Prolyl Hydroxylase Domain Protein 2 Plays a Critical Role in Diet-induced Obesity and Glucose Intolerance

Running title: Matsuura et al.; Role of PHD2 in adipocyte

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Abstract:

Background—Recent studies suggest that oxygen-sensing pathway consisting of transcription factor hypoxia inducible factor (HIF) and prolyl hydroxylase domain proteins (PHD) plays a critical role in glucose metabolism. However, the role of adipocyte PHD in the development of obesity has not been clarified. We examined whether deletion of PHD2, the main oxygen sensor, in adipocyte affects diet-induced obesity and associated metabolic abnormalities.

Methods and Results—To delete PHD2 in adipocyte, PHD2-floxed mice were crossed with aP2-Cre transgenic mice (Phd2\(^{\text{floxed}}\)/aP2-Cre). Phd2\(^{\text{floxed}}\)/aP2-Cre mice were resistant to high-fat diet-induced obesity (36.7 ± 1.7g vs 44.3 ± 2.0g in control, P<0.01) and showed better glucose tolerance and HOMA-IR index (3.6±1.0 vs 11.1±2.1 in control, P<0.01) than control mice. The weight of white adipose tissue (WAT) was lighter (epididymal fat: 758 ± 35mg vs. 1208 ± 507mg in control, P<0.01) with reduction of adipocyte size. Macrophage infiltration into WAT was also alleviated in Phd2\(^{\text{floxed}}\)/aP2-Cre mice. Target genes of HIF including glycolytic enzymes and adiponectin were upregulated in adipocytes of Phd2\(^{\text{floxed}}\)/aP2-Cre mice. Lipid content was decreased and uncoupling protein 1 expression was increased in brown adipose tissue of Phd2\(^{\text{floxed}}\)/aP2-Cre mice. Knockdown of PHD2 in 3T3L1 adipocytes induced a decrease in the glucose level and an increase in the lactate level in the supernatant with up-regulation of glycolytic enzymes and reduced lipid accumulation.

Conclusions—PHD2 in adipose tissue plays a critical role in the development of diet-induced obesity and glucose intolerance. PHD2 might be a novel target molecule for the treatment of obesity and associated metabolic abnormalities.

Key words: hypoxia, obesity, adipose tissue, prolyl hydroxylase domain protein
Introduction

Obesity is one of the critical risk factors for the development of atherosclerosis, diabetes and coronary artery disease. Previous studies have shown that obesity induces low-grade chronic inflammation in the adipose tissue, leading to dysregulated adipocytokine production and increased oxidative stress. These contribute to the pathogenesis of glucose intolerance, dyslipidemia, and insulin resistance in obesity. To prevent these adverse effects in obese patients, body weight reduction is necessary. While patient education for lifestyle modification and encouragement of physical exercise are recommended to normalize body weight, the effects are often insufficient. Therefore, alternative means to ameliorate obesity have been attempted such as development of anti-obesity drugs. Cannabinoid-1 receptor blocker rimonabant was developed with great expectation, but it has not been commonly used in clinical practices because of several side effects such as depression and anxiety disorder. Thus, a novel therapeutic target to treat obesity has been aspired.

Hypoxia has long been known to reduce body weight in both human and animals. Although hypoxia is shown to suppress fatty acid synthesis and reduce fat mass, the mechanism has not been clarified. Recently, the role of oxygen sensing pathway on metabolism has received much attention. Oxygen sensing pathway consists of a transcription factor, hypoxia-inducible factor (HIF) that is a heterodimer of HIF- and HIF-, and an oxygen sensor, prolyl hydroxylase domain protein (PHD). PHD catalyzes oxygen-dependent hydroxylation of the specific proline residues in HIF- subunits, a modification that tags HIF- for rapid polyubiquitination and subsequent proteosomal degradation. Hypoxia increases HIF expression by diminishing PHD activities, thereby activating the expression of divergent target genes involved in metabolism and angiogenesis.
Several cell culture studies have revealed that hypoxia and HIF convert cell metabolism dependent on aerobic glucose oxidation and fatty acid synthesis into that dependent on anaerobic glycolysis. HIF not only up-regulates a series of glycolytic enzymes,\textsuperscript{15,16} but also actively inhibits oxidative phosphorylation in mitochondria by inducing pyruvate dehydrogenase kinase 1 (PDK1).\textsuperscript{17,18} PDK1 inhibits pyruvate dehydrogenase activity and consequently reduces conversion of pyruvate to acetyl CoA, an essential substrate for oxidative phosphorylation.\textsuperscript{17,18} Also, HIF inhibits adipogenesis by inducing DEC1/Stra13.\textsuperscript{13} These HIF-induced metabolic alterations such as increased glucose consumption and less fatty acid synthesis might be beneficial for nutrient excess in obese or diabetic subjects. Although HIF could be a potential therapeutic target, direct manipulation of HIF is often difficult in vivo. In contrast, PHD is an ideal target to manipulate HIF levels and several chemical inhibitors of PHD have been developed.\textsuperscript{19} However, the role of adipocyte PHD in the development of obesity-induced glucose intolerance has not been determined. In the present study, we generated mice lacking PHD2 also known as Egl nine homolog 1 (EglN1) in adipocytes, because PHD2 is the most crucial isoform to regulate HIF level in vitro\textsuperscript{20} and in vivo\textsuperscript{21} among three PHD isoforms (PHD1, PHD2 and PHD3). We found that PHD2 deletion in adipocyte attenuates weight gain and alleviates glucose intolerance induced by a high-fat diet (HFD).

**Methods**

Additional details of the experimental procedures are included in the online-only Data Supplement.

All animal procedures were approved by Animal Care and Use Committee, Kyushu University and conducted in accordance with the institutional guidelines. Previously generated
Phd2-loxed mice (Phd2\textsuperscript{loxt})\textsuperscript{21} were crossed with transgenic mice expressing Cre recombinase under control of aP2 gene promoter (aP2-Cre), resulting in the generation of Phd2\textsuperscript{loxt}/aP2-Cre mice. Then, Phd2\textsuperscript{loxt}/aP2-Cre mice were generated by stepwise crossing of Phd2\textsuperscript{loxt}/aP2-Cre mice with Phd2\textsuperscript{loxt} mice. Phd2\textsuperscript{loxt} mice were served as controls. These mice were fed a HFD containing 60% kcal fat (High Fat Diet 32, Clea Japan) from 12 weeks to 18 weeks. Mice at the age of 12- and 18-week-old were analyzed. Preparation of cell lysate and total RNA, Western blot analysis, quantitative reverse transcription polymerase chain reaction (qRT-PCR), luciferase assay, and histological/imunohistochemical analysis were performed in conventional methods. The primer sequences for qRT-PCR are shown in Supplemental Table 1. Serum concentrations of glucose, cholesterol, triglyceride, insulin, lactate and cytokines were determined by commercially available kits. Oxygen consumption was measured using a computer-controlled open-circuit indirect calorimeter. Normality and homoskedasticity of the data were assessed by Shapiro-Wilk test and Levene test, respectively. A \textit{t}-test or exact binomial test was used for pair-wise comparisons. Multiple comparison was performed one-way or two-way ANOVA. Fisher’s post hoc test was used if appropriate. Data are shown as mean±SEM. P<0.05 was considered to be statistically significant. Detailed methods are indicated in the supplemental file.

Results

PHD2-deficient mice showed better glucose tolerance after HFD feeding.

PHD2 protein was reduced in WAT and BAT but not in other organs such as lung and skeletal muscle in PHD2-deficient mice (Phd2\textsuperscript{loxt}/aP2-Cre) (Figure 1A and Supplemental Figure 1A). Expression of PHD2 in heart and bone marrow derived macrophage was slightly reduced. We did not find any apparent abnormalities in the appearance of Phd2\textsuperscript{loxt}/aP2-Cre mice. Phd2 mRNA
was significantly decreased in WAT from Phd2<sup>f/f</sup>/aP2-Cre mice (Figure 1B). Then we separated an adipocyte-rich fraction (Adipocyte) and a stromal vascular fraction (SVF) of WAT (Supplemental Figure 1B) and examined the expression of Phd2 mRNA. Expression of Phd2 mRNA was significantly reduced in an Adipocyte fraction of WAT (Supplemental Figure 1C). Expression of Phd2 mRNA was modestly reduced in a SVF, but the difference was not significant. Phd1 mRNA level was not changed and Phd3 mRNA level was increased several fold in WAT of Phd2<sup>f/f</sup>/aP2-Cre mice (Supplemental Figure 1D). Both HIF-1α and HIF-2α proteins were significantly increased in PHD2-deficient WAT (Figure 1C, Supplemental Figure 1E), confirming that PHD1 and PHD3 cannot compensate for the absence of PHD2 in terms of HIF-α degradation.

Body weight in Phd2<sup>f/f</sup>/aP2-Cre mice was slightly lighter than control mice (Figure 1D). After 6 weeks of HFD, Phd2<sup>f/f</sup>/aP2-Cre mice gained significantly less body weight than controls. Since food intake was comparable between the two groups (Figure 1E) and we did not find any abnormalities in the feces, these data suggest that Phd2<sup>f/f</sup>/aP2-Cre mice were resistant to HFD-induced obesity.

Before HFD, glucose tolerance was comparable between controls and Phd2<sup>f/f</sup>/aP2-Cre mice (Supplemental Figure 2A). After 6 weeks of HFD, control mice developed severe glucose intolerance, whereas Phd2<sup>f/f</sup>/aP2-Cre mice showed significantly better glucose tolerance (Figure 1F). Although Insulin tolerance test revealed significantly lower glucose levels at all time points in Phd2<sup>f/f</sup>/aP2-Cre mice under HFD (Supplemental Figure 2B), the relative decrease in glucose level from baseline was not different between control and Phd2<sup>f/f</sup>/aP2-Cre mice (Figure 1G). Phd2<sup>f/f</sup>/aP2-Cre mice showed lower fasting glucose level with a lower insulin concentration and hence a lower homeostasis model assessment-insulin resistance (HOMA-IR) score (Table 1),
suggesting that insulin sensitivity may also be improved in Phd2^f/f/aP2-Cre mice. Serum cholesterol and triglyceride levels were not different between control and Phd2^f/f/aP2-Cre mice (Table 1).

**WAT was lighter in weight and adipocyte was smaller in size in Phd2^f/f/aP2-Cre mice.**

After 6 weeks of HFD, the epididymal WAT of Phd2^f/f/aP2-Cre mice was smaller in size and significantly lighter in weight than that of controls (Figure 2A and Supplemental Table 2). The perirenal WAT was also significantly lighter in weight in Phd2^f/f/aP2-Cre mice (Supplemental Table 2). Liver weight was slightly lighter in Phd2^f/f/aP2-Cre mice, but the difference was not statistically significant. The weight of other organs such as heart, spleen, and kidney was not significantly altered between the two groups. Histological analysis of epididymal WAT revealed that the size of adipocyte in Phd2^f/f/aP2-Cre mice was almost the same as that in control mice before HFD (Figure 2B and 2D). However, the extent of HFD-induced adipocyte hypertrophy was significantly reduced in Phd2^f/f/aP2-Cre mice compared to control mice (Figure 2C and 2D). A detailed analysis for size distribution of the adipocytes revealed that WAT from controls contained more number of larger adipocytes (>10,000 \( \mu m^2 \)) than that from Phd2^f/f/aP2-Cre mice (Figure 2E). In contrast, the number of smaller adipocytes (< 10,000 \( \mu m^2 \)) was increased in Phd2^f/f/aP2-Cre mice compared to control mice (Figure 2E).

Lipid particles of adipocytes in BAT from HFD-fed Phd2^f/f/aP2-Cre mice were apparently smaller compared to those from controls (Figure 2F).

**Macrophage infiltration was reduced in WAT of HFD-fed Phd2^f/f/aP2-Cre mice.**

Chronic inflammation is reported as a common feature in the adipose tissue of obese subjects. The number of macrophage aggregation surrounding adipocytes, often referred to as a crown-like structure, was significantly decreased in WAT from HFD-fed Phd2^f/f/aP2-Cre mice.
compared to controls (Figure 3A-3C). However, the expression of proinflammatory cytokines including monocyte chemoattractant protein (Mcp)-1, interleukin (Il)-6, and tumor necrosis factor (Tnf)-a in WAT (Figure 3D-3F) and BAT (data not shown) was not significantly different between HFD-fed controls and Phd2f/f/aP2-Cre mice.

Serum levels of these cytokines were comparable between control and Phd2f/f/aP2-Cre mice (Supplemental Figure 3).

Enhanced angiogenesis in WAT from HFD-fed Phd2f/f/aP2-Cre mice.

Since abnormal angiogenesis in WAT is reported as a common feature in obesity,2,22 we examined the state of angiogenesis in HFD-fed controls and Phd2f/f/aP2-Cre mice. Endothelial cell-specific lectin staining demonstrated that vascular density was mildly increased in WAT from Phd2f/f/aP2-Cre mice compared to controls (Figure 3G-3I). We also determined the expression of several angiogenic factors such as vascular endothelial growth factor (Vegf)-a, fibroblast growth factor (Fgf)2 and placental growth factor (Plgf) in WAT. While the expression of Vegf-a and Fgf2 remained almost the same between controls and Phd2f/f/aP2-Cre mice under HFD, the expression of Plgf was increased in HFD-fed Phd2f/f/aP2-Cre mice (Figure 3J-4L).

Adipocyte differentiation markers and glycolytic enzymes were increased in isolated adipocytes of WAT in HFD-fed Phd2f/f/aP2-Cre mice.

We determined the expression of adipogenic markers in adipocyte-rich fraction isolated from WAT of Phd2f/f/aP2-Cre mice and controls to exclude the effect of stromal vascular cells. The expression of peroxisome proliferator-activated receptor (Ppar)γ, CCAAT/enhancer binding protein (Cebp)a and adiponectin was increased in adipocyte-rich fraction of HFD-fed Phd2f/f/aP2-Cre mice (Figure 4A-4C). However, serum adiponectin concentration was not significantly different in both groups (Supplemental Figure 3).
Glucose transporter and glycolytic enzymes expression were upregulated in isolated adipocytes from WAT of HFD-fed Phd2f/f/aP2-Cre mice. Since HIF is known to activate glycolytic pathway, we analyzed the expression of genes involved in glycolysis. The expression of glucose transporter (Glut) 1, several glycolytic enzymes such as phosphoglycerate kinase (Pgk) 1, glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and lactate dehydrogenase (Ldh) a was significantly upregulated in the adipocyte-rich fraction isolated from WAT of Phd2f/f/aP2-Cre mice (Figure 4D-4G). In addition, the expression of pyruvate dehydrogenase kinase (Pdk) 1, a rate-limiting enzyme of oxidative phosphorylation, was also significantly upregulated (Figure 4H). Dec1 that inhibits adipogenesis was also upregulated in Phd2f/f/aP2-Cre mice (Figure 4I).

Unexpectedly, however, serum lactate level was rather decreased in HFD-fed Phd2f/f/aP2-Cre mice in spite of the upregulated expression of glycolytic enzymes (Table 1). We examined LDHa protein expression and found that LDHa protein was actually increased in WAT of Phd2f/f/aP2-Cre mice (Supplemental Figure 4A).

Phd2f/f/aP2-Cre mice showed increased oxygen consumption with UCP1 upregulation. The oxygen consumption (VO₂) was significantly increased in HFD-fed Phd2f/f/aP2-Cre mice in both the light and dark cycle (Figure 5A). The carbon dioxide production (VCO₂) was slightly increased in Phd2f/f/aP2-Cre mice, however, the difference was not statistically significant (Figure 5B). The respiratory exchange ratio (RER) was significantly lower in Phd2f/f/aP2-Cre mice during the dark period when mice are active, but there was no difference during the light period (Figure 5C). The expression of uncoupling protein (Ucp) 1, one of the critical genes controlling the energy expenditure was significantly upregulated in HFD-fed PHD2-deficient BAT compared to controls (Figure 5D). These data suggest that PHD2 deletion in adipocyte...
increased energy expenditure using lipid, at least partly, mediated by upregulation of *Ucp1* in BAT.

**Glut 4 in skeletal muscle in Phd2<sup>ff</sup>/aP2-Cre mice was upregulated.**

The expression of *Glut4* in skeletal muscle (quadriceps femoris muscle) of *Phd2<sup>ff</sup>/aP2-Cre* mice was significantly upregulated compared to controls (Figure 6A). Because Glut4 is downstream of insulin signaling, we examined insulin signaling pathway in the skeletal muscle. Expression of Glut4 protein and phosphorylation of Akt were increased in *Phd2<sup>ff</sup>/aP2-Cre* mice, which may support the idea that insulin sensitivity is improved in *Phd2<sup>ff</sup>/aP2-Cre* mice (Supplemental Figure 4B). The expression of genes involved in fatty acid oxidation such as acyl-CoA oxidase (ACO), carnitine palmitoyltransferase-1 (CPT-1), and medium chain acyl-CoA dehydrogenase (MCAD) in skeletal muscle was comparable between controls and *Phd2<sup>ff</sup>/aP2-Cre* mice, suggesting that fatty oxidation was not increased in skeletal muscle of *Phd2<sup>ff</sup>/aP2-Cre* mice (Figure 6B-6D). The expression of genes involved in hepatic gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase) was not different between two mice groups, suggesting that gluconeogenesis in the liver is not affected by PHD2 deletion in the adipocytes (Figure 6E and 6F).

**Glycolysis was promoted and lipid accumulation was suppressed in PHD2-deficient 3T3-L1 cells.**

To confirm that PHD2 deficiency increases glycolysis and attenuates lipid accumulation in adipocytes, we specifically knocked down *Phd2* mRNA by *Phd2*-specific shRNA in 3T3-L1 cells. The expression of both *Phd2* mRNA and PHD2 protein was significantly decreased in PHD2-deficient 3T3-L1 preadipocytes (Figure 7A and 7B). HRE-dependent luciferase activity was significantly increased (Figure 7C).
In agreement with the results of in vivo experiments, the expression of Glut1, Pdk1, Gapdh, Ldha and Pdk1 was significantly upregulated in PHD2-deficient 3T3-L1 preadipocytes (Figure 7D). After the induction of adipocyte differentiation, the expression of Glut1 was reduced in PHD2-deficient 3T3-L1 adipocytes, whereas expression of other genes was still significantly up-regulated (Figure 7E). Both glucose consumption and lactate production in the supernatant were significantly increased in PHD2-deficient 3T3-L1 preadipocytes than control 3T3-L1 preadipocytes (Figure 7F and 7G), indicating acceleration of glycolysis. We also assessed de novo lipogenesis, because PDK1 suppresses acetyl-CoA production that is essential for fatty acid synthesis. Oil red O staining revealed that PHD2-deficient 3T3-L1 cells accumulated less lipid than control 3T3L-1 cells (Figure 7H, and 1).

Discussion

In this study, we demonstrated that PHD2 deletion in adipocyte alleviates diet-induced obesity and glucose intolerance in mice. PHD2 deletion reduced fat mass and macrophage infiltration into WAT, and increased the expression of UCP-1 in BAT and oxygen consumption, all of which are supposed to be responsible for body weight reduction and better glucose tolerance in HFD-fed Phd2<sup>-/-</sup>/aP2-Cre mice. The improvement of glucose tolerance test was remarkable compared to the improvement of insulin tolerance test under HFD, indicating that improvement of insulin sensitivity may not be the primary effect of PHD2 deficiency. However, improvement in HOMA-IR suggests that insulin sensitivity in Phd2<sup>-/-</sup>/aP2-Cre mice may be improved to some extent. These data also suggest that PHD2 inhibition may improve glucose metabolism in the presence of insulin resistance.

Phd2<sup>-/-</sup>/aP2-Cre mice showed several beneficial morphological features of adipose
tissue. First, the size of adipocytes in PHD2-deficient WAT was reduced. It is generally accepted that better glucose tolerance is associated with smaller adipocyte size and conversely hypertrophied adipocytes are strongly linked to insulin resistance.\(^1\) Second, macrophage infiltration into WAT was significantly suppressed in Phd2\(^{0/0}/aP2-Cre\) mice. Although the causal relationship might be difficult to determine, alteration of morphological features by PHD2 deletion should cause better glucose tolerance and insulin sensitivity.

While hypoxia has been known to reduce body weight\(^6\) and fat mass,\(^26-28\) it is intriguing that even PHD2 deletion in adipocyte showed a similar effect. In PHD2-deficient adipocytes, glycolytic pathway becomes dominant due to HIF-induced expression of glucose transporter and glycolytic enzymes, which is often called “aerobic glycolysis.”\(^29\) Glycolysis is an inefficient way to produce energy compared to oxidative phosphorylation. Hence, the cells depending on glycolysis consume more glucose “wastefully” compared to those depending on oxidative phosphorylation when both cell types are required to generate an equal amount of ATP.\(^30\) Thereby, PHD2-deficient adipocytes may consume more glucose than normal adipocytes.

Although in vitro study showed that PHD2 knockdown increases lactate production in the supernatant, serum lactate level was rather decreased in Phd2\(^{0/0}/aP2-Cre\) mice. The reason for this discrepancy is not clear, this may, however, be due to reduction of adiposity in Phd2\(^{0/0}/aP2-Cre\) mice. Because HFD-loading increased serum lactate level even in control mice (table 1), the decrease in lactate level in Phd2\(^{0/0}/aP2-Cre\) mice may reflect the reduced total adipose tissue mass.

**PHD2** deletion attenuated fatty acid synthesis possibly through Pdk1 up-regulation. PDK1 suppresses the activity of pyruvate dehydrogenase that catalyzes conversion of pyruvate to acetyl CoA, an essential substrate for de novo fatty acid synthesis.\(^25\) As a result, lipogenesis is
expected to be reduced. In addition, PHD2 deletion in adipocyte may enhance lipid consumption. 

Phd2<sup>f/f</sup>/aP2-Cre mice consumed more oxygen with lower RER and showed reduced lipid content in BAT, which may be explained by up-regulation of Ucp1 expression, at least in part. However, the detailed mechanism for UCP-1 up-regulation is not clear at this point, because UCP-1 is not a target gene of HIF. Overall, PHD2 deletion-associated reprogramming of glucose and lipid metabolism might contribute to obesity-resistance.

It is reported that hypoxia inhibits adipogenesis through up-regulation of DEC1/Stra13. DEC1 is a transcription factor induced by HIF1α and suppresses PPAR expression resulting in the inhibition of adipogenesis. DEC1 expression in adipose tissue from Phd2<sup>f/f</sup>/aP2-Cre mice was increased. However, PPAR expression is rather increased in WAT from Phd2<sup>f/f</sup>/aP2-Cre mice (Figure 4A). Therefore, it is unlikely that DEC1 is involved in reduced adiposity in Phd2<sup>f/f</sup>/aP2-Cre mice.

Unexpectedly, we have found that Akt phosphorylation and Glut 4 expression in the skeletal muscle of Phd2<sup>f/f</sup>/aP2-Cre mice were increased. These data may suggest that insulin sensitivity is improved in HFD-fed Phd2<sup>f/f</sup>/aP2-Cre mice compared with HFD-fed control mice. It is reported that Glut 4 expression in skeletal muscle is suppressed in a rat model of insulin resistance, suggesting that Glut 4 up-regulation in Phd2<sup>f/f</sup>/aP2-Cre mice may be due to improvement of insulin sensitivity. However, it is not clear how PHD2-deficiency in adipocytes affect skeletal muscle insulin signaling pathway and further study is needed.

It is known that adipose tissues in obese patients are subjected to hypoxia and HIF is accumulated, which is explained by that hypertrophied adipocytes become physically distant from capillaries and inflammatory cells infiltrating into adipose tissue consume a substantial amount of oxygen. However, it has not been determined whether hypoxia in obese adipose plays
a causative role in obesity-associated metabolic abnormalities. Recently adipocyte-specific HIF-1α transgenic mice have been reported. The transgenic mice gained more body weight than controls under both normal diet and HFD, showing glucose intolerance and insulin resistance. The adipose tissue in HIF-1α transgenic mice developed more fibrosis in association with local inflammation. These phenotypes are opposite to our observation. We observed that PHD2 deficiency with increased HIF-1α and HIF-2α neither led to adipocyte hypertrophy or local inflammation, nor worsen HFD-induced obesity and glucose intolerance. The reason for this discrepancy is not immediately clear at this stage, but one of the differences between the previous study and our study is upregulation of HIF-2α in adipocytes in Phd2<sup>−/−</sup>/aP2-Cre mice. Interestingly, HIF-1α and HIF-2α have an opposite effect on adipogenesis; HIF-1α inhibits adipogenesis<sup>13</sup> and HIF-2α promotes it.<sup>37</sup> Therefore, the net effects by PHD2 inhibition on adipose tissue formation may be more complicated than the consequence of a single HIF-1α overexpression. Another possibility is that there may be unidentified substrate(s) of PHD2 for hydroxylation that may be related to glucose and lipid metabolism and inflammation. In contrast, our observation is supported by several lines of evidence from genetically modified mice.<sup>30, 38, 39</sup> Overexpression of dominant negative form of HIF-1α in adipocytes accelerated HFD-induced glucose intolerance and insulin resistance and induced more severe obesity.<sup>38</sup> Another study showed that factor inhibiting HIF-1α-deficient mice which have elevated HIF activity are also resistant to HFD-induced body weight gain and glucose intolerance.<sup>39</sup> These evidences consistently suggest that HIF signaling is positively linked to resistance to obesity and associated metabolic abnormalities. It is of note that our study revealed that inhibition of PHD2 in adipocytes sufficiently attenuated HFD-induced glucose intolerance and obesity without an increase in serum lactate level, which is observed in SIRT6-deficient mice.<sup>30</sup> Therefore,
inhibition of adipocytes PHD might be meritorious from the point of clinical application.

The limitation of the present study is that we have not excluded the possible involvement of PHD2-deficient macrophages, because aP2 gene is known to be expressed in not only adipocytes but also macrophages. However, the reduction of PHD2 expression in BMDM or SVF that is rich in macrophage in Phd2<sup>f/f</sup>/aP2-Cre mice was modest and not so remarkable compared with that in adipocytes. Therefore, the effect of PHD2-deletion in macrophage may play a relatively minor role in the reduction of fat mass and improvement of glucose metabolism in Phd2<sup>f/f</sup>/aP2-Cre mice.

In conclusion, we showed in this study that PHD2 in adipocytes plays a multifaceted role in the regulation of metabolism and inflammation in diet-induced obesity. Adipocyte-specific Phd2 deletion ameliorates diet-induced obesity and several obesity-associated metabolic abnormalities. Thus, PHD2 in adipocytes may be a novel target for the treatment of patients with metabolic syndrome.

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Conflict of Interest Disclosure: None.

References:


28. Reynolds RD, Lickteig JA, Deuster PA, Howard MP, Conway JM, Pietersma A, deStoppelaar...


31. Leguisamo NM, Lehnen AM, Machado UF, Okamoto MM, Markoski MM, Pinto GH, Schaan BD. Glut4 content decreases along with insulin resistance and high levels of inflammatory markers in rats with metabolic syndrome. *Cardiovasc Diabetol* 2012;11:100.


Table 1. Serum chemistry of control and Phd2^{fl/fl}/aP2-Cre mice.

<table>
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<th>Parameters</th>
<th>Control (n=6)</th>
<th>Phd2^{fl/fl}/aP2-Cre (n=6)</th>
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<td>Fasting glucose (mg/dl)</td>
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<td>Normal chow</td>
<td>138 ± 7</td>
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<td>HFD</td>
<td>230 ± 11</td>
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<td>Fasting insulin (ng/ml)</td>
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<td>89 ± 3</td>
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HFD; high fat diet
HOMA-IR; homeostasis model assessment-insulin resistance; = fasting glucose ×fasting insulin / 22.5

Figure Legends:

Figure 1. Phd2^{fl/fl}/aP2-Cre mice were resistant to diet-induced obesity with better glucose tolerance. A, Western blot analysis for PHD2 in epididymal white adipose tissue (WAT), brown adipose tissue (BAT) and lung in control and Phd2^{fl/fl}/aP2-Cre mice is shown. As a loading control, Western blotting for α-tubulin was performed. The same results were obtained in other independent experiments. n=3. B, The result of real-time qPCR analysis of WAT for Phd2 in control and Phd2^{fl/fl}/aP2-Cre mice is shown in the bar graph. n=6. **P<0.01 vs control. C, Western blot analysis for HIF-1α, HIF-2α and cAMP response element binding protein (CREB) using nuclear extracts from WAT is shown. Bar graphs indicate statistical analysis. n=3. *P<0.05. D, Twelve-week-old control or Phd2^{fl/fl}/aP2-Cre mice were fed a high-fat diet (HFD) for six weeks. Body weight (BW) at 12 weeks and 18 weeks is shown in the bar graphs. n=6~7.
*P<0.05, **P<0.01 vs control. E, The amount of food intake in both groups fed a HFD was shown in the bar graph. n=6~7. N.S. not significant. F, Control (black box) and Phd2^{f/f}/aP2-Cre mice (white box) fed a HFD for six weeks were intraperitoneally injected with glucose and blood glucose levels were measured. n=6~7. *P<0.05 vs control. G, Control (black box) and Phd2^{f/f}/aP2-Cre mice (white box) fed a HFD for six weeks were intraperitoneally injected with insulin and blood glucose levels were measured. n=6~7.

**Figure 2.** Phd2^{f/f}/aP2-Cre mice showed reduced fat mass. A, Representative pictures of epididymal white adipose tissue (WAT) from control and Phd2^{f/f}/aP2-Cre mice fed a HFD for 6 weeks are shown. Scale bar, 10 mm. B-C, Representative pictures of H&E stained sections of epididymal WAT from control and Phd2^{f/f}/aP2-Cre mice at 12 weeks (B) and after 6 weeks of a HFD (C) are shown. Scale bar, 100 μm. D, The average cross-sectional area of adipocytes in epididymal WAT is shown in the bar graph. **P<0.01. n=6~7. E, The distribution of adipocyte cross-sectional area in epididymal WAT of HFD-fed control (black bar) or Phd2^{f/f}/aP2-Cre mice (white bar) is shown in the bar graph. n=6~7. F, Representative pictures of H&E stained sections of BAT from HFD-fed control and Phd2^{f/f}/aP2-Cre mice are shown. Scale bar, 100 μm.

**Figure 3.** White adipose tissue (WAT) of Phd2^{f/f}/aP2-Cre mice showed reduced macrophage infiltration and increased angiogenesis. A and B, Representative pictures of anti-Mac3 immunohistochemical analysis of macrophage aggregation in WAT of HFD-fed control (A) and Phd2^{f/f}/aP2-Cre mice (B) are shown. Macrophage aggregation surrounding adipocytes (crown-like structure) is indicated by arrows. C, The number of crown-like structure is shown in the bar graph. n=5~6. **P<0.01 vs control. Exact binomial test was used. D-F, The results of real-time
qPCR analysis for monocyte chemoattractant protein (Mcp)-1, interleukin (Il)-6, and tumor necrosis factor (Tnf)-α of WAT from HFD-fed control and Phd2<sup>f/f</sup>/aP2-Cre mice are shown in the bar graphs. n=6. N.S. not significant. G and H, Representative pictures of epididymal WAT from HFD-fed control and Phd2<sup>f/f</sup>/aP2-Cre mice stained with endothelial cell-specific lectin (green) are shown. Nuclei were counterstained with DAPI (blue). Scale bar, 100 μm. I, Quantification of vascular density in panel G and H is shown in the bar graphs. *P<0.05 vs control. n=4~5. Exact binomial test was used. J–L, The results of real-time qPCR analyses for vascular endothelial growth factor (Vegf)-a (J), fibroblast growth factor (Fgf)2 (K) and placental growth factor (Plgf) (L) are shown in the bar graphs. n=6. N.S. not significant. *P<0.05 vs control.

**Figure 4.** Expression of adipocyte differentiation markers and glycolytic enzymes was increased in adipocyte rich fraction from Phd2<sup>f/f</sup>/aP2-Cre mice. Total RNA was extracted from adipocyte-rich fraction of HFD-fed control and Phd2<sup>f/f</sup>/aP2-Cre mice. The results of real-time qPCR analyses for (A) peroxisome proliferator activated receptor (Ppar)γ, (B) CCAAT/enhancer binding protein (Cebp)α, (C) adiponectin, (D) glucose transporter (Glut)1, (E) phosphoglycerate kinase (Pgk)1, (F) glyceraldehyde-3-phosphate dehydrogenase (Gapdh), (G) lactate dehydrogenase (Ldh)-a, (H) pyruvate dehydrogenase kinase (Pdk)1 and (I) DEC-1/Stra13 are shown in the bar graphs. n=6. *P<0.05, **P<0.01 vs control.

**Figure 5.** Oxygen consumption was increased in Phd2<sup>f/f</sup>/aP2-Cre mice. A–C, HFD-fed control and Phd2<sup>f/f</sup>/aP2-Cre mice were housed in computer-controlled open-circuit indirect calorimeter to determine (A) oxygen consumption, (B) carbon dioxide production and (C) respiratory exchange ratio (RER) during the light period (8 a.m. to 8 p.m.) and during the dark period (8 a.m. to 8 p.m.) carbon dioxide production and (C) respiratory exchange ratio (RER) during the light period (8 a.m. to 8 p.m.) and during the dark period (8 a.m. to 8 p.m.).
p.m. to 8 a.m.). * P<0.05, **P<0.01. n=3. **D, The result of real-time qPCR analysis for uncoupling protein (Ucp) 1 in brown adipose tissue from HFD-fed control and Phd2<sup>−/−</sup>/aP2-Cre mice is shown in the bar graph. n=6, *P<0.05 vs control shRNA.

**Figure 6.** Glut 4 in skeletal muscle in Phd2<sup>−/−</sup>/aP2-Cre mice was upregulated. A – D, Total RNA was extracted from skeletal muscle (quadriceps femoris muscle) of HFD-fed control and Phd2<sup>−/−</sup>/aP2-Cre mice. The results of real-time qPCR analyses for (A) Glucose transporter (Glut) 4, (B) acyl-CoA oxidase (ACO), (C) carnitine palmitoyltransferase-1 (CPT-1), and (D) medium chain acyl CoA dehydrogenase (MCAD) are shown in the bar graphs. n=6. *P<0.05 vs control. E and F, Total RNA was extracted from liver of HFD-fed control and Phd2<sup>−/−</sup>/aP2-Cre mice. The results of real-time qPCR analyses for (E) phosphoenolpyruvate carboxykinase (PEPCK), and (F) glucose-6-phosphatase (G6Pase) are shown in the bar graphs. n=6.

**Figure 7.** Knock down of PHD2 in 3T3-L1 cells induced enhancement of glycolytic and attenuation of lipid accumulation. A, Total RNA was extracted from control shRNA and Phd2 shRNA expressing 3T3-L1 preadipocytes. The result of real-time qPCR analyses for Phd2 is shown in the bar graph. n=4. ** P<0.01 vs control shRNA. B, Western blot analysis for PHD2 and α–tubulin using total cell lysates of control shRNA and Phd2 shRNA expressing 3T3-L1 preadipocytes is shown. The same results were obtained in other independent experiments. n=3. C, The luciferase activity after 24 hours of transfection of a hypoxia responsive element (HRE)-luciferase vector into control shRNA and Phd2 shRNA expressing 3T3-L1 preadipocytes is shown in the bar graph. n=3. **P<0.01 vs control shRNA. D and E, Total RNA was extracted from control and Phd2 shRNA expressing 3T3-L1 preadipocytes (D) and differentiated 3T3-L1
adipocytes (E). The results of real-time qPCR analyses for Glut1, Pgk1, Gapdh, Ldha and Pdk1 are shown in the bar graph. n=4. **P<0.01 vs control shRNA. F and G, Glucose consumption (F) and lactate concentration (G) in the culture media of control shRNA and Phd2 shRNA expressing 3T3-L1 preadipocytes after 24 hours of incubation are shown in the bar graphs. n=4. **P<0.01 vs control shRNA. H, Control and Phd2 shRNA expressing 3T3-L1 cells were differentiated, and lipid accumulation in the cytosol was determined by Oil Red O staining. Representative pictures of four independent experiments at 4, 6 and 8 days of differentiation are shown. I, Quantification of Oil red O contents of control shRNA (black bar) and Phd2 shRNA (white bar) expressing 3T3-L1 cells is shown in the bar graph. *P<0.05, **P<0.01 vs control shRNA. n=3.
Figure 1
Figure 2
Figure 3

A 18 weeks Control

B 18 weeks Phd2^{ff}/aP2Cre

C No of crown-like structure (/ low power field)

D Mcp-1

E II-6

F Tnf-α
Figure 3, cont’d
**Figure 4**

(A) **Ppar**

(B) **Cebpα**

(C) **Adiponectin**

(D) **Glut1**

(E) **Pgk1**

(F) **Gapdh**

(G) **Ldha**

(H) **Pdk1**

(I) **Dec1**
Figure 5

A) VO2

B) VCO2

C) RER

D) Ucp1

Relative mRNA level (fold)

Control Phd2f/f aP2Cre Control Phd2f/f aP2Cre

Light Dark

HFD

Control Phd2f/f aP2Cre Control Phd2f/f aP2Cre

Light Dark

HFD

Control Phd2f/f aP2Cre

HFD

* *
Figure 6

A) Glut 4

B) ACO

C) CPT-1

D) MCAD

E) PEPCK

F) G6Pase

Relative mRNA levels in Control, Phd2^ff^, aP2Cre, and HFD groups.
**Figure 7**

A) (fold) **Phd2**

B) shRNA Control Phd2 PHD2 α-Tubulin

C) (fold) **Phd2**

D) Glut1 Pgk1 Gapdh Ldha Pdk1 (fold)

E) Glut1 Pgk1 Gapdh Ldha Pdk1 relative mRNA level

Preadipocytes

Differentiated adipocytes

- **Glut1**
- **Pgk1**
- **Gapdh**
- **Ldha**
- **Pdk1**

Relative mRNA level

- Control Phd2 shRNA Phd2 shRNA
- Control Phd2 shRNA Phd2 shRNA
- Control Phd2 shRNA Phd2 shRNA
- Control Phd2 shRNA Phd2 shRNA
- Control Phd2 shRNA Phd2 shRNA

Relative HRE luciferase activity

- Control Phd2 shRNA Phd2 shRNA
- Control Phd2 shRNA Phd2 shRNA

Relative mRNA level

- Control Phd2 shRNA Phd2 shRNA
- Control Phd2 shRNA Phd2 shRNA
- Control Phd2 shRNA Phd2 shRNA
- Control Phd2 shRNA Phd2 shRNA
- Control Phd2 shRNA Phd2 shRNA
**Figure 7, cont’d**

**F**

Glucose consumption (µg/1000 cells/24hrs)

- Control shRNA
- Phd2 shRNA

**G**

Lactate production (nmol/µL)

- Control shRNA
- Phd2 shRNA

**H**

Differentiation Induction

- Day4
- Day6
- Day8

**I**

520 nm absorbance

- Control
- Phd2^f/f^/aP2Cre

*Control

**Phd2^f/f^/aP2Cre**
Prolyl Hydroxylase Domain Protein 2 Plays a Critical Role in Diet-induced Obesity and Glucose Intolerance

Hirohide Matsuura, Toshihiro Ichiki, Eriko Inoue, Masatoshi Nomura, Ryohei Miyazaki, Toru Hashimoto, Jiro Ikeda, Ryoichi Takayanagi, Guo-Hua Fong and Kenji Sunagawa

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SUPPLEMENTAL MATERIAL

Supplemental File for Supplemental Methods, Supplemental Tables, Supplemental Figures (1-4) and Legends for Supplemental Figures.

Supplemental Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM) was purchased from GIBCO BRL-Invitrogen Co. (Carlsbad, CA, U.S.A.). Fetal bovine serum (FBS) was purchased from SAFC Biosciences Inc. (Lenexa, KS, U.S.A.). A mouse monoclonal anti-α-tubulin antibody was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Horseradish peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse IgG) were purchased from Vector Laboratories, Inc. (Burlingame, CA, U.S.A.). Luciferase assay system was purchased from Promega Co. (Madison, WI, U.S.A.). Other chemical reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise stated.

Generation of adipocyte-specific PHD2-deficient mice.

To knock out Phd2 gene in adipocytes, previously generated Phd2-floxed mice were used (Phd2\textsuperscript{lox/lox}).\textsuperscript{1} Transgenic mice expressing Cre recombinase under control of aP2 gene promoter (aP2-Cre) were purchased from the Jackson Laboratory (Stock Number 5069, Bar Harbor, Maine). Phd2\textsuperscript{lox/lox} mice were crossed with aP2-Cre mice to obtain Phd2\textsuperscript{lox/lox}/aP2-Cre mice. Then, mice with PHD2 deletion in adipocyte (Phd2\textsuperscript{lox/lox}/aP2-Cre) were generated by stepwise crossing of Phd2\textsuperscript{lox/lox}/aP2-Cre mice with Phd2\textsuperscript{floxed} mice. Phd2\textsuperscript{floxed} mice were served as controls. The primers to detect Phd2-floxed gene and Cre gene were previously described.\textsuperscript{2} These mice were fed a
high-fat diet (HFD) containing 60% kcal fat (High Fat Diet 32, Clea Japan) from 12 weeks to 18 weeks. Mice at the age of 12- and 18-week-old were analyzed. All procedures were approved by Animal Care and Use Committee, Kyushu University and conducted in accordance with the institutional guidelines.

**Histological analysis**

Adipose tissues were fixed in 10% neutral buffered formaldehyde solution overnight and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E). Ten images of H&E stained sections were acquired from each animal and cross-sectional area of each adipocyte was determined using the software Dynamic cell count BZ-HIC (Keyence, Japan). To detect macrophage infiltration, the paraffin sections were immunohistochemically stained with an anti-mouse Mac-3 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and detected with DAB chromogen. For morphological analysis of blood vessels, adipose tissues were minced with scissors to small pieces (1~2 mm) and the tissues were directly stained with FITC-Conjugate *Bandeiraea simplicifolia* lectin (Sigma Sigma-Aldrich Co., St. Louis, MO, U.S.A.) for two hours at room temperature. Then, the tissues were counterstained with 4’,6-diamino-2-phenylindole (DAPI) and examined using the confocal laser scanning microscope A1R (Nikon, Japan). Capillary density was determined by counting blood vessels intersecting 1 mm line drawn in the photos of lectin-stained adipose tissues.

**Glucose tolerance test and insulin tolerance test**

Mice were starved for 6 hours. For glucose tolerance test, mice were injected intraperitoneally with glucose (1 g/kg of body weight). For insulin tolerance test, mice were injected intraperitoneally with rapid insulin (0.5 IU/kg of body weight). Blood sample was taken from tail
vein at various time points and blood glucose concentrations were determined by using Glutest Every (Sanwa Kagaku Kenkyusho, Japan).

**Measurement of serum levels of triglyceride, cholesterol, insulin, lactate and cytokines.**

Serum triglyceride and total cholesterol levels were determined by commercially available kits, Triglyceride E-test Wako (Wako) and Cholesterol E-test Wako (Wako), respectively. Serum insulin levels were determined by insulin ELISA kit (Morinaga Institute of Biological Science, Japan). Serum lactate level was determined by lactate assay kit (BioVision, Mountain View, CA). Serum cytokine levels were determined by ELISA kits (R&D systems Inc. Minneapolis, MN, USA).

**Western blot analysis**

Protein preparation and Western blot analysis for PHD2, HIF-1α, HIF-2α (Novus Biologicals, Littleton, CO USA), lactate dehydrogenase (LDH) a, Glut 4, Akt and phospho-Akt (Cell Signaling Technology, Danvers, MA, USA) were performed as described previously. α-tubulin (Sigma-Aldrich Co.) or cyclic AMP response element binding protein (CREB, Cell Signaling Technology) was used as a loading control.

**Isolation of adipocyte-enriched fraction and stromal vascular fraction (SVF) from white adipose tissues (WAT)**

The epididymal fat tissue was minced and digested with collagenase (3 mg/ml, Sigma) in phosphate-buffered saline supplemented with bovine serum albumin (2%, Sigma) at 37 °C for 60 minutes with gentle agitation. Then, the digested fat tissues were filtered through a 250 µm nylon mesh and centrifuged at 430 g for 1 minutes. The sediments were used as a SVF and floating
cells were used as an adipocyte-enriched fraction after washing with PBS for several times.

**Isolation of bone marrow derived macrophages (BMDM).**

Bone marrow cells were isolated from femurs and tibias, and were centrifuged (1000rpm, 5 min, 4°C). The sediments were resuspended in DMEM supplemented with 10% FBS and 30% L929 conditioned medium as a source of M-CSF for 7 days and attached cells were used as BMDM.4

**Oxygen consumption measurement.**

Mice were fed a HFD, maintained at a constant room temperature (21–23°C), and subjected to oxygen consumption measurements using a computer-controlled open-circuit indirect calorimeter (Oxymax; Columbus Instruments, Columbus OH) during the light period (8 a.m. to 8 p.m.) and the dark period (8 p.m. to 8 a.m.). Mice were housed individually in metabolic chamber. After a 1 hour adaptation to the chamber, VO2 was assed at 4-min intervals for 24 hours. All sample data were analyzed using Oxymax Windows software (version 1.0).

**Cell culture**

3T3-L1 murine preadipocytes were purchased from Human Science Research Resources Bank (Japan). The cells were maintained in low-glucose DMEM culture medium supplemented with 10 % FBS in an atmosphere of 5 % CO2 at 37 °C. To differentiate 3T3-L1 preadipocytes into adipocytes, the cells were cultured with high-glucose DMEM containing 10 % FBS, 10 µg/ml insulin, 0.25 µmol/L dexamethasone, and 500 µmol/L isobutylmethylxanthine for two days. Then, the cells were cultured with fresh high-glucose DMEM containing 10 % FBS and 10 µg/ml insulin for additional two days. After that, the cells were maintained in DMEM containing 10% FBS for another 8 days.

**Gene silencing by retrovirus-mediated small hairpin (sh) RNA expression**
Expression vectors of shRNA specific for Phd2 gene were generated using pSINsi-hU6 vector containing the neomycin resistant gene under control of SV40 promoter (Takara Biotechnology, Shiga, Japan). Target DNA sequences for shRNA specific for Phd2 gene were 5'-GCAATAACTGTGTGTGATT-3'. The DNA sequence of control shRNA which had no similarity for any murine genes was 5'-TCAGAACGATGACTGAGAG-3'. The constructs were introduced to Plat-E cells (Cell Biolabs, San Diego, CA, USA) with FuGene6 transfection reagent (Roche Applied Science, Basel, Switzerland). After 48 hours, the culture medium containing retrovirus particles was collected and filtered by passing through 0.45 µm filter (Schleicher&Schuell, Dassel, Germany). Then 3T3-L1 cells were infected with the retrovirus for 24 hours and subsequently selected by G418 (500 µg/ml) treatment for another 7 days.

**Oil Red O staining**

To determine the extent of lipid accumulation in cytosol, differentiated 3T3-L1 adipocytes were fixed with 10 % formaldehyde for 10 minutes and stained with 0.18 % Oil Red O solution for 15 minutes. After staining, the cells were rinsed with 60% isopropanol. After taking photos, the dye was extracted with 100% isopropanol and absorbance at 520 nm was measured.

**RNA extraction and real-time quantitative RT-PCR (qPCR) analysis**

RNA from adipose tissue, quadriceps femoris muscle, liver, and cultured cells was extracted using ISOGEN according to manufacturer's instruction (Wako Pure Chemical Industries, Ltd.). One µg of total RNA was reverse-transcribed using ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). Real-time qPCR was performed using 7500 real-time PCR system (Life Technologies Co., Carlsbad, CA, U.S.A.) and SYBR Green PCR Master Mix (Life Technologies Co.). The expression of each gene was normalized with either hypoxanthine phosphoribosyltransferase (Hprt), β-actin expression or 18S rRNA. Primer sequences are summarized in the Supplemental Table 1.
Measurements of transcriptional activity of HRE-driven promoter

A luciferase construct with 7 copies of hypoxia responsive element (HRE) was a generous gift from Dr. Masaomi Nangaku (University of Tokyo, Japan). The HRE-luciferase vector was introduced to 3T3-L1 preadipocytes by the DEAE-dextran method according to the manufacturer’s instructions (Promega Corporation, Madison, WI, U.S.A.). The luciferase activity was measured by Lumat LB9501 (Berthold, Bad Wildbad, Germany) and normalized with protein concentrations.

Statistical analysis

Normality and homoskedasticity of the data were assessed by Shapiro-Wilk test and Levene test, respectively. A t-test was used for comparing two groups. Differences between multiple groups were evaluated using one-way ANOVA followed by Fisher’s post hoc test if appropriate. The data on the number of crown-like structure (Figure 3C) and vascular density (Figure 3I) were assessed by exact binomial test. The data on insulin tolerance test and glucose tolerance test were analyzed by repeated measure two-way ANOVA. Data are shown as mean±SEM. P<0.05 was considered to be statistically significant.

References for the methods.

1. Takeda K, Ho VC, Takeda H, Duan LJ, Nagy A, Fong GH. Placental but not heart defects are associated with elevated hypoxia-inducible factor alpha levels in mice lacking prolyl hydroxylase domain protein 2. *Mol Cell Biol*. 2006;26:8336-8346


### Supplemental table 1. Sequences of the primers used for real-time qPCR

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Abbreviations
Aco; acyl CoA oxidase
C/EBP; CCAAT/enhancer binding protein
Cpt1a; carnitine palmitoyltransferase-1
DEC1; differentiated embryo chondrocyte 1
Fgf ; fibroblast growth factor
Gapdh ; glyceraldehyde-3-phosphate dehydrogenase
Glut ; glucose transporter
G6pase ; glucose-6-phosphatase
Hp rt ; hypoxanthine phosphoribosyltransferase
Il ; interleukin
Ldh ; lactate dehydrogenase
MCAD; medium chain acyl-CoA dehydrogenase
Mcp ; monocyte chemoattractant protein
Pepck; phosphoenolpyruvate carboxykinase
Pdk ; pyruvate dehydrogenase kinase
Plgf ; placental growth factor
Pgk ; phosphoglycerate kinase
Phd ; prolyl hydroxylase domain protein
Ppar ; peroxisome proliferator activated receptor
Tnf ; tumor necrosis factor
Ucp; uncoupling protein
Vegf ; vascular endothelial growth factor
**Supplemental Table 2.** Organ weight of control and Phd2<sup>ff</sup> / aP2-Cre mice.

<table>
<thead>
<tr>
<th>Organs</th>
<th>Control (n=6)</th>
<th>Phd2&lt;sup&gt;ff&lt;/sup&gt; / aP2-Cre (n=6)</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epididymal fat (mg)</td>
<td>1208 ± 50</td>
<td>758 ± 35</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Perirenal fat (mg)</td>
<td>647± 35</td>
<td>366 ± 26</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Liver (mg)</td>
<td>1496 ± 117</td>
<td>1229 ± 105</td>
<td>P=0.12</td>
</tr>
<tr>
<td>Heart (mg)</td>
<td>178 ± 3</td>
<td>190 ± 12</td>
<td>P=0.35</td>
</tr>
<tr>
<td>Spleen (mg)</td>
<td>108 ±10</td>
<td>100 ± 20</td>
<td>P=0.35</td>
</tr>
<tr>
<td>Kidney (mg)</td>
<td>200 ±10</td>
<td>176 ± 5</td>
<td>P=0.06</td>
</tr>
</tbody>
</table>
Supplemental Figure 2

A

GTT (Normal Chow)

blood glucose (mg/dl)

Control

Phd2 \textit{f/f} aP2Cre

B

ITT (HFD)

blood glucose (mg/dl)

Control

Phd2 \textit{f/f} aP2Cre
Supplemental Figure 4

A

LDHa

α-tubulin

LDHa/α-tubulin

Control  Phd2^floxed/
aP2Cre

B

p-Akt

Akt

pAkt/Akt

Control  Phd2^floxed/
aP2Cre

Glut4

Glut4/α-tubulin

Control  Phd2^floxed/
aP2Cre
Legends for supplemental figures.

Supplemental Figure 1

Expression of PHD and HIF in control and Phd2ff/aP2-Cre mice.

A, Western blot analysis for PHD2 in heart, bone marrow derived macrophage (BMDM) and skeletal muscle (SKM from quadriceps femoris) in control and Phd2ff/aP2-Cre mice is shown. As a loading control, Western blotting for α-tubulin was performed. The same results were obtained in other independent experiments. n=3. B, The result of real-time qPCR analysis for adiponectin, F4/80 and Phd2 in adipocyte rich fraction (Adipocyte) and stromal vascular fraction (SVF) in control and Phd2ff/aP2-Cre mice is shown in the bar graph. n=5. *P<0.05, **P<0.01 vs control. Adiponectin is specific for adipocytes and F4/80 is specific for macrophages. The results indicate that the two fractions are appropriately separated. C, The results of real-time qPCR analysis for Phd2 in SVF and Adipocytes from control and Phd2ff/aP2-Cre mice are shown. n=5. *P<0.05 vs control. N.S. not significant. D, The result of real-time qPCR analysis for Phd1 and Phd3 in white adipose tissue from control and Phd2ff/aP2-Cre mice is shown. n=3. **P<0.01 vs control. E, Statistical analysis of HIF-1α and HIF-2α expression in white adipose tissue from control and Phd2ff/aP2-Cre mice is shown. Western blot analysis for cAMP response element binding protein (CREB), a nuclear transcription factor, was used as a loading control. *P<0.05 vs control. n=3
Supplemental Figure 2

A. Glucose tolerance test in control and Phd2f/f/aP2-Cre mice before high-fat diet feeding.

Glucose tolerance test was performed as indicated in the method. No significant difference was observed between control and Phd2f/f/aP2-Cre mice.

B. Insulin tolerance test in control and Phd2f/f/aP2-Cre mice after high-fat diet feeding for 6 weeks.

The results of insulin tolerance test in control and Phd2f/f/aP2-Cre mice are indicated in actual glucose levels. n=6~7. **P<0.01, *P<0.05 vs control.

Supplemental Figure 3

Serum level of adipokines in control and Phd2f/f/aP2-Cre mice.

Serum concentrations of MCP-1, IL-6, TNFα and adiponectin were determined by ELISA in high-fat diet fed control and Phd2f/f/aP2-Cre mice. TNFα was not detectable in both mice and therefore the data are not shown. Although serum MCP-1 level is lower in Phd2f/f/aP2-Cre mice, the difference was not statistically significant. n=6. N.S.; not significant.

Supplemental Figure 4.

Expression of protein in white adipose tissue (WAT) and skeletal muscle.

A. Western blot analysis shows expression of lactate dehydrogenase (LDH) a in WAT from control and Phd2f/f/aP2-Cre mice. The bar graph shows the results of densitometric analysis. n=3. *P<0.05 vs control.

B. Western blot analysis shows phosphorylation of Akt and expression of Glut4 in skeletal muscle (quadriceps femoris). The bar graph shows the results of densitometric analysis. n=3. *P<0.05 vs control.