Macrophage infiltration into obese adipose tissues suppresses the induction of UCP1 level in mice

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1Laboratory of Molecular Function of Food, Division of Food Science and Biotechnology, Graduate School of Agriculture, Uji, Kyoto University, Kyoto, Japan; 2Center for Genetic Analysis of Behavior, National Institute for Physiological Sciences, Aichi, Japan; 3Research Unit for Physiological Chemistry, Center for the Promotion of Interdisciplinary Education and Research, Kyoto University, Kyoto, Japan

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Sakamoto T, Nitta T, Maruno K, YehYS, Kuwata H, Tomita K, Goto T, Takahashi N, Kawada T. Macrophage infiltration into obese adipose tissues suppresses the induction of UCP1 level in mice. Am J Physiol Endocrinol Metab 310: E676–E687, 2016. First published February 16, 2016; doi:10.1152/ajpendo.00028.2015.—Emergence of thermogenic adipocytes such as brown and beige adipocytes is critical for whole body energy metabolism. Promoting the emergence of these adipocytes, which increase energy expenditure, could be a viable strategy in treating obesity and its related diseases. However, little is known regarding the mechanisms that regulate the emergence of these adipocytes in obese adipose tissue. Here, we demonstrated that inflammation induced by infiltrated M1 macrophages (M1 MΦ) suppresses the induction of thermogenic adipocytes in obese adipose tissues of mice. Cold exposure significantly induced the expression levels of uncoupling protein-1 (UCP1), which is a mitochondrial protein unique in thermogenic adipocytes, in C57BL/6 mice fed a normal diet. However, UCP1 induction was significantly suppressed in adipose tissues of C57BL/6 mice fed a high-fat diet, into which M1 MΦ infiltrated. Depletion of M1 MΦ using clodronate liposomes eliminated the suppressive effect and markedly reduced the mRNA level of tumor necrosis factor-α (TNFα) in the adipose tissues. Importantly, consistent with the observed expression levels of marker genes for thermogenic adipocytes, combination treatment of clodronate liposome and cold exposure resulted in metabolic benefits such as lowered body weight and blood glucose level in obese mice. Moreover, intraperitoneal injection of recombinant TNFα protein suppressed UCP1 induction in lean adipose tissues of mice. Collectively, our data indicate that infiltrated M1 MΦ suppress the induction of thermogenic adipocytes in obese adipose tissues via TNFα. This report suggests that inflammation induced by infiltrated MΦ could cause not only insulin resistance but also reduction of energy expenditure in adipose tissues.

uncoupling protein 1; thermogenic adipocyte; inflammation; mitochondrial biogenesis; macrophage; tumor necrosis factor-α

BROWN ADIPOCYTES CARRY A UNIQUE MITOCHONDRIAL PROTEIN named uncoupling protein 1 (UCP1). The activation of UCP1 has a significant role in dissipating energy in the form of heat in response to external stimuli such as cold. This process is called nonshivering thermogenesis (34). Many studies have demonstrated that changes in brown adipose tissue (BAT) activity can profoundly affect fuel (such as glucose and lipid) metabolisms and body weight (4, 5, 11, 13, 16, 18). Increasing whole body energy expenditure is an effective strategy for combating obesity and obesity-related metabolic diseases such as type 2 diabetes (40). Numerous recent studies have indicated that adult humans maintain potentially active BAT, making this highly energetic tissue a promising therapeutic target for the treatment of obesity (8, 35, 42, 44).

Recent advancements in the field have led to the identification of “brown-like” adipocytes, also called “beige” adipocytes, that exhibit thermogenic activity in white adipose tissues (WAT) in response to certain pharmacological stimulations or external stimuli such as cold (3, 7, 12, 22). The induction of beige adipocytes in WAT is called “browning.” Their origin is different from that of classical brown adipocytes, which are exemplified by the interscapular BAT of mice (37). Notably, beige adipocytes increase the oxygen consumption rate in response to adrenergic stimulation (28), and browning is highly correlated with insulin sensitivity and improvement of whole body energy metabolism (4, 6, 38). These reports strongly suggest the involvement of antiobesity and antidiabetic activities in the browning of WAT.

Chronic inflammation of obese WAT can lead to the development of metabolic disorders (14, 15). Macrophages (MΦ) are attracted by chemokines such as monocyte chemoattractant protein-1 (MCP1) released from these hypertrophied adipocytes. Two populations of MΦ have been recognized in adipose tissues: the classically activated MΦ (M1 MΦ) that exhibit proinflammatory properties and the alternatively activated MΦ (M2 MΦ) that display anti-inflammatory properties (19). M1 MΦ have been shown to secrete a variety of proinflammatory cytokines, such as TNFα (19, 39), leading to insulin resistance (47). In addition, we reported previously that TNFα derived from activated RAW 264.7 MΦ suppresses the induction of Ucp1 expression level in C3H10T1/2 adipocytes differentiated from mesenchymal stem cells (36). This finding suggests that inflammation induced by infiltrated M1 MΦ could suppress the emergence of thermogenic adipocytes in obese adipose tissues.

In the present study, we investigated the effects of TNFα on the induction of thermogenic adipocytes in C57BL/6 and KK-Ay mice with the obese condition. We found a possible role of TNFα in mediating the suppression of the emergence of thermogenic adipocytes in vivo.

MATERIALS AND METHODS

Animal experiments. We used 5-wk-old male C57BL/6 mice (CLEA Japan, Tokyo, Japan) fed either the standard diet (CRF-1; Charles River Japan, Tokyo, Japan) or 60% high-fat diet (HFD; 193-1849/16 Copyright © 2016 the American Physiological Society http://www.ajpendo.org
D12492; Research Diets) for 16 wk for the diet-induced obese model. We also used 5-wk-old male KK-Ay mice (CLEA Japan), a useful model of obesity and diabetes, and KK mice (CLEA Japan), a control for KK-Ay mice, and fed them the normal diet (ND) (MF; Oriental Yeast, Tokyo, Japan) for 5–7 wk. All animals were maintained in a temperature-controlled (23°C) facility with a constant 12-h light-dark cycle and free access to water and the above-mentioned diets. For the induction of UCP1 expression due to cold exposure, the mice were exposed to 4°C for 24 h, with other conditions the same as those described above. After euthanization, interscapular BAT, inguinal WAT (iWAT), and epididymal WAT (eWAT) were dissected and frozen in liquid nitrogen.

To examine the effects of inflammation induced by infiltrated Mφ on UCP1 expression in the adipose tissues, we used Clophosome-Liposomal Clodronate (Neutral) (FormuMAX Scientific) to induce apoptosis in Mφ. Eight-week-old male KK-Ay mice fed a HFD for 3 wk were injected with 30 mg/kg of clodronate liposomes or an equal volume of control liposomes containing PBS (PBS-liposome) intraperitoneally. After 48 h, the mice were exposed to 4°C for 24 h. After euthanization, BAT, iWAT, and eWAT were dissected and frozen in liquid nitrogen.

For measurements of locomotor activity and food intake, the mice were acclimated to the single housing environment for 48 h, locomotor activity data was collected with ACTIMO-100 (Shin factions, Japan), and the food intake data were collected manually. Cumulative ambulatory counts were recorded at 5-min intervals during the 48-h period following the administration of the clodronate liposomes.

Rectal temperature was monitored using a thermos recorder TR-715 (T & D, Matsumoto, Japan) in indicated time.

Blood samples were harvested during the fed state. Plasma insulin levels were measured using enzyme-linked immunosorbent assay kits (Morinaga Institute of Biological Science, Tokyo, Japan), and plasma glucose levels were measured using the glucose CII-test Wako kit (Wako Pure Chemicals, Osaka, Japan). To investigate the effects of TNFα on the induction of UCP1 expression in adipose tissues, 5-wk-old C57BL/6 mice were intraperitoneally injected with ~100 μg/kg body wt recombinant TNFα protein (Peprotech). After 24 h, another injection was administered, and the mice were exposed at 4°C for 24 h. After euthanization, BAT, iWAT, and eWAT were dissected and frozen in liquid nitrogen.

All animal care procedures and methods were approved by the Animal Care Committee of Kyoto University.

Histochemistry. Adipose tissues removed from each animal were fixed in 4% paraformaldehyde in PBS and maintained at 4°C until use. The fixed samples were embedded in paraffin. They were cut into 5- or 8-μm sections using a microtome and mounted on silanized slides. For hematoxylin-eosin staining, the sections were deparaffinized and stained with hematoxylin and eosin (Merck Millipore, Billerica, MA). For immunohistochemistry, tissues were deparaffinized and treated with 3% hydrogen peroxide in Tris-buffered saline (TBS) to inactivate endogenous peroxidases and blocked with 5% normal goat serum in TBS to reduce nonspecific staining. Subsequently, sections were incubated overnight at 4°C with anti-mouse F4/80 (AbD Serotec) or anti-mouse CD11c hamster antibody (Abcam) at 1:100 dilution as primary antibodies. After three washes in TBS with Tween-20 (TBST) buffer, incubation was performed at room temperature for 1 h with biotinylated anti-rat IgG antibody (Vector Laboratories) or anti-hamster IgG antibody (Vector Laboratories) at 1:200 dilution as secondary antibodies. The sections were further incubated with HRP-conjugated streptavidin (Merck Millipore) at 1:100 dilution, and immunoreactive proteins were detected using the Peroxidase Stain DAB Kit (Nakalai Tesque, Kyoto, Japan). The slides were observed using a light microscope (IX71N-22FL/PH-SP; Olympus, Tokyo, Japan), and images were captured using the Olympus DP Controller software (version 3.2.1.276; Olympus).

RNA preparation and quantification of gene expression. Total RNA was prepared from adipose tissues using the Sepasol-RNA I Super G reagent (Nacalai Tesque) according to the manufacturer’s instructions. Total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer’s instructions using a thermal cycler (Takara PCR Thermal Cycler SP; Takara, Shiga, Japan). To quantify mRNA expression, real-time RT-PCR was performed with a LightCycler System (Roche Diagnostics, Mannheim, Germany) using SYBR Green fluorescence signals, as described previously (36). The oligonucleotide primers were designed using Primer-BLAST, a primer designing software tool publicly available at http://www.ncbi.nlm.nih.gov/tools/primer-blast. The primers used for measuring the mRNA expression levels of genes are listed in Table 1. To compare mRNA expression levels among the samples, the copy numbers of all transcripts were divided by that of mouse 36B4, showing a constant expression level in adipose tissues and adipocytes. All mRNA expression levels were represented as a ratio relative to that of the control in each experiment. The mRNA expression level of 36B4 was stable under all conditions.

Immunoblotting. Isolation of the mitochondrial fraction from adipose tissues was performed as described previously (22). Mitochondrial proteins were solubilized in the lysis buffer [50 mM Tris·HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS (pH 7.4), and a protease inhibitor cocktail]. Protein concentrations of the samples were determined using a protein assay kit (Bio-Rad Laboratories). Mitochondrial protein samples (10 μg for iWAT and eWAT, 0.5 μg for BAT) were subjected to SDS-PAGE on a 12.5% gel. Separated proteins were transferred electrophoretically to PVDF membranes (Merck Millipore), which were blocked with 5% nonfat dried milk in TBS with Tween-20. The membranes were incubated with an antibody against UCP1 (Sigma-Aldrich) or cytochrome c oxidase subunit IV (COX4; Cell Signaling Technology) and then with peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies (Santa Cruz Biotechnology). Blots were developed using an Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) and detected using the Alpha Innotech Multigem II Light Cabinet With Camera & Filters (Alpha Innotech, San Leandro, CA).

To examine the effects of inflammation induced by infiltrated Mφ on UCP1 expression in the adipose tissues, we used Clophosome-Liposomal Clodronate (Neutral) (FormuMAX Scientific) to induce apoptosis in Mφ. Eight-week-old male KK-Ay mice fed a HFD for 3 wk were injected with 30 mg/kg of clodronate liposomes or an equal volume of control liposomes containing PBS (PBS-liposome) intraperitoneally. After 48 h, the mice were exposed to 4°C for 24 h. After euthanization, BAT, iWAT, and eWAT were dissected and frozen in liquid nitrogen.

Table 1. Primer Sequences For RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ to 3’)</th>
<th>Official Full Name</th>
</tr>
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<tbody>
<tr>
<td>36B4</td>
<td>TGT TGT TCT GCA GAT GGA GTA C</td>
<td>Ribosomal protein, large, P0</td>
</tr>
<tr>
<td>Arg1</td>
<td>GAT GAG ATT ACA CAT GAC GGA ATT C</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>Cox4i1</td>
<td>GAC CAT TGG CAC GAG CAT CTT</td>
<td>Cytochrome c oxidase subunit IV</td>
</tr>
<tr>
<td>Cpt1b</td>
<td>CTG TGG GGC CTC AAG ACC GAA C</td>
<td>Carnitine palmityltransferase A1b (muscle)</td>
</tr>
<tr>
<td>Cycs</td>
<td>CCA ATT GCT CAC GCT CTT TT</td>
<td>Cytochrome c somatic</td>
</tr>
<tr>
<td>F4/80</td>
<td>TTT CTT CCT GTG CTT CTT C</td>
<td>EGF-like module containing, mucin-like, hormone receptor-like sequence 1</td>
</tr>
<tr>
<td>Mepl</td>
<td>GAC CCC AAG AAG GAA TGA GT</td>
<td>Chemokine (C-C motif) ligand 2</td>
</tr>
<tr>
<td>Pparc1a</td>
<td>CCG TGC CAT TGT TAA GAC C</td>
<td>Peroxisome proliferator-activated receptor-γ coactivator-1α</td>
</tr>
<tr>
<td>Tbx1</td>
<td>GGC AGG CAG GAG AAT GCT C</td>
<td>T-box 6</td>
</tr>
<tr>
<td>Tmem26</td>
<td>ACC TGG TCA TCC CAC AGA G</td>
<td>Transmembrane protein 26</td>
</tr>
<tr>
<td>Tnfα</td>
<td>ACA TGA CAT GAT CTT CTC AAA ATT C</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>Ucp1</td>
<td>GTC CTC TGT CAG ATG TAC TAG T</td>
<td>Uncoupling protein 1</td>
</tr>
<tr>
<td>Ym1</td>
<td>ACA AGG GAG TTA CAC ACC TG</td>
<td>Chitinase-like 3</td>
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Images were captured using the AlphaEaseFc software (version 3.1.2, Alpha Innotech, San Leandro, CA). Immunoreactive protein bands were quantified by using the ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analyses. Means of two groups were compared by Student’s t-test. To compare the means of several groups, two-way ANOVA was used, followed by Tukey-Kramer’s multiple comparison test. Data are expressed as means ± SE. Differences were considered significant at *P* < 0.05.

**RESULTS**

The induction of UCP1 expression was suppressed in obese adipose tissues. To examine the induction of UCP1 expression in obese adipose tissues, we exposed C57BL/6 mice fed a HFD for 16 wk to cold temperature (4°C) for 24 h. Body weights and adipose tissue weights of HFD-fed mice were markedly increased (data not shown). Cold exposure increased the expression levels of *Ucp1* in iWAT and BAT of mice fed the ND. However, the induction was significantly suppressed in adipose tissues of obese mice (Fig. 1A). Based on the Cq values from quantitative PCR, under the basal condition, the *Ucp1* mRNA levels are ~8,000-fold higher in BAT than in iWAT and eWAT. Under the cold exposure condition, the levels are ~180-fold higher in iWAT than in eWAT and some 30,000-fold higher in BAT than in eWAT. The protein expression levels of *Ucp1* were consistent with the mRNA expression levels, except for *Ucp1* level in the BAT of the HFD group (Fig. 1B). The internal control 36B4 levels were not changed under each condition in each adipose tissue. These data indicate that the changes in *Ucp1*/36B4 levels under each condition were true induction and reduction of *Ucp1* transcript level (Fig. 1C).

We also tested KK-Ay mice, which are a useful model of obesity and diabetes, in the cold exposure experiments. When 9-wk-old KK-Ay mice were used, the results obtained were similar to those observed with HFD-fed C57BL/6 mice. In contrast to KK mice, which are a control model for KK-Ay mice, the induction of *Ucp1* expression by cold exposure was significantly suppressed in adipose tissues of KK-Ay mice (Fig. 1D). The UCPI protein levels were not changed in the BAT (Fig. 1E), but the trends in the UCPI protein levels were consistent with the *Ucp1* mRNA levels in iWAT (Fig. 1E). UCPI protein was not detected in the eWAT (Fig. 1E). These observations were consistent with the results derived from the diet-induced obese model, except for BAT. These data suggest that the induction of UCPI levels and the emergence of thermogenic adipocytes are suppressed in obese adipose tissues.

Obesity was associated with an increase in Mφ infiltration into adipose tissues. We reported previously that inflammation induced by activated RAW Mφ suppresses the induction of *Ucp1* expression in C3H10T1/2 adipocytes (36). In this study, we examined whether chronic inflammation due to HFD could suppress the induction of UCPI expression in obese adipose tissues. First, we examined Mφ content in the obese adipose tissues. Hematoxylin-eosin staining revealed that the numbers of crown-like structures, which were considered to be the accumulation of immune cells such as Mφ (19), were increased in iWAT of HFD-fed C57BL/6 mice. In contrast, such infiltration was rarely detected in mice fed the ND (Fig. 2A). Consistent with the observed increase in the numbers of crown-like structures in the obese adipose tissues, HFD feeding for 16 wk increased the mRNA expression levels of *F4/80* and *Tnfa*, which are macrophage marker genes (Fig. 2B). We also confirmed the higher levels of *F4/80* in the adipose tissues of KK-Ay mice compared with KK mice under ND feeding conditions (data not shown).

**Clodronate liposomes preferentially eliminated M1 Mφ in obese adipose tissues of KK-ay mice.** To examine the effects of inflammation induced by the infiltrated Mφ on the induction of thermogenic adipocytes in obese adipose tissues, we used clodronate liposomes to specifically promote apoptosis in Mφ (43), using the protocol as represented in Fig. 3A. HFD feeding promoted Mφ infiltration in the iWAT of KK-Ay mice in a time-dependent manner, and 3-wk HFD feeding significantly increased Mφ infiltration in iWAT of KK-Ay mice based on the hematoxylin-eosin staining (Fig. 3B) and the mRNA expression levels of *F4/80* and *Tnfa* (Fig. 3C). As shown in Fig. 3D, hematoxylin-eosin staining showed that the intraperitoneal injection of 30 mg/kg clodronate liposomes resulted in lower numbers of crown-like structures than the injection of PBS-liposomes in iWAT of obese KK-Ay mice fed a HFD. Immunohistochemistry also showed lower amounts of *F4/80*- and CD11c-positive (Fig. 3D) macrophages, which are considered to be M1 Mφ (19) in the crown-like structures of the clodronate liposome-injected group. In addition, the injection of clodronate liposomes significantly decreased the mRNA expression levels of *F4/80* and *Mcp-1*, which are global Mφ marker genes, and *Tnfa* and *Il-6*, which are typical M1 Mφ marker genes in iWAT (Fig. 3E). The mRNA expression level of *Tnfa* in the clodronate liposome-injected group was the same level as in the non-HFD feeding group (Fig. 3C). *F4/80* level was not the same as the level in the non-HFD feeding group, but the level was significantly reduced by the clodronate liposomes (Fig. 3C). In BAT, the injection of clodronate liposomes also decreased the mRNA expression levels of *F4/80* and *Tnfa* significantly (Fig. 3E).

In contrast, mRNA expression levels of chitinase-like 3 (Ym1) and arginine-1 (Arg1), which are marker genes for M2 Mφ, were not changed by the injection of clodronate liposomes (Fig. 3E). Taken together, these data indicate that clodronate liposomes can preferentially eliminate M1 Mφ in obese adipose tissues of KK-Ay mice. Interestingly, in the BAT, the clodronate liposomes tended to decrease the Arg1 level but not Ym1. This might suggest that the clodronate liposomes decreased both the M1 and M2 macrophages in the BAT (Fig. 3E). This result is partially consistent with a previous report (23).

**M1 Mφ suppressed the induction of thermogenic adipocytes in obese adipose tissues.** To examine whether the depletion of M1 Mφ could promote the emergence of thermogenic adipocytes in obese adipose tissues, we challenged KK-Ay mice depleted of M1 Mφ (by treatment with clodronate liposomes) with cold temperature (Fig. 4A). The injection of clodronate liposomes markedly restored the suppression of *Ucp1* induction in iWAT, but not BAT, of KK-Ay mice (Fig. 4B). Moreover, the trend of UCPI protein expression levels is the same as the *Ucp1* mRNA expression levels (Fig. 4C). These data suggest that in iWAT, M1 Mφ suppresses the induction of thermogenic adipocytes.

We measured the expression levels of mitochondrial genes, including *cytochrome c*, somatic (*Cycs*), carnitine palmitoyl-
transferase 1B (muscle) (Ctp1b), and peroxisome proliferator-
activated receptor-γ coactivator-1α (Ppargc1a), which is the
master regulator for mitochondrial biogenesis, to examine
whether the injection of clodronate liposomes increases mito-
chondrial biogenesis in iWAT. As shown in Fig. 4D, in the
iWAT of obese KK-Ay mice, cold exposure did not signifi-
cantly increase the mRNA levels of mitochondrial genes and
Ppargc1a. The combination treatment with clodronate lipo-
somes and cold exposure did not induce the mitochondrial gene
expression levels compared with cold exposure alone. The

**Fig. 1.** Cold exposure-induced uncoupling protein 1 (UCP1) expression levels are sup-
pressed in obese adipose tissues of C57BL/6 and KK-Ay mice. A and B: mRNA (A) and
protein (B) expression levels of UCP1 in obese adipose tissues of C57BL/6 mice fed a
high-fat diet (HFD) for 16 wk. Values are means ± SE for 5–8 mice. Average $C_q$ values
are 12.67 [normal diet (ND) room temperature (RT) in brown adipose tissue (BAT)], 11.55
(HFD RT in BAT), 11.40 (ND cold in BAT), 10.61 (HFD cold in BAT), 28.27 [ND RT in
inguinal white adipose tissue (iWAT)], 25.07 (HFD RT in iWAT), 20.13 (ND cold in
iWAT), 23.26 (HFD cold in iWAT), 25.93 (ND RT in eWAT), 24.80 (HFD RT in
eWAT), 24.24 (ND cold in eWAT), and 25.10 (HFD cold in eWAT). C: $2^C_q$ of 36B4 in
obese adipose tissues of C57BL/6 mice fed HFD for 16 wk. Values are means ± SE for
5–8 mice. D: mRNA expression level of Ucp1 in obese adipose tissues of KK and KK-Ay
mice. Values are means ± SE for 4 mice. E: protein expression levels of UCP1 in KK and
KK-Ay mice fed a ND for 5–7 wk. Average $C_q$ values are 11.21 (BAT in KK RT), 9.77
(BAT in KK cold), 9.73 (BAT in KK-Ay RT), 10.73 (BAT in KK-Ay cold), 9.73 (BAT in
KK-Ay cold), 14.87 (iWAT in KK RT), 13.04 (iWAT in KK cold), 17.56 (iWAT in KK-Ay RT), 14.29 (iWAT in
KK-Ay cold), 24.86 (eWAT in KK RT), 22.88 (eWAT in KK cold), 24.08 (eWAT in KK-Ay RT),
and 23.80 (KK-Ay cold). *$P < 0.05$, compared between indicated groups. COX4, cytochrome
$\alpha$ oxidase subunit IV isoform 1.
after the injection of clodronate liposomes, the subjected the shown experimental condition (Fig. 5). Forty-eight hours obese KK-Ay mice, we measured the rectal temperature under liposomes and cold exposure affect the thermogenic ability in with clodronate liposomes.

mitochondrial biogenesis.

thermogenic adipocytes through mechanisms independent of these data suggest that M1 M\textsubscript{1}/H9278

Expression of these genes was significantly increased by cold exposure and clodronate liposomes (Fig. 4). Taken together, these data support the restoration of UCP1 expression level by the cold exposure, but this induction was not significant. These data indicate that the restoration of UCP1 expression level by the injection of clodronate liposomes was not due to mitochondrial biogenesis.

Cold tolerance was improved in obese KK-Ay mice treated with clodronate liposomes. To examine whether the clodronate liposomes and cold exposure affect the thermogenic ability in obese KK-Ay mice, we measured the rectal temperature under the shown experimental condition (Fig. 5). Forty-eight hours after the injection of clodronate liposomes, the subjected obese KK-Ay mice were exposed to cold temperature for 24 h (1st cold exposure) for the induction of thermogenic adipocytes and then to room temperature for 2 h. After the room temperature exposure, we again exposed the KK-Ay mice to cold temperature for 6 h (2nd cold exposure). As shown in Fig. 5, during the first cold exposure, the rectal temperature in the clodronate liposome-injected group was slightly higher than in the PBS-injected group, but the differences were not significant. However, consistent with the observed induction of UCP1 and the other browning marker levels, during the second cold exposure the rectal temperatures in the clodronate liposome-injected group were significantly higher compared with the PBS-injected group. Taken together, these data suggest that the clodronate liposome-mediated M\textsubscript{1} M\textsubscript{1}/H9278 elimination can help the induction of thermogenic adipocytes, leading to increase in the thermogenic ability in obese KK-Ay mice.

Combination treatment of clodronate liposomes and cold exposure reduced blood glucose level and body weight of obese KK-ay mice. Recently, the activation of thermogenic adipocytes has been shown to be involved in the improvement of whole body metabolism in humans and rodents (6, 21, 38, 48).
To investigate the effects of M1 Mφ depletion in obese adipose tissues on whole body metabolism, we measured some metabolic parameters, including the body weight, blood glucose, and insulin levels of KK-Ay mice. Neither cold exposure nor the injection of clodronate liposomes changed the blood glucose levels or body weights of obese KK-Ay mice. Levels of marker genes for thermogenic adipocytes, the combination treatment with cold exposure and clo-
Clodronate liposomes significantly reduced both body weight of obese KK-Ay by 4.0 g (data not shown) and the glucose level in their blood (Fig. 6A). The clodronate/liposome injection did not affect the food consumption 48 h after the injection (Fig. 6B) or their locomotor activity at 24, 36, and 45 h after the injection (Fig. 6C).

Cold exposure significantly reduced the insulin level of the KK-Ay mice. The combination treatment tended to
expression levels of Cys and Cox4i1, which are marker genes for mitochondrial biogenesis. The effects of cold exposure and TNFα administration on mitochondrial biogenesis were tissue specific. In BAT, cold exposure, but not TNFα administration, induced the expression level of Cys. Neither cold exposure nor TNFα injection affected the mRNA expression levels of Cox4i1 (Fig. 8A). In iWAT, cold exposure increased the mRNA expression level of Cys, but not Cox4i1, whereas TNFα administration had no effect on the mRNA expression levels of either gene (Fig. 8B). In eWAT, the administration of TNFα significantly decreased the mRNA expression levels of Cys and Cox4i1, but cold exposure did not affect these levels (Fig. 8C). These data suggest that suppression of UCP1 induction by TNFα is not associated with the suppression of mito-

decrease the insulin level compared with only the cold exposure group, but the difference was not significant (Fig. 6D). Taken together, these data suggest that chronic inflammation induced by M1 Mφ leads to dysregulation of whole body energy metabolism, which could be partially caused by the suppression of thermogenic adipocyte induction.

Intraperitoneally administered recombinant TNFα protein suppressed the induction of UCP1 protein expression in BAT and iWAT of lean C57BL/6 mice. Our previous study has indicated that TNFα derived from activated Mφ as a potential proinflammatory cytokine that suppresses the induction of Ucp1 mRNA expression in white adipocytes (36). In this study, we sought to confirm the effects of TNFα on the induction of UCP1 expression by intraperitoneal administration of 100 μg/kg recombinant TNFα protein into lean 5-wk-old C57BL/6 mice. Administration of recombinant TNFα protein suppressed the cold-induced Ucp1 expression in adipose tissues of C57BL/6 mice (Fig. 7A). The protein expression level of UCP1 was also suppressed by the TNFα administration in BAT and iWAT (Fig. 7, B and C). Cold exposure increased Ucp1 mRNA expression level, but we could not observe protein expression in the eWAT (Fig. 7B). These data support the role of TNFα as a proinflammatory cytokine that suppress the induction of UCP1 expression in adipose tissues. Moreover, TNFα released by M1 Mφ into obese adipose tissues could act as a mediator in suppressing the induction of thermogenic adipocytes.

Recombinant TNFα protein did not suppress mitochondrial biogenesis in iWAT or BAT of lean C57BL/6 mice. Next, we examined the effects of TNFα administration on mitochondrial biogenesis in adipose tissues. We investigated the mRNA

Fig. 5. M1 Mφ depletion using clodronate liposomes improves cold tolerance in obese KK-Ay mice fed HFD. Rectal temperature under the shown experimental condition in obese KK-Ay mice indicated hours after the 1st cold exposure was started. Values are means ± SE for 4–5 mice. *P < 0.05 compared with PBS-liposome-treated mice.

Fig. 6. Combination treatment with clodronate-liposomes and cold exposure improves the abnormalities in blood glucose levels caused by obesity in KK-Ay mice. Blood glucose levels (A), food intake (B), locomotor activity (C), and blood insulin level (D) of KK-Ay mice were measured. Blood glucose and blood insulin levels were measured under the experimental condition, as shown in Fig. 4A. Food intake was measured 48 h after the PBS-liposome or clodronate-liposome injection. Locomotor activity was assessed as indicated hours after the injections. Values are means ± SE for 4–7 mice. *P < 0.05, compared between indicated groups.
Fig. 7. Recombinant TNFα protein ip suppresses the UCP1 induction caused by cold exposure in adipose tissues of lean C57BL/6 mice. Five-week-old C57BL/6 mice were injected with ~100 μg/kg body wt recombinant TNFα protein. Twenty-four hours after the first injection, the mice were injected again and exposed to temperatures of 4°C for 24 h to induce UCP1 expression in adipose tissues. A and B: the mRNA (A) and protein expression levels (B) of UCP1 in adipose tissues of C57BL/6 mice. Values are means ± SE for 3–4 mice. Average Cq values are 17.87 (RT saline in BAT), 17.43 (RT TNFα in BAT), 14.82 (cold saline in BAT), 15.09 (cold TNFα in BAT), 21.59 (RT saline in iWAT), 22.58 (RT TNFα in iWAT), 15.00 (cold saline in iWAT), 16.79 (cold TNFα in iWAT), 25.74 (RT saline in eWAT), 25.87 (RT TNFα in eWAT), 24.06 (cold saline in eWAT), and 25.07 (cold TNFα in eWAT). C: the band intensities in B were quantified using ImageJ software. Values are means ± SE for 5 mice. *p < 0.05, compared between indicated groups.

DISCUSSION

In the present study, we showed that the induction of UCP1 expression is suppressed in obese adipose tissues of diet-induced obese C57BL/6 mice and KK-Ay mice. This suppression of UCP1 induction was partially canceled by the ablation of M1 Mφ using clodronate liposomes. Importantly, consistent with the observed changes in the levels of marker genes for thermogenic adipocytes, cold exposure and the disruption of M1 Mφ using clodronate liposomes cooperated to improve thermogenic activity and decrease the blood glucose level and body weight in obese KK-Ay mice. Therefore, M1 Mφ could suppress UCP1 induction, probably beige adipocyte emergence, and contribute to the metabolic dysregulations in obese adipose tissues.

M2 Mφ have been shown to produce catecholamine to trigger adrenergic signaling in brown and white adipocytes, leading to increased energy expenditure via thermogenesis (23, 30, 31). Interestingly, in the BAT of obese KK-Ay, the injection of clodronate liposomes did not significantly restore UCP1 induction (Fig. 3E). Our data suggest that the clodronate liposomes could reduce both M1 and M2 Mφ in the BAT (Fig. 4A). The result is partially consistent with a previous report (23). Considering the M2 Mφ is important for the activation of thermogenesis in BAT of mice (23), the loss of restoration of UCP1 induction by clodronate liposomes could make sense in our study.

Recently, nuclear receptor interacting protein 1 (also known as RIP140) has been shown to control the M1 and M2 Mφ polarization in obese adipose tissue, which control browning (17). The present study is the first report suggesting that infiltrated M1 Mφ suppressed the induction of thermogenic adipocytes in obese adipose tissues such as iWAT, since in our experimental condition clodronate liposomes preferentially eliminated M1 Mφ and did not affect M2 Mφ population in obese iWAT. Accordingly, M1 and M2 Mφ could act by coordinately regulating energy metabolism in adipocytes in a paracrine manner (26).
Much more Mφ content has been shown to infiltrate into visceral fat than subcutaneous fat (1). In obese eWAT, we could observe neither the UCP1 mRNA nor protein induction by cold exposure (Fig. 1A). In addition, we could observe the Ucp1 mRNA induction by cold exposure but not detect UCP1 protein induction by cold exposure in the eWAT of lean mice, although the induction of UCP1 level was observed in the iWAT (Fig. 7A). The inflammatory condition caused by more M1 Mφ content might partially contribute to the loss of UCP1 induction in eWAT compared with BAT and iWAT.

Previously, we reported that TNFα derived from activated RAW 266.7 Mφ suppresses the induction of Ucp1 expression in C3H10T1/2 adipocytes in vitro (36). In this study, we confirmed that the expression level of Tnfa was increased in obese adipose tissues of mouse, and the increase was significantly downregulated by the injection of clodronate liposomes (Figs. 2B and Fig. 3D). Notably, TNFα administration into lean mice suppressed the induction of Ucp1 expression caused by cold exposure in the BAT, iWAT, and eWAT (Fig. 7, A–C). Therefore, TNFα derived from M1 Mφ could serve as a potential proinflammatory cytokine that suppresses the induction of thermogenic adipocytes in vivo. TNFα has been demonstrated to suppress the expression of UCP1 in BAT of rodents as well as primary mouse brown adipocytes (20, 24, 29, 33). In addition to being consistent with the previously reported study, our study is the first report that describes the presence of the same regulatory mechanism in iWAT. A recent paper has shown that TNFα is able to inhibit browning in isolated human adipocytes (10). This might suggest that the same mechanisms are also operative in human obese adipose tissues.

Surprisingly, our data suggest that the mechanisms controlling the suppression of thermogenic adipocytes by M1 Mφ are independent of mitochondrial biogenesis, as we detected no effect of clodronate liposomes or TNFα on the mitochondrial biogenesis induced by cold exposure in iWAT (Figs. 4 and 8). Hence, intracellular mechanisms involved in M1 Mφ suppression of thermogenic adipocytes remain to be elucidated.

Treatment with the clodronate liposomes has been reported to deplete Mφ not only in adipose tissues but also in other tissues such as pancreatic tissues (9, 25). We could not completely exclude the possibility of Mφ depletion in other tissues as a factor for the reduced blood glucose level and body weight observed in obese KK-Ay mice. The combination treatment of clodronate liposomes and cold exposure tended to decrease the insulin level compared with only the cold exposure group, but the difference was not significant. These data might suggest that the decrease in the blood glucose level by the combination treatment could be independent of the improvement of insulin secretion in the pancreatic tissues.

In this study, we have shown that the combination treatment of clodronate liposomes and cold exposure improves hyperglycemia and hyperinsulinemia, suggesting that it ameliorates insulin resistance in HFD-fed KK-Ay mice. Insulin has been reported to enhance Ucp1 transcriptional activity (27, 41). Thus, the recovery of cold-induced UCP1 expression by clodronate liposome in obese adipose tissues might concern the improvement of insulin sensitivity. However, in a previous study, we have shown that conditioned medium from lipopolysaccharide-activated RAW 266.7 macrophages decreased β-adrenoreceptor-stimulated Ucp1 induction in C3H10T1/2 adipocytes under the insulin-free condition (36). Therefore, we believe that elimination of macrophages in adipose tissues seems to be important for the recovery of cold-induced UCP1 induction by clodronate liposomes, at least partially. Further studies investigating the detailed mechanisms underlying this observation are warranted.

Fig. 8. Recombinant TNFα protein ip suppresses mitochondrial biogenesis in the eWAT but not in the iWAT or BAT of lean C57BL/6 mice. mRNA expression levels of Cycs and Cox4i1 in BAT (A), iWAT (B), and eWAT (C) of C57BL/6 mice were measured under the same conditions as those stated in Fig. 7. Values are means ± SE for 4 mice. *P < 0.05, compared between indicated groups.
It has been known that TNFα promotes apoptosis of primary brown adipocyte and downregulates the β3-adrenergic receptor level, which is important to activate brown adipocyte oxidative functions (24). This mechanism could be involved in the downregulation of UCP1 and the marker genes for beige adipocytes in our study. However, we think that TNFα could also affect the downstream signaling of the β3-adrenergic receptor. This is because we have already reported that activation of forskolin, a typical adenylyl cyclase activator-induced Ucp1 promoter, is also downregulated by TNFα (36). Since the adenylyl cyclase is the downstream of the β-adrenergic receptors, this could suggest that TNFα attenuates Ucp1 promoter activity and expression independently of β-adrenergic receptor levels.

In the present study, Ucp1 induction was not activated by cold exposure in eWAT of 21-wk-old C57BL/6 mice fed the ND (Fig. 1A). In contrast, in eWAT of 5-wk-old C57BL/6 mice, Ucp1 expression level was significantly induced by cold exposure (Fig. 7A). The effects of aging on adipose tissues might explain this phenomenon. Aging leads to a loss of brown adipocytes in murine white adipose tissues (32); furthermore, aging is known to increase the levels of proinflammatory mediators such as TNFα in mouse adipose tissues (2, 45). Taken together, promotion of proinflammatory conditions by aging could underlie the loss of thermogenic adipocytes, leading to a loss of BAT activity in rodents as well as humans (49).

In conclusion, inflammation induced by infiltrated M1 Mφ in obese adipose tissues could suppress the induction of brown/beige adipocytes through mechanisms independent of mitochondrial biogenesis. We also identified TNFα as a possible proinflammatory cytokine derived from M1 Mφ that suppresses the emergence of thermogenic adipocytes in vivo. Results from this study also suggest that inflammation induced by Mφ may deteriorate the conditions of obesity and obesity–related metabolic diseases. Therefore, anti-inflammatory agents could be more than just insulin sensitizers; they could serve to increase energy expenditure to improve metabolic disorders.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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