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

Tomoya Sakamoto1*, Takahiro Nitta1*, Koji Maruno1, Yu-Sheng Yeh1, Hidetoshi Kuwata1, Koichi Tomita2, Tsuyoshi Goto1,3, Nobuyuki Takahashi1,3, and Teruo Kawada1,3

1 Laboratory of Molecular Function of Food, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan
2 Center for Genetic Analysis of Behavior, National Institute for Physiological Sciences, 5-1 Higashiyama, Myodaiji-cho, Okazaki, Aichi 444-8787, Japan
3 Research Unit for Physiological Chemistry, the Center for the Promotion of Interdisciplinary Education and Research, Kyoto University, Uji, Kyoto, Japan

* These authors contributed equally to this work

Running title: Inflammation suppresses induction of UCP1 level in vivo

Correspondence: T. Kawada
Laboratory of Molecular Function of Food, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University
Uji 51-0011, Japan

Copyright © 2016 by the American Physiological Society.
Tel: +81-774-38-3751
Fax: +81-774-38-3752
E-mail: fat@kais.kyoto-u.ac.jp
Inflammation suppresses induction of UCP1 level in vivo

ABSTRACT

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 classically activated macrophages (M1 Mφ) suppress 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 changes in the 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
Inflammation suppresses induction of UCP1 level in vivo 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.

Keywords: Thermogenic adipocyte, Inflammation, Mitochondrial biogenesis, Macrophage, TNFα

INTRODUCTION

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 non-shivering 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 anti-obesity and anti-diabetic 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 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 has been shown to secrete a variety of proinflammatory cytokines such as TNFα (19, 39), leading to cause insulin resistance (47). In addition, we previously...
Inflammation suppresses induction of UCP1 level in vivo

reported that TNFα derived from activated RAW 264.7 Mφ suppresses the induction of \textit{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 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-week-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) (D12492, Research Diets, NJ, USA) for 16 weeks for the diet-induced obese model. We also used 5-week-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 weeks. 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
Inflammation suppresses induction of UCP1 level in vivo

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 same as those described above. After euthanization, interscapular BAT (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, CA, USA) to induce apoptosis in Mφ. Eight-week-old male KK-Ay mice fed HFD for 3 weeks were injected with 30 mg/kg of clodronate liposomes or 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, and locomotor activity data was collected with ACTIMO-100 (Shinfactory, Fukuoka, Japan), and the food intake data was collected manually. Cumulative ambulatory counts were recorded at 5-minute intervals during the 48 h period following the administration of the clodronate liposomes.

Rectal temperature was monitored using a thermos recorder.
Inflammation suppresses induction of UCP1 level in vivo

TR-71S (T&D Corporation, Matsumoto, Japan) in indicated time.

Blood samples were harvested on 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-week-old C57BL/6 mice were intraperitoneally injected with approximately 100 μg/kg body weight recombinant TNFα protein (Peprotech, NJ, USA). After 24 h, another injection was administered, and the mice were exposed to 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, sections 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 rat antibody (AbD Serotec, NC, USA) or anti-mouse CD11c hamster antibody (Abcam, NC, USA) 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, CA, USA) 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 (ver. 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
Inflammation suppresses induction of UCP1 level in vivo

205 the manufacturer’s instructions. Total RNA was
206 reverse-transcribed using M-MLV reverse transcriptase
207 (Promega, Madison, WI, USA) according to the manufacturer's
208 instructions using a thermal cycler (Takara PCR Thermal
209 Cycler SP, Takara, Shiga, Japan). To quantify mRNA
210 expression, real-time RT-PCR was performed with a
211 LightCycler System (Roche Diagnostics, Mannheim, Germany)
212 using SYBR Green fluorescence signals as previously
213 described (36). The oligonucleotide primers were designed
214 using Primer-BLAST, a primer designing software tool publicly
216 The primers used for measuring the mRNA expression levels of
217 genes are listed in Table 1. To compare mRNA expression
218 levels among the samples, the copy numbers of all transcripts
219 were divided by that of mouse 36B4 showing a constant
220 expression level in adipose tissues and adipocytes. All mRNA
221 expression levels were represented as a ratio relative to that of
222 the control in each experiment. The mRNA expression level of
223 36B4 was stable under all conditions.
224
225 Immunoblotting
226 Isolation of the mitochondrial fraction from adipose tissues
227 was performed as previously described (22). Mitochondrial
228 proteins were solubilized in the lysis buffer [50 mM Tris-HCl,
229 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1%
Inflammation suppresses induction of UCP1 level in vivo

SDS (pH 7.4), and a protease inhibitor cocktail]. Protein concentrations of the samples were determined using a protein assay kit (Bio-Rad Laboratories, CA, USA). 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% non-fat dried milk in TBS with Tween 20. The membranes were incubated with an antibody against UCP1 (Sigma-Aldrich, MO, USA) or cytochrome c oxidase subunit IV isoform 1 (COX4) (Cell Signaling Technology, MA, USA) and then with peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies (Santa Cruz, CA, USA), respectively. Blots were developed using an Immobilon™ Western Chemiluminescent HRP Substrate (Merck Millipore) and detected using the Alpha Innotech MultiimageII Light Cabinet With Camera & Filters (Alpha-Innotech, CA, USA). Images were captured using the AlphaEaseFc software (ver. 3.1.2, Alpha-Innotech, San Leandro, CA). Immunoreactive protein bands were quantified by using the Image J software (NIH, Bethesda, MD, USA).

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.
Inflammation suppresses induction of UCP1 level in vivo

Data are expressed as means ± S.E.M. 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 HFD for 16 weeks to cold temperature ($4^\circ$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 qPCR, under the basal condition, the $Ucp1$ mRNA levels are about 8000 fold higher in BAT than in IWAT and EWAT. Under the cold exposure condition, the levels are about 180 fold higher in IWAT than in EWAT, and some 30000 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 BAT of HFD group (Fig. 1B). The internal control $36B4$ levels were not changed under each condition in each adipose tissue. This data indicates that the changes of $Ucp1/36B4$ 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-week-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 UCP1 protein levels were not changed in the BAT (Fig. 1E) but the trend in the UCP1 protein levels were consistent with the *Ucp1* mRNA levels in IWAT (Fig. 1E). UCP1 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 UCP1 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 previously reported 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 UCP1 expression in obese adipose tissues. First, we examined Mφ content in the obese adipose
Inflammation suppresses induction of UCP1 level in vivo

tissues. Hematoxylin-eosin staining revealed that the numbers
of crown-like structures, which are considered to be
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 weeks 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
condition (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 time-dependent manner and
3-week 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
Inflammation suppresses induction of UCP1 level in vivo

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 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 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 liposomes 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 significantly decreased the mRNA expression levels of F4/80 and Tnfa (Fig. 3F).

In contrast, mRNA expression levels of chitinase-like 3 (Ym1) and arginase-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
Inflammation suppresses induction of UCP1 level in vivo

might suggest that the clodronate liposomes decreased the both
M1 and M2 macrophages in the BAT (Fig. 3F). This result is
partially consistent with the previous report (23).

M1 Mφ suppressed the induction of thermogenic adipocytes in
obese adipose tissues.

To examine if 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 UCP1 protein
expression levels is same as 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
palmitoyltransferase 1B (muscle) (Ctp1b) and peroxisome
proliferator-activated receptor gamma, coactivator 1 alpha
(Ppargc1a), which is the master regulator for mitochondrial
biogenesis, to examine if the injection of clodronate liposomes
increases mitochondrial biogenesis in IWAT. As shown in Fig.
4D, in the IWAT of obese KK-Ay mice, cold exposure did not
significantly increase the mRNA levels of mitochondrial genes
Inflammation suppresses induction of UCP1 level in vivo

and Ppargc1a. The combination treatment with clodronate liposomes and cold exposure did not induce the mitochondrial gene expression levels as compared with cold exposure alone. The Ppargc1a level tended to be induced by the combination treatment 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.

Next, to examine the effects of inflammation caused by Mφ on browning in detail, we measured the levels of marker genes for beige adipocytes such as T-box 1 (Tbx1) and transmembrane protein 26 (Tmem26) (46) in IWAT of KK-Ay mice. Neither cold exposure nor the injection of clodronate liposomes significantly induced the expression levels of these genes. However, they were highly induced by the combination treatment with cold exposure and clodronate liposomes (Fig. 4E). Taken together, these data suggest that M1 Mφ suppresses the induction of thermogenic adipocytes through mechanisms independent of mitochondrial biogenesis.

Cold tolerance was improved in obese KK-Ay mice treated with clodronate liposomes.

To examine if the clodronate liposomes and cold exposure affects the thermogenic ability in obese KK-Ay mice, we measured the rectal temperature under the shown experimental
Inflammation suppresses induction of UCP1 level in vivo

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 expose the KK-Ay mice to cold temperature for 6 h (2nd
cold exposure). As shown in Fig. 5, during the 1st cold
exposure, the rectal temperatures in the clodronate
liposome-injected group were 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 2nd cold exposure,
the rectal temperatures in the clodronate liposomes injected
group were significantly higher compared with the
PBS-injected group. Taken together, these data suggest that the
clodronate liposomes-mediated Mφ 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 level and body weight of obese KK-Ay mice. However, consistent with the changes in the levels of marker genes for thermogenic adipocytes, the combination treatment with cold exposure and 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) and their locomotor activity at 24, 36, 45 h after the injection (Fig. 6C).

Cold exposure significantly reduced the insulin level of the KK-Ay mice. The combination treatment tended to decrease the insulin level compared with only 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 adipocytes induction.

Intraperitoneally administered recombinant TNFα protein suppressed the induction of UCP1 protein expression in BAT
Inflammation suppresses induction of UCP1 level in vivo

and IWAT of lean C57BL/6 mice.

Our previous study has indicated 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 i.p. administration of 100 μg/kg recombinant TNFα protein into lean 5-week-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, IWAT (Fig. 7B, 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 and BAT of lean C57BL/6 mice.

Next, we examined the effects of TNFα administration on mitochondrial biogenesis in adipose tissues. We investigated the mRNA expression levels of Cycs and Cox4i1, which are
Inflammation suppresses induction of UCP1 level in vivo

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 Cycs. 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 Cycs, but not Cox4i1, whereas TNFα administration had no effect on the mRNA expression levels of both genes (Fig. 8B). In EWAT, the administration of TNFα significantly decreased the mRNA expression levels of Cycs 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 mitochondrial biogenesis in BAT and IWAT. This is in accordance with the restoration of UCP1 expression level by clodronate liposomes (Fig. 4B), which is also independent of mitochondrial biogenesis (Fig. 4D).

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
Inflammation suppresses induction of UCP1 level in vivo

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 adipocytes
emergence and contribute 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 the 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
Inflammation suppresses induction of UCP1 level in vivo

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 to BAT and IWAT.

We previously reported that TNFα derived from activated RAW264.7 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 (Fig. 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. 7A, B, 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. The recent paper has shown that TNFα is able to inhibit browning in isolated human adipocyte (10). This might suggest that 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 and TNFα on the mitochondrial biogenesis induced by cold exposure in IWAT (Fig. 4 and Fig. 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 in not only adipose tissues but also 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
Inflammation suppresses induction of UCP1 level in vivo

insulin level compared with only cold exposure group but the
difference was not significant. This 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 \textit{Ucp1} transcriptional activity (27, 41). Thus, the recovery of cold-induced UCP1 expression by
clodronate liposome in obese adipose tissues might concern to
the improvement of insulin sensitivity. However, in previous
study, we have shown that conditioned medium from
lipopolysaccharide-activated RAW264.7 macrophages
decreased \(\beta\)-adrenoreceptor-stimulated \textit{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.

It has been known that TNF\(\alpha\) promotes apoptosis of primary
brown adipocyte and downregulates \(\beta3\)-adrenergic receptor
level, which is important to activate brown adipocyte functions
Inflammation suppresses induction of UCP1 level in vivo (24). This mechanism could be involved in the downregulation of UCP1 and the marker genes for beige adipocytes in our study. However, we think TNFα also could affect the downstream signaling of β3-adrenergic receptor. This is because we have already reported that forskolin, a typical adenylate cyclase activator-induced Ucp1 promoter activation is also down-regulated by TNFα (36). Since the adenylate cyclase is the downstream of the β-adrenergic receptors, this could suggest TNFα attenuates Ucp1 promoter activity and expression independently of β-adrenergic receptors levels.

In the present study, Ucp1 induction was not activated by cold exposure in EWAT of 21-week-old C57BL/6 mice fed the ND (Fig. 1A). In contrast, in EWAT of 5-week-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
Inflammation suppresses induction of UCP1 level in vivo 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.

ACKNOWLEDGEMENTS

The authors thank Ms. Sayoko Shinoto for secretarial assistance and Ms. Manami Sakai for technical assistance.

GRANTS

This work was largely supported by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No., 22228001, 22380075, 24229 and 24688015).

REFERENCE


Inflammation suppresses induction of UCP1 level in vivo


Inflammation suppresses induction of UCP1 level in vivo


26. Parinandi NL, and Magalang UJ. Avatars of adipose tissue: the saga of transformation of white fat, the villain into brown fat, the protector. Focus on "Inflammation induced by RAW macrophages suppresses the UCP1 mRNA induction via ERK activation in 10T1/2 adipocytes". Am J Physiol Cell Physiol 304:
Inflammation suppresses induction of UCP1 level in vivo


32. Rogers NH, Landa A, Park S, and Smith RG. Aging leads to a programmed loss of brown adipocytes in murine
Inflammation suppresses induction of UCP1 level in vivo


38. Seale P, Conroe HM, Estall J, Kajimura S, Frontini A,
Inflammation suppresses induction of UCP1 level in vivo


45. Wu D, Ren Z, Pae M, Guo W, Cui X, Merrill AH, and
Inflammation suppresses induction of UCP1 level in vivo


**FIGURE LEGEND**

Figure 1. Cold exposure-induced UCP1 expression levels are suppressed in obese adipose tissues of C57BL/6 mice
Inflammation suppresses induction of UCP1 level in vivo and KK-Ay mice.

(A) mRNA expression level and (B) protein expression level of UCP1 in obese adipose tissues of C57BL/6 mice fed with a HFD for 16 weeks. Values are means ± SEM for 5-8. *p<0.05, compared between indicated groups. Average Cq values are 12.67 (ND RT in BAT), 11.55 (HFD RT in BAT), 11.40 (ND Cold in BAT), 10.61 (HFD Cold in BAT), 28.27 (ND RT in 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 Cold in EWAT), 24.24 (ND Cold in EWAT) 25.10 (HFD Cold in EWAT). (C) $2^{\Delta\Delta C_q}$ of 36B4 in obese adipose tissues of C57BL/6 mice fed with a HFD for 16 weeks. Values are means ± SEM for 5-8. (D) mRNA expression level of Ucp1 in obese adipose tissues of KK and KK-Ay mice. Values are means ± SEM for 4. *p<0.05, compared between indicated groups. (E) Protein expression levels of UCP1 in KK and KK-Ay mice fed with a ND for 5-7 weeks. Average Cq values are 11.21 (BAT in KK RT), 9.77 (BAT in KK Cold), 10.73 (BAT in KK-Ay RT), 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).

Figure 2. Mφ infiltrates obese adipose tissues of C57BL/6 mice.
Inflammation suppresses induction of UCP1 level in vivo

(A) Representative images of H&E-stained sections from the IWAT of C57BL/6 mice with or without HFD feeding for 16 weeks. Scale bar = 100 μm. (B) mRNA expression levels of F4/80 and Tnfa in each obese adipose tissues of C57BL/6 mice fed with a HFD for 16 weeks. Values are means ± SEM for 4-7.

*p<0.05, compared between indicated groups.

Figure 3. The intraperitoneal injection of clodronate liposomes depletes M1 Mφ in obese IWAT of KK-Ay mice.

(A) Schematic of the experimental procedure used to inject clodronate liposomes into KK-Ay mice. (B) Representative images of H&E-stained sections from IWAT of obese KK-Ay with or without HFD for 3 weeks. Scale bar = 100 μm (C) mRNA expression levels of F4/80 and Tnfa in KK-Ay mice fed HFD for 0-3 weeks with PBS liposomes or clodronate liposomes treatment. Values are means ± SEM for 4-6. *p<0.05, compared between indicated groups. (D) H&E-stained sections and immunostaining section with anti-F4/80 or -CD11c from IWAT of KK-Ay mice with PBS liposomes or clodronate liposomes treatment. Scale bar = 100 μm. mRNA expression levels of marker genes for global Mφ, M1 and M2 Mφ in obese (E) IWAT and (F) BAT following treatment with PBS liposomes or clodronate liposomes treatment. Clodronate liposomes or PBS liposomes (30 mg/kg for each) were intraperitoneally injected into KK-Ay mice fed with a HFD for
Inflammation suppresses induction of UCP1 level in vivo

3 weeks. RNA samples were collected from adipose tissues 48 h after injection. Values are means ± SEM for 3-6. *p<0.05, compared to the PBS-liposomes group.

**Figure 4. M1 Mφ depleting using clodronate liposomes cancels the suppression of UCP1 and marker genes for beige adipocyte induction in the IWAT of obese KK-Ay mice independently of mitochondrial biogenesis.**

(A) Schematic of the experimental procedure used for the injection of clodronate liposomes into KK-Ay mice (B) the mRNA expression level and (C) the protein expression level of UCP1 and (D) mRNA expression level of *Cycs*, *Cpt1b* and *Ppargc1a* in IWAT of KK-Ay mice under experimental conditions as shown in Fig. 4A. Average Cq values of *Ucp1* are 11.98 (RT PBS in BAT), 11.49 (RT Clo in BAT), 10.52 (Cold PBS in BAT), 10.65 (Cold Clo in BAT), 15.87 (RT PBS in IWAT), 15.80 (RT Clo in IWAT), 16.93 (RT PBS in IWAT), 13.75 (Cold Clo in IWAT). (E) mRNA expression levels of *Tbx1* and *Tmem26* in the IWAT of KK-Ay mice under experimental conditions as shown in Fig. 4A. Values are means ± SEM for 6-8. *p<0.05, compared between indicated groups.

ND means non-detected.

**Figure 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
Inflammation suppresses induction of UCP1 level in vivo

obese KK-Ay mice indicated hours after starting 1st cold exposure. Values are means ± SEM for 4-5. *p<0.05, compared to PBS-liposomes treated mice.

Figure 6. Combination treatment with clodronate-liposomes and cold exposure improves the abnormalities in blood glucose level 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. (A) and (D) were measured under the experimental condition as shown in Fig. 4A (B) was measured 48 h after the PBS-liposomes or clodronate liposomes injection. (C) was assessed indicated hours after the injections. Values are means ± SEM for 4-7. *p<0.05, compared between indicated groups.

Figure 7. Recombinant TNFα protein i.p. 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 approximately 100 μg/kg body weight 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) The mRNA expression level and (B) the protein expression level of UCP1 in adipose tissues of C57BL/6 mice. Values are means ±
Inflammation suppresses induction of UCP1 level in vivo

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), 25.07 (Cold TNFα in EWAT). (C) The band intensities of Fig. 7B were quantified using ImageJ software. Values are means ± SEM for 5 *p<0.05, compared between indicated groups.

Figure 8. Recombinant TNFα protein i.p. suppresses mitochondrial biogenesis in the EWAT but not in the IWAT or the BAT of lean C57BL/6 mice.

The mRNA expression levels of Cycs and Cox4i1 in (A) BAT, (B) IWAT, and (C) EWAT of C57BL/6 mice were measured under the same conditions as stated in Fig. 7. Values are means ± SEM for 4 *p<0.05, compared between indicated groups.
Figure 1

A. Relative Ucp1 mRNA expression level

B. Western blot analysis of UCP1 and COX4

C. Relative $2^{(-\Delta\Delta C_t)}$ of 36B4 relative value

D. Relative Ucp1 mRNA expression

E. Western blot analysis of UCP1 and COX4
Figure 2
Figure 3

A) KK-Ay 5 week-old mice were fed HFD for 3 weeks. After 48 h of HFD feeding, Clodronate was administered. 

B) Hematoxylin-eosin staining of IWAT from mice fed HFD for 0 weeks or 3 weeks. 

C) Relative mRNA expression levels of F4/80 and Tnfa in mice fed HFD for 0, 1, 2, 3 weeks. 

D) Immunohistochemistry of F4/80 and CD11c in IWAT from mice fed HFD for 3 weeks. 

E) Relative mRNA expression levels of genes related to M1 and M2 macrophages in IWAT and BAT. 

PBS, Clodronate treatments are indicated.

N.S.: Not significant
Figure.4
Figure 5

KK-Ay 5-week-old mice were fed a high-fat diet (HFD) for 3 weeks, followed by cold exposure at 4°C for 24 h. Clodronate injection was given 48 h before the cold exposure. Rectal temperature was measured at 4°C and 23°C for 1st and 2nd cold exposures, respectively.

- **First Cold Exposure (4°C):** Rectal temperature decreased over time, with a significant drop at 4°C.
- **Second Cold Exposure (23°C):** Rectal temperature decreased significantly at 23°C compared to 4°C.

Data are presented as mean ± SEM. *p < 0.05 compared to PBS.
Figure 6

A. Comparison of plasma glucose (mg/dl) between RT and Cold conditions in PBS and Clodronate groups.

B. Comparison of food intake (g) between PBS and Clodronate groups.

C. Comparison of food intake (g) at 24, 36, and 45 hours between PBS and Clodronate groups.

D. Comparison of plasma insulin (ng/ml) between RT and Cold conditions in PBS and Clodronate groups.
Figure.7
Figure 8
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ to 3’)</th>
<th>Official Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>36B4</strong></td>
<td>TGT GTG TCT GCA GAT CGG GTA C</td>
<td>Ribosomal protein, large, P0</td>
</tr>
<tr>
<td><strong>Arg1</strong></td>
<td>GTG AGC ATC CAC CCA AAT GAC</td>
<td>Arginase 1</td>
</tr>
<tr>
<td><strong>Cox4i1</strong></td>
<td>CTC CGC CAA GTG GGA CTA TG GAC CAT TGG ATA CGG CAG CTT</td>
<td>Cytochrome c oxidase subunit IV</td>
</tr>
<tr>
<td><strong>Cpt1b</strong></td>
<td>CTG TTA GGC CTC AAC ACC GAA C CTG TCA TGG CTA GGC GGT ACA T</td>
<td>Carnitine palmitoyltransferase 1b, muscle</td>
</tr>
<tr>
<td><strong>Cycls</strong></td>
<td>CCA AAT CTC CAC GGT CTT C</td>
<td>Cytochrome c Somatic</td>
</tr>
<tr>
<td><strong>F4/80</strong></td>
<td>TTT CCT CGC CTG CTT CTT C CCC CGT CTC TGT ATT CAA C</td>
<td>EGF-like module containing, mucin-like, hormone receptor-like sequence 1</td>
</tr>
<tr>
<td><strong>Mcp1</strong></td>
<td>GAC CCC AAG AAG GAA TGG GT ACC TTA GGG CAG ATG CAG TT</td>
<td>Chemokine (C-C motif) ligand 2</td>
</tr>
<tr>
<td><strong>Ppargc1a</strong></td>
<td>CCC TGC CAT TGT TAA GAC C TGC TGC TGT TCC TGT TTT C</td>
<td>Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha</td>
</tr>
<tr>
<td><strong>Tbx1</strong></td>
<td>GGC AGG CAG ACG AAT GTT C TTG TCA TCT AGG GGC ACA AAG</td>
<td>T-box 1</td>
</tr>
<tr>
<td><strong>Tmem26</strong></td>
<td>ACC CTG TCA TCC CAC AGA G TGT TTG GTG GAG TCC TAA GGT C</td>
<td>Transmembrane protein 26</td>
</tr>
<tr>
<td><strong>Tnfa</strong></td>
<td>ACA TCA GAT CAT CTT CTC AAA ATT C GTG TGG GTG AGG AGG AGG TAG T</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td><strong>Ucp1</strong></td>
<td>ACT GCC ACA CCT CCA GTC ATT CTT TGC CTC ACT CAG GAT TGG</td>
<td>Uncoupling Protein 1</td>
</tr>
<tr>
<td><strong>Ym1</strong></td>
<td>AGA AGG GAG TTT CAA ACC TG GTC TTG CTC ATG TGT GTA AG</td>
<td>Chitinase-like 3</td>
</tr>
</tbody>
</table>