Triiodothyronine induces UCP-1 expression and mitochondrial biogenesis in human adipocytes

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In both rodents and humans, brown adipose tissue (BAT) is specialized for energy expenditure through thermogenesis (7, 11). Uncoupling protein (UCP)-1 is considered to play an important role in thermogenesis in BAT. UCP-1 generates heat by leaking proton across the mitochondrial inner membrane, thus uncoupling oxidative phosphorylations without ATP production (32). Ablating UCP-1 causes cold sensitivity (10) and obesity in mice (11). Thus UCP-1 in BAT is essential for thermogenesis (12). However, Nagase et al. (26) reported that chronic stimulation of β-adrenergic receptor induces the ectopic expression of UCP-1 in white adipose tissue (WAT) and skeletal muscle. Indeed, some brown adipocytes arise from progenitor cells in WAT under certain physiological and pharmacological conditions (27). The increase in UCP-1 expression level in WAT has been suggested as the mechanism that prevents obesity (13). These brown-like adipocytes show an increase in oxygen consumption rate (OCR) in response to noradrenalin stimulation (27) and are abundant in an obesity-resistant strain of mice (1), suggesting a crucial role of these cells in the regulation of body weight. However, the mechanism underlying UCP-1 induction in white adipocytes is poorly known. Human multipotent adipose-derived stem (hMADS) cells are a suitable human fat cell model for investigating the mechanism because they are able to differentiate into various lineages, including adipocytes with molecular and functional characteristics of human adipocytes (30, 31). In addition, chronic peroxisome proliferator-activated receptor (PPAR)-γ activation leads to the conversion of these cells to functional brown adipocytes (9). Thus hMADS cells are suitable for investigating the mechanism underlying the regulation of UCP-1 expression in WAT.

The thyroid hormone (TH) is an important physiological modulator for energy homeostasis in the entire body (21). The physiological effects of TH are exerted at the level of transcription through interaction with specific TH receptors (TRs), TR-α and TR-β (3). TH induces mitochondrial biogenesis by modulating multiple mitochondrial respiratory gene expression, thereby enhancing coupled oxidative phosphorylations in a tissue-specific manner (34). In adipose tissue, TH regulates multiple aspects of lipid metabolism, including lipogenesis, lipolysis, and thermogenesis (2, 19, 43). TH treatment induces UCP-1 expression in brown adipocytes (14) by interaction with a specific TR in the nucleus (28). TR-β mediates triiodothyronine (T3)-induced UCP-1 expression, whereas the TR-α isoform regulates adaptive thermogenesis in BAT (29). The effect is also dependent on the induction of type 2 iodothyronine deiodinase (D2), which increases the amount of active TH (T3) in brown adipocytes (8). Furthermore, activating this enzyme increases energy expenditure in BAT (45), suggesting a crucial role of TH in thermogenesis in the tissue. These observations suggest that TH plays a pivotal role in the regulation of energy homeostasis through direct interaction with adipocytes. However, the action of TH on UCP-1 expression in white adipocytes has not been elucidated.

In this study, we investigate whether TH could regulate UCP-1 expression in WAT using multipotent cells isolated from human adipose tissue. T3 treatment increased the mRNA expression level of mitochondrial biogenesis genes, including PPAR-γ coactivator-1α (PGC-1α), nuclear respiratory factor 1 (NRF1), and cytochrome c (Cyt c), in adipocyte differentiation of hMADS cells. T3 treatment also increased UCP-1 mRNA expression and mitochondrial biogenesis in human adipocytes.
expression level via a TR-mediated pathway, resulting in the increase in OCR.

MATERIALS AND METHODS

Materials and cell culture. 3,3′,5-Triiodo-l-thyronine sodium salt was purchased from Sigma and dissolved in DMSO as a stock solution. All of the other chemicals used were from Sigma or Nacalai Tesque (Kyoto, Japan) and guaranteed to be of reagent or tissue-culture grade.

hMADS cells were established by Rodriguez et al. (30) and supplied by Stem Cell Sciences KK (Kobe, Japan). We modified the culture protocol from the published protocol (9, 30). hMADS cells were maintained in a maintenance medium (MM; 10% FBS and 10 mg/ml penicillin/streptomycin in DMEM) at 37 °C in 5% CO₂ 95% air under a humidified condition. Under this condition, the concentrations of free T₃ and thyroxine in FBS were 2.1 ± 0.006 and 165.6 ± 0.285 nM, respectively. The determinations of total and free T₃ concentrations were performed by Mitsubishi Chemical Mediense (Tokyo, Japan). For white adipocyte differentiation, hMADS cells were seeded at a high density (25,000 cells/cm²). Two days after the seeding, the culture medium was changed with a fresh medium supplemented with 1 μM insulin, and 1 μM pioglitazone. Three days after the incubation, the cell culture medium was changed to a post-DM, which was MM supplemented with 0.85 μM insulin and 1 μM pioglitazone, and then the medium was changed with a fresh one every 2 days. Cells cultured on 6- or 12-well tissue culture plates were prepared for biochemical assays.

RNA preparation and quantification of gene expression. RNA samples of the differentiated hMADS cells were prepared using cells cultured on 12-well tissue culture plates 10 days after the differentiation induction by Sepasol-RNA-I Super (Nacalai Tesque), in accordance with the manufacturer’s protocol. Ten days after the differentiation induction, hMADS cells were incubated at 37 °C for 30 min with 200 nM MitoTracker Red and washed twice with PBS. Fluorescence signals were imaged using an IX71 fluorescence microscope (Olympus, Tokyo, Japan).

Mitochondrial content and microscopic analysis. Staining for analyzing the mass of mitochondria was performed using an oxidized MitoTracker Red probe (CM-XRos, Invitrogen), in accordance with the manufacturer’s protocol. Ten days after the differentiation induction, hMADS cells were incubated at 37 °C for 30 min with 200 nM MitoTracker Red and washed twice with PBS. Fluorescence signals were imaged using an IX71 fluorescence microscope (Olympus, Tokyo, Japan).

To examine the effects of T₃ on mitochondrial content, mitochondrial DNA (mtDNA) and genomic DNA samples of hMADS cells were prepared using cells cultured 10 days after differentiation induction. Nuclear and mitochondrial extracts were prepared in accordance with a protocol described by Busch et al. (4). The differentiated hMADS cells (2 × 10⁵) were homogenized using a motor-driven homogenizer in 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 1% protease inhibitor cocktail. The homogenates were centrifuged at 228 g for 5 min at 4°C. Pellets (nuclear fraction) were stored for genomic DNA isolation. Supernatants were centrifuged at 20,000 g for 20 min at 4°C. Pellets (mitochondrial and

Table 1. Primers for real-time PCR

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αP2, adipocyte lipid-binding protein; PPAR-γ, peroxisome proliferator-activated receptor-γ; C/EBP-α, CCAAT/enhancer binding protein-α; FATP1, fatty acid transporter-1; LPL, lipoprotein lipase; UCP-1, uncoupling protein-1; UCP-2, uncoupling protein-2; NRF1, nuclear respiratory factor 1; TFAM, mitochondrial transcription factor-A; PGC-1α, PPAR-γ coactivator-1α; PRDM16, PR domain containing-16; Cidea, cell death-inducing DFFA-like effector-a; Elov3, ELOVL fatty acid elongase-3; Cyt c, cytochrome c; COX4, Cyt c oxidase subunit IV; COX8a, Cyt c oxidase subunit VIIIa; CPT-1b, human carnitine palmitoyltransferase-1b; AOX, acyl-CoA oxidase; 36B4, ribosomal protein large P0.
cytosol fraction) were stored for mtDNA isolation. Each fraction was resuspended in cell lysis buffer (containing 50 mM NaCl, 5 mM EDTA, 0.1% SDS, 10 mM HEPES; pH 7.9). The fractions were incubated with Protease K (60 µg/ml) at 55°C for 30 min. Phenol-chloroform-isooamyl alcohol (25-24-1; Nacalai Tesque) was added using the same volume for each fraction. After centrifugation (at 12,000 g for 10 min), isopropanol (Nacalai Tesque) was added using the same volume for each fraction. After centrifugation, the pellets containing mtDNA and genomic DNA were solubilized in DNase-free water containing RNase A. The concentrations of mtDNA and genomic DNA were measured using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The concentration of mtDNA was normalized by those of genomic DNA. The mtDNA levels are presented as a ratio compared with a control in each experiment.

**RESULTS**

*T3* treatment did not induce adipocyte differentiation but induced *UCP-1* mRNA expression in hMADS cells. To determine the effects of *T3* on the mRNA expression of *UCP-1*, confluent hMADS cells were incubated in DM with 250 nM *T3* for 3 days. Although the concentrations of *T3* were very high, the concentrations of free *T3*, an active form of *T3*, were 1.20 and 2.30 nM in the medium containing 100 and 250 nM *T3*, respectively. Ten days after the *T3* treatment to induce adipocyte differentiation, the mRNA expression level of CCAAT/enhancer binding protein (C/EBP-α) significantly increased, whereas the white adipocyte marker genes, adipocyte lipid-binding protein (apo2, also known as fatty acid binding protein 4, FABP4), adiponectin, FA transporter-1 (fatty acid transport protein 1, FATP1), lipoprotein lipase (LPL), and *UCP-2* were not affected by *T3* treatment (Fig. 1A). On the other hand, treatment with *T3* increased the mRNA expression levels of *UCP-1* (4.6-fold increase), cell death-inducing DFFA-like effector-a (*Cidea*), and *ELOVL* FA elongase-3 (*Elovl3*), as shown in Fig. 1B. The *T3* treatment increased the mRNA expression levels of *PGC-1α* (3.2-fold increase) and *NRF1*, a specific marker gene involved in mitochondrial biogenesis (1.9-fold increase). The mRNA expression level of PR domain containing-16 (*PRDM16*), a marker of brown adipocytes, was not altered by *T3* treatment (Fig. 1B). The mRNA expression of D2, a gene encoding an enzyme that converts *T4* to *T3*, was not detected on days 0, 3, and 10 of adipocyte differentiation of hMADS cells (data not shown). Next, to examine the phase dependence of the effects of *T3* treatment, we treated hMADS cells with 50 nM *T3* in different phases (Fig. 2A). No significant induction of *UCP-1* mRNA expression was observed in the treatment in the absence of either of its cofactors: *T3*, *T3* (in the absence of *T3*), or *T3* (in the absence of *T3*). The mRNA expression levels of *PGC-1α* and *NRF1* were significantly increased, whereas the white adipocyte marker genes, adipocyte lipid-binding protein (apo2, also known as fatty acid binding protein 4, FABP4), adiponectin, FA transporter-1 (fatty acid transport protein 1, FATP1), lipoprotein lipase (LPL), and *UCP-2* were not induced by *T3* treatment. These findings indicate that *T3* induces the mRNA expression of *UCP-1* and other genes related to mitochondria biogenesis in hMADS cells in a differentiation stage-dependent manner. Importantly, the effects of *T3* treatment between day 0 and day 3 on *UCP-1* mRNA expression examined on day 10 suggest that adipocyte transcriptional factors may be involved in the *T3*-induced *UCP-1* expression.

UCP-1 mRNA expression was induced in hMADS cells in a T3 dose-dependent manner. To examine the details of the *T3* effects on mRNA induction, we exposed hMADS cells to *T3* at various concentrations for 3 days (from day 0 to day 3). The *T3* treatment increased the mRNA expression level of *UCP-1* in a dose-dependent manner (Fig. 3A). The mRNA expression levels of *PGC-1α* and *NRF1* were increased only at the highest concentration of *T3* (250 nM) (Fig. 3, B and C). The mRNA expression of mitochondrial transcription factor A (*TFAM*), a key regulator of mitochondrial biogenesis (5), tended to be higher in the presence of 250 nM *T3*, although the difference was not statistically significant (Fig. 3D). Next, we examined *UCP-1* mRNA expression at various time points (Fig. 4A). *T3* treatment increased the mRNA expression level of *UCP-1* significantly (Fig. 4B). The mRNA expression levels of *PGC-1α* and *NRF1* were increased only at the highest concentration of *T3* (250 nM) (Fig. 4, B and C). The mRNA expression of mitochondrial transcription factor A (*TFAM*), a key regulator of mitochondrial biogenesis (5), tended to be higher in the presence of 250 nM *T3*, although the difference was not statistically significant (Fig. 4D). Next, we examined *UCP-1* mRNA expression at various time points (Fig. 4A).
(50 nM) treatment for 3 days (from day 0 to day 3) significantly increased the mRNA expression levels of UCP-1 and C/EBP-α on day 8 (Fig. 4, A and E). These expression levels were maintained up to day 10. The mRNA expression of TFAM was decreased from day 8 in hMADS cells. The decrease was inhibited by the T3 treatment. On the other hand, those of the other genes did not show significant differences at this concentration of T3.

T3 treatment increased mitochondrial-related gene expression and mitochondrial biogenesis. To further clarify the effects of T3 on mitochondrial biogenesis, nucleus-encoded mitochondrial genes, Cyt c, Cyt c oxidase subunit IV (COX4), and Cyt c oxidase subunit VIIIa (COX8a), were measured by real-time PCR. The Cyt c mRNA level at the 10th day was decreased in the control cells, and the decrease was significantly inhibited in the T3-treated cells. At protein levels, Cyt c expression was also higher in cultured cells treated with T3 than in control at the 10th day (Fig. 5C). The protein levels of Cyt c and UCP-1 showed 1.65- and 3.34-fold amounts, respectively, in the T3-treated cells. The mRNA levels of COX4 and COX8a were higher in the T3-treated cells than in control (Fig. 5D). Moreover, the treatment with T3 also increased amounts of mtDNA, indicating higher levels of mitochondrial amounts (Fig. 5E). These findings suggest that the T3 treatment augments mitochondrial biogenesis in hMADS cells. Indeed, staining of differentiated hMADS cells with MitoTracker Red revealed stronger staining of T3-treated cells than that of control cells (Fig. 5F), indicating higher levels of membrane potential by T3 treatment. Increased membrane potential is created by the respiratory chain reaction to drive ATP synthesis, suggesting that T3 treatment may increase glucose and FA oxidation in hMADS cells.

T3-induced UCP-1 mRNA expression was mediated by TR. To investigate the involvement of TR in UCP-1 mRNA expression, the TR antagonist 1–850 (5 μM) was added to the medium throughout the T3 treatment period (50 nM). As expected, T3 in the absence of 1–850 significantly increased the UCP-1 mRNA expression level in cultured cells. The addition of 1–850 significantly decreased the T3-mediated UCP-1 mRNA expression level (Fig. 6A). To further verify the TR dependence of the effects of T3, we decreased TR expression level by siRNA-mediated knockdown. Quantitative RT-PCR revealed the decrease of the TR-α mRNA level by siRNA treatment, although that of TR-β mRNA level was not signif-

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**Fig. 1. Effects of triiodothyronine (T3) treatment on mRNA expression levels of adipogenic marker genes, UCP-1, and mitochondrial biogenesis genes in human multipotent adipose-derived stem (hMADS) cells.** hMADS cells were incubated in differentiation medium (DM) supplemented with vehicle or T3 (250 nM) for 3 days. Ten days after adipocyte differentiation, the mRNA expression levels of adipogenic marker genes (PPAR-γ, aP2, adiponectin, C/EBP-α, FATP1, LPL, and UCP-2) and thermogenic genes and mitochondrial biogenesis genes (UCP-1, PGC-1α, NRF1, TFAM, PRDM16, Cidea, and Elovl3) were determined. Values are means ± SE (n = 4). *P < 0.05 compared with control, as analyzed by the unpaired t-test.

**Fig. 2. Phase-dependent effects of T3 treatment on mRNA expression levels of UCP-1 and mitochondrial biogenesis genes in adipocyte differentiation.** A: schematic diagram of protocols to examine phase dependence of effects of T3 treatment (50 nM). hMADS cells were exposed to DM in the absence or presence of T3, as indicated. B: T3 was added between indicated days. Ten days after adipocyte differentiation, the mRNA expression level of UCP-1 was determined. Values are means ± SE (n = 4). *P < 0.05 compared with control, as analyzed by the unpaired t-test.

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**Fig. 3. Effects of T3 on mitochondrial biogenesis, nucleus-encoded mitochondrial genes, Cyt c and UCP-1 showed 1.65- and 3.34-fold amounts, respectively, in the T3-treated cells. The mRNA levels of COX4 and COX8a were higher in the T3-treated cells than in control (Fig. 5D). Moreover, the treatment with T3 also increased amounts of mtDNA, indicating higher levels of mitochondrial amounts (Fig. 5E). These findings suggest that the T3 treatment augments mitochondrial biogenesis in hMADS cells. Indeed, staining of differentiated hMADS cells with MitoTracker Red revealed stronger staining of T3-treated cells than that of control cells (Fig. 5F), indicating higher levels of membrane potential by T3 treatment. Increased membrane potential is created by the respiratory chain reaction to drive ATP synthesis, suggesting that T3 treatment may increase glucose and FA oxidation in hMADS cells.

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**Fig. 4. T3 treatment increased mitochondrion-related gene expression and mitochondrial biogenesis.** To further clarify the effects of T3 on mitochondrial biogenesis, nucleus-encoded mitochondrial genes, Cyt c, Cyt c oxidase subunit IV (COX4), and Cyt c oxidase subunit VIIIa (COX8a), were measured by real-time PCR. The Cyt c mRNA level at the 10th day was decreased in the control cells, and the decrease was significantly inhibited in the T3-treated cells. At protein levels, Cyt c expression was also higher in cultured cells treated with T3 than in control at the 10th day (Fig. 5C). The protein levels of Cyt c and UCP-1 showed 1.65- and 3.34-fold amounts, respectively, in the T3-treated cells. The mRNA levels of COX4 and COX8a were higher in the T3-treated cells than in control (Fig. 5D). Moreover, the treatment with T3 also increased amounts of mtDNA, indicating higher levels of mitochondrial amounts (Fig. 5E). These findings suggest that the T3 treatment augments mitochondrial biogenesis in hMADS cells. Indeed, staining of differentiated hMADS cells with MitoTracker Red revealed stronger staining of T3-treated cells than that of control cells (Fig. 5F), indicating higher levels of membrane potential by T3 treatment. Increased membrane potential is created by the respiratory chain reaction to drive ATP synthesis, suggesting that T3 treatment may increase glucose and FA oxidation in hMADS cells.

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**Fig. 5. T3-induced UCP-1 mRNA expression was mediated by TR.** To investigate the involvement of TR in UCP-1 mRNA expression, the TR antagonist 1–850 (5 μM) was added to the medium throughout the T3 treatment period (50 nM). As expected, T3 in the absence of 1–850 significantly increased the UCP-1 mRNA expression level in cultured cells. The addition of 1–850 significantly decreased the T3-mediated UCP-1 mRNA expression level (Fig. 6A). To further verify the TR dependence of the effects of T3, we decreased TR expression level by siRNA-mediated knockdown. Quantitative RT-PCR revealed the decrease of the TR-α mRNA level by siRNA treatment, although that of TR-β mRNA level was not signif-

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**Fig. 6. TR dependence of the effects of T3, we decreased TR expression level by siRNA-mediated knockdown.**
However, the TR-β protein level was significantly decreased (43.1%), as shown in Fig. 6C. The knockdown of TR attenuated the TR-dependent induction of UCP-1 mRNA expression (Fig. 6D). Specificity of TR isoforms was not observed under our experimental conditions. These findings indicate that T3 induces UCP-1 mRNA expression by TR activation, and TR mediates the T3-induced UCP-1 mRNA expression.

OCR was significantly increased by the T3 treatment (Fig. 7A). The T3 treatment showed a 1.2-fold increase in OCR. To determine the UCP-1-mediated respiration, hMADS cells were treated with isoproterenol. OCR was increased in T3-treated cells (Fig. 7B).

Next, we measured the mRNA expression levels of FA oxidation-related genes. T3 treatment increased the mRNA expression levels of UCP-1 and mitochondrial biogenesis genes. hMADS cells were differentiated into adipocytes and exposed to the indicated concentrations of T3 from day 0 to day 3. Ten days after adipocyte differentiation, the mRNA expression levels of UCP-1 (A), PGC-1α (B), NRF1 (C), and TFAM (D) were determined. Values are means ± SE (n = 4). *P < 0.05 compared with control, as analyzed by one-way ANOVA and Bonferroni test.

Fig. 3. Dose-dependent effects of T3 treatment on mRNA expression levels of UCP-1 and mitochondrial biogenesis genes. hMADS cells were differentiated into adipocytes and exposed to the indicated concentrations of T3 from day 0 to day 3. Ten days after adipocyte differentiation, the mRNA expression levels of UCP-1 (A), PGC-1α (B), NRF1 (C), and TFAM (D) were determined. Values are means ± SE (n = 4). *P < 0.05 compared with control, as analyzed by one-way ANOVA and Bonferroni test.

Fig. 4. Time course of mRNA expression levels of UCP-1 and mitochondrial biogenesis genes during adipocyte differentiation. hMADS cells were differentiated into adipocytes and exposed to T3 (50 nM) from day 0 to day 3. The mRNA expression levels of UCP-1 (A), PGC-1α (B), NRF1 (C), TFAM (D), and C/EBP-α (E) were quantified by a real-time PCR at indicated time points. Values are means ± SE (n = 4). *P < 0.05 compared with control, as analyzed by the unpaired t-test. The control at day 10 was set in each experiment to 100%.
In this study, T3 induced the UCP-1 expression in hMADS cells under the conditions for white adipocyte differentiation. UCP-1, a brown adipocyte-specific protein, consumes lipids without ATP production in brown adipocytes, suggesting that the increase in UCP-1 expression level suppresses the accumulation of lipids, thus regulating body weight (1, 13). Therefore, understanding of the mechanism of the increase in UCP-1 expression level contributes to the management of obesity and obesity-related diseases. The expression of UCP-1 is classically considered to be a physiological difference between brown and white adipocytes, which have distinct developmental origins (38). However, recent studies have shown that UCP-1 expression is observed in WAT under chronic β-agonist receptor activation or PPAR-γ activation in animals (17, 26). White preadipocytes isolated from WAT can differentiate into UCP-1-expressing cells without mRNA expression of brown adipocyte markers (27). Elabd et al. (9) have also shown that hMADS cells differentiate into functional brown adipocytes, depending on the duration of PPAR-γ activation. The observations suggest that the UCP-1-expressing cells in WAT are descendants of white adipocyte precursors, but not of brown adipocyte precursors. In this study, T3-treated hMADS cells after differentiation showed the phenotype of white adipocytes, indicating the involvement of TH in the regulation of UCP-1 expression in white adipocytes. The concentrations of T3 used in this study (50–250 nM) were higher than the physiological concentrations. However, the concentrations of free T3 were much lower than expected. This is because changes of culture medium increase D3 activity to a great extent and lead to a rapid decrease in T3 concentrations in a few hours under in vitro conditions (15). In brown adipocytes, the amount of T3 is increased by D2. The increase in the local

Values are means ± SE (n = 4). *P < 0.05 compared with control, as analyzed by the unpaired t-test. E: the concentration of mitochondrial DNA (mtDNA) was determined by spectrophotometry on day 10 of culture. The concentration of mtDNA was normalized by those of genomic DNA. The value of a control was set at 100%, and the relative value was presented as fold induction with respect to that of the control. F: differentiated hMADS cells were analyzed for mitochondrial abundance by MitoTracker Red staining. The center and right panels show images of the same areas. The yellow scale bars in panels represent 100 μm. See text for definition of gene acronyms.

DISCUSSION

In this study, T3 induced the UCP-1 expression in hMADS cells under the conditions for white adipocyte differentiation. UCP-1, a brown adipocyte-specific protein, consumes lipids without ATP production in brown adipocytes, suggesting that the increase in UCP-1 expression level suppresses the accumulation of lipids, thus regulating body weight (1, 13). Therefore, understanding of the mechanism of the increase in UCP-1 expression level contributes to the management of obesity and obesity-related diseases. The expression of UCP-1 is classically considered to be a physiological difference between brown and white adipocytes, which have distinct developmental origins (38). However, recent studies have shown that UCP-1 expression is observed in WAT under chronic β-agonist receptor activation or PPAR-γ activation in animals (17, 26). White preadipocytes isolated from WAT can differentiate into UCP-1-expressing cells without mRNA expression of brown adipocyte markers (27). Elabd et al. (9) have also shown that hMADS cells differentiate into functional brown adipocytes, depending on the duration of PPAR-γ activation. The observations suggest that the UCP-1-expressing cells in WAT are descendants of white adipocyte precursors, but not of brown adipocyte precursors. In this study, T3-treated hMADS cells after differentiation showed the phenotype of white adipocytes, indicating the involvement of TH in the regulation of UCP-1 expression in white adipocytes. The concentrations of T3 used in this study (50–250 nM) were higher than the physiological concentrations. However, the concentrations of free T3 were much lower than expected. This is because changes of culture medium increase D3 activity to a great extent and lead to a rapid decrease in T3 concentrations in a few hours under in vitro conditions (15). In brown adipocytes, the amount of T3 is increased by D2. The increase in the local

expression levels of human carnitine palmitoyltransferase-1b (CPT-1b) and acyl-CoA oxidase (AOX), which are rate-limiting enzymes in mitochondria and peroxisomes, respectively (Fig. 7C). Moreover, the evaluation of CO2 and ASM releases using [14C]palmitic acid revealed that T3 treatment increased the amounts of released CO2 and ASMs, which are products of FA oxidation, in hMADS cells (Fig. 7, D and E). These findings suggest that T3 treatment augmented oxidative capacity in addition to mitochondrial biogenesis in hMADS cells.
concentration of T3 in brown adipocytes is essential for BAT activation (8). In addition, it has been reported that D2 is expressed in WAT, and its expression level is increased by cold exposure (48), although hMADS cells showed no mRNA expression of D2 (data not shown). Moreover, there are many reports showing that important genomic actions of T3 usually require higher concentrations (10–100 nM) in in vitro experiments than in intact organisms (22, 35). Although further investigations are required to elucidate the in vivo effects of T3

Fig. 6. Involvement of thyroid hormone receptors (TR) in T3-induced UCP-1 expression in white adipocytes. A: hMADS cells were differentiated into adipocytes and treated with T3 (50 nM) from day 0 to day 3. Ten days after differentiation induction, the mRNA expression levels of UCP-1 were determined by real-time PCR. Values are means ± SE (n = 4). B: TRs were knocked down with a small interfering RNA (siRNA), as described in MATERIALS AND METHODS. The mRNA expression levels of adipocyte differentiation marker genes (CPT-1B and AOX; C), CO2 release (D), and acid-soluble metabolite (ASM; E) release in T3-treated hMADS cells were determined. The value of a control was set at 100%, and the relative value was presented as fold induction with respect to that of the control. Values are means ± SE (n = 10). *P < 0.05 compared with control, as analyzed by the unpaired t-test. See text for definition of gene acronyms.

Fig. 7. Effects of T3 treatment on oxygen consumption rate (OCR) and fatty acid oxidation in hMADS cells. hMADS cells were differentiated into adipocytes and treated with T3 (250 nM) from day 0 to day 3. Ten days after differentiation induction, OCR (A) and β-agonist-stimulated respiration (the ratio of OCR before and after addition of 1 μM isoproterenol; B) were determined by the method described in MATERIALS AND METHODS. The mRNA expression levels of adipocyte differentiation marker genes (CPT-1B and AOX; C), CO2 release (D), and acid-soluble metabolite (ASM; E) release in T3-treated hMADS cells were determined. The value of a control was set at 100%, and the relative value was presented as fold induction with respect to that of the control. Values are means ± SE (n = 10). *P < 0.05 compared with control, as analyzed by the unpaired t-test. See text for definition of gene acronyms.
and the requirement of high concentrations in in vitro experiments. Our findings may provide a clue to understanding the effects of T3 on UCP-1 induction in white adipocytes.

TH has been shown to regulate a wide range of genes involved in metabolisms in adipose tissues (14, 19, 43). Chronic TH treatment has increased heat production in rodent animals, indicating that TH is involved in thermogenesis (16, 24). The effects of TH on thermogenesis are both direct and indirect. Several transcriptional factors have been suggested as intermediary factors for the indirect effects (18). For example, C/EBP-α is a transcriptional activator of the promoter sequences of UCP-1 (49) and ablating C/EBP-α causes defects in UCP-1 expression (44) in BAT of mice. The overexpression of C/EBP-α induces UCP-1 expression in fetal brown adipocytes (42). These findings suggest a possibility that C/EBP-α can play an important role in the induction of UCP-1 expression in white adipocytes. In the present study, T3 treatment enhanced the induction of C/EBP-α mRNA expression, and C/EBP-α mRNA expression level was continuously increased during adipocyte differentiation of hMADS cells. The findings suggest that T3 exposure in the early phase of adipocyte differentiation induces UCP-1 expression examined on day 10 via the induction of C/EBP-α mRNA expression. It has been reported that T3 can induce C/EBP-α expression by a direct transcriptional regulation in a brown adipocyte cell line (25). Moreover, C/EBP-α permits autoregulation by direct binding of the C/EBP-α promoter region (6), as well as by an indirect mechanism (37). Thus our findings may provide evidence that transcriptional factors regulated by T3 may play an intermediary role in T3-induced gene expression. On the other hand, PRDM16 has been reported as an important factor indispensable for differentiation into brown adipocytes (20). Recently, it has been demonstrated that PRDM16 determines the thermogenic program of WAT, as well as BAT (20). However, the PRDM16 mRNA expression was not changed by the T3 treatment in our study (Fig. 1). The data might suggest a possibility that there is a PRDM16-independent pathway for inducing UCP-1 expression in white adipocytes or that the basal expression of PRDM16 in hMADS cells is enough to stimulate the T3-dependent UCP-1 expression. Petrovic and colleagues (27) have demonstrated that chronic PPAR-γ activation of mouse primary culture of white adipocytes induces UCP-1 expression, but only modest increase of PRDM16, and described that it is apparently not PRDM16 gene expression that confers white adipocytes with the ability to express UCP-1. Further investigations are needed for examining a role of PRDM16 in the UCP-1 expression in white adipocytes.

In this study, we demonstrated that PGC-1α mRNA expression was also induced by T3 treatment. Many studies have shown that PGC-1α, which is predominantly expressed in BAT and skeletal muscle, is a regulator of mitochondrial biogenesis (41, 47). The overexpression of PGC-1α in a mouse muscle cell line induces NRF1 mRNA expression and stimulates mitochondrial biogenesis via the induction of NRF1 (47). Although the PGC-1α mRNA expression level is lower in white adipocytes than that in brown adipocytes and muscle cells, a similar transcriptional regulation of PGC-1α expression has also been observed in human white adipocytes (39). Thus the regulation of PGC-1α and NRF1 expression may also play an important role in mitochondrial biogenesis in white adipocytes. In this study, T3 treatment increased the mRNA expression levels of PGC-1α, NRF1, and UCP-1 in hMADS cells. These findings suggest that T3 treatment enhances mitochondrial biogenesis in human white adipocytes. The results of MitoTracker probe staining and immunoblotting of UCP-1 and Cyt c also supported the idea that T3-mediated gene regulation induces mitochondrial biogenesis in human white adipocytes. The ectopic expression of PGC-1α in human adipocytes leads to an increase in UCP-1 mRNA expression level (39). Importantly, when comparing the mRNA expression levels of UCP-1, the minor T3 effects on the mRNA expression levels of Cyt c are contrasted with the general concern that UCP-1 expression and mitochondrial biogenesis should overlap to a certain extent. However, Petrovic et al. (27) recently showed that a proportion of mitochondrion-rich cells are UCP-1 positive, whereas numerous mitochondrion-rich cells are negative for UCP-1 during the induction of brown adipocytes in WAT by PPAR-γ activation. A recent study also showed that, in a primary culture of β-adrenoceptor knockout brown adipocytes, UCP-1 mRNA expression level is markedly decreased, whereas PGC-1α and TFAM mRNA expression levels are not (23). Moreover, in the same experiment, UCP-1, PGC-1α, and Cox IV protein expression levels are decreased by 97, 62, and 22%, respectively. The previous observations suggest that the amount of mitochondria is not a limiting factor for the regulation of UCP-1 expression. These findings suggest that the T3-mediated mitochondrial biogenesis in human white adipocytes is partly due to the induction of PGC-1α and NRF1 expression, in addition to the direct effect of T3 through TR activation. However, the results shown in the present study could not demonstrate the increase in mitochondrial uncoupling induced by the T-dependent induction of UCP-1 expression. It is very important for understanding the thermogenic effect of T3 on energy consumption in white adipocytes to elucidate changes in the uncoupling of mitochondria. Therefore, further investigations must be performed for clarifying the changes in white adipocytes.

In conclusion, the present study demonstrated that T3 treatment induced UCP-1 expression and mitochondrial biogenesis accompanied by the induction of PGC-1α and NRF1 in differentiated hMADS white adipocytes. The effects of T3 on UCP-1 induction were dependent on TR-β. Moreover, T3 treatment resulted in the increase in cellular OCR. The findings suggest a possibility that TH is a physiological modulator that induces energy utilization in white adipocytes through the induction of UCP-1 expression. However, the molecular mechanism underlying the effects of T3 on the regulation of gene expression remains to be clarified.

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