Hypoxia-inducible factor-1α regulates Lipin1 differently in pre-adipocytes and mature adipocytes

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Abstract. Hypoxia-inducible factor (HIF)-1α is a transcription factor that is activated in low oxygen conditions. Adipose tissues are poorly oxygenated in patients with obesity. The low oxygen conditions in obese adipose tissues induce HIF-1α in adipocytes. Previous studies using genetically modified mice suggest that HIF-1α contributes to dysfunction in adipocytes. Lipin1 is a bifunctional protein that works as a phosphatidate phosphatase and transcriptional coactivator, which regulates lipid metabolism and adipogenesis, respectively. HIF-1α directly regulates Lipin1 in hepatocytes. However, the regulation of Lipin1 by HIF-1α in adipocytes is not well determined. Therefore, the present study investigated the regulation of Lipin1 by HIF-1α in adipocytes. Expression levels of Lipin1 were reduced in epididymal adipose tissues of adipocyte-specific HIF-1α knockout mice, indicating that HIF-1α regulates Lipin1 in adipocytes. In differentiated mature adipocytes, a HIF-1α activator, dimethyloxallyl glycine (DMOG), was demonstrated to increase Lipin1, and a HIF-1α inhibitor, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), reversed this increase, indicating that HIF-1α regulates Lipin1 in differentiated adipocytes. However, during differentiation of pre-adipocytes into adipocytes, YC-1 increased Lipin1 even though HIF-1α was decreased. The differentiation efficiency increased with YC-1 treatment. In addition, DMOG reduced Lipin1 expression levels during differentiation despite increased HIF-1α. Under these conditions, differentiation efficiency was reduced. These results suggest that Lipin1 is negatively regulated by HIF-1α in pre-adipocytes. Our results show that regulation of Lipin1 by HIF-1α is different in adipocytes and pre-adipocytes.

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Introduction

Obesity is one of the causes of type 2 diabetes. In obesity, adipocytes are hypertrophied, and their functions are aberrant, leading to glucose intolerance or type 2 diabetes. It is widely suggested that adipose tissues are poorly oxygenated in obesity (1-5). Hypoxia activates many genes for cellular adaptation to hypoxic environments. Hypoxia-inducible factor (HIF)-1α is a transcription factor that responds to low oxygen conditions (6). Prolyl hydroxylase enzymes (PHDs) sense cellular oxygen, leading to degradation of HIF-1α under normoxic conditions. However, HIF-1α is stabilized under hypoxic conditions and is transferred to the nucleus where it activates its target genes (7,8). HIF-1α is expressed in adipocytes from obese adipose tissue under hypoxic conditions (4,9). Because HIF-1α regulates glucose metabolism, cell survival, and inflammation (6), it is expected that HIF-1α expression in adipocytes is dysregulated causing inflammation in adipose tissue, thereby inducing the onset of type 2 diabetes. Indeed, previous reports show expression of HIF-1α in adipocytes leads to inflammation and fibrosis in adipose tissue, secretion defects of hormones and cytokines from adipocytes, increased lipid storage, and whole-body glucose intolerance (5,10-12). In addition, adipocyte-specific knockout of HIF-1α shows reduced adipocyte sizes in obese adipose tissues (11,12). Transgenic HIF-1α mice show increased adipocyte size in subcutaneous white adipose tissues (5). These results indicate that HIF-1α participates in adipogenesis.

Lipin1 plays important roles in lipid homeostasis and metabolism as an enzyme for lipid synthesis and as a nuclear receptor coactivator (13). The enzymatic function of Lipin1 is as a phosphatidate phosphatase (PAP), which catalyzes phosphatidic acid to diacylglycerol, contributing to lipid storage in adipocytes (14,15). As a transcriptional coactivator, Lipin1 forms a complex with peroxisome proliferator-activated receptor (PPAR)α and PPARγ coactivator 1 (PGC-1) regu-

lating gene expression for fatty acid oxidation (16). In addition, Lipin1 is expressed in differentiating pre-adipocytes. Lipin1 activation during differentiation of adipocyte requires the adipogenic transcription factors PPARγ and CCAAT/enhancer binding protein α (C/EBPα) (17). Knockdown of Lipin1 in pre-adipocytes inhibits differentiation into adipocytes, whereas Lipin1 overexpression enhances adipocyte differentiation (18,19). Thus, Lipin1 is important for adipogenesis. Lipin1 is regulated by HIF-1α in cells of non-adipocyte origin (20). However, the regulation of Lipin1 by HIF-1α in adipocytes is unknown.

In the present study, we investigated the HIF-1α regulation of Lipin1 in adipocytes. Lipin1 expression levels in epididymal adipose tissue of adipocyte-specific HIF-1α knockout mice were significantly decreased relative to wild type mice, indicating that HIF-1α regulates Lipin1. In differentiated 3T3-L1 adipocytes, HIF-1α activation induced Lipin1 and HIF-1α inhibition reduced Lipin1. However, during differentiation, HIF-1α activation reduced Lipin1 and HIF-1α inhibition induced Lipin1. Lipin1 expression levels correlated with adipocyte differentiation efficiency. Together, our results indicate that regulation of Lipin1 by HIF-1α is different in pre-adipocytes and mature adipocytes.

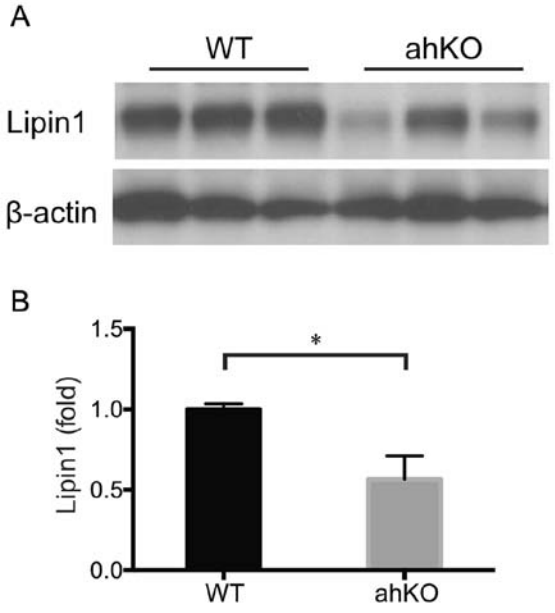

Figure 1. Lipin1 expression in epididymal adipose tissues in wild type and adipocyte-specific HIF-1α knockout (ahKO) mice. WT and ahKO mice were fed a high fat diet for 15 weeks. Then, epididymal fat pads were resected. The expression levels of Lipin1 were observed using western blot. (A) Representative western blot and (B) means expression levels (mean ± S.E.M; n =3) of Lipin1. *P<0.05. HIF, Hypoxia-inducible factor; WT, wild-type.

Materials and methods

Chemicals and antibodies. 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) and dimethyloxallyl glycine (DMOG) were purchased from Cayman Chemical. Anti-HIF-1α antibody (Cayman Chemical), anti-Lipin1 antibody (Cell Signaling), and anti-β-actin antibody (Cell Signaling) were used.

Adipocyte-specific HIF-1α knockout mice. All the experimental procedures were performed in accordance with the guidelines of the Animal Research Committee, Tokushima University. The protocol was approved by the Animal Research Committee, Tokushima University (approval no: 14129). HIF-1α-floxed mice containing loxP sites flanking exons 13–15 of the HIF-1α gene (21) were crossed with mice harboring the Cre recombinase under the control of the aP2 promoter (aP2-Cre mice; a gift from Ronald M. Evans, Salk Institute for Biological Studies), generating adipocyte-specific HIF-1α knockout (ahKO) mice. All mice were C57BL/6 and only male mice were used for experiments. The mice were maintained under temperature- and light-controlled environmental settings with free access to water. Six-week-old mice were fed a high fat diet (HFD) (57% kcal consisting of fat; high fat diet 32 (CLEA Japan)) for 15 weeks. The epididymal fat pads were resected from the wild type and ahKO mice.

Cell culture. 3T3-L1 cells were cultured until confluence, allowed to grow for 2 days postconfluency, and then differentiated with the addition of 500 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 10 μg/ml insulin for 2 days. The medium was changed to growth medium supplemented with 10 μg/ml insulin (differentiation medium) every 2 days. For chemical treatment of differentiated 3T3-L1 adipocytes, the adipocytes were cultured in serum-free media for 24 h and then treated with 500 μM DMOG or 50 μM YC-1. For measurement of differentiation efficiency, the area of differentiated adipocytes was divided by the total area in a microscopic field taken at magnification, x100.

Western blot analysis. Epididymal fat pads and cells were lysed in lysis buffer (20 mM Tris-HCl, pH 8.0), 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, protease inhibitor mixture (2 g/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain, and 10 μg/ml benzamidine), and phosphatase inhibitor mixture (10 mM NaF, 60 mM β-glycerophosphate, 10 mM sodium pyrophosphate, and 2 mM sodium orthovanadate). Proteins were separated on SDS polyacrylamide gels and electrophoretically transferred to polyvinylidene fluoride membranes. The membranes were incubated with a primary antibody overnight at 4˚C and probed with an HRP-conjugated secondary antibody (KPL). Immunoreactive bands were detected with ECL (GE Healthcare) and visualized by exposing the membranes to X-ray films (GE Healthcare). The proteins were quantified by densitometric analysis using ImageJ analysis software.

Statistical analysis. Data are presented as mean values ± standard error of the mean (S.E.M.). Statistical significance was assessed using the Student's t-test or two-way analysis of variance (ANOVA) with Sidak's multiple comparisons test, where values of P<0.05 were considered to indicate a statisically significant difference. Prism version 6.0h (GraphPad Software) was used for data analysis.

Results

Lipin1 is decreased in ahKO mice. Lipin1 is regulated by HIF-1α in cells of non-adipocyte origin (20). However, the regulation of Lipin1 by HIF-1α in adipocytes is unknown.

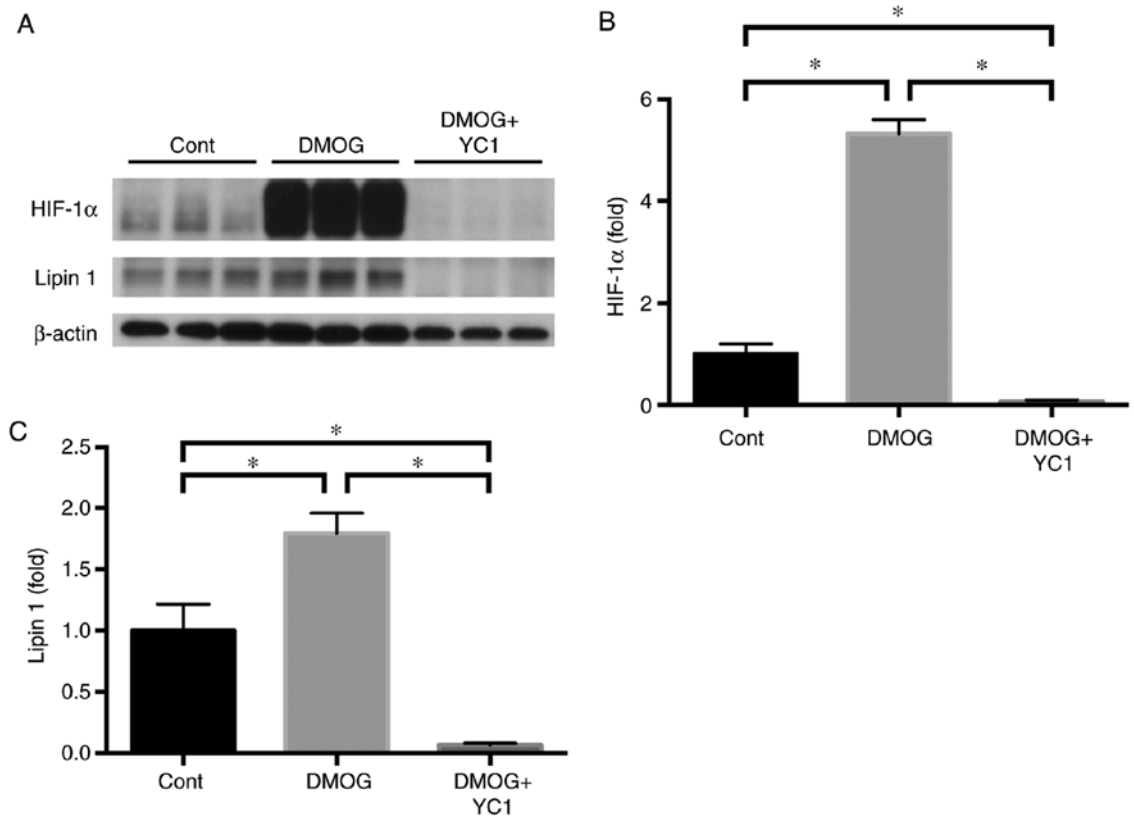

Figure 2. Effects of activation and inhibition of HIF-1α on Lipin1 expression in differentiated adipocytes. Differentiated 3T3-L1 cells were treated with 500 μM DMOG and 50 μM YC-1 for 4 h. (A) Representative western blot analysis. Expression levels (mean ± S.E.M; n=3) of (B) HIF-1α and (C) Lipin1, respectively. *P<0.05. HIF, Hypoxia-inducible factor; DMOG, dimethyloxallyl glycine; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole.

Therefore, we investigated the expression of Lipin1 in adipose tissue from ahKO mice. Wild type (WT) and ahKO mice were fed an HFD for 15 weeks and then the epididymal adipose tissues were resected. Western blots showed that the expression levels of Lipin1 in the epididymal adipose tissues of ahKO mice significantly decreased compared with WT mice (Fig. 1). This result indicates that HIF-1α regulates Lipin1 in adipocytes.

Lipin1 regulation by HIF-1α in differentiated 3T3-L1 adipocytes. To assess whether HIF-1α regulates Lipin1 in adipocytes, 3T3-L1 cells were differentiated to adipocytes and treated with a HIF-1α activator, DMOG, for 4 h. DMOG treatment significantly increased HIF-1α expression levels in the adipocytes (Fig. 2A and B). In addition, DMOG also elevated Lipin1 expression levels in the differentiated adipocytes under the same conditions (Fig. 2A and C). To confirm the regulation of Lipin1 by HIF-1α, the effect of YC-1, an inhibitor of HIF-1α, on the elevation of Lipin1 was studied. The DMOG-induced increases of HIF-1α and Lipin1 were decreased by YC-1 (Fig. 2A-C), suggesting that HIF-1α regulates Lipin1 in the differentiated adipocytes.

DMOG suppresses the differentiation of 3T3-L1 pre-adipocytes into adipocytes. Lipin1 has two different functions: one is as an enzyme regulating lipid metabolism (14,15) and the other is as a mediator of differentiation into adipocytes (17). Therefore, we studied the relationship between HIF-1α and Lipin1 along with the effect of DMOG on the differentiation of 3T3-L1 pre-adipocytes into adipocytes. we studied the effect of DMOG on differentiation of 3T3-L1 cells. Treatment with DMOG significantly increased HIF-1α expression levels in 3T3-L1 cells on days 2, 4, and 8 during differentiation (Fig. 3A and B). In contrast, the expression levels of Lipin1 significantly decreased on day 6 and 8. The differentiation efficiency of 3T3-L1 cells was significantly reduced with DMOG (Fig. 3D and E). These results show that DMOG reduces Lipin1 expression levels and differentiation efficiency, whereas HIF-1α expression levels increase.

YC-1 accelerates the differentiation of 3T3-L1 pre-adipocytes into adipocytes. The effect of the HIF-1α inhibitor, YC-1, during differentiation of 3T3-L1 cells, was investigated. HIF-1α was induced in the initial 24 h and then gradually decreased under the normal conditions for differentiation (Fig. 4A and B). In contrast, Lipin1 increased at day 4 after differentiation initiation. Next, the effect of YC-1 on differentiation of 3T3-L1 pre-adipocytes into adipocytes was investigated. 3T3-L1 pre-adipocytes were differentiated in differentiation medium containing YC-1. Addition of YC-1 during differentiation of 3T3-L1 cells reduced HIF-1α expression levels in the initial 24 h of the differentiation. Under the same conditions, Lipin1 increased at day 6 and day 8 after differentiation initiation. YC-1 treatment significantly increased the differentiation efficiency of 3T3-L1 cells (Fig. 4D and E). The expression of PPARγ (an adipogenic transcription factor) and adiponectin (an adipose-secreted protein) was significantly increased in YC-1 treated adipocytes at day 8 (Fig. 5).

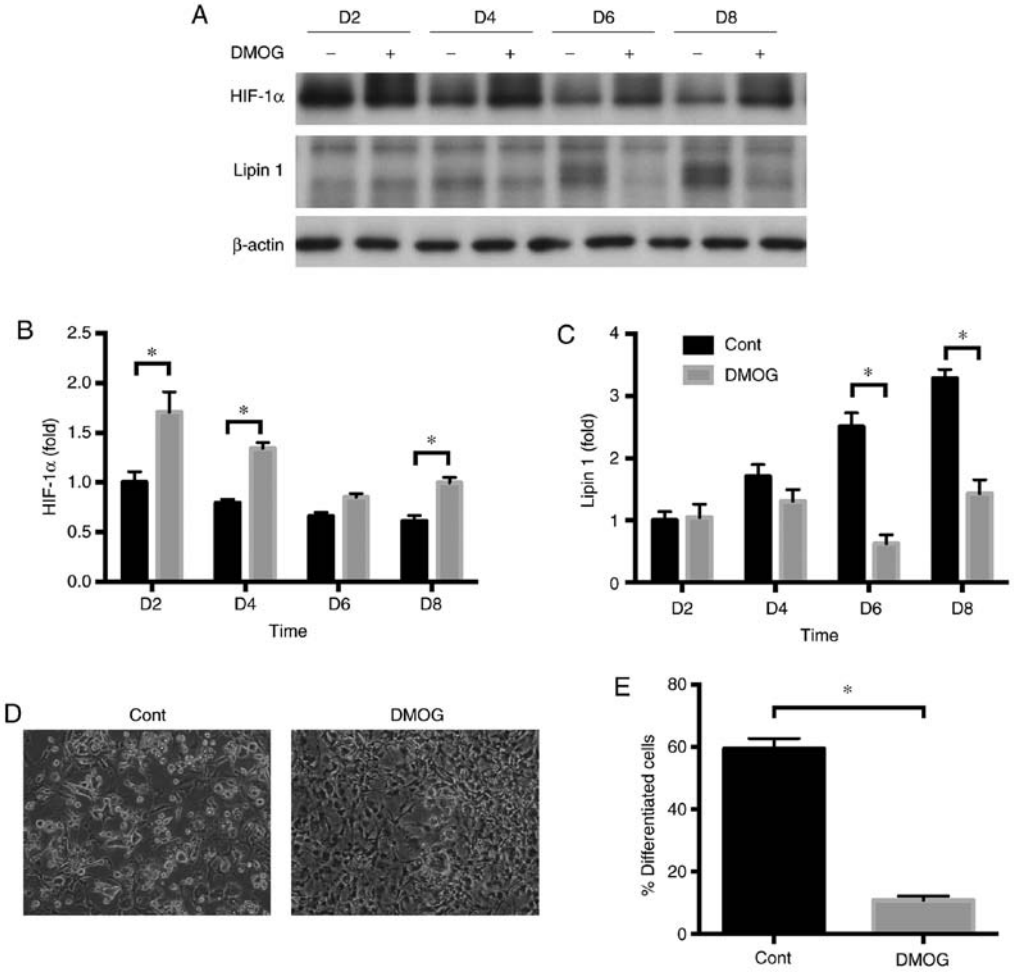

Figure 3. Effect of activation of HIF-1α on expression of Lipin1 and differentiation efficiency of pre-adipocytes. 3T3-L1 pre-adipocytes were differentiated in differentiation medium with or without 500 μM DMOG. (A) Representative western blot. The day after differentiation initiation is indicated on the top of the blot. (B and C) Expression levels of HIF-1α and Lipin1, respectively. Values are mean ± S.E.M; (n=3). (D) Cells 8 days after differentiation initiation. Magnification, x100. (E) Differentiation efficiency. *P<0.05. HIF, Hypoxia-inducible factor; DMOG, dimethyloxallyl glycine; S.E.M., standard error of the mean.

Discussion

Lipin1 is regulated by HIF-1α in cells of non-adipocyte origin (20). However, the regulation of Lipin1 by HIF-1α in adipocytes is unknown. Therefore, in the present study, we focused on the regulation of Lipin1 by HIF-1α. We found that Lipin1 was significantly decreased in epididymal adipose tissues of ahKO mice (Fig. 1). This result indicates that HIF-1α regulates Lipin1 in adipocytes in the adipose tissue of mice. In addition, HIF-1α upregulates Lipin1 in mature adipocytes but downregulates Lipin1 in pre-adipocytes.

We found that DMOG, a HIF-1α activator, induced Lipin1 in differentiated adipocytes (Fig. 2). In addition, YC-1, a HIF-1α inhibitor, canceled the induction of Lipin1 with DMOG (Fig. 2). These results indicate that HIF-1α regulates Lipin1 in differentiated adipocytes. Previously, it was shown that Lipin1 is regulated by HIF-1α in cells of non-adipocyte origin (20,22,23). Our results show that Lipin1 is regulated by HIF-1α in differentiated adipocytes as well. Previously, it has been shown that HIF-1α-induced Lipin1 causes the accumulation of lipids in hepatocytes (20). Therefore, HIF-1α activation in adipocytes can also cause excess lipid accumulation, leading to metabolic disorders.

During differentiation of pre-adipocytes into adipocytes, HIF-1α levels gradually decreased (Figs. 3 and 4). However, although HIF-1α expression had gradually decreased by day 6 in the DMOG-treated cells its expression increased at day 8 (Fig. 3). HIF-1α is constantly synthesized and degraded (6). Therefore, the balance between synthesis and degradation of HIF-1α changes to synthesis dominant around day 8 after increased differentiation, suggesting that the effects of DMOG is increased. In addition, because HIF-1α mRNA levels increase in the initial 3 to 6 h after differentiation (24), mRNA regulation partly contributed to the initial increased expression of HIF-1α protein after differentiation.

During differentiation, YC-1, an inhibitor of HIF-1α, reduced HIF-1α expression levels in the pre-adipocytes. However, regulation of Lipin1 by HIF-1α was opposite in differentiating pre-adipocytes compared with differentiated adipocytes. YC-1 reduced HIF-1α expression levels during the initial 24 h, whereas it increased Lipin1 expression levels 6 to 8 days after differentiation initiation (Fig. 4). This result indicates that HIF-1α inhibition indirectly participated in the upregulation of Lipin1. To activate Lipin1 during differentiation of adipocytes, the adipogenic transcription factors PPARγ and C/EBPα are required (17). In the study, PPARγ was increased

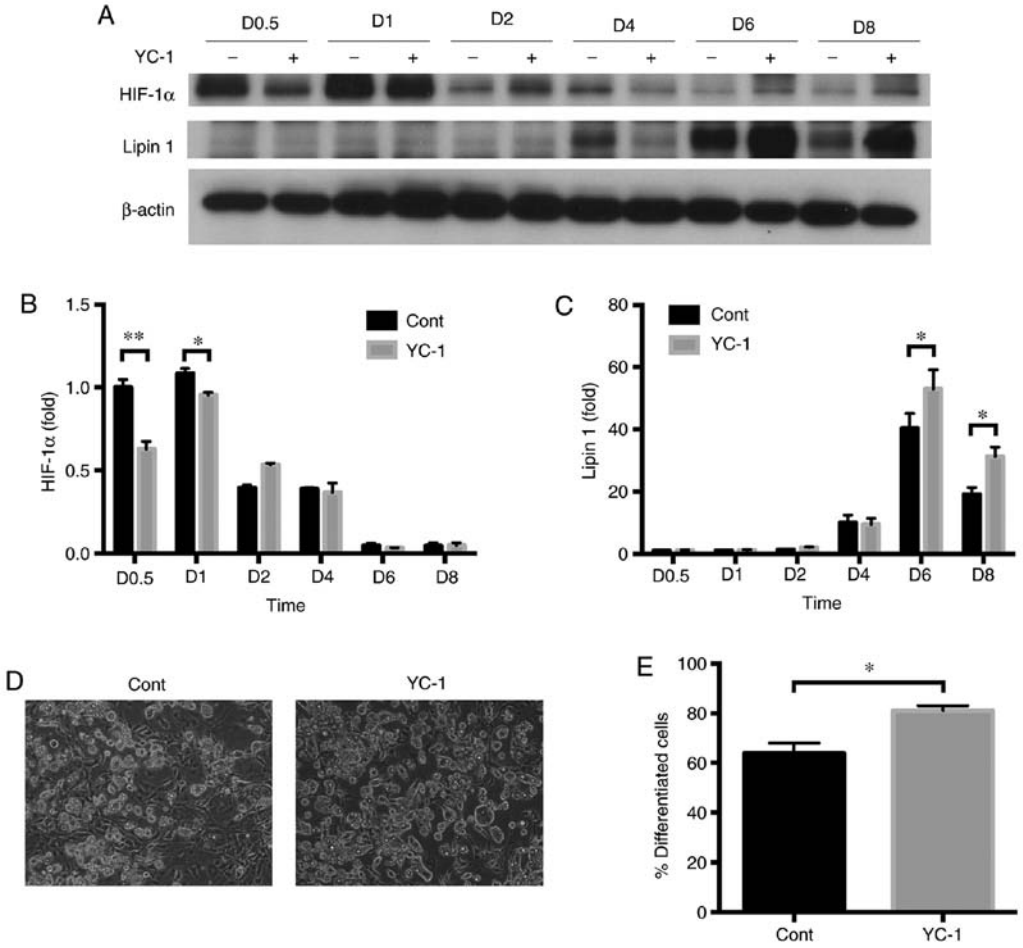

Figure 4. Effect of HIF-1α inhibition on expression of Lipin1 and differentiation efficiency of pre-adipocytes. 3T3-L1 pre-adipocytes were differentiated in differentiation medium with or without 50 μM YC-1. (A) Representative western blot analysis. The day after in itiating differentiation is indicated on the top of the blot. Expression levels of (B) HIF-1α and (C) Lipin1, respectively. Values are mean ± S.E.M. (n=3). (D) Cells 8 days after differentiation initiation. Magnification, x100. (E) Differentiation efficiency. *P<0.05, **P<0.01. HIF, Hypoxia-inducible factor; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole, S.E.M., standard error of the mean.

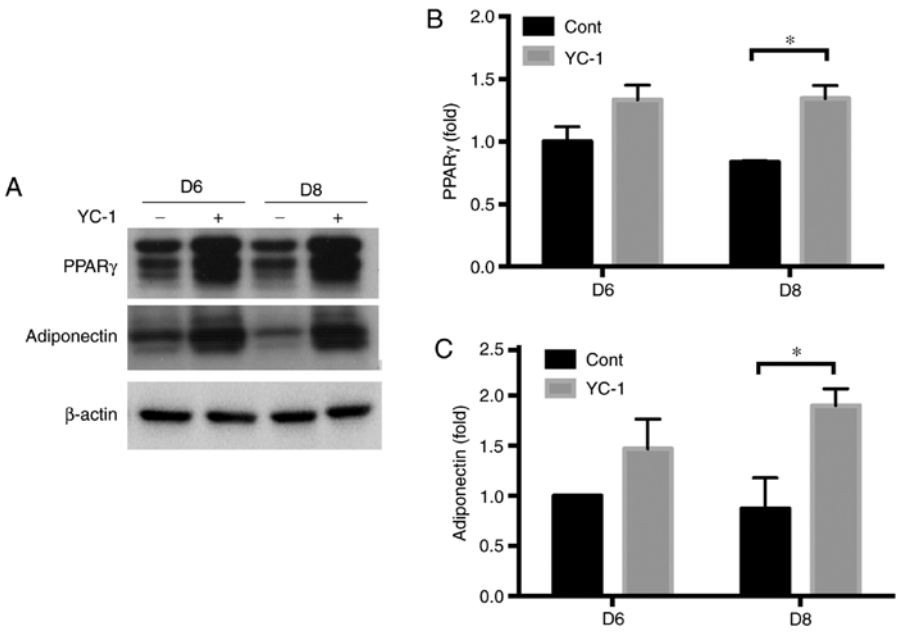

Figure 5. Effect of HIF-1α inhibition on the expression of PPARγ and adiponectin. 3T3-L1 pre-adipocytes were differentiated in a differentiation medium with or without 50 μM YC-1. (A) Representative western blot. The day after initiating differentiation is indicated on the top of the blot. (B and C) Expression levels of PPARγ and adiponectin, respectively. Values are mean ± S.E.M (n=3). *P<0.05. YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole; HIF, Hypoxia-inducible factor; PPARγ, peroxisome proliferator-activated receptor; S.E.M., standard error of the mean.

in YC-1 treated adipocytes (Fig. 5). Further, it was reported that HIF-1α indirectly downregulates PPARγ (25). Therefore, the inhibition of HIF-1α probably contributes to upregulation of PPARγ, leading to Lipin1 induction during YC-1-affected differentiation of adipocytes. Adiponectin is an adipokine derived from the adipocytes and has anti-inflammatory and insulin-sensitizing effects (26). In obese adipose tissues, the secretion of adiponectin is decreased (27-30). YC-1-affected differentiation increased adiponectin expression, indicating that the differentiated adipocytes are healthy. In addition, DMOG increased HIF-1α expression levels 2, 4, and 8 days after starting differentiation. On the contrary, Lipin1 was decreased 6 and 8 days after differentiation initiation. In this condition, differentiation efficiency was quite low (Fig. 3). Previous reports showed that hypoxia inhibits adipogenesis of 3T3-L1 cells through HIF-1α (25,31,32). Our results suggest that the hypoxic inhibition of adipogenesis may participate in Lipin1 downregulation.

In *in vivo* studies, adipocyte-specific knockout of HIF-1α protects obese mice from insulin resistance and inflammation (11,12), whereas transgenic mice expressing a constitutively active form of HIF-1α have insulin resistance and tissue fibrosis (5). In the studies, the size of adipocytes were smaller in the adipocyte-specific HIF-1α knockout mice than in WT mice (11,12). In the overexpression study of a constitutively active form of HIF-1α, the transgenic mice showed increased adipocyte size in subcutaneous white adipose tissues (5). In addition, HIF overexpression with PHD2 deletion in mice reduces lipolysis and increases lipid storage (33). Our results suggest that lipid accumulation in the knockout mice might be decreased by reduced Lipin1 expression in differentiated adipocytes.

Some limitations exist in the present study. Our study showed that HIF-1α upregulates Lipin1 in mature adipocytes but downregulates it in pre-adipocytes. However, the regulation of Lipin1 by HIF-1α in ahKO mice was not clear. To further study this, the effects of YC-1 on the adipose tissue of ahKO mice, or HIF-1α knockdown as reported in the previous study (34), should be observed.

It is possible that regulation mechanisms of Lipin1 by HIF-1α are different between pre-adipocytes and adipocytes. HIF-1α reduces Lipin1 during differentiation of pre-adipocytes and reduces differentiation efficiency. HIF-1α directly increases Lipin1 in differentiated adipocytes and regulates lipid metabolism. Regulation of the HIF-1α - Lipin1 system may be a potential therapeutic target for the treatment of obesity and type 2 diabetes.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YK and ST contributed to the conception and design of the study, acquired and analyzed the data and drafted the manuscript. YF contributed to acquiring and analyzing the data. TT and ES contributed to the design of the study, revised the manuscript and approved the final manuscript.

Ethics approval and consent to participate

All the experimental procedures were performed in accordance with the guidelines of the Animal Research Committee, Tokushima University. The protocol was approved by the Animal Research Committee, Tokushima University (approval no. 14129).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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