 expression, as compared with LacZ expression, in subcutaneous fat (Figure 3A), but the effects were much smaller than those produced by UCP1 expression in Epi (Figure 2C). Furthermore, there were no statistically significant decreases in blood glucose (Figure 3B), insulin (Figure 3C), or leptin (Figure 3D) levels. Thus, exogenous UCP1 expression in subcutaneous fat was far less effective in improving insulin and leptin resistance than that in intra-abdominal fat tissue. These findings suggest the anatomical location of the manipulated adipose tissue to be involved in the observed therapeutic effects, which would appear to be important for understanding the metabolic differences between visceral fat-dominant and subcutaneous fat-dominant obesity.

How does the signal (or signals) from intra-abdominal fat tissue exert these remote effects? The importance of anatomical fat-tissue location suggests the involvement of neuronal signaling. The afferent activity from Epi is reportedly transmitted through the nerve bundle, which runs alongside blood vessels supplying Epi, in rats (Niijima, 1998). To study the possible involvement of neuronal signals from Epi, we dissected this nerve bundle in mice with dietary obesity and diabetes. Ten days after bilateral nerve-bundle dissection, adenoviruses were injected into Epi. No significant differences in body weights or Epi weights were observed between sham-operated and nerve-dissected mice (data not shown). While UCP1 adenoviral administration significantly decreased food intake in sham-operated mice, nerve dissection blunted this decrease in food intake such that it was no longer statistically significant (Figure 3E). Similarly, nerve dissection blunted a decrease in hypothalamic NPY mRNA expression, rendering it statistically insignificant (NPY; LacZ versus UCP1: 12.06 ± 6.16 versus 6.39 ± 3.10; $p = 0.15$). These findings suggest that neuronal signals from intra-abdominal fat tissue are involved in food-intake regulation. In contrast, in nerve-dissected mice, blood glucose (Figure 3F) as well as serum insulin (Figure 3G) and leptin (Figure 3H) levels were significantly suppressed in a fashion similar to in sham-operated mice. Thus, improved insulin resistance is largely independent of this neuronal pathway.

To confirm that afferent-nerve signals are involved in UCP1-expression-mediated suppression of food intake, we next examined the effects of functional deafferentation by administering capsaicin (Fu et al., 2003), a selective neurotoxin for unmyelinated C fibers. In LacZ mice, food intake was not altered by capsaicin treatment 10 days prior to adenoviral administration. In contrast, capsaicin pretreatment significantly reversed the food-intake suppression induced by UCP1 expression in Epi (Figure 3I). The inhibitory effect of capsaicin pretreatment was very similar to that of local-nerve dissection (Figure 3E). Taken together, these observations suggest that afferent-nerve signals from Epi are involved in food-intake regulation. To elucidate the molecular mechanism whereby UCP1 expression in Epi modulates neuronal activity, we searched for genes upregulated by adipose UCP1 expression. Using the DNA microarray technique, gene expressions were examined in LacZ- and UCP1-adenovirus-treated Epi (Table S1) and in 3T3-L1 adipocytes (Table S2). With the exception of UCP1, however, there was no overlap in genes showing significantly increased expression. Although further expression profiling including proteomic approaches might elucidate the underlying mechanisms, the apparent lack of genes showing increased expression raises the possibility that the activation of afferent nerves does not involve gene-expression alterations. For instance, UCP1 generates heat, and a capsaicin receptor, TRPV1, is activated by a slightly above normal body temperature (Caterina et al., 1997). Capsaicin treatment affected UCP1-induced food-intake suppression (Figure 3I), raising the possibility that UCP1 expression activates capsaicin-sensitive nerves via TRPV1 activation. Another possibility is involvement of reactive oxygen species, which are affected by mitochondrial uncoupling (Bernal-Mizrachi et al., 2005; Jezek et al., 2004) and reportedly regulate capsaicin-sensitive afferent fibers (Ruan et al., 2005). Further studies are required to examine these hypotheses.

In this study, very limited UCP1 expression in Epi markedly improved insulin and leptin resistance, thereby improving glucose tolerance and decreasing food intake. UCP1 mice were more insulin sensitive than pair-fed LacZ mice. In addition, in db/db mice, despite no food-intake suppression, blood glucose

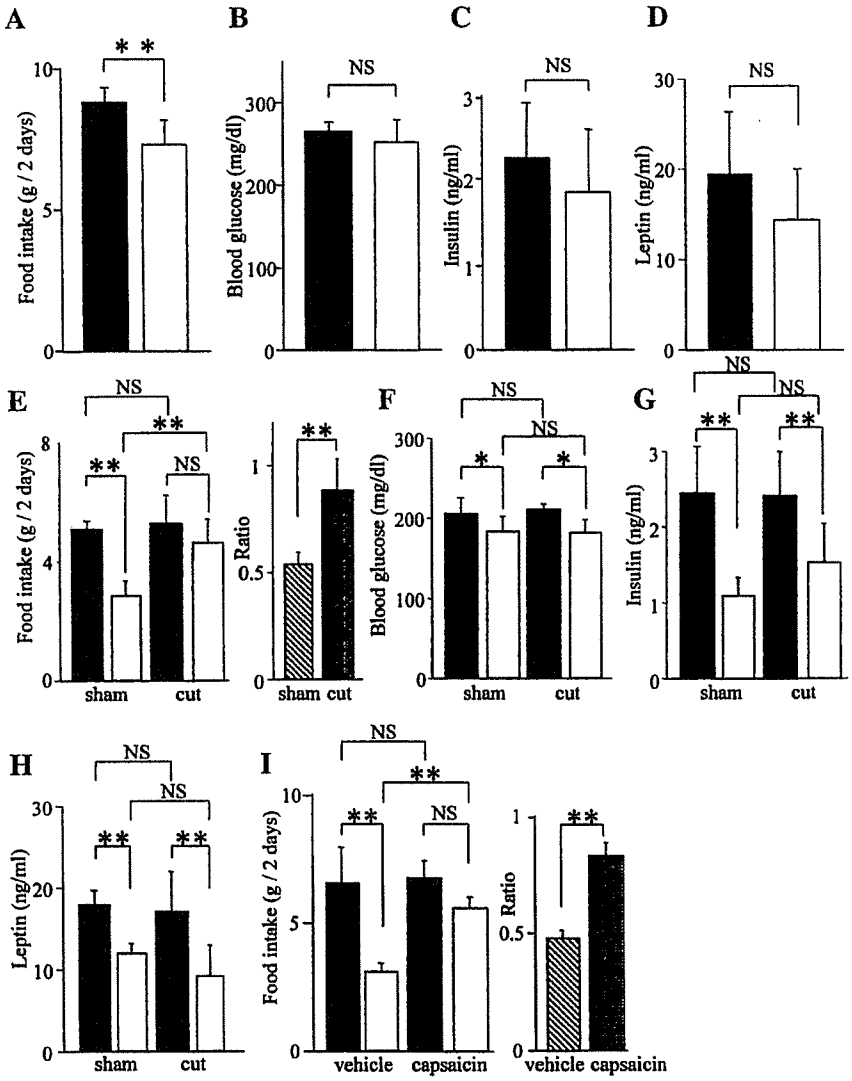

Figure 3. Neuronal signals are likely to be involved in food-intake regulation

A–D) LacZ (black bars) or UCP1 (white bars) adenovirus was injected into subcutaneous fat, and metabolic markers were measured. Total food intakes on days 2 and 3 after adenoviral administration are presented. Blood glucose (**B**), insulin (**C**), and leptin (**D**) levels were determined after a 10 hr fast on day 3 after adenoviral administration. $^{**}p < 0.01$ by unpaired t test.

E–H) Mice were subjected to local-nerve dissection 10 days prior to adenoviral injection into Epi. Total food intakes of sham-operated (sham) and nerve-dissected (cut) mice (**E**) on days 2 and 3 are presented graphically. Blood glucose (**F**), serum insulin (**G**), and leptin (**H**) levels were determined on day 3.

I) Mice were treated with capsaicin or vehicle 10 days prior to adenoviral injection into Epi. Total food intakes on days 2 and 3 after administration of LacZ (black bars) or UCP1 (white bars) adenovirus are presented. In (**E**) and (**I**), the food intakes of UCP1 mice are expressed in the right graph as ratios to those of LacZ mice. $^{**}p < 0.01$ assessed by one-factor ANOVA. Data are presented as means ± SD.

and insulin levels were modestly but significantly decreased by UCP1 expression in Epi. Thus, the mechanism underlying improved insulin sensitivity with UCP1 expression in Epi is, in part, independent of leptin signaling and food-intake suppression (Figure 4). Dissection of the nerve bundle from Epi did not alter the decreases in blood glucose and insulin levels. Taken together with the findings that UCP1 expression in subcutaneous fat did not significantly decrease blood glucose or insulin levels, our observations indicate that nonneuronal signals including humoral factors from intra-abdominal adipose tissue possibly participate in systemic improvement of insulin resistance. Since UCP1 expression was observed in a very limited population of adipocytes in Epi, suppression of insulin-resistant adipocytokine secretion is unlikely to explain the beneficial effects. Serum adiponectin levels were not altered, suggesting involvement of other unknown insulin-sensitizing factor (or factors).

On the other hand, decreased food intake is likely to be, at least partially, mediated by afferent-nerve signals from Epi (Figure 4). Afferent-nerve signals from Epi to the central nervous system reportedly result in a reflex from epididymal fat to white adipose tissues via efferent sympathetic-nerve activation (Niijima, 1998; Tanida et al., 2000). In addition, vagal afferent

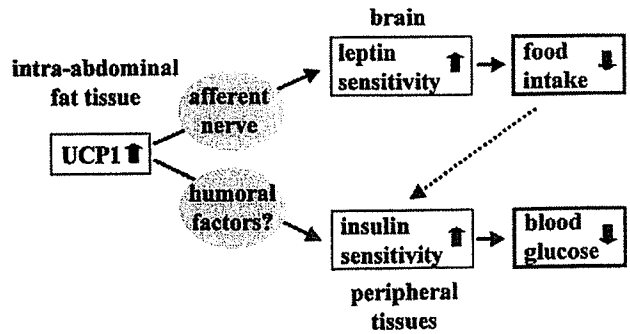

Figure 4. The proposed mechanism whereby UCP1 expression in Epi decreases food intake and improves glucose tolerance

neuronal signals from intra-abdominal tissues, including the gut (Fu et al., 2003; Smith et al., 1981) and the liver (Friedman, 1998; Scharrer, 1999), are known to play a part in regulating food intake. We also reported that UCP1 gene administration into the liver modulates food intake (Ishigaki et al., 2005). Herein we report that intra-abdominal fat tissue is likely to convey metabolic signals to the brain via a neuronal pathway, in addition to via the circulation, resulting in modulation of food intake. Although the precise molecular mechanism remains to be elucidated, this neuronal pathway might play a role in development of the metabolic syndrome, making it a potentially novel therapeutic target.

Experimental procedures

Preparation of recombinant adenovirus

Recombinant adenovirus containing murine UCP1 cDNA (Ishigaki et al., 2005) was constructed as described previously (Katagiri et al., 1996). Recombinant adenoviruses bearing the bacterial β-galactosidase gene (*Adex1CAlacZ*) and green fluorescent protein (AdCMV-GFP) were used as controls.

Animals and in vivo adenovirus injection into fat pad

Animal studies were conducted in accordance with the institutional guidelines for animal experiments at Tohoku University. Male C57BL/6N and AKR/N mice were housed individually, and high-fat-chow feeding (32% safflower oil, 33.1% casein, 17.6% sucrose, and 5.6% cellulose) (Ishigaki et al., 2005) was initiated at 5 weeks of age. After 4 weeks of high-fat-chow loading, body-weight-matched mice were anesthetized prior to dissection of the skin and body wall. The adenoviral preparation (1×10^8 plaque-forming units in a volume of 20 μl) was injected at two points each on each side of the epididymal fat pad or subcutaneous fat tissues in the flank, i.e., a total of four points. KK mice and KK-Ay mice maintained on a standard diet (65% carbohydrate, 4% fat, 24% protein) were similarly administered adenoviruses at 9 weeks and 5 weeks of age, respectively.

Immunoblotting

Tissue protein extracts (250 μg total protein) were boiled in Laemmli buffer containing 10 mM dithiothreitol, subjected to SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose filters. The filters were incubated with anti-UCP1 antibody (Santa Cruz Biotechnology, Santa Cruz, California) and then with anti-goat immunoglobulin G coupled to horseradish peroxidase. The immunoblots were visualized with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK). The intensities of bands were quantified with the NIH Image 1.62 program.

Histological analysis

Mouse epididymal fat and BAT were immunostained as previously reported (Ishigaki et al., 2005). Mature white adipocytes were identified by their characteristic unilocular appearance. Diameters of 100 or more white adipocytes per mouse in each group were traced manually and analyzed.

Oxygen consumption

Oxygen consumption was measured as previously reported (Ishigaki et al., 2005).

Pair-feeding experiments

Pair-feeding experiments were performed as previously described (Ishigaki et al., 2005).

Blood analysis

Blood glucose and serum insulin, leptin, adiponectin, TNFα, total cholesterol, triglyceride, and free-fatty-acid levels were determined as previously described (Ishigaki et al., 2005).

Measurement of quantitative RT-PCR-based gene expression

The skull was reflected from the brain and the hypothalamus was isolated by snap freezing in liquid nitrogen as previously reported (Bjorbaek et al., 1998). Total RNA was isolated from mouse hypothalamus, fat tissues, or 3T3-L1 adipocytes with ISOGEN (Wako Pure Chemical Co., Osaka, Japan), and cDNA synthesized from total RNA was evaluated with a real-time PCR quantitative system (Light Cycler Quick System 350S; Roche Diagnostics GmbH, Mannheim, Germany).The relative amount of mRNA was calculated with β-actin mRNA as the invariant control. The primers used are shown in Table S3.

Glucose-, insulin-, and leptin-tolerance tests

Glucose-tolerance tests were performed on fasted (10 hr, daytime) mice. Mice were given glucose (2 g/kg of body weight) intraperitoneally, followed by measurement of blood glucose levels. Insulin-tolerance tests were performed on ad libitum-fed mice. Mice were intraperitoneally injected with human regular insulin (0.75 U/kg of body weight; Eli Lilly Co., Kobe, Japan).

Leptin-tolerance tests were carried out as described in a previous report (Igel et al., 1997), with slight modification. Fasted (12 hr) mice were injected with mouse leptin (7.2 mg/kg of body weight; R&D Systems, Inc.) intraperitoneally, and food intakes were monitored for 12 hr after the injection. To examine effects on body-weight change, these two groups of mice were given leptin daily starting on the day of adenoviral administration. Each mouse was then weighed.

Capsaicin treatments

Capsaicin treatment was performed as described in a previous report (Fu et al., 2003), with minor modification. Mice were anesthetized prior to subcutaneous injection of capsaicin solution (50 mg/kg, 12.5 mg/ml dissolved in vehicle). The control group received vehicle treatment (10% Tween 80, 10% ethanol, and 80% saline) under identical administration conditions. Adenoviral administration into Epi was carried out 10 days later.

Local-nerve dissection

The small nerve bundle which runs along side blood vessels supplying Epi was dissected as previously reported (Nijima, 1998). Ten days after bilateral dissection of this nerve bundle, adenoviruses were injected into epididymal fat pad.

Measurement of ATP

Fully differentiated 3T3-L1 adipocytes were infected with recombinant adenoviruses as previously described (Katagiri et al., 1996). Intracellular ATP levels were measured using an ATP determination kit (TOYO B-Net, Tokyo, Japan).

Microarray experiments

Total RNA from epididymal fat or 3T3-L1 adipocytes was used to synthesize cRNA, which was then hybridized to an HG-U133A *oligonucleotide* array (Affymetrix, Santa Clara, California) according to standard protocols, as described previously (Hippo et al., 2002).

Statistical analysis

All data were expressed as means ± SD. The statistical significance of differences was assessed by the unpaired t test and one-factor ANOVA.

Supplemental data

Supplemental Data include four figures and three tables and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/3/3/223/DC1/.

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A negative feedback system between brain serotonin systems and plasma active ghrelin levels in mice

Katsunori Nonogaki *, Kana Ohashi-Nozue, Yoshitomo Oka

Center of Excellence, Division of Molecular Metabolism and Diabetes, Tohoku University Graduate School of Medicine, Japan

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Abstract

Brain serotonin (5-hydroxytryptamine; 5-HT) systems contribute to regulate eating behavior and energy homeostasis. 5-HT2C receptors and 5-HT1B receptors have been shown to mediate anorexic effects of 5-HT drugs such as D-fenfluramine, which stimulates 5-HT release and inhibits 5-HT reuptake, and *m*-chlorophenylpiperazine (mCPP), a 5-HT2C receptor agonist. Here, we report that 24-h fasting increased the expression of hypothalamic 5-HT2C receptor and 5-HT1B receptor genes in association with increases in plasma active ghrelin levels compared with fed state in mice. Treatment with mCPP or fenfluramine significantly inhibited the increases in plasma active ghrelin levels. mCPP or fenfluramine significantly increased the expression of hypothalamic pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript genes while having no significant effects on the expression of hypothalamic neuropeptide Y, agouti-related protein, and ghrelin genes. These results suggest that there is a negative feedback system between brain 5-HT systems and plasma active ghrelin levels in energy homeostasis in mice.

Keywords: Serotonin; 5-HT2C receptor; 5-HT1B receptor; Ghrelin; Neuropeptide; POMC; CART; AGRP; Hypothalamus; Eating behavior; Energy homeostasis; Gene expression; mCPP; Fenfluramine

Mice with a mutated serotonin (5-hydroxytryptamine; 5-HT) 5-HT2C receptor gene display leptin-independent hyperphagia and hyperactivity that leads to a late onset of obesity associated with the secondary leptin resistance [1–3]. Several lines of evidence indicate that 5-HT2C receptors contribute to mediate the anorexic actions of 5-HT agonists such as *m*-chlorophenylpiperazine (mCPP) and 5-HT reuptake inhibitors such as D-fenfluramine and D-norfenfluramine [1,4,5]. These finding indicate that brain 5-HT systems via 5-HT2C receptors contribute to the regulation of eating behavior and energy homeostasis. In addition, 5-HT1B receptors have been suggested to mediate the anorexic actions of mCPP and D-fenfluramine in mice [6]. Activation of 5-HT2C receptor and 5-HT1B receptor signaling might therefore coordinate to suppress food intake.

* Corresponding author. Fax: +81 22 717 7612.
E-mail address: knonogaki-tky@umin.ac.jp (K. Nonogaki).

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The link between brain 5-HT systems and neuropeptides or gut peptides in the regulation of feeding and energy homeostasis, however, is still unclear. Ghrelin is an orexigenic peptide, secreted by stomach [7–9]. Fasting elevates plasma ghrelin levels, while feeding reduces the levels in animals and humans [8–10]. There are two forms of ghrelin, an active ghrelin with bioactivity and des-acyl ghrelin without biologic activity [10–12]. Because only the acylated form of ghrelin is active, measuring the acylated form of ghrelin might be advantageous.

In the present study, to determine the relationship between brain 5-HT systems and plasma ghrelin levels in the regulation of energy homeostasis, we investigated: (1) alterations of the expression of hypothalamic 5-HT2C and 5-HT1B receptor genes induced by fasting and feeding, (2) the effects of mCPP or fenfluramine on plasma active ghrelin levels, and (3) the effects of mCPP or fenfluramine on the gene expression of hypothalamic neuropeptides that

are involved in regulating feeding behavior and energy homeostasis in mice.

Materials and methods

Animals and drug treatment. Four-week-old male C57BL/6J mice were purchased from Japan CLEA. Mice were individually housed in cages with free access to water and chow pellets in a light- (12 h on/12 h off; lights on at 08:00 h and lights off at 20:00 h) and temperature (20–22 °C)-controlled environment. Animals were acclimatized to the laboratory environment for 1 week before the experiment. mCPP and fenfluramine were purchased from Sigma Chemical (St. Louis, MO, USA). mCPP (5 mg/kg) and fenfluramine (3 mg/kg) were dissolved in saline. Drugs were administered at 10 a.m. to12 p.m. mCPP and fenfluramine were purchased from Sigma Chemical, Japan. mCPP (5 mg/kg) and fenfluramine (3 mg/kg) were dissolved in saline. The doses of mCPP (5 mg/kg) and fenfluramine (3 mg/kg) were set on the evidence that either mCPP or fenfluramine-induced hypophagia is attenuated in 5-HT2C receptor-deficient mice [1,4].

In the first experiment, 5-week-old male C57BL/6J mice in either a fed or 24-h fasted state were decapitated and blood was collected. Whole blood was mixed with EDTA-2Na (2 mg/ml) and aprotinin (500 kIU/ml) to determine the plasma active ghrelin levels. The hypothalamus was removed for RNA extraction.

In the second experiment, 5-week-old male C57BL/6J mice were deprived of food for 23 h. The following morning, the animals were intraperitoneally injected with saline or mCPP (5 mg/kg) or fenfluramine (3 mg/kg). Sixty minutes later, the animals were decapitated and blood was drawn; the animals were not fed. The hypothalamus was removed for RNA extraction.

The animal studies were conducted under protocols in accordance with the Institutional Guidelines for Animal Experiments at Tohoku University.

Real-time quantitative RT-PCR. Total RNA was isolated from mouse hypothalamic tissue using the RNeasy Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's directions, and cDNA synthesis was performed using a Super Script III First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Rockville, MD) using 1 μg total RNA. cDNA synthesized from total RNA was evaluated in a real-time polymerase chain reaction (PCR) quantitative system (Light Cycler Quick System 350S; Roche Diagnostics, Mannheim, Germany). The primers used were as follows. For mouse 5-HT1B receptor, sense, 5′-TGC CTG CTG GTT TCA CAT-3′, 5′-ATA GAT GTG TGG AGC TGG TG-3′, antisense, 5′-GCG CAC TTA AAG CGT ATC A-3′; 5-HT2C receptor, sense, 5′-CTG AGG GAC GAA AGC AAA G-3′, antisense, 5′-CAC ATA GCC AAT CCA AAC AAA C-3′; pro-opiomelanocortin (POMC), sense, 5′-ATA GAT GTG TGG AGC TGG TG-3′, antisense, 5′-GGC TGT TCA TCT CCG TTG-3′; for mouse cocaine- and amphetamine-regulated transcript (CART), sense, 5′-CTG GAC ATC TAC TCT GCC GTG G-3′, antisense, 5′-GTT CCT CGG GGA CAG TCA CAC AGC-3′; for mouse neuropeptide Y (NPY), sense, 5′-GCT TGA AGA CCC TTC CAT TGG TG-3′, antisense, 5′-GGC GGA GTC CAG CCT AGT GG-3′; for mouse agouti-related protein (AGRP), sense 5′-CAG ACC GAG CAG AAG AAG-3′, antisense, 5′-GAC TCG TGC AGC CTT ACA-3′; for mouse ghrelin, sense, 5′-GAA AGG AAT CCA AGA AGC CA-3′, antisense, 5′-GCT TGA TGC CAA CAT CGA A-3′; for mouse agouti-related peptide (AGRP), sense, 5′-CAG ACC GAG CAG AAG AAG-3′, antisense, 5′-GAC TCG TGC AGC CTT ACA-3′; and for mouse β-actin, sense, 5′-TTG TAA CCA ACT GGG ACG ATA TGG-3′, antisense, 5′-GAT CTT GAT CTT CAT GGT GCT AGG-3′. The relative amount of mRNA was calculated with β-actin mRNA as the invariant control. The data are shown as the percentage of mean values of the control group which received saline.

Plasma active ghrelin assay. Plasma active ghrelin levels were measured by enzyme-linked immunosorbent assay (ELISA; Active Ghrelin ELISA kit, Mitsubishi Kagaku Iatron, Japan) according to the manufacturer's instructions. For the ELISA of active ghrelin, 1 N hydrochloric acid was added to the samples at a final concentration of 0.1 N immediately after plasma separation.

Statistical methods. Data are presented as mean values ± SEM. Statistical significance of differences between two groups was determined using Student's t test. A value of $P < 0.05$ was considered statistically significant.

Results and discussion

Hypothalamic 5-HT1B receptor and 5-HT2C receptor mRNA levels in fed and fasted mice

The hypothalamic 5-HT1B receptor mRNA levels and 5-HT2C receptor mRNA levels were significantly increased in 24-h fasted mice compared with fed mice (2-fold and 2-fold, respectively; Fig. 1A). Plasma active ghrelin levels were also significantly increased in 24-h fasted mice compared with fed mice (2.5-fold; Fig. 1B). These results indicate that increases in the expression of hypothalamic 5-HT1B receptor and 5-HT2C receptor genes are

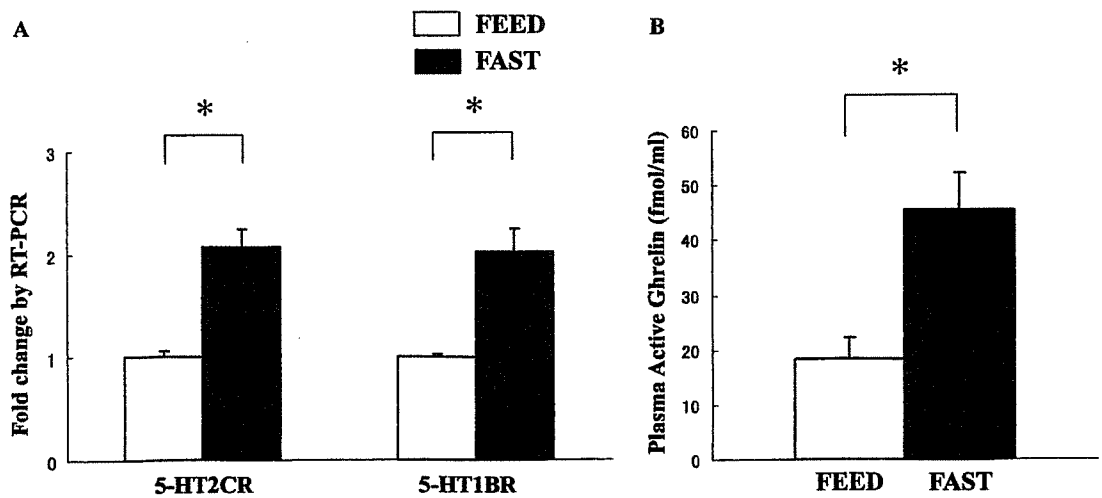

Fig. 1. Hypothalamic 5-HT2C receptor and 5-HT1B receptor mRNA levels (A) and plasma active ghrelin levels (B) in fed and 24-h fasted C57BL6J mice. Each column and bar represents the mean value ± SEM of six mice. *$P < 0.05$.

proportional to the increases in plasma active ghrelin levels after a 24-h fast.

Effects of mCPP or fenfluramine on plasma active ghrelin levels

To determine the effects of activation of brain 5-HT systems on plasma active ghrelin levels, we examined the effects of mCPP or fenfluramine on plasma levels of active ghrelin. Administration of either mCPP or fenfluramine significantly inhibited plasma active ghrelin levels after a 24-h fast (to 56% and 59% that of saline controls, respectively) (Fig. 2). These findings indicate that brain 5-HT systems via 5-HT2C receptors and/or 5-HT1B receptors play an inhibitory role in the regulation of plasma active ghrelin levels.

Effects of mCPP or fenfluramine on the expression of hypothalamic genes involved in the regulation of energy homeostasis

To determine the effects of mCPP or fenfluramine on the expression of the hypothalamic genes involved in the regulation of energy homeostasis, we examined the expression of hypothalamic POMC, CART, NPY, AGRP, and ghrelin genes that have an important role in the regulation of energy homeostasis [10,13,14]. mCPP slightly but significantly increased hypothalamic POMC and CART mRNA levels compared with saline controls (1.3- and 1.6-fold, respectively) while it had no significant effects on hypothalamic NPY, AGRP, and ghrelin mRNA levels (Fig. 3A). Fenfluramine significantly increased hypothalamic POMC and CART mRNA levels compared with saline controls (1.8- and 1.7-fold, respectively; Fig. 3B) while it had no significant effects on hypothalamic NPY, AGRP, and ghrelin mRNA levels (Fig. 3B). These results indicate that mCPP and fenfluramine increase the expression of hypothalamic POMC and CART genes without affecting the expression of hypothalamic NPY, AGRP, and ghrelin genes, and that

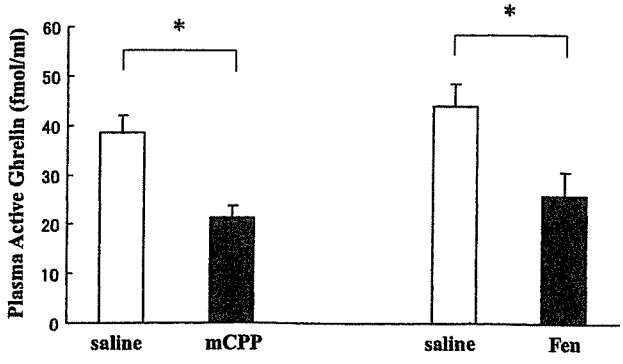

Fig. 2. Effects of mCPP (5 mg/kg) or fenfluramine (3 mg/kg) on plasma active ghrelin levels in C57BL6J mice. mCPP, fenfluramine, and saline were administered as described in Materials and methods. Each column and bar represents the mean value ± SEM of 8–12 mice. mCPP, *m*-chlorophenylpiperazine; Fen, fenfluramine. $^*P < 0.05$.

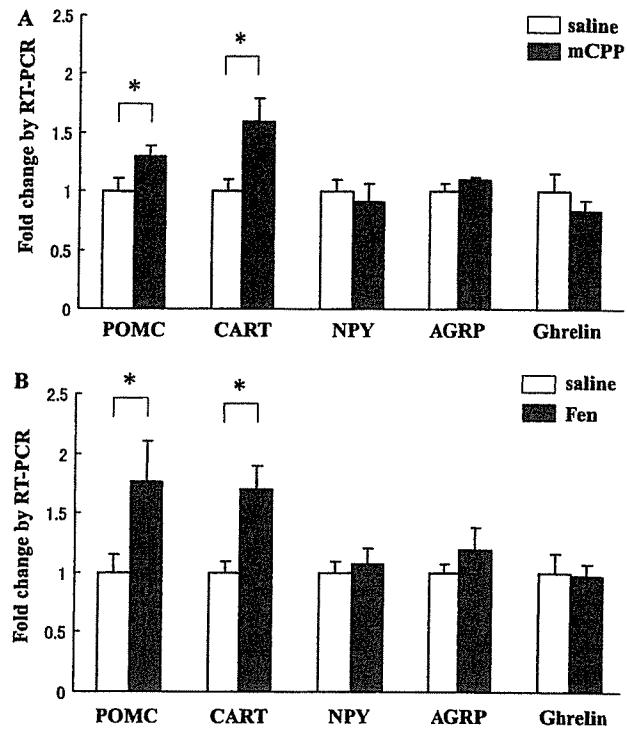

Fig. 3. Effects of mCPP (5 mg/kg) (A) or fenfluramine (3 mg/kg) (B) on hypothalamic POMC, CART, NPY, AGRP, and ghrelin mRNA levels in C57BL6J mice. mCPP, fenfluramine, and saline were administered as described in Materials and methods. Each column and bar represents the mean values ± SEM of five mice. POMC, pro-opiomelanocortin; CART, cocaine- and amphetamine-regulated transcript; NPY, neuropeptide Y; AGRP, agouti-related protein; mCPP, *m*-chlorophenylpiperazine; Fen, fenfluramine. $^*P < 0.05$.

mCPP increases hypothalamic CART gene expression more than POMC gene expression.

The present results of our study demonstrate that 24-h fasting increases the expression of hypothalamic 5-HT1B receptor and 5-HT2C receptor genes associated with increases in plasma active ghrelin levels, and that mCPP and fenfluramine, which exert the anorexic effects via 5-HT2C receptors and/or 5-HT1B receptors, inhibit the increases in plasma active ghrelin levels induced by fasting. These results indicate that there is be a negative feedback system between brain 5-HT systems via 5-HT2C receptors and/or 5-HT1B receptors and plasma ghrelin levels in the regulation of energy homeostasis in mice.

Ghrelin is localized to the hypothalamic arcuate nucleus (ARC) and the stomach [10,15,16]. In the hypothalamus, ghrelin neurons contact the cell bodies and dendrites of NPY/AGRP and POMC neurons [10,16]. Hypothalamic NPY has been shown to mediate the hyperphagic action of ghrelin [17]. However, our present results demonstrate that either mCPP or fenfluramine inhibits plasma active ghrelin levels without altered the expression of hypothalamic NPY, AGRP, and ghrelin gene. Because 5-HT2C receptors are located in the central nervous system, but not the stomach [1,18], the inhibitory effects of mCPP and fenfluramine on plasma active ghrelin levels might be

mediated by the central 5-HT system. Direct effects of 5-HT on ghrelin secretion by the stomach, however, cannot be completely ruled out.

POMC neurons have been shown to express 5-HT2C receptors in the ARC [19]. POMC neurons are depolarized in response to either fenfluramine or mCPP in coronal slices of the hypothalamus of transgenic mice expressing green fluorescent protein under control of the POMC promoter [19]. The present results demonstrate that both fenfluramine and mCPP significantly increase the expression of hypothalamic POMC gene, although fenfluramine increases it more than mCPP. However, all of 5-HT2C receptors are not expressed on POMC neurons, although some of them are expressed on POMC neurons in the ARC [19]. Moreover, 5-HT2C receptors are expressed in a variety of hypothalamic nuclei other than the ARC that are involved in the regulation of feeding and energy homeostasis [18]. Accordingly, not all signals induced by the activation of 5-HT2C receptors are transferred to the melanocortin pathway.

The genes for CART, an anorexigenic peptide, are expressed in the hypothalamus, including the ARC, paraventricular nucleus, lateral hypothalamus, supraoptic nucleus, and dorsomedial nucleus of the hypothalamus [20–22]. The present results demonstrate that either mCPP or fenfluramine induces an increase in the expression of the hypothalamic CART gene, the downstream pathway of which is different from the melanocortin pathway [21–23]. Not only POMC but also CART neurons in the hypothalamus might therefore contribute to the inhibition of plasma active ghrelin levels induced by mCPP or fenfluramine.

In summary, these results suggest that there is a negative feedback system between brain 5-HT systems via 5-HT2C and/or 5-HT1B receptors and plasma active ghrelin levels, and that mCPP or fenfluramine increases the expression of hypothalamic POMC and CART genes without altering the expression of hypothalamic NPY, AGRP, and ghrelin genes in mice. This is the first report of interactions between brain 5-HT systems and plasma active ghrelin, and of the effects of activation of 5-HT systems on POMC, CART, NPY, AGRP, and ghrelin gene expression in the hypothalamus in vivo.

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Cold Exposure Suppresses Serum Adiponectin Levels through Sympathetic Nerve Activation in Mice

Junta Imai,†‡¶ Hideki Katagiri,†‡¶ Tetsuya Yamada,*¶ Yasushi Ishigaki,* Takehide Ogihara,† Kenji Uno,*† Yutaka Hasegawa,*† Junhong Gao,*†‡ Hisamitsu Ishihara,* Hironobu Sasano,§ and Yoshitomo Oka**

Abstract

IMAI, JUNTA, HIDEKI KATAGIRI, TETSUYA YAMADA, YASUSHI ISHIGAKI, TAKEHIDE OGIHARA, KENJI UNO, YUTAKA HASEGAWA, JUNHONG GAO, HISAMITSU ISHIHARA, HIRONOBU SASANO, AND YOSHITOMO OKA. Cold exposure suppresses serum adiponectin levels through sympathetic nerve activation in mice. *Obesity.* 2006;14:1132–1141.

Objective: Several lines of evidence suggest important roles for adiponectin in glucose and lipid metabolism and atherosclerosis. However, the mechanisms regulating serum adiponectin levels and adiponectin production are still not completely understood. Our aim was to determine whether adiponectin synthesis is physiologically regulated by the sympathetic nervous system (SNS).

Research Methods and Procedures: Mice were exposed to cold (4 °C) for 12 hours and for 24 hours with or without inhibition of noradrenaline synthesis or pan-β adrenergic function, followed by measurement of serum adiponectin concentrations and levels of adiponectin and uncoupling protein (UCP) 1 expressions in various white adipose tissues (WATs).

Results: Cold exposure significantly reduced serum adiponectin concentrations without changing body weights or WAT sizes in either subcutaneous or intra-abdominal fat tissues. The serum adiponectin reduction was associated with a decrease in adiponectin mRNA expression in subcutaneous, epididymal, and mesenteric fat tissues. In these adipose tissues, UCP1 expression was markedly enhanced, suggesting SNS activation in these tissues. Administration of α-methyl-*p*-tyrosine or a combination of SR59230A and propranolol reversed the cold-exposure–induced decreases in serum adiponectin concentrations and adiponectin mRNA expression in these tissues. In contrast, in retroperitoneal fat, the effects of cold exposure on adiponectin and UCP1 expressions were strikingly weak but were not reversed by SNS inhibitors.

Discussion: SNS physiologically regulates serum adiponectin levels and adiponectin synthesis in WATs in vivo, although responsiveness to SNS stimulation differs markedly among WATs. Sympathetic activation might be involved in development of the metabolic syndrome by modulation of serum adiponectin concentrations.

Key words: white adipose tissue, adipocytokine, α-methyl-*p*-tyrosine, SR59230A, metabolic syndrome

Introduction

Obesity results from disruption of the balance between caloric intake and energy expenditure and is associated with insulin resistance (1). Insulin resistance is a fundamental contributor to the metabolic syndrome associated with type 2 diabetes, hypertension, hyperlipidemia, and atherosclerosis. Among the major advancements in this field were the discoveries of adipocyte-derived factors, such as leptin, adiponectin, free fatty acids (2), tumor necrosis factor-α (3), and plasminogen-activator inhibitor-1 (4). These adipocyte-derived factors play crucial roles in insulin sensitivity. Free fatty acids, tumor necrosis factor-α, and plasminogen-activator inhibitor-1 reportedly contribute to development of insulin resistance in vivo and in vitro (2–4), whereas adiponectin increases insulin sensitivity.

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¶ These authors contributed equally to the paper.
Address correspondence to Hideki Katagiri, Division of Advanced Therapeutics for Metabolic Diseases, Center for Translational and Advanced Animal Research, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan.
E-mail: katagiri@mail.tains.tohoku.ac.jp

Adiponectin was originally identified as a protein induced during adipogenesis (5–8). Low serum adiponectin levels were demonstrated in murine models of obesity and insulin resistance (6). In humans, serum adiponectin levels are inversely correlated with body weights and adiposity (9–11), suggesting important roles of adiponectin in the development of insulin resistance in obese subjects. In addition, adiponectin is reportedly involved in protection against atherosclerosis (12–14). Low serum levels of adiponectin are associated with atherogenesis in obese and insulin-resistant subjects (15,16). Therefore, to understand the pathophysiology of metabolic syndrome associated with insulin resistance and to develop novel therapeutic targets for the metabolic syndrome, it is essential to elucidate the mechanisms regulating adiponectin production and the resultant serum adiponectin levels.

There is considerable evidence that leptin is regulated by many types of stimuli (17–21). Notably, the sympathetic nervous system (SNS)[1] reportedly regulates leptin synthesis both in vitro and in vivo. In isolated brown and white adipocytes, leptin synthesis is suppressed by β3 adrenergic receptor (AR) agonists (18). In addition, SNS stimulation by acute cold exposure decreases leptin mRNA in white and brown adipose tissues (BATs) (22,23). Blockade of noradrenaline synthesis by α-methyl-*p*-tyrosine (α-MPT) causes marked increases in leptin mRNA in white adipose tissue (WAT) and plasma leptin levels (24). Thus, SNS activation suppresses leptin synthesis, thereby decreasing serum leptin in vivo.

In contrast, SNS involvement in adiponectin regulation is somewhat controversial. In 3T3-L1 adipocytes, isoproterenol, a β-adrenergic stimulant, reportedly reduced adiponectin mRNA, and this inhibitory effect was reversed by pretreatment with propranolol, a β-adrenergic antagonist (25). In mice, administration of a β-adrenergic stimulant reduced adiponectin mRNA expression in adipose tissue and serum adiponectin concentrations (26), suggesting SNS involvement in adiponectin regulation in vivo. However, Puerta et al. (22) reported that cold-exposure (18 hours at 6 °C)–induced SNS activation did not significantly affect adiponectin expression in WAT or the serum adiponectin concentrations in rats, although adiponectin mRNA levels were reduced in BAT. Conversely, cold exposure (24 hours at 4 °C) also reportedly increases serum adiponectin levels (27). Thus, the physiological role of SNS in adiponectin regulation is unclear and remains controversial. Therefore, to determine whether SNS is involved in physiological regulation of adiponectin synthesis in vivo, we exposed mice to cold environments with or without inhibition of noradrenaline synthesis or pan-β adrenergic function. To achieve inhibition of noradrenaline synthesis and β adrenergic function, mice were treated with α-MPT and a combination of SR59230A and propranolol, a β3 AR antagonist and a β1/β2 AR antagonist, respectively. We then examined the effects of these treatments on adiponectin expression in various WATs and on serum adiponectin concentrations. Herein, we report that, although the degrees of effects differed among WATs, cold exposure decreased adiponectin expression in subcutaneous, epididymal, and mesenteric fat tissues, resulting in significantly decreased serum adiponectin concentrations. Furthermore, the effects of cold exposure were suppressed by inhibition of noradrenaline synthesis or β adrenergic function. To our knowledge, this is the first study demonstrating that SNS activation physiologically influences serum adiponectin concentrations in vivo.

[1] Nonstandard abbreviations: SNS, sympathetic nervous system; AR, adrenergic receptor; BAT, brown adipose tissue; α-MPT, α-methyl-*p*-tyrosine; WAT, white adipose tissue; UCP, uncoupling protein; SE, standard error; PPAR, peroxisome proliferator activator.

Research Methods and Procedures

Animals

Animal studies were conducted under protocols in accordance with the institutional guidelines for animal experiments at Tohoku University. Ten-week-old male C57BL/6N mice were purchased from Clea (Tokyo, Japan), housed in an air-conditioned environment, with a 12-hour light-dark cycle, and fed a regular unrestricted diet. Mice were divided into two body-weight–matched groups; mice in the cold-stimulated group were placed in a cold room (4 °C) for 12 hours or for 24 hours with the same light-dark schedule as control mice, whereas for the control group, the temperature was 25 °C. In several experiments, 150 mg/kg body weight α-MPT (Sigma, St. Louis, MO) was intraperitoneally injected immediately before the exposure to 4 °C or 25 °C. In several other experiments, a combination of 1.5 mg/kg body weight SR59230A (Sigma) and 1.5 mg/kg body weight (*S*)-(−)-propranolol (Sigma) was intraperitoneally injected immediately before starting the experiment and then every 4 hours for the 24-hour duration of exposure to 4 °C or 25 °C. Body weights were measured before and after cold exposure. Food intake amounts during these periods were also determined. Blood samples were obtained before and immediately after cold exposure. The mice were then sacrificed and epididymal fat, subcutaneous fat, mesenteric fat, and retroperitoneal fat were rapidly removed, immediately frozen in liquid N_2, and stored at −80 °C until analysis.

Histological Analysis

Each adipose tissue was stained with hematoxylin and eosin. Total adipocyte areas were traced manually and analyzed. Cell diameters were measured for 100 or more adipocytes in each group.

Immunoassay

Serum adiponectin concentrations were measured using a mouse/rat adiponectin ELISA Kit (Otsuka Pharmacy, To-

kyo, Japan). The limit of sensitivity was 0.25 ng/mL, and the linearity limit was 8.0 ng/mL. Serum samples were diluted 1:10,000 with assay buffer before analysis.

Quantitative Reverse-Transcription-Polymerase Chain-Reaction-Based Gene Expression

Total RNA was isolated from 100 mg of mouse WAT and 50 mg of BAT with ISOGEN (Wako Pure Chemical Co., Osaka, Japan), and cDNA synthesis was performed with a First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN) using 1.0 μg of total RNA. cDNA synthesized from total RNA was evaluated in a real-time polymerase chain reaction quantitative system (Light Cycler Quick System 350S; Roche Diagnostics GmbH, Mannheim, Germany). The relative amount of mRNA was calculated with β-actin mRNA as the invariant control. The following oligonucleotide primers were used: adiponectin, CTGGAGAGAAGGGAGAGAAAGG (sense) and ACATTGGGAACAGTGACGCGGG (antisense); uncoupling protein (UCP) 1, TACCAAGCTGTGCGATGT (sense) and AAGCCCAATGATGTTCAGT (antisense); and β-actin, TTGTAACCAACTGGGACGATATGG (sense) and GATCTTGATCTTCATGGTGCTAGG (antisense).

Statistical Analysis

The statistical significance of differences between groups was assessed by unpaired Student's t test. Data are presented as means ± standard error (SE).

Results

Effects of Cold Exposure on Serum Adiponectin Concentration

First, to determine the effects of cold exposure on adiponectin synthesis and serum adiponectin concentrations, mice were exposed to cold (4 °C). Twenty-four-hour cold exposure did not affect the fat content of WAT. For example, epididymal fat weight did not differ between the two groups (Table 1). In addition, histological analysis revealed that there were no significant differences in cell diameters of white adipocytes, including subcutaneous, epididymal, mesenteric, and retroperitoneal fat tissues, between control and cold-stimulated mice (Figure 1).

Although 24-hour cold exposure did not significantly affect cell sizes in WAT, where adiponectin is mainly produced, serum adiponectin concentrations were significantly decreased in response to 24-hour cold exposure (Figure 2A). We therefore examined the time course of alterations in serum adiponectin levels in response to cold stimulation, measuring adiponectin levels at 3, 6, 9, 12, and 24 hours of cold stimulation. Serum adiponectin levels decreased gradually throughout the experiment, and the difference in serum adiponectin levels between control mice and cold-stimulated mice reached statistical significance by 24 hours (Figure 2B). These data suggest that sympathetic nerve activation is involved in the regulation of serum adiponectin levels, irrespective of white adipocyte sizes.

Body weights of 12-hour cold-stimulated mice were significantly lower than those of control mice that had been exposed to 25 °C for 12 hours, whereas those of 24-hour cold-stimulated mice had been restored to the control level (Table 1). Food intake amounts of cold-stimulated mice were significantly higher than those of control mice (Table 1). These results suggest that cold stimulation accelerates energy expenditure and that hyperphagia after 12 hours restores body weight. Serum insulin and leptin levels were increased after 24- but not 12-hour cold exposure, whereas no significant changes were seen in lipid levels. In

Table 1. Parameters of 12-hr and 24-hr cold-stimulated mice

	12 hr		24 hr	
Parameter (units)	Room temperature (n = 6)	Cold stimulation (n = 7)	Room temperature (n = 6)	Cold stimulation (n = 8)
Total body weight (g)	23.20 ± 0.18	21.38 ± 0.25*	23.83 ± 0.28	24.00 ± 0.39
Food intake (g)	ND	ND	3.46 ± 0.17	7.20 ± 0.13*
Epididymal fat weight (mg)	ND	ND	273.00 ± 11.9	268.67 ± 7.32
TG (mg/dl)	31.21 ± 5.21	25.19 ± 1.73	36.39 ± 2.88	38.31 ± 1.79
T-Chol (mg/dl)	86.88 ± 3.15	93.97 ± 2.11	82.72 ± 1.65	88.34 ± 1.19
FFA (mEq/l)	0.64 ± 0.10	0.72 ± 0.07	0.54 ± 0.029	0.21 ± 0.024*
Insulin (ng/ml)	1.20 ± 0.16	1.39 ± 0.12	1.37 ± 0.16	3.35 ± 0.77†
Leptin (ng/ml)	2.68 ± 0.18	2.22 ± 0.34	2.20 ± 0.40	4.33 ± 0.48†
Glucose (mg/dl)	160.20 ± 5.16	167.00 ± 4.09	161.00 ± 2.24	167.40 ± 4.50

Data are presented graphically as means ± standard error of the mean.
* $p < 0.01$, † $p < 0.05$ vs. control as assessed by unpaired t test.

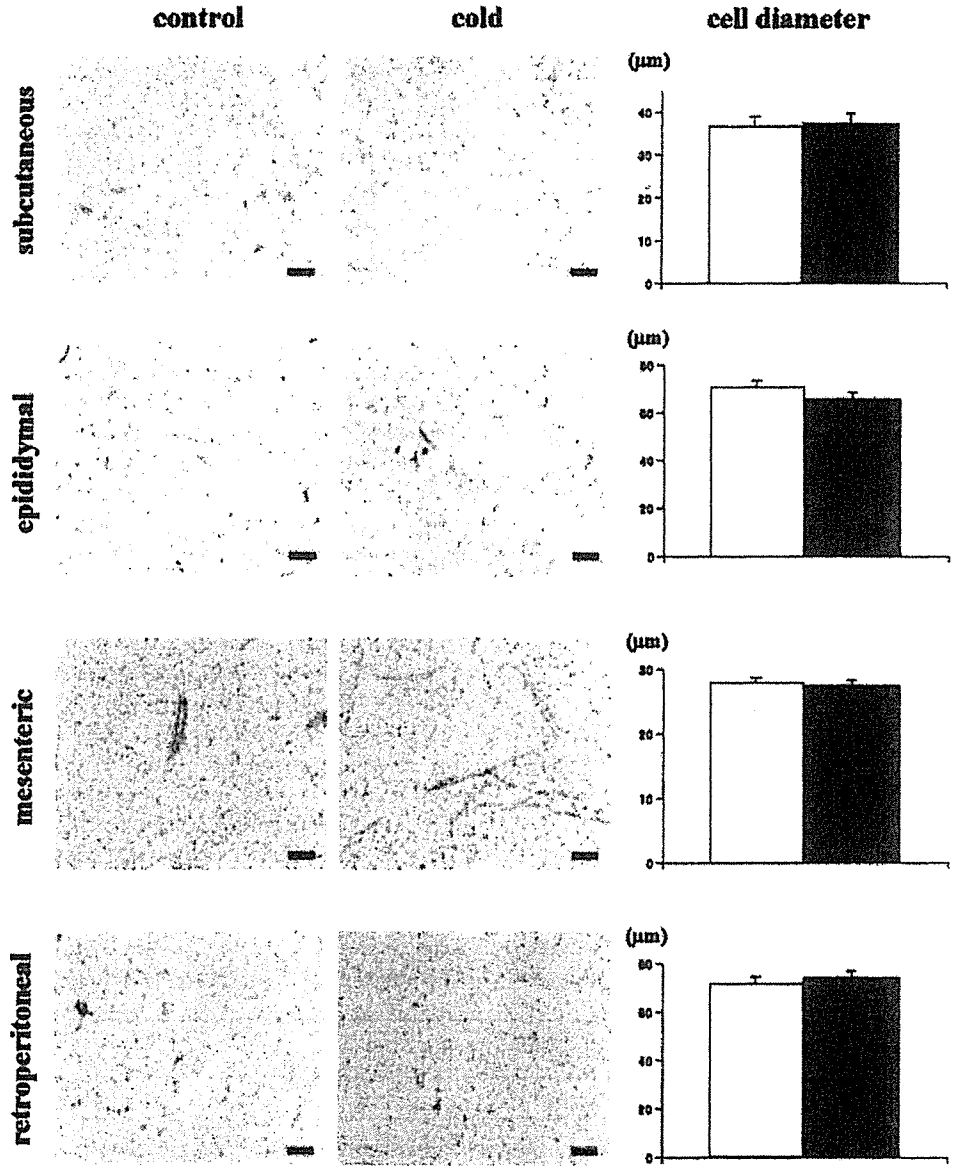

Figure 1: Effects of cold exposure on cell diameter in various WATs. Morphologies and cell diameters of adipocytes in subcutaneous, epididymal, mesenteric, and retroperitoneal fat tissues from control (white bars) and cold-stimulated (black bars) mice. Original magnification, ×100. Scale bar, 100 μm.

contrast, serum adiponectin levels were persistently decreased during cold stimulation (Figure 2B), suggesting it to be unlikely that these lipids and hormones have any major involvement in the adiponectin regulation observed in our experiments.

Next, to examine whether the serum adiponectin decrease induced by cold stimulation is due to decreased adiponectin production, we examined adiponectin mRNA expression levels in various WATs after 12- or 24-hour cold stimulation. Without cold stimulation, the adiponectin expression levels did not differ significantly among subcutaneous, mesenteric, epididymal, and retroperitoneal fat tissues (Figure 2C). Consistent with the results from the time course study of serum adiponectin levels, after 12-hour cold stimulation, adiponectin mRNA expressions in all adipose tissues examined tended to be decreased, but the differences did not reach statistical significance when compared with those in control mice. With 24-hour cold stimulation, de-

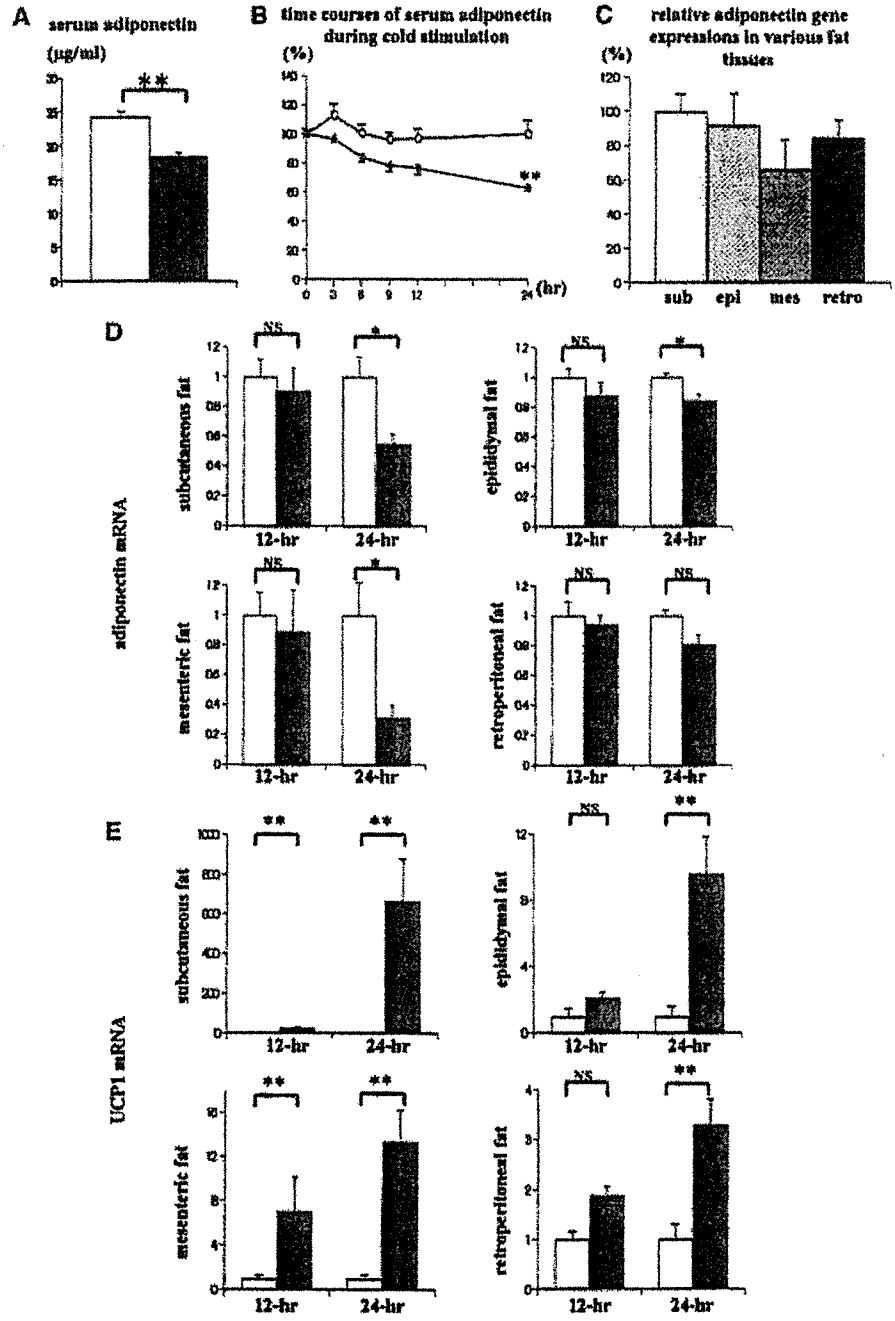

Figure 2: Effects of cold exposure on serum adiponectin concentrations, adiponectin, and UCP1 mRNA expressions in various WATs. (A) Serum adiponectin concentrations of control (open bars) and 24-hour cold-stimulated mice (gray bars) (control mice exposed to 25 °C, cold-stimulated mice to 4 °C). (B) Time courses of serum adiponectin in control (open circles) and cold-stimulated (filled circles) mice. *y*-Axis indicates percentage changes from baseline. (C) Relative adiponectin gene expressions in subcutaneous (sub), epididymal (epi), mesenteric (mes), and retroperitoneal (retro) fat. *y*-Axis indicates percent expressions of subcutaneous fat tissue. In each fat tissue, the relative amounts of adiponectin mRNA were calculated with β-actin mRNA as the invariant control. Data are presented graphically as means ± SE (n = 15 per each fat tissue). (D) Adiponectin mRNA expression levels in various WATs of control (open bars) and cold-stimulated (gray bars) mice after a 12- or 24-hour exposure to cold. (E) UCP1 mRNA expression levels in various fat tissues of control (open bars) and cold-stimulated (gray bars) mice after a 12-or 24-hour exposure to cold. Data are presented graphically as means ± SE (n = 6 per group). * $p < 0.05$ and ** $p < 0.01$ vs. control as assessed by unpaired Student's t test.

clines in adiponectin mRNA expressions in subcutaneous, epididymal, and mesenteric fat tissues became significant, whereas those in retroperitoneal fat tissue did not (Figure 2D). Thus, cold stimulation decreased adiponectin expression in various fat tissues, resulting in a decrease in serum adiponectin, although the effects of cold stimulation on adiponectin synthesis seem to differ markedly among various WATs. Taken together with the report that the number of cells in subcutaneous fat is ~75% of that in all adipose tissue of lean mice (28), adiponectin expression in subcutaneous fat is likely to contribute greatly to its serum concentration.

SNS activation reportedly increases UCP1 expression in WAT (29,30). Therefore, to investigate the degrees of activation of sympathetic nerves by various fat tissues in response to cold stimulation, we next examined UCP1 mRNA expressions in various WATs. In control mice (at 25 °C), the levels of UCP1 mRNA did not differ significantly among WATs, including subcutaneous, epididymal, mesenteric, and retroperitoneal fat (data not shown). In subcutaneous fat tissue, cold stimulation markedly increased UCP1 mRNA expression levels, 29- and 664-fold with 12- and 24-hour cold stimulation, respectively. With 12-hour cold stimulation, modest increases in UCP1 expression were observed in epididymal and mesenteric fat tissues, 2- and 7-fold; with 24-hour cold stimulation, there were further increases of 10- and 13-fold, respectively. On the other hand, the magnitude of the increment in UCP1 expression in retroperitoneal fat tissue was much smaller than those in other WATs (Figure 2E). Thus, cold stimulation activated sympathetic nerves to WATs, but the degree of the effects of cold stimulation, such as UCP1 induction, differed among WATs.

Effects of SNS Inhibitors on Serum Adiponectin Concentration after Cold Exposure

Next, to examine directly whether the serum adiponectin concentration decrease is due to SNS activation, mice were treated with α-MPT, a specific inhibitor of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of noradrenaline (31). In a previous study, to completely inhibit the SNS activation, mice were administered α-MPT intraperitoneally at a dose of 300 mg/kg body weight (24). In our preliminary study, therefore, we also administered 300 mg/kg body weight α-MPT. At this dose, however, all of the mice died during cold exposure, presumably due to diminished heat production resulting from complete blockage of SNS activation. Therefore, we administered 150 mg/kg body weight α-MPT. At this dose, ~70% of mice survived the full duration of cold exposure. We examined serum adiponectin concentrations and adiponectin expression levels in WAT using the mice that had survived throughout cold exposure. It is noteworthy that the inhibitory effects of α-MPT on SNS activation were partial, not complete.

With α-MPT administration, cold stimulation had no effect on body weights. The increment in food intake with cold stimulation was also observed in α-MPT-administered mice. Similarly, cold exposure had no impact on WAT weights (data not shown).

Serum adiponectin concentrations tended to be decreased in response to cold exposure with α-MPT administration, but the decreases were not statistically significant (Figure 3A). The magnitude of cold-exposure–induced decrements in serum adiponectin levels was significantly blunted by α-MPT administration (Figure 3B). Thus, partial inhibition of SNS activation reversed the serum adiponectin reduction, which clearly indicates involvement of SNS in regulating serum adiponectin levels. To further elucidate the mechanisms whereby SNS activation affects serum adiponectin concentrations, alterations in adiponectin mRNA levels in response to cold stimulation were examined in WAT with and without α-MPT administration. Administration of α-MPT reversed the cold-stimulation–induced decreases in adiponectin expression in subcutaneous, epididymal, and mesenteric fat tissues (Figure 3C). In retroperitoneal fat tissue, however, α-MPT administration had no effect on adiponectin expression (Figure 3C). These findings suggest that cold stimulation decreases adiponectin expression in various WATs by SNS activation, resulting in decreased serum adiponectin levels, although the degrees of the responses to cold stimulation differ markedly among WATs.

To confirm the diverse effects of SNS activation on various WATs, alterations in UCP1 mRNA levels in response to cold stimulation were examined in WAT with and without α-MPT administration. In subcutaneous fat tissue, although cold stimulation markedly enhanced UCP1 expression, this enhancement was inhibited with α-MPT administration (664- vs. 34-fold) (Figure 3D). In epididymal and mesenteric fat tissues, α-MPT administration clearly reversed the increase in UCP1 expression induced by cold stimulation (Figure 3D). In contrast, in retroperitoneal fat tissue, α-MPT administration did not significantly suppress UCP1 expression (Figure 3D). These findings indicated SNS involvement in the regulation of adiponectin expression in adipose tissues and the resultant serum adiponectin concentration, although the effects of cold stimulation were relatively small in retroperitoneal fat tissue.

Next, to determine whether β adrenergic function is involved in adiponectin regulation, mice were simultaneously administered 1.5 mg/kg body weight SR59230A, a β3 AR antagonist, and 1.5 mg/kg body weight (*S*)-(−)-propranolol, a β1/β2 AR antagonist. In a previous study, to inhibit sympathetic stimulation, these inhibitors were administered at the same doses that we used, and the effects were ana-

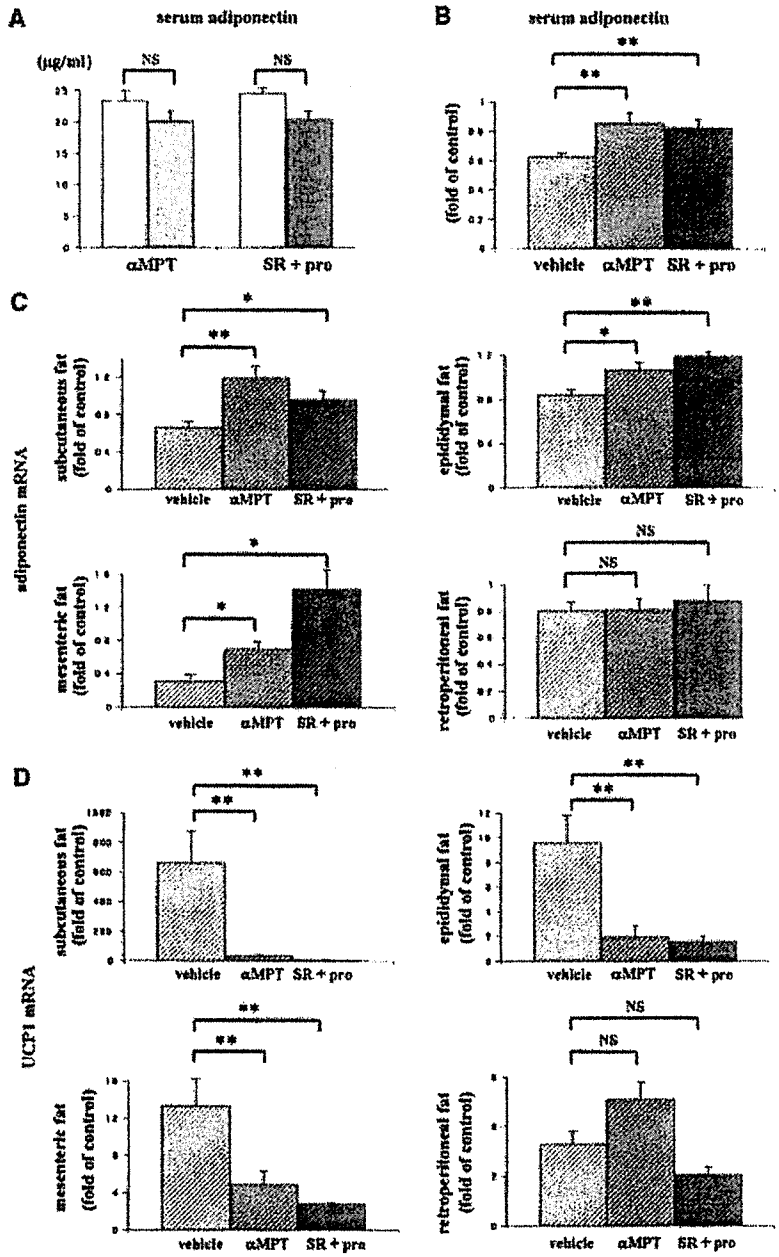

Figure 3: Effects of cold exposure with SNS inhibitor administration on serum adiponectin concentrations and adiponectin and UCP1 mRNA expressions in various WATs. (A) Serum adiponectin concentrations of control (open bar) and cold-stimulated mice given α-MPT (light gray bar) or a combination of SR59230A and propranolol (dark gray bar). Data are presented graphically as means ± SE (α-MPT-control; $n = 6$, α-MPT-cold stimulated; $n = 10$, SR + pro-control; $n = 4$, SR + pro-cold stimulated; $n = 5$). * $p < 0.05$ and ** $p < 0.01$ vs. control as assessed by unpaired Student's t test. (B) Magnitudes of serum adiponectin concentration change after cold exposure (vehicle) and those with α-MPT (α-MPT) or a combination of SR59230A and propranolol (SR + pro) administration. (C) Magnitudes of adiponectin mRNA expression changes after cold exposure (vehicle) and those with α-MPT (α-MPT) or a combination of SR59230A and propranolol (SR + pro) administration. (D) Magnitudes of UCP1 mRNA expression change after cold exposure (vehicle) and those given α-MPT (α-MPT) or a combination of SR59230A and propranolol (SR + pro). (B to D) Each parameter of 24-hour cold-stimulated mice was divided by the mean level in room-temperature-conditioned mice given the same treatment. Data are presented graphically as means ± SE (α-MPT-control, $n = 6$; α-MPT-cold-stimulated, $n = 10$; SR + pro-control, $n = 4$; SR + pro-cold-stimulated, $n = 5$). * $p < 0.05$ and ** $p < 0.01$ vs. cold exposure with vehicle.

lyzed 4 hours after administrations (32). Therefore, we administered these drugs to control and cold-stimulated mice every 4 hours for 24 hours. In our experiment, all mice survived after 24-hour cold exposure. The results were very similar to those obtained with α-MPT administration. Suppression of increased UCP1 expression in subcutaneous, epididymal, and mesenteric fat tissues confirmed effective blockade of β3 adrenergic function in these adipose tissues (Figure 3D). Adrenergic blockade inhibited the effects of cold stimulation on adiponectin expression in these fat tissues (Figure 3C) and serum adiponectin concentrations (Figure 3, A and B). In retroperitoneal fat tissue, administration of these β AR inhibitors did not significantly affect cold-exposure–induced alterations in the expression of adiponectin (Figure 3C) or UCP1 (Figure 3D). Our findings clearly show that the aforementioned adiponectin regulation by SNS is mediated mainly by β ARs. In addition, in retroperitoneal fat tissue, there appears to be little, if any, SNS involvement in metabolic activities.

Discussion

Several lines of evidence have recently suggested that adiponectin is involved in glucose and lipid metabolism. In mice, systemic adiponectin treatment increased fatty acid oxidation in muscle (33), decreased plasma glucose by enhancing the ability of insulin to suppress hepatic glucose production (34), and improved insulin sensitivity in insulin-resistant models (35). In addition, loss of the adiponectin gene in mice reportedly decreased insulin responsiveness and enhanced atherogenesis (16,36). Furthermore, a central action of adiponectin was reported: a decrease in body weight due to stimulation of energy expenditure (37). Together, these data strongly suggest that adiponectin plays an important role in preventing development of the metabolic syndrome.

Despite the physiological significance of adiponectin, the mechanism regulating serum adiponectin is still not completely understood. In the present study, cold exposure, which activates SNS physiologically, reduced adiponectin expression in various WATs, resulting in a significant decrease in the serum adiponectin concentration. In addition, administration of a noradrenaline synthesis inhibitor or β AR inhibitors inhibited cold-exposure–induced reductions in serum adiponectin levels and adiponectin expression in WATs. These findings clearly show that SNS activation, especially β stimulation, is involved in regulating the production of adiponectin and its serum levels. There is considerable evidence that adiponectin correlates negatively with body weight (9–11). In addition, adiponectin expression is reportedly decreased in enlarged adipocytes (38). However, in the present study, it is noteworthy that body weights and adipocyte sizes did not differ, regardless of 24-hour cold exposure and/or SNS inhibitor administration. These findings indicate that, independently of alterations in adiposity, SNS regulates adiponectin production and the resultant serum adiponectin levels.

Peroxisome proliferator activator (PPAR) γ reportedly increases adiponectin expression in WAT (39). However, in this study, no significant alterations were observed in PPARγ mRNA expression in various WATs of mice subjected to cold stimulation (data not shown), suggesting that cold-stimulated alteration of adiponectin mRNA expression in WAT is not mediated by increased PPARγ expression.

In contrast to our study, Puerta et al. (22) reported that, in rats, acute cold exposure did not significantly change adiponectin expression in WAT or the serum adiponectin concentration. The reasons for this discrepancy are not known, but differences in the magnitude and periods of cold exposure might explain the differing results. In our study, mice were exposed to 4 °C for 24 hours, whereas in the previous report, rats were subjected to milder cold stimulation, i.e., 6 °C for 18 hours. Alternatively, mice might be more sensitive to cold stimulation than rats. In any case, in the present study, administering a noradrenaline synthesis inhibitor or β AR inhibitors reversed the effects of cold stimulation, confirming involvement of SNS, especially β adrenergic function, in the regulation of adiponectin synthesis in WAT. Because it is well known that SNS is activated in obese subjects (40,41), decreased serum adiponectin levels in obese subjects might be, at least partly, attributable to SNS hyperactivity.

Another interesting finding in this study is the markedly different degrees of responsiveness to cold exposure among WATs. In response to cold stimulation, in subcutaneous fat tissue, UCP1 expression was increased 664-fold. In contrast, in retroperitoneal fat tissue, the increase was only 3- to 4-fold, although without cold stimulation, the UCP1 mRNA levels did not differ significantly among these WATs. In addition, although SNS inhibitor administration reversed the increases in UCP1 expression in subcutaneous, epididymal, and mesenteric fat tissues, such inhibitors did not affect UCP1 expression in retroperitoneal fat tissue. Furthermore, in contrast to other fat tissues studied, the decrease in adiponectin expression after cold exposure was not significant in retroperitoneal fat tissue. Thus, the metabolic alteration induced by SNS activation differs markedly among various fat tissues and is especially small in retroperitoneal fat tissue.

The differences in responsiveness among various WATs might be explained by how abundant the nerve supply to these fat tissues is. Alternatively, sympathetic nerves to retroperitoneal fat might not be fully activated by cold stimulation. Differential activations of SNS innervating different tissues, in response to various stimuli, have reportedly been observed. For instance, in contrast to cold stimulation, intracerebroventricular injection of 2-deoxyglucose, which activates many sympathetic nerves, suppresses the activation of sympathetic nerves to BAT (42). In addition, it

was recently reported that hypothalamic phosphoinositide 3-kinase and mitogen-activated protein kinase differentially regulate different regional sympathetic activations in response to intracerebroventricular injection of insulin (43). In the present study, we recognized that responsiveness to cold stimulation differs mechanistically among various WATs according to anatomical location. Although further studies are necessary for elucidating the mechanisms in detail, the different responses to SNS stimulation might be involved in the diverse characteristics of adipocytes according to anatomical location. Increased adiposity in various fat tissues contributes in different ways to the development of the metabolic syndrome (44,45), which might, at least in part, be explained by the magnitude of activation of sympathetic nerves to each fat tissue.

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