Induction of Beige-Like Adipocytes in 3T3-L1 Cells

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ABSTRACT. There are two types of brown adipocytes: classical brown adipocytes that form the brown fat depots and beige adipocytes that emerge in the white fat depots. Beige adipocytes have a low level of uncoupling protein 1 (Ucp1) expression in the basal state, but Ucp1 expression is increased in response to β adrenergic receptor activation. The present study explored the factors responsible for the differentiation of 3T3-L1 white preadipocytes to beige adipocytes. Significant expression of Ucp1 was not detected under any tested conditions in the absence of isoproterenol (Iso), an agonist of β adrenergic receptor. Iso-induced Ucp1 expression was significantly higher in the cells treated with a mixture of triiodothyronine (T3) and 3-isobutyl-1-methylxanthine (IBMX) for days 0–8 than in the control cells. Chronic IBMX treatment was indispensable for the enhanced Iso-induced Ucp1 expression, and treatment with additional rosiglitazone (Rosi) for days 0–8 further increased the Ucp1 expression. Recently, genes were identified that are predominantly expressed in beige adipocytes, which were induced from stromal vascular cells in white fat depots. However, the expression levels of the beige adipocyte-selective genes in the adipocytes induced by the mixture of T3, IBMX and Rosi did not differ from those in the control adipocytes. The present study indicates that 3T3-L1 cells can differentiate to beige-like adipocytes by prolonged treatment with the mixture of T3, IBMX and Rosi and that the gene expression profile of the adipocytes is distinct from those previously induced from white fat depots.

KEY WORDS: adipocyte, beige adipocyte, cell culture, cellular differentiation, Ucp1.

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There are two major types of adipocytes in mammals: white and brown. White adipocytes are specialized for the storage of excess energy [31]. In contrast, brown adipocytes dissipate chemical energy in the form of heat as a reaction against cold exposure or excess feeding [1, 4, 14, 20, 21, 38]. This thermogenic function of brown adipocytes results from the expression of a series of genes related to a high mitochondrial content and elevated cellular respiration that is largely uncoupled from ATP synthesis [35]. This uncoupling occurs through mitochondrial uncoupling protein 1 (Ucp1), a mammalian brown adipocyte-specific protein that promotes proton leak across the inner mitochondrial membrane [4, 13].

There are at least 2 origins of Ucp1-positive adipocytes in mice: brown adipocytes consisting of the classical brown fat depots, which are located in the interscapular region, and beige adipocytes residing in white fat depots. Both cell types up-regulate Ucp1 expression in response to β adrenergic receptor activation [3, 28, 37, 46]. However, beige adipocytes resemble white adipocytes in having extremely low basal expression of Ucp1, whereas classical brown adipocytes constitutively express Ucp1 [46]. In accordance with the differential regulation of Ucp1 expression, a distinct commitment/differentiation process is suggested between classical brown adipocytes and beige adipocytes in mice; classical brown adipocytes are derived from Myf-5-positive myoblast precursors, whereas beige adipocytes arise from non-Myf-5 lineage cells [36]. Certain studies have explored the cell origin of beige adipocytes and showed a direct conversion from white adipocytes [16], differentiation from beige pre-adipocytes located in white fat depots [46], commitment/differentiation from Sca1+/CD45−/Mac1− stem cells [33] and commitment/differentiation from Pdgfrα+/CD34−/Sca1+ stellate-like cells, which can be bipotentially differentiated into white adipocytes and beige adipocytes [18]. Thus, beige adipocytes may be induced from multiple types of cells.

Recent findings that adult humans have functional brown adipocytes [6, 26, 32, 43, 45] have triggered a focus on brown adipocyte activation as a novel therapeutic treatment for obesity [5]. In fact, the activation of human brown adipocytes is responsible for energy expenditure during acute cold exposure [29]. Comprehensive profiles of gene expression indicate a similar pattern between human brown adipocytes and mouse beige adipocytes but not mouse classical brown adipocytes, suggesting that human brown adipocytes have compatible characteristics to mouse beige adipocytes [37, 46]. In mice, increases in the number of beige adipocytes in the white fat depots are associated with protection against diet-induced obesity and metabolic dysfunction, including insulin resistance in mice [34, 44]. Therefore, clarification of the factors affecting the development of beige adipocytes is a prerequisite to basic information on beige adipocyte-me-
MATERIALS AND METHODS

exogenous gene transfer in 3T3-L1 white preadipocytes. The present study investigated the conditions to induce beige adipocytes without exogenous gene transfer in 3T3-L1 white preadipocytes.

MATERIALS AND METHODS

Materials: The following reagents were purchased from Sigma (St. Louis, MO, U.S.A.): dexamethasone (Dex), 3-isobutyl-1-methylxanthine (IBMX), insulin (Ins), triiodothyronine (T3), rosiglitazone (Rosi) and isoproterenol (Iso).

Cell culture: The 3T3-L1 preadipocytes were cultured as described previously [40]. The standard protocol of differentiation in 3T3-L1 cells [39] was treated as the control: two days after reaching confluence (day 0), the cells were cultured in DMEM with 10% FBS and antibiotics (growth medium) in the presence of differentiation inducers (Dex (0.25 µM), IBMX (0.5 mM) and Ins (10 µg/ml)) for 2 days, followed by culture in growth medium supplemented with Ins (5 µg/ml). According to the protocol, 3T3-L1 cells are differentiated to white adipocytes on day 8 [41]. In addition to the control protocol, Rosi (1 µM) for 5 to 7 days induced Ucp1 expression in stromal vascular cells isolated from white fat depots [28, 30]. Treatment with T3 (50 nM) enhanced norepinephrine-induced Ucp1 expression in primary brown adipocytes [22]. In addition, T3 is frequently used during brown adipocyte differentiation at concentrations of 1–250 nM [12, 15, 19, 28, 42].

Real-time RT-quantitative PCR: RNA isolation and real-time RT-quantitative PCR (qPCR) were performed as described previously [2, 11, 25]. The oligonucleotide primers for RT-qPCR are presented in Table 1. The Ct value was determined, and the abundance of gene transcripts was analyzed using the ∆∆Ct method, using TATA-binding protein (Tbp) as the normalization gene [8].

Statistical analyses: The data are expressed as the mean ± SEM. The data on gene expression were log-transformed to provide an approximation of a normal distribution before analysis. The differences between the groups were examined by ANOVA. *P* < 0.05 was considered to be significant.

RESULTS

The 3T3-L1 preadipocytes were differentiated by treatments with Rosi and T3 in addition to the reagents used in the control protocol for differentiation to white adipocytes [39]. Oil red O staining on day 8 showed that the 3T3-L1 cells were efficiently differentiated to adipocytes, irrespective of the treatment (Fig. 1A). Expression of aP2, a fatty-acid binding protein expressed in adipocytes, was comparable among groups, which was verified by RT-qPCR analyses (Fig. 1B) [47].

We also examined expression level of transcription factors related to adipogenesis [41]. The expression level of Ppary2 in treatments D and E was significantly lower than that in treatment B (Fig. 2A). Compared to the control treatment A, the gene transcript levels of Cebpa were lower in treatments C, B and E (Fig. 2B). The expression level of Cebpf was comparable among treatments (Fig. 2C).

Ucp1 expression is restricted in brown/beige adipocytes in mammals [4, 13]. The expression of Ucp1 was not reproducibly detected in any cells without β adrenergic activation (data not shown). In contrast, significant Ucp1 expression was detected in all the cells treated with Iso (Fig. 3). Treatment with the mixture of T3, IBMX and Rosi (treatment E) enhanced Iso-induced Ucp1 expression; the expression in treatment D, which was not reproducibly detected in any cells without β adrenergic activation (data not shown), was ~8-fold higher than that in the control treatment A (*P* = 0.003). The prolonged IBMX treatment was essential for the increased expression of Ucp1 in response to Iso treatment; the expression in treatment D,

| Oligonucleotide | 5′-primer | 3′-primer | GenBank accession number |
|-----------------|-----------|-----------|-------------------------|
| Ucp1            | 5′-ACTGCCACACCTCCAGTCATT-3′ | 3′-CTTTGCCTCACTCAGGATTGG-3′ | NM_024406 |
| Pparγ2          | 5′-CTGACTGCTACCTCGAATGTCG-3′ | 3′-AGGCTGAGCTTCAGCTCCCG-3′ | NM_011977 |
| Ear2            | 5′-GCTGGGAATCTCTGGAACCTG-3′ | 3′-GCTCTGGAACCTGTAATTTCT-3′ | NM_008904 |
| Slc27a1         | 5′-TGGTTATGGTGAAACTCTG-3′ | 3′-CTGTGTCAACCATGGTAATTCTT-3′ | NM_133249 |
| Cidea           | 5′-TTCTTGACACAGGCTGTTTCC-3′ | 3′-TGTGGAACTCTCTGGAACTGC-3′ | NM_024406 |
| C/ebpα          | 5′-CAAAGGATGATTCGGCTCAG-3′ | 3′-AAGCTGAATATATGCCTGCTTTC-3′ | NM_011146 |
| Pgc1f           | 5′-GGGCTGCAAGACCTCAGCTCAG-3′ | 3′-AAGCTGAATATATGCCTGCTTTC-3′ | NM_009360 |
| C/ebpβ          | 5′-CCAATGACTTCTATGACCTCCTTA-3′ | 3′-GCCTTGAAAGGTTATCTTG-3′ | NM_011977 |
| aP2             | 5′-AACGTTGAGGAGACCTCACAAC-3′ | 3′-GAGGCCAGTTGTGATGACTAAGAC-3′ | NM_024406 |
| Tee1            | 5′-CAACCAGCCCTAAGTTCACGTA-3′ | 3′-TGAGGCAAGGCTTAGAGGACAA-3′ | NM_007895 |
| Ear2            | 5′-CGCTGGGAATCTCTGGAACCTG-3′ | 3′-GCTCTGGAACCTGTAATTTCT-3′ | NM_008904 |
| Pgc1f           | 5′-CTGACTGCTACCTCGAATGTCG-3′ | 3′-AGGCTGAGCTTCAGCTCCCG-3′ | NM_011977 |
| Ucp1            | 5′-ACTGCCACACCTCCAGTCATT-3′ | 3′-CTTTGCCTCACTCAGGATTGG-3′ | NM_009360 |
which lacked the IBMX used in treatment E, was not different from that in the control treatment A. Ucp1 expression in treatment C, which is devoid of Rosi unlike treatment E, was still higher than that in the control treatment A \( (P=0.04) \), although the extent of the induction in treatment C was smaller than that in treatment E \( (P=0.03) \).

The expression of genes that are predominantly expressed in brown fat depots compared with white fat depots [35]...
was next examined (Fig. 4). The expression levels of 
Pgc1α, 
Pgc1β, 
Cited1
and 
Ear2
were comparable among treatments, whereas the expression of 
Cidea
was higher in the cells of treatments C (P=0.02) and E (P=0.04) than in treatment A.

Wu et al. [46] identified genes expressed selectively in beige adipocytes, but not brown adipocytes and white adipocytes, including 
CD137, 
Slc27a1, 
Ear2, 
Tbx1 and 
Tmem26.
Among these genes, significant expression of 
CD137, 
Tbx1 and 
Tmem26 was not detected in the 3T3-L1 cells, irrespective of the treatment (data not shown). The expression level of 
Slc27a1
was not higher in treatments B-E than in treatment A (Fig. 5A); rather, it was significantly lower in treatments C (P=0.004), D (P=0.005) and E (P=0.001) than in the control treatment A. The expression level of 
Ear2
was higher in treatment B (P=0.04) than in the control treatment A (Fig. 5B). However, the expression was lower in treatments C (P=0.001) and D (P=0.03). Sharp et al. [37] independently identified the beige adipocyte-selective gene in cells following prolonged treatment with 
T3 and 
Rosi; they revealed 
Cited1
as a novel beige adipocyte marker. The gene transcript level of 
Cited1
in treatment D was significantly higher than that in the other treatments (Fig. 5C).

DISCUSSION

The present results indicate that 3T3-L1 adipocytes treated with 
T3, 
Rosi and 
IBMX
express higher 
Ucp1 in response to β adrenergic activation. Basal expression of 
Ucp1
in beige adipocytes is as low as that in white adipocytes, whereas 
Ucp1 expression is enhanced in response to β adrenergic activation [46]. Significant expression of 
Ucp1 was also detected in the control 3T3-L1 adipocytes (treatment A) when the cells were treated with 
Iso; the result is consistent with that by Mottillo and Granneman [24]. Thus, the control 3T3-L1 adipocytes meet the definition of beige adipocytes by Wu et al. [46]. It is possible that the differences between white adipocytes and beige adipocytes are not discrete, but continuous. Our results suggest that 3T3-L1 cells chronically treated with the mixture of 
T3, 
Rosi and 
IBMX
are closer to mature beige adipocytes.

T3, 
IBMX
and 
Rosi
are all needed for the efficient induction of 
Ucp1
in response to β adrenergic receptor activation. However, whether 
T3 is essential is not known, because the observed 
Iso-induced 
Ucp1 expression was not examined in cells treated with 
IBMX
and 
Rosi, but without 
T3. In addition, the present results suggest that 
Rosi augments the effects of 
T3 and 
IBMX on the 
Ucp1 induction in response to 
Iso treatment. The increase in 
Ucp1 expression in white fat depots has been shown in mice chronically treated with 
Rosi [7, 28], implying a role of 
Rosi
as an enhancer of beige adipocyte induction.

We focused on 
T3, 
Rosi
and 
IBMX in view of the following evidence: prolonged treatment with 
Rosi with or without 
T3 in stromal vascular cells from white fat depots resulted in 
Iso-induced 
Ucp1 induction [28, 30]. The overexpression of 
Cebpβ enhanced cAMP-mediated 
Ucp1 induction in 3T3-L1 cells [17]. Furthermore, IBMX is responsible for 
Cebpβ induction during mitotic clonal expansion, i.e., days 0–2, in 3T3-L1 cells, which allows for the cells to differentiate to white adipocytes [47]; therefore, we expected up-regulation of the 
Cebpβ expression in treatments C and E that were treated with the prolonged IBMX. However, the expression level of 
Cebpβ was comparable among treatments A, C and E (Fig. 2C), suggesting an activity of IBMX other than the regulation of 
Cebpβ expression. There are nearly 100 cyclic nucleotide phosphodiesterases that catalyze cAMP or cGMP or both [10]. The non-selective phosphodiesterase inhibitor IBMX actually increased cytosolic concentration of cAMP in 3T3-L1 cells [9] and possibly increases cGMP concentration. It was recently revealed that cGMP-dependent protein kinase I in white adipocytes acts induces beige adipocytes [23] and may be involved in the IBMX-induced development of beige adipocytes.

Expression level of 
Cebpα
was lower in treatments B, C and E than in the control treatment A (Fig. 2B); the transcript level of 
Cebpα reflects adipocyte differentiation [41]. The precise reason for the decreased expression is unknown, although there are at least 2 possibilities: 1) the adipocyte differentiation was partially inhibited by treatments B, C and E, although lipid accumulation was unaffected; or 2) the decreased expression is partially related to the induction of
beige (brown) adipocytes. The expression level of *C/ebpa* in beige (brown) adipocytes may be lower than that in white adipocytes. The results of the transcriptomic analyses (NCBI gene expression omnibus accession number: GSE8044), which were performed in the study by Seale et al. [35], indicated that the expression level of *C/ebpα* was lower in brown fat depots than in white fat depots.

Wu et al. [46] showed that the expression level of genes highly expressing in brown fat depots [35] was comparable between beige adipocytes and white adipocytes, although others observed higher expression of these genes in beige adipocytes [28, 30, 33]. Thus, the expression pattern of the brown fat-selective genes in 3T3-L1 cells treated with the mixture of T3, IBMX and Rosi essentially resembles that of the beige adipocytes identified by Wu et al. [46]. However, the expression pattern of beige adipocyte-selective genes was different from the results by Wu et al. [46], suggesting that the characteristics of the beige-like adipocytes induced in this study are distinct from those developed by Wu et al. [46].

Our results also suggest the distinct cell context of the T3- and Rosi-induced beige-like adipocytes from those developed by Sharp et al. [37]. *Cited1* expression was increased by prolonged treatment with T3 and Rosi (treatment D: \( P < 0.001 \)) in the 3T3-L1 cells, which was similar to the results by Sharp et al. [37]. Thus, *Cited1* is likely to be induced by the activation of Pparγ, and the expression level of *Cited1* does not reflect the development of beige-like adipocytes, at least in the 3T3-L1 cell model. In addition, the T3- and Rosi-induced *Cited* expression is blocked by co-treatment with IBMX.

The present study clarifies the differentiation of 3T3-L1 white preadipocytes into beige-like adipocytes. As described above, beige adipocytes could be differentiated from white
adipocytes [16], beige preadipocytes [46], Sca1+/CD45−/Mac1− stem cells [33], pluripotent stem cells [27] and Pdgfrα+/Sca1+ stellate-like cells [18]. Considering all the previous results with the present results, beige adipocytes could be developed from a variety of cells through their specific regulation. It was recently reported that a hematopoietin cocktail composed of stem cell factor, interleukin-6, fms-related tyrosine kinase 3 ligand and vascular endothelial growth factor efficiently differentiates human pluripotent stem cells to brown adipocytes [27]. Further studies are needed to pursue efficient beige adipocyte development, which would provide basic information on the differentiation of white preadipocytes to beige adipocytes.

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