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Regulatory Role of Endothelial PHD2 in the Hepatic Steatosis

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Abstract

Background/Aims: Liver disease is a leading cause of high mortality and morbidity worldwide. The aim of the present study is to investigate the regulatory role of prolyl hydroxylase-2 (PHD2)-hypoxia-inducible factor-2α (HIF-2α) axis on nonalcoholic fatty liver disease (NAFLD) and to explore the potential mechanisms by which endothelial (EC)-specific PHD2 deficiency regulates hepatic steatosis and fibrosis.

Methods: In the endothelial-specific PHD2 knockout ($PHD2^{EC}KO$) mouse fed with normal diet or high fat diet (HFD), liver lipid accumulation and fibrosis were measured by Oil Red O and Masson trichrome staining. The fat and body weight (FW/BW) ratio and glucose tolerance were measured. The expression of HIF-2α, atrial natriuretic peptide (ANP), angiopoietin-2 (Ang-2), and transforming growth factor-β (TGF-β) were analyzed by western blot analysis.

Results: The steatosis and fibrosis were significantly increased in the $PHD2^{EC}KO$ mice. FW/BW ratio was significantly increased in the $PHD2^{EC}KO$ mice. Moreover, knockout of endothelial PHD2 resulted in an impairment of glucose tolerance in mice. Western blot analysis showed that the expression of HIF-2α in liver tissues was not significantly increased. Interestingly, the expression of ANP was decreased, and Ang-2 and TGF-β levels were significantly increased in the liver of $PHD2^{EC}KO$ mice. The FW/BW ratio was also significantly increased in the $PHD2^{EC}KO$ mice fed with HFD for 16 weeks. Feeding HFD resulted in a significant increase in hepatic steatosis in the control $PHD2^{f/f}$ mice, but did not further enhance hepatic steatosis in the $PHD2^{EC}KO$ mice.

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Conclusions: We concluded that the endothelial PHD2 plays a critical role in hepatic steatosis and fibrosis, which may be involved in the regulation of ANP and Ang-2/TGF-β signaling pathway, but not the HIF-2α expression.

Keywords

Phd2; HIF-2α; Endothelium; Hepatic Steatosis; Atrial natriuretic peptide (ANP)

Introduction

The prevalence of nonalcoholic fatty liver disease (NAFLD) is increasing at an alarming rate, therefore, it is urgent to identify potential therapeutic targets. Fatty liver disease (FLD), which is a major cause of chronic liver disease worldwide [1, 2], initially begins with simple hepatic steatosis but can irreversibly progress to steatohepatitis, fibrosis, cirrhosis, or hepatocellular carcinoma. Recent studies have revealed that fat accumulation in the liver clearly predisposes the liver to injury and progresses to FLD [3, 4]. Thus, hepatic steatosis is a key step in the development and progression of NAFLD. Hypoxia is closely associated with lipid homeostasis. Studies reveal that ischemic and hypoxic stress increases cellular lipid deposition both *in vitro* and *in vivo* [5–7]. Hypoxia also upregulates genes involved in lipogenesis, lipid uptake, and lipid droplet formation [8–10]. In addition, several studies have implicated the crucial role of hypoxia and hypoxia inducible factors (HIFs) in lipid homeostasis [11–13].

Prolyl hydroxylase domain enzymes (PHD) are the oxygen sensing molecules that form a complex von Hippel-Lindau protein (VHL) and degrade HIF-α [14–17]. In three PHD isoforms, PHD2 is considered as the most important HIF-α-regulating isoform [18–20]. Our recent studies indicate the expression of PHD2 in endothelial cells has a critical role in the regulation of vascular remodeling and the development of fibrosis by the mechanism involving in upregulation of HIF-2α expression in lung and kidney [21, 22]. Accumulating evidence suggests some of hypoxia-associated genes were not regulated by HIFs, implicating potential HIF-independent pathways that are controlled by prolyl hydroxylase domain (PHD) enzymes [23]. PHD2 has many aspects that are not explained by HIF-mediated mechanisms alone. A recent study revealed that PHD2 could control both HIF-dependent and HIF-independent hypoxia responses [24]. In the present study, we test whether specific knockout of endothelial PHD2 regulates hepatic steatosis and fibrosis via a HIF-2α-independent pathway in adult mice.

Using an endothelial-specific PHD2 knockout (PHD2^{EC}KO) mouse with normal diet or high-fat diet (HFD), we investigate the regulatory role of endothelial PHD2-HIF-2α signaling pathway in NAFLD and explore the potential mechanisms endothelial PHD2 regulates hepatic steatosis and fibrosis. Our data demonstrated that specific deletion of PHD2 in endothelium promoted hepatic steatosis and fibrosis via downregulation of atrial natriuretic peptide (ANP) and upregulation of angiopoietin-2 (Ang-2)/transforming growth factor-b (TGF-β) in a HIF-2α-independent mechanism.

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Materials and Methods

Ethics Statement

Experimental animals were fed with laboratory standard chow and water and housed in individually ventilated cages in the Laboratory Animal Facilities (LAF) at the University of Mississippi Medical Center. All procedures conformed to the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were approved by the University of Mississippi Medical Center Animal Care and Use Committee (Protocol ID: 1280B). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Generation of the PHD2flox/flox(f/f) and PHD2ECKO mice

PHD2$^{flox/flox}$ (PHD2$^{f/f}$) mice were obtained from Dr. Guo-hua Fong at University Connecticut. PHD2^{EC}KO mice were generated using the Cre-LoxP system as previous described [21, 22]. The absence of PHD2 in vascular endothelium was confirmed by western blot analysis using cultured endothelial cells (EC) isolated from PHD2^{EC}KO mice.

HFD model

PHD2$^{f/f}$ and PHD2^{EC}KO mice (8 weeks of age) were fed with normal chow diet or high-fat (60% kcal) diet (D12492; Research Diets (New Brunswick, NJ, USA) for 16 weeks to produce a HFD model. Mice were housed in the LAF and were given free access to water throughout the study.

Western Blot Analysis

Mouse liver tissues were homogenized in 300 μL of an ice-cold lysis buffer. The homogenates were centrifuged at 4, 500rpm for 5 minutes at 4°C and the total protein concentrations were determined using a BCA protein assay kit (Pierce Co, IL). An aliquot (30 μg) of the protein lysate was separated on 10% SDS-PAGE gel and transferred to a membrane by electrophoresis. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and incubated with the following primary antibodies overnight: HIF-2α, ANP, Akt, and 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase-3 (PFKFB3) (1:1000, Novus Bio, CO); transforming growth factor (TGF)-β and Ang-2 (1:1000, Sigma, MO). The membranes were then washed and incubated for 2 hrs with an anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase (1:5000, Santa Cruz, CA). Densitometric analysis of the bands was carried out using image acquisition and analysis software (TINA 2.0).

Histological Analysis

Live tissues were fixed with buffered 10% formalin solution (SF93– 20; Fisher Scientific, Pittsburgh, PA), embedded in frozen optimal-cutting-temperature compound, and 10μm frozen sections prepared. Some sections were stained with Hematoxylin & eosin (H & E), and some sections were stained with Oil Red O working solution for lipid analysis [25, 26]. Masson trichrome staining was also performed on adjacent sections to measure the degree of

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fibrosis. The area percentage of lipid or fibrosis was quantified by measuring 6 random microscopic fields using image-analysis software (Image J, NIH).

Measurement of body fat accumulation

At the end of experiments, animals were sacrificed and fat from abdominal tissue was collected and weighted. Fat weight/body weight ratio (FW/BW) was calculated.

Glucose tolerance test

PHD2$^{f/f}$ and PHD2^{EC}KO mice (24 weeks of age) were subjected to glucose tolerance test. Glucose tolerance test was carried out after a 12 hour fast by intraperitoneal injection with D-glucose (1 mg/g) in sterile saline. Blood was obtained from experimental mice by tail snip, and blood glucose levels were measured with One Touch SureStep test strips. Glucose levels were expressed as mg/dL.

Statistical Methods

Mean ± SD are presented. The significance of differences in the means of corresponding values between groups were determined using the independent samples t test. A $p<0.05$ was considered to be significant.

Results

PHD2^{EC}KO increases hepatic steatosis and downregulates ANP, PFKFB3, and Akt expression

Oil Red O staining of liver tissues showed that the fat is significantly increased in the PHD2^{EC}KO mice (Fig. 1A). We examined HIF-2α in liver tissues and found there was no significantly increased HIF-2α levels in the PHD2^{EC}KO mice (Fig. 1B). In contrast, the expression of PFKFB3 and Akt was significantly decreased in the PHD2^{EC}KO mice (Fig. 1C and 1D). Most intriguingly, atrial natriuretic peptide (ANP), a peptide hormone secreted from cardiomyocyte and endothelium, was significantly reduced in the liver of the PHD2^{EC}KO mice (Fig. 1E).

PHD2^{EC}KO upregulates Ang-2/TGF-β pathway and increases hepatic fibrosis

We used Masson trichrome staining to detect the fibrosis of liver. Fibrosis was found in the perivascular region of small arteries and venous, especially in the PHD2^{EC}KO mice. The percentage of fibrotic area increased significantly compared with the control PHD2$^{f/f}$ mice (Fig. 2A). The expression of Ang-2 increased significantly by PHD2 knockout in endothelium. TGF-β, which has contributed to liver fibrosis, was increased significantly by PHD2^{EC}KO (Fig. 2B and 2C). To confirm PHD2 was specific knockout in EC, EC was isolated from PHD2^{EC}KO mice and control PHD2$^{f/f}$ mice, and PHD2 was analyzed. As shown in Fig. 2D, PHD2 expression was abolished in the EC from PHD2^{EC}KO mice.

PHD2^{EC}KO increases body fat accumulation and impairs glucose tolerance in mice

We measured body fat accumulation using the index of fat weight and body weight ratio (FW/BW ratio). As shown in Fig. 3A, PHD2^{EC}KO mice fed with normal diet had a

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significant increase in FW/BW ratio as compared with the control PHD2$^{f/f}$ mice. Glucose tolerance test further showed that glucose tolerance was impaired in PHD2^{EC}KO mice compared with the control PHD2$^{f/f}$ mice challenged with glucose on normal diet (Fig. 3B).

Roles of PHD2^{EC}KO on HFD-induced body fat accumulation and hepatic steatosis

Next, we examined the effects of endothelial PHD2 on HFD-induced hepatic steatosis. As shown in Fig. 3A, PHD2^{EC}KO mice fed with high-fat diet for 16 weeks led to a significant increase in FW/BW ratio as compared with PHD2^{EC}KO mice fed with normal diet. PHD2$^{f/f}$ mice fed with high-fat diet resulted in a significant increase in hepatic steatosis (red oil stain) and FW/BW ratio (Fig. 1A and Fig. 3A). Surprisingly, the hepatic steatosis was not further exacerbated in the PHD2^{EC}KO mice fed with HFD as compared with PHD2^{EC}KO mice fed with normal diet (Fig. 1A). Also, hepatic fibrosis was not significantly different between the control PHD2$^{f/f}$ mice and PHD2^{EC}KO mice fed with HFD (Fig. 2B). Western blot analysis revealed that the expression of HIF-2α and ANP was significantly decreased in the PHD2^{EC}KO mice fed with HFD (Fig 4A and 4B). The expression of PFKFB3 and Akt had no significant difference between the control PHD2$^{f/f}$ mice and PHD2^{EC}KO mice fed with HFD (Fig. 4C and 4D). The expression of Ang-2 and TGF-β was increased but did not reach a significant difference between the control PHD2$^{f/f}$ mice and PHD2^{EC}KO mice fed with HFD (Fig. 4E and 4F).

Discussion

In the present study, we found that specific deletion of PHD2 in the endothelium resulted in a significant hepatic steatosis. This was accompanied by a significant increase in body fat accumulation and impaired glucose tolerance in the mice. Furthermore, knockout of PHD2 in EC led to perivascular fibrosis. Mechanistically, knockout of PHD2 in EC downregulates ANP and upregulates Ang-2/TGF-β expression in liver that may promote hepatic steatosis and fibrosis. Our results suggest a regulatory role of endothelial PHD2 in the hepatic steatosis and fibrosis via a HIF-2α-independent pathway.

Activation of HIF-2α has been shown to result in suppression of fatty acid β-oxidation and lipid synthesis as well as an increase in lipid storage [27]. It is HIF-2α, not HIF-1α, which suppresses fatty acid oxidation and promotes lipid accumulation [27] HIF-2α controls at least three components of hepatic lipid metabolism: synthesis, oxidation, and storage [27]. Our present study showed that deletion of PHD2 in EC led to a lipid accumulation and fibrosis in the liver. Moreover, HIF-2α seemed to not be involved in this process since we failed to detect an increase in HIF-2α expression in the liver tissues of PHD2^{EC}KO mice. This may be due to PHD2 was a specific knockout in the endothelium and HIF-2α was increased only in EC but not in the liver. PHD2 has been shown to regulate HIF-independent pathways by interacting with other substrates such as NDRG3 [24]. Our present study also indicates that the regulatory role of endothelial PHD2 on hepatic steatosis is mediated by a HIF-2α-independent pathway.

In the present study, we found that ANP was significantly suppressed in the liver of PHD2^{EC}KO mice as well as in the PHD2^{EC}KO mice fed with high fat diet. ANP is a peptide-hormone produced mainly by cardiac atrium, ventricles of the heart, and vascular

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endothelial cells. ANP plays an important role in cardiovascular homeostasis by regulation of blood pressure [28–30]. Emerging evidence indicates a critical role of ANP in the regulation of lipid metabolism. ANP has been shown to reduce lipid accumulation by the mechanisms involved in promoting lipogenesis, lipid oxidation, and lipid mobilization [31–33]. ANP deficiency was observed in metabolic syndrome including type 2 diabetes, obesity, and NAFLD suggesting an involvement of ANP in the pathophysiology of metabolic disease [29–31]. Moreover, reduction of ANP expression has been contributed to lipid accumulation in diabetes [32]. ANP is also the most potent activator of adipocyte lipolysis, and ANP-mediated lipolysis is significantly impaired in type 2 diabetic patients and in obese humans [33–35]. Our data showed that $PHD2^{EC}KO$ mice fed with HFD had similar degree of lipid accumulation in the liver as fed with normal diet. In addition, ANP was downregulated in the liver of $PHD2^{EC}KO$ mouse either on normal diet or high fat diet which suggested a direct role of endothelial PHD2 on ANP expression that may not be associated with HFD-mediated lipid accumulation. Based upon these results, we postulated that reduction of ANP levels in $PHD2^{EC}KO$ mice may be attributed to hepatic lipid accumulation. In addition, the downregulation of ANP not only impacted the liver but may also be associated with body fat accumulation.

Ang-2 is predominately released by endothelial cells. Studies have shown that endothelial derived Ang-2 expression is regulated primarily by endothelial HIF-2α [36]. Our previous studies demonstrated that specific knockout of endothelial PHD2 promotes HIF-2α accumulation and upregulates Ang-2 expression. Upregulation of endothelial Ang-2 increases perivascular region of fibrosis in the lung and kidney of the $PHD2^{EC}KO$ mice [21, 22]. Consistent with these studies, the expression of Ang-2 in liver tissues was also increased significantly in the $PHD2^{EC}KO$ mice. This may result in significant hepatic fibrosis in the perivascular region of $PHD2^{EC}KO$ mice. TGF-β is a ‘‘master switch’’ in the differentiation of myofibroblasts in many tissues. We found that the expression of TGF-β was significantly increased in the $PHD2^{EC}KO$ mice. Our previous studies have provided evidence that activation of TGF-β signaling pathway may contribute to pericyte differentiation into myofibroblasts and fibrosis in the $PHD2^{EC}KO$ mice [21, 22]. Therefore, increased Ang-2 and TGF-β in the liver tissues may be responsible for hepatic fibrosis in the $PHD2^{EC}KO$ mice. In addition, inhibition of PFKFB3 has been shown to increase Ang-2 expression in the EC [37]. Treatment with ANP has been shown to reduce liver fibrosis [38]. Downregulation of ANP and PFKFB3 expression in the $PHD2^{EC}KO$ mice may also contribute, at least in part, to the development of hepatic fibrosis.

Our data also showed that knockout of PHD2 in EC dramatically increased body fat accumulation under high-fat diet stress. Surprisingly, deletion of endothelial PHD2 did not accentuate HFD-induced hepatic steatosis and fibrosis. So far, the underlying mechanism of endothelial PHD2 in HFD stress remains unknown. Upregulation of liver HIF-2α has been reported to increase lipid accumulation [27]. In contrast to $PHD2^{EC}KO$ mice on normal diet, $PHD2^{EC}KO$ mice fed with HFD had a significant reduction of HIF-2α in the liver. Further investigations are warrant to clarify the mechanisms and determine whether reduction of liver HIF-2α contributes HFD-induced hepatic steatosis.

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In summary, endothelium is one of the most complicated systems of the body; its function is regulated precisely at the gene levels as well as by metabolic stress. Further investigations should be done to clarify the precise mechanism of the regulation of endothelial PHD2 on lipid metabolism. PHD2 and HIF-2α-independent pathway in the endothelium may be a therapeutic target candidate for chronic liver disease inducing to steatosis and fibrosis.

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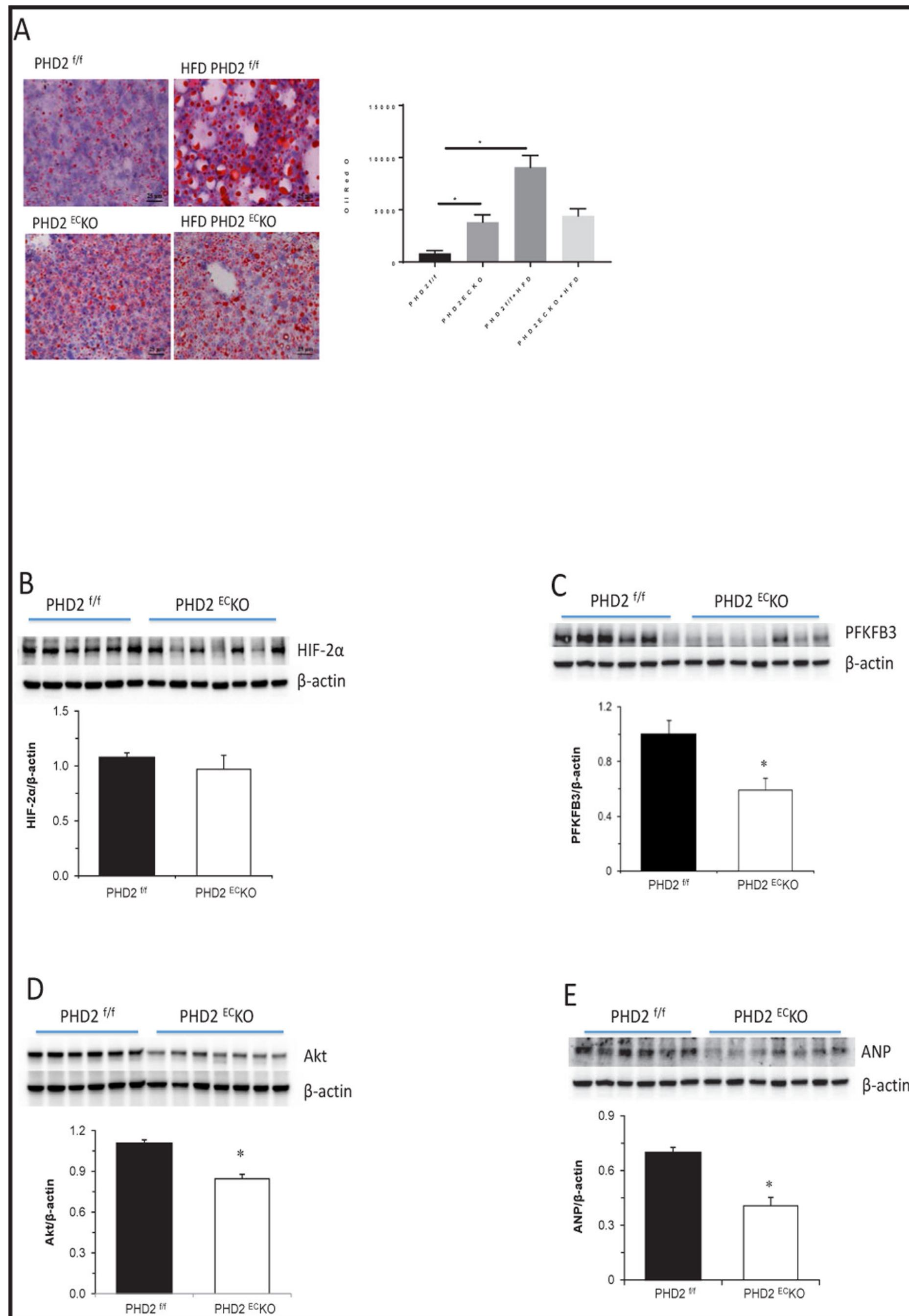

Fig. 1.

PHD2^{EC}KO increases hepatic steatosis and regulates PFKFB3/Akt pathway. A) PHD2^{EC}KO mice displayed a significant increase of red oil staining in liver (n=6–7 mice, *$p<0.05$). PHD2$^{f/f}$ mice + HFD led to a significant increase of red oil staining in liver (n=6–7 mice, *$p<0.05$). B) There was not a significant difference of HIF-2α expression between the PHD2$^{f/f}$ and PHD2^{EC}KO mice. (n=6–7 mice, *$p<0.05$). C) The expression of PFKFB3 was significantly decreased in the PHD2^{EC}KO mice. (n=6–7 mice, *$p<0.05$). D) The expression of Akt was significantly decreased in the PHD2^{EC}KO mice. (n=6–7 mice, *$p<0.05$). E) The

expression of ANP was significantly decreased in the PHD2^{EC}KO mice. (n=6–7 mice, *$p<0.05$). Mean ± SD, *$p<0.05$.

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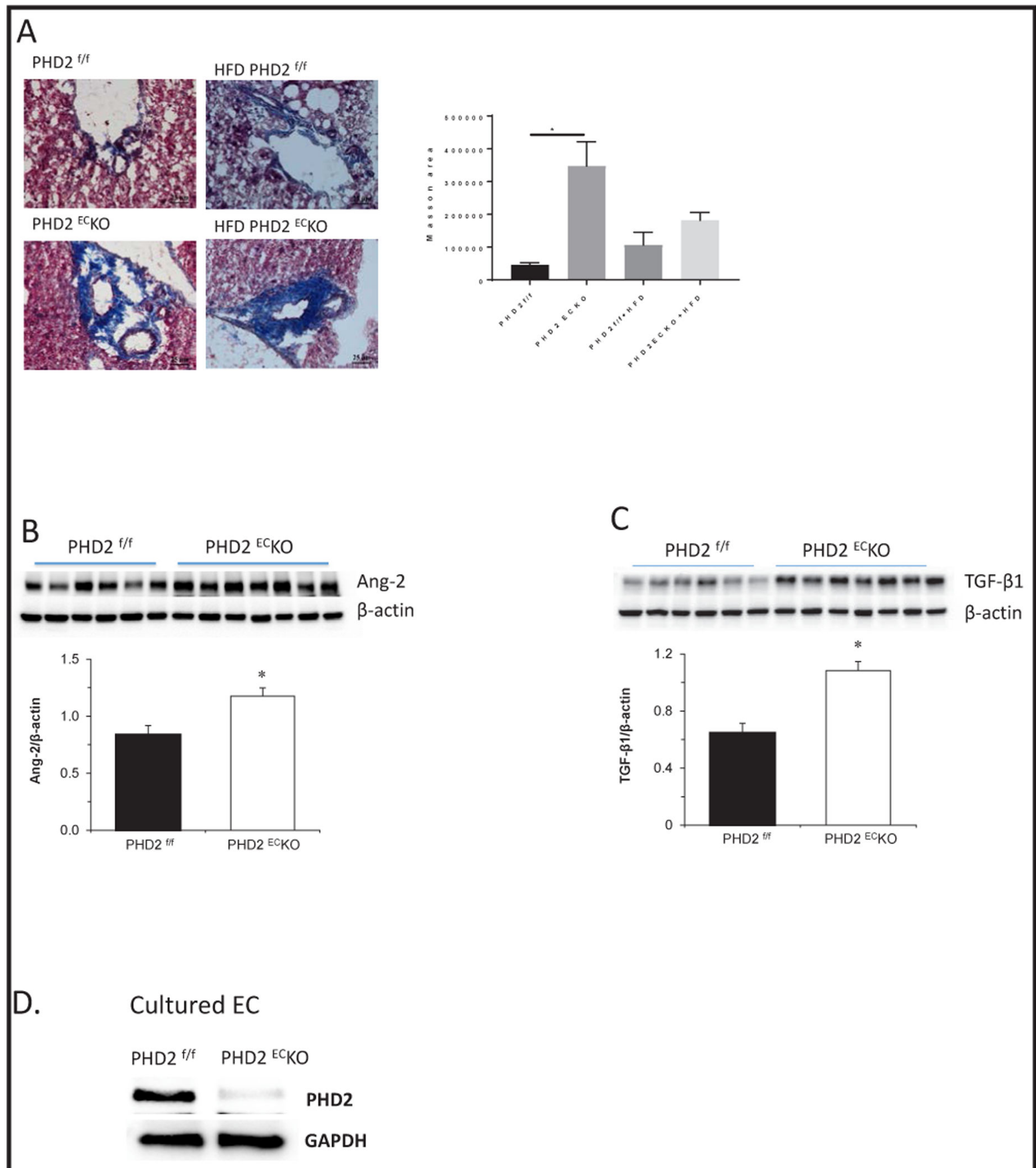

Fig. 2.

PHD2^{EC}KO increases hepatic fibrosis by regulating Ang-2/TGF-β pathway. A) Masson trichrome staining showed that fibrosis around small arteries and venous, especially in the PHD2^{EC}KO mice. The percentage of fibrotic area was significantly increased in the PHD2^{EC}KO mice compared with the PHD2$^{f/f}$ mice. (n=6–7 mice, *$p<0.05$). B) The expression of Ang-2 was increased significantly in the PHD2^{EC}KO mice. (n=6–7 mice, *$p<0.05$). C) The expression of TGF-β was significantly upregulated in the PHD2^{EC}KO

mice. (n=6–7 mice, *p<0.05). D) The expression of PHD2 was abolished in cultured EC from the PHD2^{EC}KO mice. Mean ± SD, *p<0.05.

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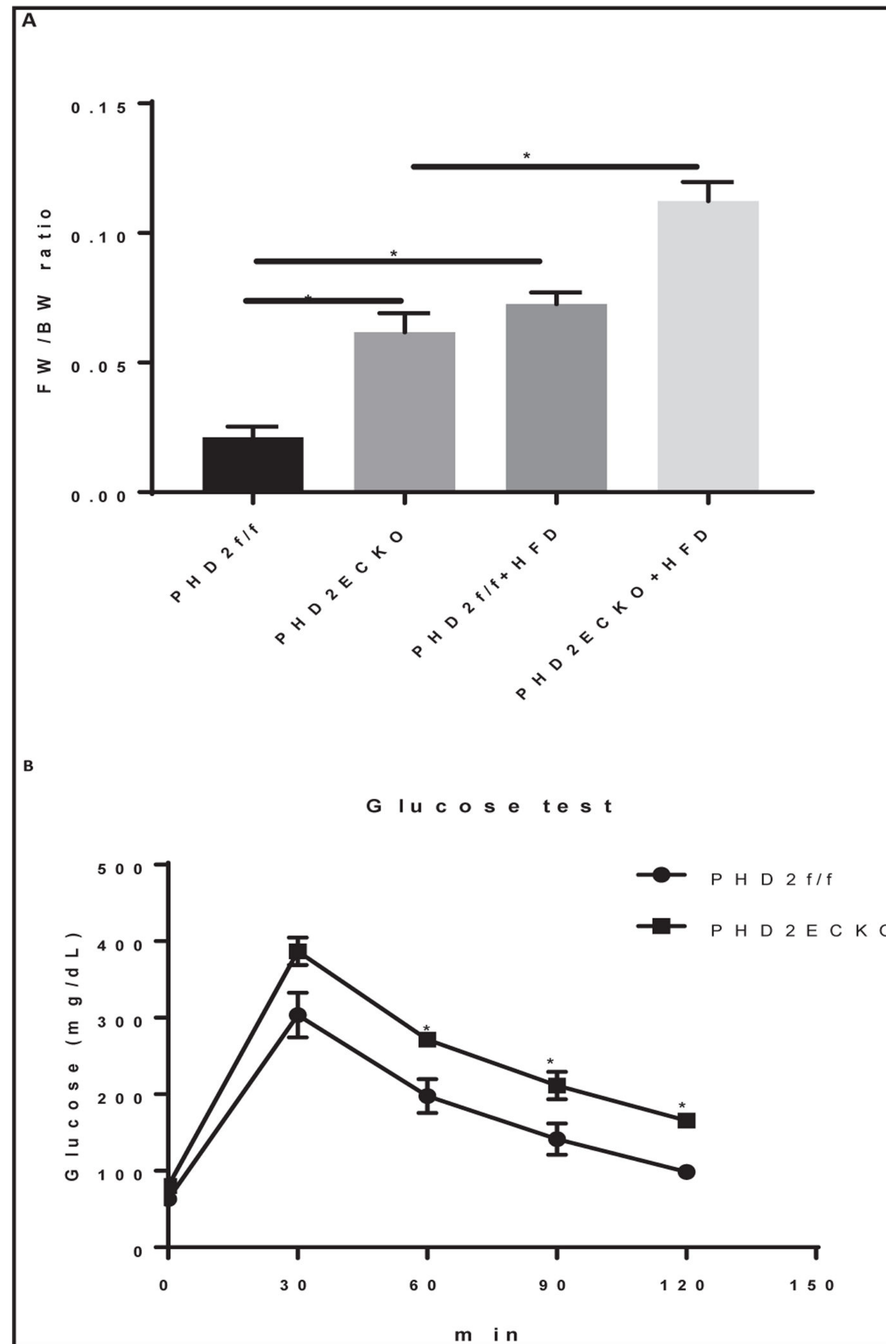

Fig. 3.

PHD2^{EC}KO increases body fat accumulation and impairs glucose tolerance. A) FW/BW ratio was significantly increased in the PHD2^{EC}KO mice and PHD2$^{f/f}$ mice + HFD compared with the PHD2$^{f/f}$ mice. FW/BW ratio was significantly increased in the PHD2^{EC}KO mice + HFD compared with the PHD2^{EC}KO mice (n=7–9 mice, *$p<0.05$). B) Glucose tolerance was impaired in the PHD2^{EC}KO mice compared with the PHD2$^{f/f}$ mice (n=4 mice, *$p<0.05$).

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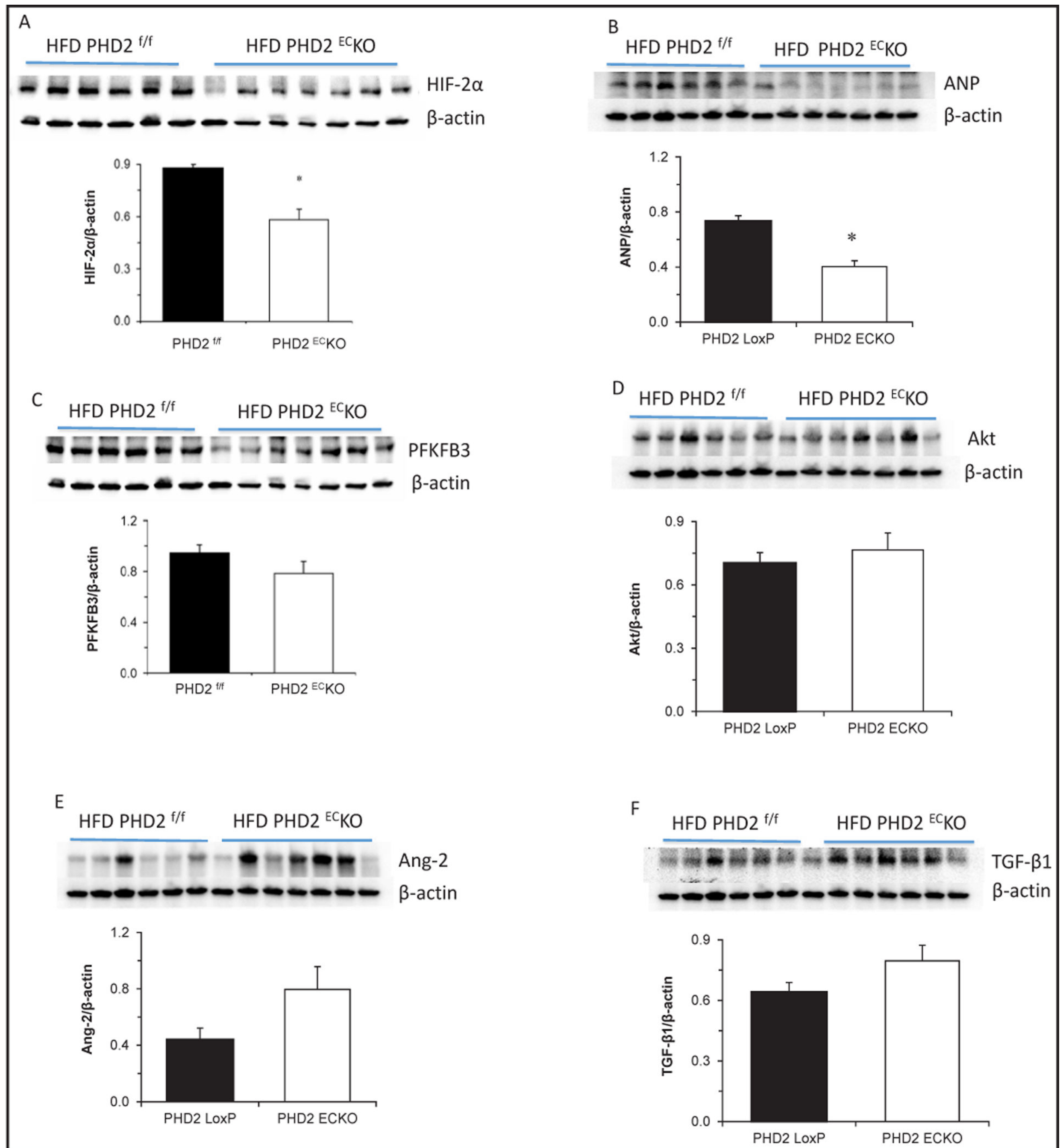

Fig. 4.

PHD2^{EC}KO on HFD-induced liver gene expression. A) The expression of HIF-1α was significantly decreased in the PHD2^{EC}KO mice + HFD compared with the PHD2$^{f/f}$ mice + HFD (n=6–7 mice, *p<0.05). B) The expression of ANP was significantly decreased in the PHD2^{EC}KO mice + HFD compared with the PHD2$^{f/f}$ mice + HFD (n=6–7 mice, *p<0.05). C-F) No significant difference of PFKFB3, Akt, Ang-2 and TGF-β are found between the

PHD2^{EC}KO mice + HFD and the PHD2$^{f/f}$ mice + HFD groups. (n=6–7 mice, *$p<0.05$). Mean ± SD,*$p<0.05$.

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