Coronary Heart Disease

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

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Background—Recent studies suggest that the oxygen-sensing pathway consisting of transcription factor hypoxia-inducible factor and prolyl hydroxylase domain proteins (PHDs) 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 adipocytes 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%aP2-Cre). Phd2%aP2-Cre mice were resistant to high-fat diet–induced obesity (36.7±1.7 versus 44.3±2.0 g in control; P<0.01) and showed better glucose tolerance and homeostasis model assessment–insulin resistance index than control mice (3.6±1.0 versus 11.1±2.1; P<0.01). The weight of white adipose tissue was lighter (epididymal fat, 758±35 versus 1208±507 mg in control; P<0.01) with a reduction in adipocyte size. Macrophage infiltration into white adipose tissue was also alleviated in Phd2%aP2-Cre mice. Target genes of hypoxia-inducible factor, including glycolytic enzymes and adiponectin, were upregulated in adipocytes of Phd2%aP2-Cre mice. Lipid content was decreased and uncoupling protein-1 expression was increased in brown adipose tissue of Phd2%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 upregulation of glycolytic enzymes and reduced lipid accumulation.


Key Words: adipocytes ■ cell hypoxia ■ obesity ■ prolyl hydroxylase domain protein

Obesity is one of the critical risk factors for the development of atherosclerosis, diabetes mellitus, and coronary artery disease. Previous studies have shown that obesity induces low-grade chronic inflammation in 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. Although patient education on lifestyle modification and the 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 the development of antiobesity drugs. The cannabinoid-1 receptor blocker rimonabant was developed with great expectation, but it has not been commonly used in clinical practice because of side effects such as depression and anxiety disorder. Thus, a novel therapeutic target to treat obesity is sought.

Clinical Perspective on p 2087

Hypoxia has long been known to reduce body weight in both humans and animals. Although hypoxia is shown to suppress fatty acid synthesis and to reduce fat mass, the mechanism has not been clarified. Recently, the role of the oxygen-sensing pathway in metabolism has received much attention. The oxygen-sensing pathway consists of a transcription factor, hypoxia-inducible factor (HIF), that is a heterodimer of HIF-α and HIF-β and an oxygen sensor, prolyl hydroxylase domain protein (PHD). PHD catalyzes oxygen-dependent hydroxylation of the specific proline residues in HIF-α subunits, a modification that tags HIF-α for rapid polyubiquitination and subsequent proteasomal degradation. Hypoxia increases HIF expression by diminishing PHD activities, thereby activating the expression of divergent target genes involved in metabolism and angiogenesis.

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Several cell culture studies have revealed that hypoxia and HIF convert cell metabolism that is dependent on aerobic glucose oxidation and fatty acid synthesis into that which is dependent on anaerobic glycolysis. HIF not only upregulates a series of glycolytic enzymes but also actively inhibits oxidative phosphorylation in mitochondria by inducing pyruvate dehydrogenase kinase 1 (PDK1). PDK1 inhibits pyruvate dehydrogenase activity and consequently reduces the conversion of pyruvate to acetyl CoA, an essential substrate for oxidative phosphorylation. In addition, HIF inhibits adipogenesis by inducing DEC1/Stra13. 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. 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 in adipocytes, determined. In the present study, we generated mice lacking PHD2 in adipocytes, determined. In the present study, we generated mice lacking PHD2 in adipocytes, determined. In the present study, we generated mice lacking PHD2 in adipocytes, determined. In the present study, we generated mice lacking PHD2 in adipocytes, determined. In the present study, we generated mice lacking PHD2 in adipocytes, determined.

Methods

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

All animal procedures were approved by the Animal Care and Use Committee of Kyushu University and conducted in accordance with the institutional guidelines. Previously generated Phd2-floxed mice (Phd2(f/f)) were crossed with transgenic mice expressing Cre recombinase under control of the alpha2 promoter (αP2-Cre), resulting in the generation of Phd2(αP2-Cre) mice. Then, Phd2(αP2-Cre) mice were generated by stepwise crossing of Phd2(αP2-Cre) mice with Phd2(−/−) mice. Phd2(−/−) mice served as controls. These mice were fed an HFD containing 60% kcal fat (High Fat Diet 32, Clea Japan, Inc) from 12 to 18 weeks of age. Mice 12 and 18 weeks of age were analyzed. Preparation of cell lysate and total RNA, Western blot analysis, quantitative reverse transcription–polymerase chain reaction, luciferase assay, and histological/immunohistochemical analysis were performed using conventional methods. The primer sequences for quantitative reverse transcription–polymerase chain reaction are shown in Table I in the online-only Data Supplement. Serum concentrations of glucose, cholesterol, triglyceride, insulin, lactate, and cytokines were determined by commercially available kits. Oxygen consumption was measured with a computer-controlled open-circuit indirect calorimeter. Normality and homoscedasticity of the data were assessed by the Shapiro-Wilk test and Levene test, respectively. A t test or exact binomial test was used for pairwise comparisons. Multiple comparisons were performed with 1-way or 2-way ANOVA. The Fisher post hoc test was used if appropriate. Data are shown as mean±SEM. Values of P<0.05 were considered significant. Detailed methods are given in the online-only Data Supplement.

Results

PHD2-Deficient Mice Showed Better Glucose Tolerance After HFD Feeding

PHD2 protein was reduced in white adipose tissue (WAT) and brown adipose tissue (BAT) but not in other organs such as lung and skeletal muscle in PHD2-deficient mice (Phd2(−/−)αP2-Cre; Figure 1A and Figure 1A in the online-only Data Supplement). Expression of PHD2 in heart and bone marrow–derived macrophages was slightly reduced. We did not find any apparent abnormalities in the appearance in Phd2(−/−)αP2-Cre mice. Phd2 mRNA was significantly decreased in WAT from Phd2(−/−)αP2-Cre mice (Figure 1B). We then separated...
Table. Serum Chemistry of Control and Phd2<sup>f/f</sup>/aP2-Cre Mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n=6)</th>
<th>Phd2&lt;sup&gt;f/f&lt;/sup&gt;/aP2-Cre (n=6)</th>
<th>P Values</th>
</tr>
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<tbody>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>Normal chow</td>
<td>138±7</td>
<td>122±9</td>
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<tr>
<td></td>
<td>HFD</td>
<td>230±11</td>
<td>158±16</td>
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<tr>
<td>Fasting insulin, ng/mL</td>
<td>Normal chow</td>
<td>0.71±0.10</td>
<td>0.65±0.12</td>
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<tr>
<td></td>
<td>HFD</td>
<td>2.10±0.34</td>
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<td>HOMA-IR</td>
<td>Normal chow</td>
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<td></td>
<td>HFD</td>
<td>11.10±2.14</td>
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<td>Total cholesterol, mg/dL</td>
<td>Normal chow</td>
<td>163±11</td>
<td>171±16</td>
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<td></td>
<td>HFD</td>
<td>230±11</td>
<td>158±16</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>Normal chow</td>
<td>84±3</td>
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<td></td>
<td>HFD</td>
<td>11.3±0.5</td>
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</table>

HFD indicates high-fat diet; and HOMA-IR, homeostasis model assessment–insulin resistance: (fasting glucose × fasting insulin)/22.5.

an adipocyte-rich fraction and a stromal vascular fraction of WAT (Figure IB in the online-only Data Supplement) and examined the expression of Phd2 mRNA. Expression of Phd2 mRNA was significantly reduced in the adipocyte-rich fraction of WAT (Figure IC in the online-only Data Supplement). Expression of Phd2 mRNA was modestly reduced in the stromal vascular fraction, but the difference was not significant. The Phd1 mRNA level was not changed and the Phd3 mRNA level was increased several-fold in WAT of Phd2<sup>f/f</sup>/aP2-Cre mice (Figure ID in the online-only Data Supplement). Both HIF-1α and HIF-2α proteins were significantly increased in PHD2-deficient WAT (Figure IC and Figure IE in the online-only Data Supplement), 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 in control mice (Figure 1D). After 6 weeks of HFD, Phd2<sup>f/f</sup>/aP2-Cre mice gained significantly less body weight than controls. Food intake was comparable between the 2 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 (Figure IIA in the online-only Data Supplement). 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 1E). Although an insulin tolerance test revealed significantly lower glucose levels at all time points in Phd2<sup>f/f</sup>/aP2-Cre mice on an HFD (Figure IIB in the online-only Data Supplement), the relative decrease in the 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 score (Table), suggesting that insulin sensitivity may also be improved in Phd2<sup>f/f</sup>/aP2-Cre mice. Serum cholesterol and triglyceride levels were not different between control and Phd2<sup>f/f</sup>/aP2-Cre mice (Table).

WAT Was Lighter in Weight and Adipocytes Were Smaller in Phd2<sup>f/f</sup>/aP2-Cre Mice

After 6 weeks of HFD, the epididymal WAT of Phd2<sup>f/f</sup>/aP2-Cre mice was smaller in size and significantly lighter in weight than that of controls (Figure 2A and Table II in the online-only Data Supplement). The perirenal WAT was also significantly lighter in weight in Phd2<sup>f/f</sup>/aP2-Cre mice (Table II in the online-only Data Supplement). Liver weight was slightly smaller in Phd2<sup>f/f</sup>/aP2-Cre mice, but the difference was not statistically significant. The weight of other organs such as heart, spleen, and kidney was not significantly different between the 2 groups. Histological analysis of epididymal WAT revealed that the size of adipocytes in Phd2<sup>f/f</sup>/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<sup>f/f</sup>/aP2-Cre mice compared with control mice (Figure 2C and 2D). A detailed analysis of the size distribution of the adipocytes revealed that WAT from controls contained a greater number of larger adipocytes (>10000 μm<sup>2</sup>) than that...
from Phd2\textsuperscript{f/f}/aP2-Cre mice (Figure 2E). In contrast, the number of smaller adipocytes (<10,000 μm\textsuperscript{2}) increased in Phd2\textsuperscript{f/f}/aP2-Cre mice compared with control mice (Figure 2E).

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

**Macrophage Infiltration Was Reduced in WAT of HFD-Fed Phd2\textsuperscript{f/f}/aP2-Cre Mice**

Chronic inflammation is reported as a common feature in the adipose tissue of obese subjects.\textsuperscript{22,23} The macrophage aggregation surrounding adipocytes, often referred to as a crown-like structure,\textsuperscript{23,24} was significantly decreased in WAT from HFD-fed controls and Phd2\textsuperscript{f/f}/aP2-Cre mice compared with control mice (Figure 3A–3C). However, the expression of proinflammatory cytokines, including monocyte chemotactic protein-1 (McP-1), interleukin-6 (Il-6), and tumor necrosis factor-α (Tnf-α), in WAT (Figure 3D–3F) and BAT (data not shown) was not significantly different between HFD-fed controls and Phd2\textsuperscript{f/f}/aP2-Cre mice.

Serum levels of these cytokines were comparable between control and Phd2\textsuperscript{f/f}/aP2-Cre mice (Figure III in the online-only Data Supplement).

**Enhanced Angiogenesis in WAT From HFD-Fed Phd2\textsuperscript{f/f}/aP2-Cre Mice**

Because abnormal angiogenesis in WAT is reported as a common feature in obesity,\textsuperscript{22} we examined the state of angiogenesis in HFD-fed controls and Phd2\textsuperscript{f/f}/aP2-Cre mice. Endothelial cell–specific lectin staining demonstrated that vascular density was mildly increased in WAT from Phd2\textsuperscript{f/f}/aP2-Cre mice compared with controls (Figure 3G–3I). We also determined the expression of several angiogenic factors such as vascular endothelial growth factor-a (Vegf-a), fibroblast growth factor 2 (Fgf2), and placental growth factor (Plgf) in WAT. Although the expression of Vegf-a and Fgf2 remained almost the same between controls and Phd2\textsuperscript{f/f}/aP2-Cre mice fed an HFD, the expression of Plgf was increased in HFD-fed Phd2\textsuperscript{f/f}/aP2-Cre mice (Figures 3J–3L).
Adipocyte Differentiation Markers and Glycolytic Enzymes Were Increased in Isolated Adipocytes of WAT in HFD-Fed Phd2%/aP2-Cre Mice

We determined the expression of adipogenic markers in an adipocyte-rich fraction isolated from WAT of Phd2%/aP2-Cre mice and controls to exclude the effect of stromal vascular cells. The expression of peroxisome proliferator-activated receptor-γ (Pparγ), CCAAT/enhancer binding protein α (Cebpα), and adiponectin was increased in the adipocyte-rich fraction of HFD-fed Phd2%/aP2-Cre mice (Figure 4A–4C). However, the serum adiponectin concentration was not significantly different between control and Phd2%/aP2-Cre mice (Figure III in the online-only Data Supplement).

Expression of Glucose Transporter and Glycolytic Enzymes Was Upregulated in Isolated Adipocytes From WAT of HFD-Fed Phd2%/aP2-Cre Mice

Because HIF is known to activate glycolytic pathway,17 we analyzed the expression of genes involved in glycolysis. The expression of glucose transporter 1 (Glut1) and several glycolytic enzymes such as phosphoglycerate kinase (Pgk1), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), and lactate dehydrogenase-a (Ldha) was significantly upregulated in the adipocyte-rich fraction isolated from WAT of Phd2%/aP2-Cre mice (Figure 4D–4G). In addition, the expression of pyruvate dehydrogenase kinase 1 (Pdk1), a rate-limiting enzyme of oxidative phosphorylation, was also significantly upregulated (Figure 4H). Dec1, which inhibits adipogenesis,13 was also upregulated in Phd2%/aP2-Cre mice (Figure 4I).

Unexpectedly, however, the serum lactate level was rather decreased in HFD-fed Phd2%/aP2-Cre mice despite the upregulated expression of glycolytic enzymes (Table). We examined LDHa protein expression and found that LDHa protein was actually increased in WAT of Phd2%/aP2-Cre mice (Figure IVA in the online-only Data Supplement).

Phd2%/aP2-Cre Mice Showed Increased Oxygen Consumption With Uncoupling Protein-1 Upregulation

Oxygen consumption (V\textsubscript{O\textsubscript{2}}) was significantly increased in HFD-fed Phd2%/aP2-Cre mice in both the light and dark periods (Figure 5A). Carbon dioxide production (V\textsubscript{CO\textsubscript{2}}) was slightly increased in Phd2%/aP2-Cre mice, but the difference was not statistically significant (Figure 5B). The respiratory exchange ratio was significantly lower in Phd2%/aP2-Cre mice during the dark period when mice were active, but there was no difference during the light period (Figure 5C). The expression of Ucp1, one of the critical genes controlling the energy expenditure, was significantly upregulated in HFD-fed PHD2-deficient BAT compared with controls (Figure 5D). These data suggest that PHD2 deletion in adipocytes increased energy expenditure using lipid at least partly mediated by upregulation of Ucp1 in BAT.
Glut4 in Skeletal Muscle in Phd2f/f/aP2-Cre Mice Was Upregulated

The expression of Glut4 in skeletal muscle (quadriceps femoris muscle) of Phd2f/f/aP2-Cre mice was significantly upregulated compared with controls (Figure 6A). Because Glut4 is downstream of insulin signaling, we examined the insulin signaling pathway in skeletal muscle. Expression of Glut4 protein and phosphorylation of Akt were increased in Phd2f/f/aP2-Cre mice, which may support the idea that insulin sensitivity is improved in Phd2f/f/aP2-Cre mice (Figure IVB in the online-only Data Supplement). The expression of genes involved in fatty acid oxidation such as acyl-CoA oxidase, carnitine palmitoyltransferase-1, and medium-chain acyl-CoA dehydrogenase in skeletal muscle was comparable between controls and Phd2f/f/aP2-Cre mice, suggesting that fatty acid oxidation was not increased in skeletal muscle of Phd2f/f/aP2-Cre mice (Figure 6B–6D). The expression of genes involved in hepatic gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase was not different between the 2 mouse 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). Hypoxia responsive element–dependent luciferase activity was significantly increased (Figure 7C).

In agreement with the results of in vivo experiments, the expression of Glut1, Pgk1, 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 the expression of other genes was still significantly upregulated (Figure 7E). Both glucose consumption and lactate production in the supernatant were significantly increased in PHD2-deficient 3T3-L1 preadipocytes compared with control 3T3-L1 preadipocytes (Figure 7F and 7G), indicating acceleration of glycolysis. We also assessed de novo lipogenesis because PDK1 suppresses acetyl-CoA production, which is essential for fatty acid synthesis. Oil Red O staining revealed that PHD2-deficient 3T3-L1 cells accumulated less lipid than control 3T3-L1 cells (Figure 7H and 7I).

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 Phd2f/f/aP2-Cre mice. The improvement in the glucose tolerance test was remarkable compared with the improvement in the insulin tolerance test under HFD, indicating that an improvement of insulin sensitivity may not be the
primary effect of PHD2 deficiency. However, improvement in the homeostasis model assessment—insulin resistance suggests that insulin sensitivity in Phd2f/f/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.

Phd2f/f/aP2-Cre mice showed several beneficial morphological features of adipose tissue. First, the size of adipocytes in PHD2-deficient VAT was reduced. It is generally accepted that better glucose tolerance is associated with smaller adipocyte size and conversely that hypertrophied adipocytes are strongly linked to insulin resistance. Second, macrophage infiltration into VAT was significantly suppressed in Phd2f/f/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.

Although hypoxia has been known to reduce body weight and fat mass, it is intriguing that even PHD2 deletion in adipocytes showed a similar effect. In PHD2-deficient adipocytes, the glycolytic pathway becomes dominant because of the HIF-induced expression of glucose transporter and glycolytic enzymes, which is often called aerobic glycolysis. Glycolysis is an inefficient way to produce energy compared with oxidative phosphorylation. Hence, the cells depending on glycolysis consume more glucose wastefully compared with those depending on oxidative phosphorylation when both cell types are required to generate an equal amount of ATP. Therefore, PHD2-deficient adipocytes may consume more glucose than normal adipocytes. Although an in vitro study showed that PHD2 knockdown increases lactate production in the supernatant, the serum lactate level was rather decreased in Phd2f/f/aP2-Cre mice. The reason for this discrepancy is not clear but may be due to a reduction in adiposity in Phd2f/f/aP2-Cre mice. Because HFD loading increased serum lactate levels even in control mice (Table), the decrease in lactate levels in Phd2f/f/aP2-Cre mice may reflect the reduced total adipose tissue mass.

PHD2 deletion attenuated fatty acid synthesis possibly through Pdk1 upregulation. PDK1 suppresses the activity of pyruvate dehydrogenase, which catalyzes the conversion of pyruvate to acetyl CoA, an essential substrate for de novo fatty acid synthesis. As a result, lipogenesis is expected to be reduced. In addition, PHD2 deletion in adipocytes may enhance lipid consumption. Phd2f/f/aP2-Cre mice consumed more oxygen with a lower respiratory exchange ratio and showed reduced lipid content in BAT, which may be explained, at least in part, by the upregulation of Ucp1 expression. However, the detailed mechanism for UCP-1 upregulation 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 upregulation of DEC1. DEC1 is a transcription factor induced by HIF-1α that suppresses peroxisome proliferator–activated receptor-γ expression, resulting in the inhibition of adipogenesis. DEC1 expression in adipose tissue from Phd2f/f/aP2-Cre mice was increased. However, peroxisome proliferator–activated receptor-γ expression is rather increased in VAT from Phd2f/f/aP2-Cre mice (Figure 4A). Therefore, it is unlikely that DEC1 is involved in the reduced adiposity in Phd2f/f/aP2-Cre mice.

Unexpectedly, we have found that Akt phosphorylation and Glut4 expression in the skeletal muscle of Phd2f/f/aP2-Cre mice were increased. These data may suggest that insulin sensitivity is improved in HFD-fed Phd2f/f/aP2-Cre mice compared with HFD-fed control mice. It is reported that Glut4 expression in skeletal muscle is suppressed in a rat model of insulin resistance, suggesting that Glut4 upregulation in Phd2f/f/aP2-Cre mice may be due to an improvement in insulin sensitivity. However, it is not clear how PHD2 deficiency in adipocytes affects the skeletal muscle insulin signaling pathway; further study is needed.
It is known that adipose tissue in obese patients is subjected to hypoxia and HIF is accumulated, which is explained by the facts that hypertrophied adipocytes become physically distant from capillaries and that inflammatory cells infiltrating into adipose tissue consume a substantial amount of oxygen.

However, it has not been determined whether hypoxia in obese adipose tissue 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 on both normal diet

Figure 7. Knockdown of prolyl hydroxylase domain protein 2 (PHD2) in 3T3-L1 cells induced enhancement of glycolysis 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 quantitative polymerase chain reaction (qPCR) analyses for Phd2 is shown in the bar graph. n=4. ** P<0.01 vs control shRNA. B, Western blot analysis for PHD2 (α-Tubulin) is shown in the bar graph. n=4. ** P<0.01 vs control shRNA. 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 and differentiated 3T3-L1 adipocytes (E). The results of real-time qPCR analyses for glucose transporter 1 (Glut1), phosphoglycerate kinase 1 (Pgk1), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), lactate dehydrogenase a (Ldha), and pyruvate dehydrogenase kinase 1 (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 shRNA 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 4 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. n=3. * P<0.05, ** P<0.01 vs control shRNA.
and an 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 of our observation. We observed that PHD2 deficiency with increased HIF-1α and HIF-2α neither led to adipocyte hypertrophy or local inflammation nor worsened 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 opposite effects 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 substrates 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 a 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 that have elevated HIF activity are also resistant to HFD-induced body weight gain and glucose intolerance.<sup>39</sup> This evidence consistently suggests 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>40</sup> Therefore, inhibition of PHD in adipocytes might be meritorious in terms of clinical application.

The limitation of the present study is that we have not excluded the possible involvement of PHD2-deficient macrophages because the ap2 gene is known to be expressed in not only adipocytes but also macrophages.<sup>40</sup> However, the reduction in PHD2 expression in bone marrow–derived macrophages or stromal vascular fraction that is rich in macrophages in Phd2<sup>+/-</sup>/aP2-Cre mice was modest and not so remarkable compared with that in adipocytes. Therefore, the effect of PHD2-deletion in macrophages may play a relatively minor role in the reduction of fat mass and the improvement in glucose metabolism in Phd2<sup>+/-</sup>/aP2-Cre mice.

Conclusions
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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Disclosures
None.

References
21. Takeda K, Ho VC, Takeda H, Duan L, Nagy A, Fong GH. Placental but not heart defects are associated with elevated hypoxia-inducible factor

CLINICAL PERSPECTIVE

Obesity is associated with low-grade chronic inflammation, dysregulated adipocytokine production, and increased oxidative stress in visceral adipose tissue, which is believed to result in insulin resistance, high blood pressure, and acceleration of atherosclerosis. Although hypoxia has long been known to reduce body weight in both humans and animals, the role of the hypoxia response system, including hypoxia-inducible factor and an oxygen sensor, prolyl hydroxylase domain protein (PHD), in the regulation of fat mass and glucose metabolism remains controversial. Therefore, in the present study, we sought to determine whether deletion of PHD2, a main isoform of PHD, in adipose tissue affects high-fat diet–induced obesity and glucose intolerance. We showed that PHD2 deficiency in adipocyte resulted in upregulation of hypoxia-inducible factor and attenuated high-fat diet–induced body weight gain and glucose intolerance compared with control mice. These effects seemed to be mediated by upregulation of glycolytic enzymes in white adipose tissue and uncoupling protein-1 in brown adipose tissue in the PHD2-deficient mice. The PHD2-deficient mice also showed modest improvement in insulin sensitivity. The improvement in glucose metabolism is associated with a decrease in adipocyte size, macrophage infiltration, and abnormal angiogenesis of white adipose tissue in the PHD2-deficient mice. Because of the worldwide pandemic of obesity and diabetes mellitus, a novel strategy that is effective for the treatment of both conditions has been sought. The present study suggests that PHD2 inhibition in adipocyte may be a new therapeutic approach to reduce body weight and to improve glucose tolerance simultaneously.
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 knockout Phd2 gene in adipocytes, previously generated Phd2-floxed mice were used (Phd2<sup>f/f</sup>).<sup>1</sup> 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<sup>f/+</sup> mice were crossed with aP2-Cre mice to obtain Phd2<sup>f/+</sup>/aP2-Cre mice. Then, mice with PHD2 deletion in adipocyte (Phd2<sup>f/+</sup>/aP2-Cre) were generated by stepwise crossing of Phd2<sup>f/+</sup>/aP2-Cre mice with Phd2<sup>f/f</sup> mice. Phd2<sup>f/f</sup> mice were served as controls. The primers to detect Phd2-floxed gene and Cre gene were previously described.<sup>2</sup> 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'-GCAATAACTGTTTGGTATT-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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<th>Reverse primer</th>
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<td>5'-ACTCAACACGGGAAACTCTCA-3'</td>
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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
Hprr; 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.

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<th>Organs</th>
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<td>647± 35</td>
<td>366 ± 26</td>
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<td>Liver (mg)</td>
<td>1496 ± 117</td>
<td>1229 ± 105</td>
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<td>Heart (mg)</td>
<td>178 ± 3</td>
<td>190 ± 12</td>
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<td>Spleen (mg)</td>
<td>108 ±10</td>
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<td>Kidney (mg)</td>
<td>200 ±10</td>
<td>176 ± 5</td>
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Supplemental Figure 1

A

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</table>
Supplemental Figure 2

A

GTT (Normal Chow)

blood glucose (mg/dl)

Control

Phd2<sup>+/αP2Cre</sup>

0 30 60 90 120 (min)

B

ITT (HFD)

blood glucose (mg/dl)

Control

Phd2<sup>+/αP2Cre</sup>

0 30 60 90 (min)
Legends for supplemental figures.

**Supplemental Figure 1**

Expression of PHD and HIF in control and \textit{Phd2}/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 \textit{Phd2}/aP2-Cre mice is shown. As a loading control, Western blotting for \(\alpha\)-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 \textit{Phd2}/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 \textit{Phd2}/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 \textit{Phd2}/aP2-Cre mice is shown. \(n=3\). **P<0.01 vs control.  

**E,** Statistical analysis of HIF-1\(\alpha\) and HIF-2\(\alpha\) expression in white adipose tissue from control and \textit{Phd2}/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 Phd2\textsuperscript{f/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 Phd2\textsuperscript{f/f}/aP2-Cre mice.

B. Insulin tolerance test in control and Phd2\textsuperscript{f/f}/aP2-Cre mice after high-fat diet feeding for 6 weeks.

The results of insulin tolerance test in control and Phd2\textsuperscript{f/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 Phd2\textsuperscript{f/f}/aP2-Cre mice.

Serum concentrations of MCP-1, IL-6, TNF\textalpha and adiponectin were determined by ELISA in high-fat diet fed control and Phd2\textsuperscript{f/f}/aP2-Cre mice. TNF\textalpha was not detectable in both mice and therefore the data are not shown. Although serum MCP-1 level is lower in Phd2\textsuperscript{f/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 Phd2\textsuperscript{f/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.