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Delayed liver regeneration after partial hepatectomy in adiponectin knockout mice

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ABSTRACT

We previously demonstrated that adiponectin has anti-fibrogenic and anti-inflammatory effects in the liver of mouse models of various liver diseases. However, its role in liver regeneration remains unclear. The aim of this study was to determine the role of adiponectin in liver regeneration. We assessed liver regeneration after partial hepatectomy in wild-type (WT) and adiponectin knockout (KO) mice. We analyzed DNA replication and various signaling pathways involved in cell proliferation and metabolism. Adiponectin KO mice exhibited delayed DNA replication and increased lipid accumulation in the regenerating liver. The expression levels of peroxisome proliferator-activated receptor (PPAR) α and carnitine palmitoyltransferase-1 (CPT-1), a key enzyme in mitochondrial fatty acid oxidation, were decreased in adiponectin KO mice, suggesting possible contribution of altered fat metabolism to these phenomena. Collectively, the present results highlight a new role for adiponectin in the process of liver regeneration.

The liver is one of few adult organs that can regenerate following acute injury, such as toxins, viral hepatitis and hepatic resection. To understand the mechanism of liver regeneration, many investigators have induced liver regeneration by two-thirds partial hepatectomy (PHx) in rodent models [1]. Liver regeneration induced by PHx results in synchronous induction of hepatocyte DNA replication and mitosis. This proliferative response is initiated by the release of various cytokines and growth factors. During the regeneration process, immediate early transcription factors such as c-Jun, JunB or c-Myc are activated to promote hepatoproliferation [2].

Adiponectin (also known as ACRP30 and GBP28) is a plasma protein secreted from adipose tissue with known anti-diabetic and anti-atherogenic properties [3,4]. Adiponectin is expressed in the plasma (range, 5–30 μg/ml), and low levels are found in obesity and type 2 diabetes [3]. So far, two types of receptors for adiponectin, AdipoR1 and AdipoR2, have been identified [4]. It is reported that AdipoR1 is expressed mainly in skeletal muscles, whereas AdipoR2 is expressed mainly in the liver [4]. Mouse genetic analysis also demonstrated that AdipoR1 is strongly related to the activation of AMP-activated protein kinase (AMPK) signaling pathways, whereas AdipoR2 is linked to the stimulation of peroxisome proliferator-activated receptor (PPAR) α signaling pathways [5]. Studies from our laboratory have demonstrated that adiponectin has anti-fibrogenic and anti-inflammatory effects in mouse models of hepatic fibrosis [6] and acute hepatitis [7], respectively. Although hypoadiponectinemia is associated with several liver diseases [8], the role of adiponectin in liver regeneration remains unclear so far.

In this present study, we demonstrated that adiponectin KO mice exhibit delayed liver regeneration and show accumulation of triglyceride in hepatocytes after PHx. The results suggest that these changes might be attributed to impaired fatty acid oxidation.

Materials and methods

Mice. Generation of mice carrying homozygote mutation for mouse adiponectin gene, which was bred into the C57BL/6 background for five generations, was described previously [9]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

Partial hepatectomy. Adiponectin KO mice and wild-type (WT) littermates (males, weighing 22–30 g, 12 weeks of age) were subjected to 70% PHx to induce liver regeneration. PHx was performed according to the method of Higgins and Anderson [10]. Six to eight mice at each time points were sacrificed at 0, 12, 24, 48, 72, and 96 h after PHx. At the time of sacrifice, the mice were weighed, blood was harvested from the inferior vena cava and the remnant liver was removed en bloc. The removed livers were rapidly weighed and frozen in liquid nitrogen and stored at −80 °C.

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Sections from the removed liver were fixed in 10% buffered formalin for further histological analysis.

BrdU labeling and immunohistochemical staining. Hematoxylin and eosin (H&E) stained liver sections were assessed for hepatocyte lipid accumulation. Hepatocyte DNA replication was assessed by nuclear incorporation of bromodeoxyuridine (BrdU), a thymidine analog incorporated into newly synthesized DNA, on immunostained liver sections using Cell Proliferation Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). BrdU (30 mg/kg body weight) was injected intraperitoneally 4 h before harvesting the remnant regenerating livers. In each regenerating livers, we counted the number of BrdU-positive nuclei per 1000 hepatocytes to calculate the BrdU labeling index.

Western immunoblotting. For Western immunoblot analysis, total extracts prepared from liver tissues were separated on sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and transferred onto polyvinylidene fluoride membrane as described previously [6]. We used primary antibodies specific to cyclin D1 (Santa Cruz Biotechnologies, Santa Cruz, CA). The density of each band was measured by a densitometer.

Real-time RT-PCR. Total RNA extraction from whole liver, reverse-transcription polymerase chain reaction (RT-PCR) and real-time PCR were performed as described previously [7]. The Quantitect gene assay kit was used for analysis of murine cyclin A2, cyclin B1, carnitine palmitoyl transferase (CPT-1), PPARα, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative gene expression was quantified using GAPDH as an internal control.

Analysis of hepatic triglyceride content. Total lipids were extracted from the liver as described previously [11]. Hepatic triglyceride contents were measured by using Wako Test kit (Wako Pure Medical, Tokyo, Japan), and the results were expressed in μg per mg liver weight.

Statistical analysis. The results are presented as means ± SD. Differences between two groups were examined for statistical significance using the Mann–Whitney U test. A P value less than 0.05 denoted the presence of a statistically significant difference.

Results

Adiponectin KO mice show delayed liver regeneration after PHx

To determine the role of adiponectin in liver regeneration, we compared the kinetics of DNA replication in hepatocytes and liver restitution in WT and KO mice at 24–96 h after PHx. DNA replication was monitored by immunohistochemical staining of BrdU nuclear incorporation. The restitution of liver mass was assessed by liver/body weight ratio. Compared with WT mice, the BrdU labeling index of KO mice was significantly lower at 48 and 72 h but was significantly higher at 96 h after PHx (Fig. 1A and B). These results showed that adiponectin KO livers show a lag in DNA replication after PHx, compared with the wild-type mice. We also found that the liver/body weight ratio was significantly lower in KO mice at 48 and 72 h after PHx than in WT mice (Fig. 1C). These results suggest delayed DNA replication of hepatocytes and impaired liver restitution after PHx in KO mice.

Underexpression of cell cycle regulators in regenerating livers of adiponectin KO mice

Next, we investigated the roles of cell cycle regulators; cyclin D1, cyclin A2, and cyclin B1, in impaired liver regeneration of KO mice. We evaluated the expression of cyclin D1 protein in the regenerating livers by Western immunoblotting at 0 and 24 h after PHx. Densitometric analysis showed a significantly low expression

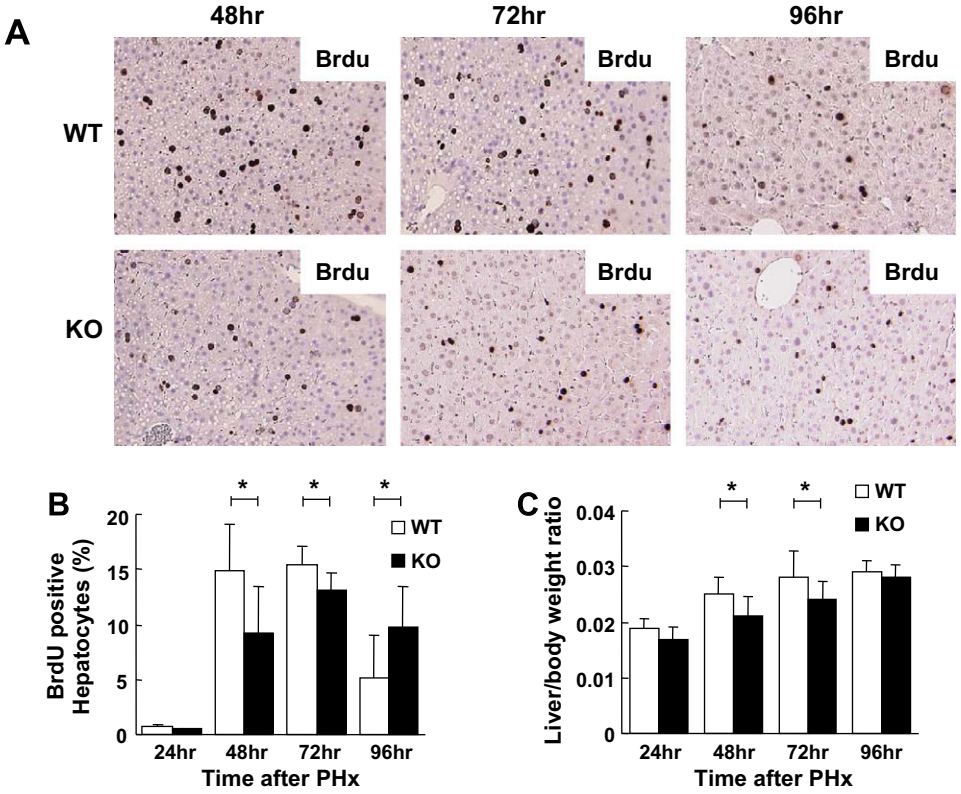

Fig. 1. Delayed hepatocytes proliferation and restitution of liver mass after PHx in adiponectin KO mice. (A) Representative micrographs of liver sections harvested from WT and KO mice after PHx, immunostained with BrdU antibody. (B) Quantitative analysis of BrdU labeling index (percentage of BrdU-positive hepatocytes) in the regenerating liver. (C) Serial changes in liver/body weight ratio after PHx. Data are means ± SD values of 6–8 mice per group and time point. $^{*}P < 0.05$.

of cyclin D1 protein in KO mice compared with WT mice at 24 h after PHx (Fig. 2A and B). Furthermore, the mRNA levels of cyclin A2 and cyclin B1 measured by real-time RT-PCR in KO mice were significantly lower at 48 and 72 h after PHx than in WT mice (Fig. 2C and D).

Increased fatty change and triglyceride content in regenerating livers after PHx in adiponectin KO mice

Lipid droplets accumulate in hepatocytes of the regenerating livers and this process is known to be essential for hepatocyte proliferation during liver regeneration [12]. We examined whether adiponectin deficiency affects this process by histological analysis and measurement of triglyceride content in the regenerating livers of WT and KO mice after PHx. We assessed lipid droplet formation in H&E stained liver sections at 0, 48, and 72 h after PHx. Lipid droplets were found in many hepatocytes at 48 and 72 h after PHx in both genotypes. At 72 h after PHx, KO mice showed increased fat accumulation compared with WT mice (Fig. 3A). Quantitative analysis confirmed that hepatic triglyceride content were significantly higher in KO mice than WT mice at 72 h after PHx (Fig. 3B).

Reduced mRNA expression levels of fatty acid oxidation-related genes in regenerating livers after PHx in adiponectin KO mice

To determine the molecular mechanism responsible for the increased hepatic fat accumulation in KO mice after PHx, we investigated fatty acid metabolism during liver regeneration by analyzing the mRNA expression level of CPT-1, a rate-limiting enzyme of fatty acid β-oxidation, at 0, 12, 24, 48, and 72 h after PHx. We also examined the mRNA expression level of PPARα, which is involved in fatty acid metabolism, at 0, 24, 48, 72, and 96 h after PHx. Real-time RT-PCR analysis showed downregulation of CPT-1 and PPARα in KO mice compared with WT mice at all time points. The differences in CPT-1 expression between the control and KO mice were significant at 24 and 72 h after PHx (Fig. 4A). Likewise, the differences in PPARα expression between the two groups were significant at 72 and 96 h after PHx (Fig. 4B). These results indicate that impaired fatty acid oxidation results in increased fat accumulation in the regenerating livers of adiponectin KO mice.

Discussion

In this study, we tested the hypothesis that adiponectin influences hepatocyte proliferation during liver regeneration. Our results in mice demonstrated that adiponectin deficiency caused delays in hepatocyte proliferation and restitution of liver mass during liver regeneration.

During the first few hours after PHx, various genes involved in cytokine signaling are induced to prime the resting hepatocytes for cell division [1]. TNFα and IL-6 are strongly induced in this process [1] and mouse genetic studies confirmed that liver regeneration is impaired in mice with homozygote mutation of IL-6 or TNF receptor type 1 [2]. Previous studies also demonstrated that leptin KO mice show disturbed liver regeneration and low expression of TNFα and IL-6 after carbon tetrachloride-induced liver injury [13]. Furthermore, we reported previously that adiponectin has anti-inflammatory properties as it suppressed the secretion of TNFα from Kupffer cells in lipopolysaccharide- and D-galactosamine-induced liver injury model in mice [7]. To further analyze the contribution of adiponectin to the priming phase of liver regeneration, we first determined whether impaired liver regeneration in adiponectin KO mice is due to abnormal production of these cytokines. However, there was no significant difference in serum levels and hepatic expression levels of TNFα and IL-6 between adiponectin KO and WT mice (data not shown). This was also confirmed by the fact that the expression levels of immediate early genes were not altered in the regenerating adiponectin KO livers (data not shown). These data suggest that adiponectin is not involved in the priming phase of liver regeneration.

It is reported that overexpression of cyclin D1 is associated with the onset of hepatocyte DNA replication in rodent models of liver regeneration [14]. We next examined the expression of cyclin D1 in the regenerating livers and showed a significant reduction of cyclin D1 expression in adiponectin KO mice compared with wild-type.

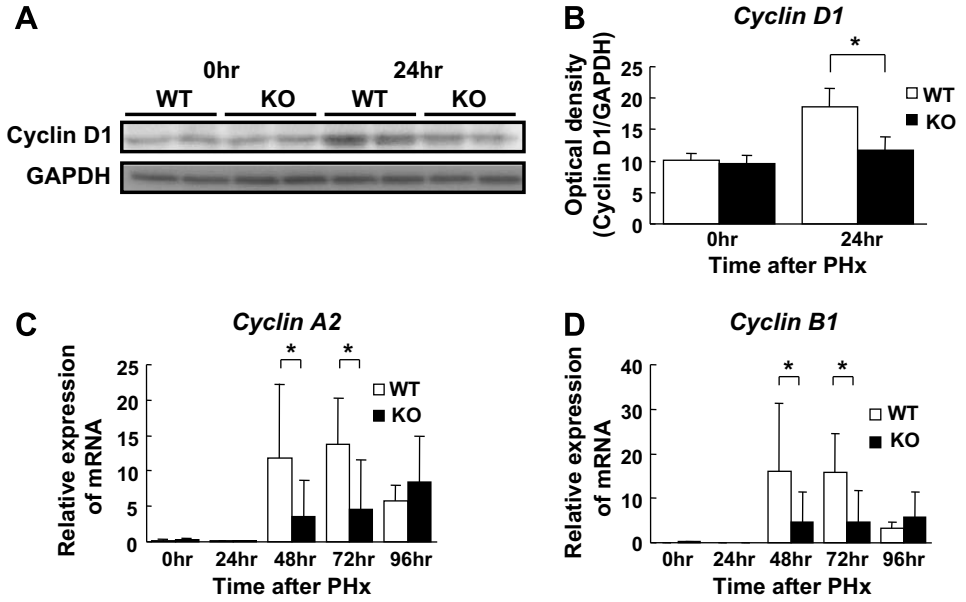

Fig. 2. Reduced expression levels of cell cycle regulatory proteins in adiponectin KO livers after PHx. (A) Western immunoblot analysis of cyclin D1 expression in the liver. Representative blots are presented. (B) Results of densitometric analysis. Data were expressed relative to GAPDH expression. Expression levels of cyclin A2 (C) and cyclin B1 (D) mRNAs in the regenerating liver after PHx measured by real-time RT-PCR. Data were expressed relative to GAPDH expression. Data are means ± SD values of 6–8 mice per group and time point. $^{*}P < 0.05$.

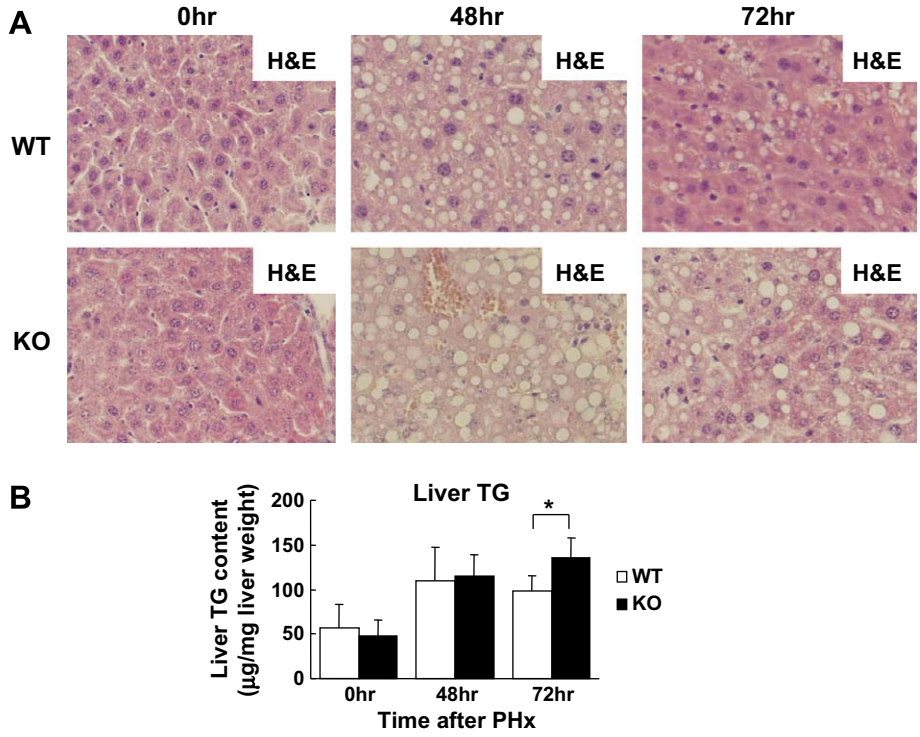

Fig. 3. Increased lipid droplet formation and triglyceride accumulation in hepatocytes of adiponectin KO mice after PHx. (A) Microphotographs of liver sections harvested from WT and KO mice after PHx stained with hematoxylin and eosin. Magnification, 200×. (B) Triglyceride contents in the regenerating livers. Data are means ± SD values of 6–8 mice per group and time point. $^{*}P < 0.05$.

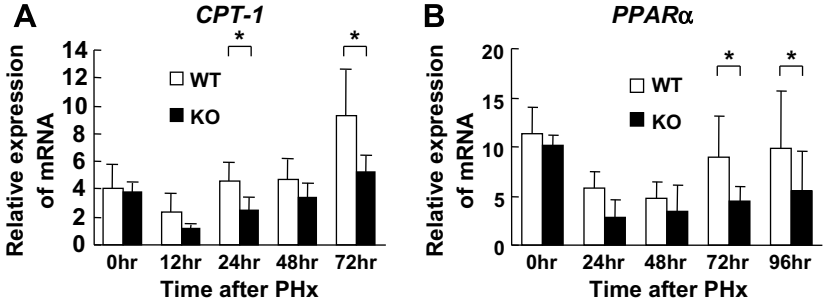

Fig. 4. Decreased expression levels of CPT-1 and PPARα in the regenerating liver of adiponectin KO mice after PHx. The expression levels of CPT-1 (A) and PPARα (B) mRNAs in the regenerating liver of WT and KO mice after PHx by real-time RT-PCR. Data were expressed relative to GAPDH expression. Data are means ± SD values of 6–8 mice per group and time point. $^{*}P < 0.05$.

We also showed reduced expression of cyclin A2 in adiponectin KO regenerating livers, indicating that adiponectin deficiency caused disturbances of the G1/S transition (DNA replication) of cell cycle progression during liver regeneration. Stimulation of Cdk1–cyclin B1 complex kinase activity is essential for G2/M transition (mitosis) during liver regeneration [15]. The current data also demonstrated decreased expression of cyclin B1 in adiponectin KO livers, suggesting that adiponectin deficiency resulted in abnormal S-phase progression and entry into mitosis in the process of liver regeneration.

The liver regeneration process requires complex signaling that includes the mobilization of nutrients and hepatocyte proliferation [16,17]. Accumulation of triglycerides in the liver manifests as fat droplet formation transiently after PHx and is believed to be essential for proper liver regeneration. Interestingly, a recent study showed that liver regeneration was impaired in mice lacking Caveolin1, an essential component of caveolae in the plasma membrane [12]. Impaired liver regeneration in Caveolin1 KO mice was associated with disrupted lipid droplet formation and was rescued by glucose feeding in advance, suggesting the important role of free fatty acid as an energy source during liver regeneration [12]. In the present study, we also observed fat accumulation in the regenerating livers and this phenomenon was more evident in hepatocytes of adiponectin KO than WT mice. This phenomenon was confirmed by increased triglyceride content in the regenerating livers of adiponectin KO mice. In the liver, adiponectin increases fatty acid oxidation through the AdipoR2–PPARα signaling pathway, which was recently confirmed in various mouse genetic models [4]. Therefore, we tested the hypothesis that the increased fat accumulation in adiponectin KO livers is due to impairment of fatty acid catabolism, leading to impaired energy supply required for proper hepatocyte proliferation. Although the expression levels of AdipoR1 and AdipoR2 in the regenerating livers of two genotypes were not different (data not shown), the expression of PPARα was reduced in the regenerating livers of adiponectin KO mice.

PPARα is known to stimulate the expression and enzyme activity of CPT-1, a rate-limiting enzyme of fatty acid oxidation, which regulates the uptake of acyl-CoA into the mitochondria [18]. Our study also showed reduced expression of CPT-1 in the regenerating livers of adiponectin KO, indicating that adiponectin deficiency caused impaired mitochondrial fatty acid oxidation.

Interestingly, there were no significant differences in serum glucose, free fatty acid and triglyceride levels in the partially hepatectomized mice of both genotypes (data not shown). These results support our hypothesis that delayed regeneration of adiponectin KO liver is mainly due to impaired mitochondrial fatty acid oxidation in hepatocytes of the regenerating liver.

The PPARα is the downstream target of adiponectin–AdipoR signaling pathway [5]. Homozygote mutation in mouse PPARα gene is reported to result in delayed liver regeneration with increased lipid accumulation in hepatocytes [19]. In addition, PPARα is known to be associated with cell proliferation through the regulation of cell cycle regulators, such as cyclin D1 [20]. These findings are in agreement with our results in adiponectin KO mice. Take together, low expression of PPARα might cause altered fat metabolism and cell cycle control in adiponectin KO mice during liver regeneration.

Clinically, low serum adiponectin levels are reported in obese patients with fatty liver [21], suggesting the possible contribution of adiponectin in the mechanism of impaired regeneration of fatty liver disease, such as non-alcoholic fatty liver disease and alcoholic liver disease. Further studies are needed to enhance our understanding of impaired liver regeneration.

In conclusion, this study provides new data linking adiponectin to liver regeneration.

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