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Oleic acid activates peroxisome proliferator-activated receptor δ to compensate insulin resistance in *steatotic* cells☆,☆☆,★

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Abstract

Nonalcoholic fatty liver disease is frequently associated with type 2 diabetes; however, this idea is challenged by recent studies because hepatic steatosis is not always associated with insulin resistance (IR). Oleic acid (OA) is known to induce hepatic steatosis with normal insulin sensitivity; however, the mechanism is still unknown. Previous studies depict that activation of peroxisome proliferator-activated receptor δ (PPARδ) improves hepatic steatosis and IR, whereas the role of PPARδ in the improvement of insulin sensitivity by OA is unknown. Here we induced steatosis in HepG2 cells by incubation with OA and OA significantly increased the expression of PPARδ through a calcium-dependent pathway. OA also induced the expression of G protein-coupled receptor 40 (GPR40), and deletion of GPR40 by small interfering ribonucleic acid transfection partially reversed the effect of OA on PPARδ. Inhibition of phospholipase C (PLC) by U73122 also reversed OA-induced PPARδ expression. Otherwise, deletion of PPARδ augmented the OA-induced steatosis in HepG2 cells. Furthermore, IR was developed in OA-treated HepG2 cells with PPARδ deletion, while insulin-related signals and insulin-stimulated glycogen synthesis were reduced through increase of phosphatase and tensin homolog (PTEN) expression. In conclusion, OA activates GPR40-PLC-calcium pathway to increase the expression of PPARδ and PPARδ further decreased the expression of PTEN to regulate insulin sensitivity in hepatic steatosis.

Keywords: Hepatic steatosis; Insulin resistance; Oleic acid; Peroxisome proliferator-activated receptor δ; PTEN

1. Introduction

Nonalcoholic fatty liver diseases (NAFLD) encompass histological features ranging from hepatic steatosis, steatohepatitis, fibrosis and cryptogenic cirrhosis [1]. Previous studies have suggested that NAFLD are commonly associated with insulin resistance (IR) [2,3]; however, the underlying mechanisms linking these two phenomena are still unclear [4–6]. Although 21% to 78% diabetic patients have hepatic steatosis [7,8], it is unsuccessful to link NAFLD to impaired glucose utility. The prevalence of type 2 diabetes varies from 2% to 55% in patients with NAFLD [9], and the amount of steatosis is more closely related to the degree of obesity [10]. In contrast, some patients with NAFLD are lean and have normal fasting glucose levels with normal glucose tolerance [11]. Moreover, mice fed a methionine–choline-deficient diet develop hepatic steatosis without IR [12]. Although lipid accumulation in hepatocytes is often associated with IR; however, it is unclear whether it is steatosis that can cause the development of IR, and the mechanisms are poorly defined.

Free fatty acids (FFAs) not only play a major role in the storage of excess energy, but also exert multiple physiological functions directly through cell membrane receptors [13,14], such as G protein-coupled receptor 40 (GPR40). Because of the various types of FFAs, the bio-activities differ greatly. Although the functions of the SFA and UFA are reported in numerous reports, the mechanisms of the bio-functions in

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detail remain unclear. Saturated fatty acids (SFA) are toxic to cells, whereas the unsaturated fatty acids (UFA) are either nontoxic or cytoprotective [14]. In addition, UFA is more steatogenic than SFA; however, SFA, but not UFA, impairs insulin signaling [15]. Insulin-related signals, such as Akt phosphorylation, were significantly increased in oleic acid (OA)-treated hepatocytes [16].

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor family and are responsible for the regulation of lipid homeostasis. PPARα is highly expressed in the liver, kidney and muscle. PPARγ is enriched in adipose tissue and PPARδ appears to be ubiquitously expressed [17]. PPARα is responsible for lipid degradation [18], whereas PPARγ is the transcriptional regulator of adipogenesis and plays an important role in the process of lipid storage [19]. PPARδ regulates the degradation of lipids [20]. The roles of PPARα [21] and PPARγ [22] in hepatic lipid homeostasis are well established. But the function of PPARδ in the liver is still unclear.

Overexpression of PPARδ in adipose tissue produced a lean phenotype due to increased degradation of lipids in adipocytes [20]. Cardiac metabolism is dependent on PPARδ, and knockdown of PPARδ in mouse results in reduced expression of β-oxidation genes and cardiomyopathy with reduced survival [23]. The role of PPARδ in the therapy of metabolic diseases has also been explored recently. Hepatic expression of PPARδ was suppressed in mice fed with a hypercaloric diet [24], and overexpression of PPARδ by adenovirus improves hepatic lipid accumulation in animals [25]. Although the functions of PPARδ in muscle and adipose tissue are well investigated, the role of PPARδ in liver is still obscure. In addition, a previous study indicated that linoleic acid, an UFA, up-regulates the expression of PPARδ in liver to stimulate gluconeogenesis [26]. OA decreases the expression of PPARα [27] and increases the expression of PPARγ [28] to facilitate hepatic steatosis. However, the potential role of PPARδ in OA-improved insulin sensitivity in hepatic steatosis is still unknown.

The cause of hepatic steatosis is increased influx of FFAs into hepatocytes, and the excess FFAs are then converted to triglycerides and stored in lipid droplets with less cytotoxicity [29]. Hepatic steatosis in humans is associated with accumulation of excess OA [30]. HepG2 cells offer an alternative and reliable model in studies of hepatic lipid metabolism [31], and treatment with OA in HepG2 cells induces morphological similarities to steatotic hepatocytes [32,33]. Thus, HepG2 cells were used in the present study to investigate the mechanism of OA-induced PPARδ expression and the possible role of PPARδ in hepatic IR.

2. Methods and materials

2.1. Cell cultures

HepG2 and Huh7 cell lines were purchased from Bioresource Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan). The cells were maintained at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum. Primary hepatocytes were prepared according to a previous study [34]. For the induction of steatosis, the cells were starved in serum-free medium overnight. Stock solutions of 100 mM OA (Sigma-Aldrich) were diluted in culture medium containing bovine serum albumin (BSA; Sigma-Aldrich) to obtain the desired final concentrations. OA was used for treatment of HepG2 cells after conjugation with the appropriate concentration of BSA. The final molar ratio of FFA/BSA was ~2:1, which was close to the ratio observed in human serum [35]. Control cells were treated with OA-free medium containing 0.5 % BSA. Staining of intracellular neutral lipids was performed with oil red O (Sigma-Aldrich). The content of glycogen in cells was determined by a kit performed by the manufacturer's protocol (BioVision; Mountain View, CA, USA).

2.2. Western blotting analysis

Total protein lysates from tissues or cells were extracted in lysis buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.5), 5 mM ethylenediaminetetraacetic acid] containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The protein concentration was determined by BCA assay kit (Pierce Biotechnology, Rockford, IL, USA). Protein lysates (50 μg) were separated using 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked at 25°C for 1 h in TBS-T [10 mM Tris (pH 7.6), 150 mM NaCl and 0.05% Tween 20], containing 3% BSA, and probed with 1:1000 primary antibodies, such as PTEN, phospho-Akt, Akt (Cell Signaling Technology, Beverly, MA, USA), insulin receptor (Neomarkers, Fremont, CA, USA), phospho-insulin receptor and PPARδ (Abcam, Cambridge, UK) at 4°C overnight. After the membrane had been washed with TBS-T, the blots were incubated with a 1/5000 dilution of horseradish peroxidase-conjugated secondary antibodies at 25°C for 1 h. The protein bands were visualized using an enhanced chemiluminescence kit (PerkinElmer, Boston, MA, USA). Actin (Millipore) was an internal control. The optical densities of the bands were determined using software (Gel-Pro Analyzer 4.0; Media Cybernetics, Silver Spring, MD, USA).

2.3. Determination of Ca^{2+} concentration

The cells were incubated with fluorescent Ca^{2+}-sensitive dye (2 μM, Fura-2/AM; Calbiochem, San Diego, CA, USA) for 30 min at 37°C in culture medium. Fura-2 fluorescence measurements were done in a cuvette containing 10^6 cells in 0.5 ml of buffer [140 mM NaCl, 5.9 mM KCl, 1.2 mM NaH_2PO_4, 5 mM $NaHCO_3$, 1.4 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES (pH 7.4)] with continuous stirring. Fluorescence was monitored using a spectrofluorophotometer (F-2000; Hitachi, Tokyo, Japan) by recording excitation signals at 340 and 380 nm and emission signals at 510 nm at 1-s intervals. Maximum and minimum fluorescence values were obtained by adding 10% Triton X-100 and 100 mM of ethylene glycol-bis(aminoethylether)-*N*,*N*′-tetraacetic acid sequentially at the end of each experiment.

2.4. Flow cytometry

The procedure was performed following that in a previous study [16]. HepG2 cells were fixed in 4% paraformaldehyde for 20 min at 25°C. Intracellular neutral lipids were labeled with 200 nM BODIPY505/515 (Invitrogen, Carlsbad, CA, USA) for 30 min in the dark. After washing with phosphate-buffered saline, flow cytometry was performed using FACScan (BD Biosciences San Diego, CA, USA). The data was analyzed by WinMDI 2.9 software (Joseph Trotter, Salk Institute for Biological Studies, La Jolla, CA, USA).

2.5. Small interfering ribonucleic acid transfection

HepG2 cells were grown in six-well culture plates at a density of 2×10^5 cells/well. Cells were transfected with duplexed RNA oligonucleotides (Stealth RNAi; Invitrogen)

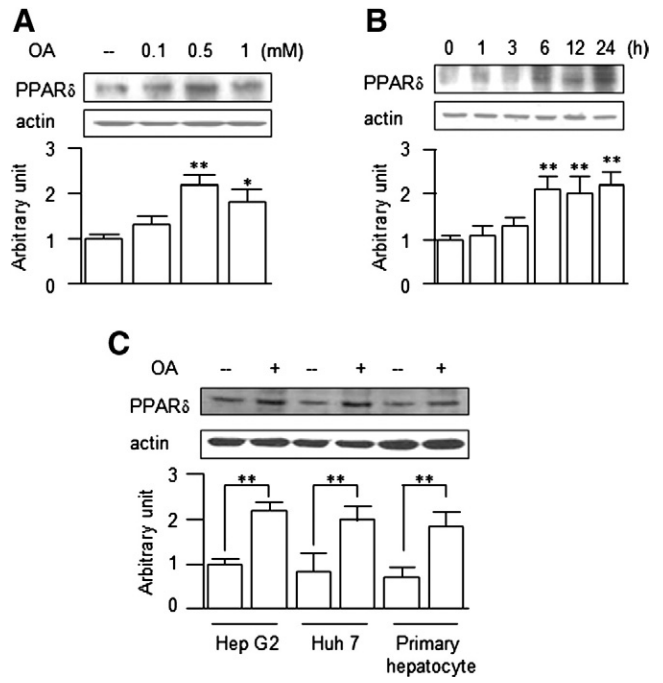

Fig. 1. Oleic acid (OA) up-regulated the expression of PPARδ in liver cells. (A) HepG2 cells were treated with OA at various doses and the expression of PPARδ was detected at 6 h. (B) HepG2 cells were treated with 0.5 mM OA and harvested at the indicated times for the detection of PPARδ levels. (C) HepG2, Huh7 and mouse primary liver cells were treated with 0.5 mM OA and harvested at 6 h for the detection of PPARδ levels. Results are means±S.E.M. of three independent experiments. *P<.05, **P<.01 as compared with the control group.

of human PPARδ, GPR40 or scramble siRNA (as negative control) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The cells were treated with OA at 48 h post-transfection.

2.6. Statistical analysis

Data are expressed as means±S.E.M. ANOVA and Dunnett range *post hoc* comparisons were used to determine the source of significant differences where appropriate. Significance was declared when the *P* value was less than .05.

3. Results

3.1. OA increased PPARδ expression in liver cells

The effective treatment time and concentration of OA for the increment in PPARδ expression were determined in HepG2 cells at various times (0–24 h) (Fig. 1A) with various concentrations (0–1.0 mM) (Fig. 1B). The effect of OA at 0.1 mM does not have any significant difference on the expression of PPARδ. Therefore, higher concentration of OA at 0.5 mM for 6 h was selected as the treatment condition in the present study. In addition, OA not only increased the expression of PPARδ in Hep G2 cells but also in Huh7 cells and mouse primary hepatic cells (Fig. 1C).

3.2. OA increased PPARδ expression through GPR40-mediated and calcium-dependent pathway

FFAs can signal directly via the FFA receptor, GPR40, which is also known as free fatty acid receptor-1. To determine the involvement of GPR40 activation in OA-induced PPARδ expression, Western blot analysis was performed. OA increased GPR40 protein expression (Fig. 2A). Transfection of GPR40-specific siRNA significantly decreased the expression of GPR40 in HepG2 cells (Fig. 2B), but partially blocked OA-induced PPARδ expression, whereas nonspecific siRNA had no effect (Fig. 2C). We further investigated the role of GPR40-related signals in OA-induced PPARδ expression. To assess the involvement of Ca^{2+} in OA-induced PPARδ expression, we confirmed that OA increased the Ca^{2+} influx either in calcium-containing (upper panel) or in calcium-deprived condition (lower panel) (Fig. 2D). OA-induced PPARδ expression was completely blocked by BAPTA-AM (intracellular calcium chelator) (Fig. 2E). To determine the involvement of phospholipase C (PLC) on OA-induced PPARδ expression, cells were treated with a PLC inhibitor. U73122 (PLC inhibitor) inhibited OA-induced PPARδ expression (Fig. 2E). Moreover, OA-induced PPARδ expression was blocked by KN93 (calmodulin-dependent kinase inhibitor) and cyclosporine A (calcineurin inhibitor) (Fig. 2E). The effective

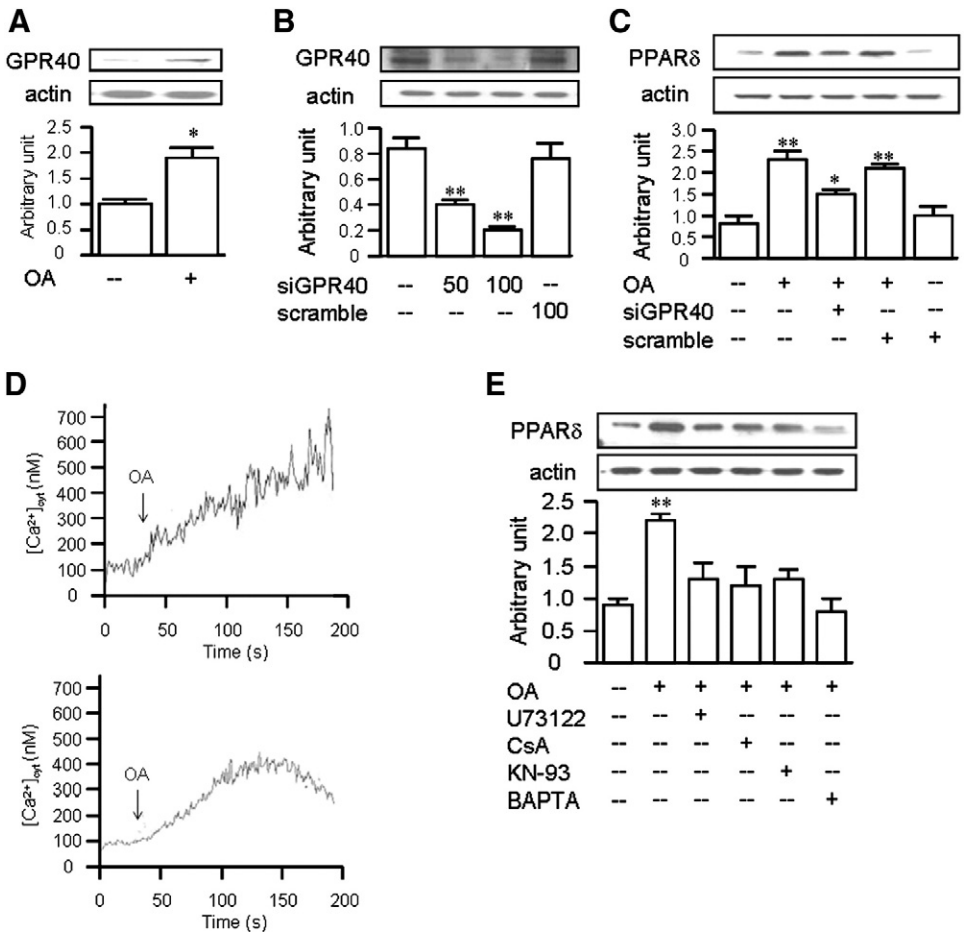

Fig. 2. PPARδ expression was regulated by OA through a GPR40-mediated and calcium-dependent mechanism. (A) HepG2 cells were treated with 0.5 mM OA for 6 h, and the total protein was extracted and blotted with GPR40 antibody. (B) Cells were transfected for 48 h with GPR40 siRNA (50 or 100 pmol) or a scramble control siRNA (100 pmol) before OA treatment for 6 h. The total protein was extracted and blotted with PPARδ or actin antibodies. (C) Cells were transfected for 48 h with 100 pmol GPR40 siRNA or 100 pmol scramble siRNA before OA treatment for 6 h. The total protein was extracted and blotted with GPR40 or actin antibodies. (D) Cells were treated with 2 μM Fura-2/AM in calcium-containing (upper panel) or calcium-free (lower panel) buffer for 30 min and treated with 0.5 mM OA. Cells were pretreated with 1 μM U73122 or 25 μM BAPTA-AM, 1 μM KN93 or cyclosporine A (CsA) (E) for 30 min and treated with OA for another 6 h. The total protein was extracted and blotted with PPARδ or actin antibodies. Values represent means±S.E.M. of three independent experiments. *P<.05, **P<.01 as compared with the control group.

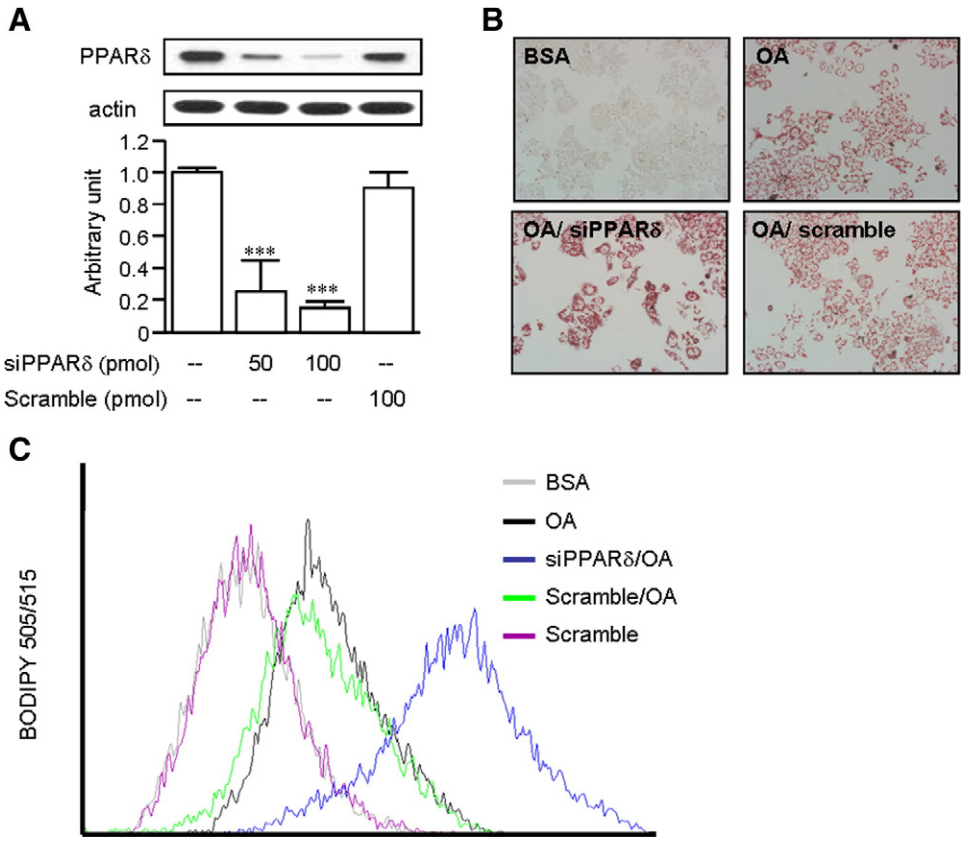

Fig. 3. Deletion of PPARδ in HepG2 cells aggravated OA-induced steatosis. (A) Cells were transfected for 48 h with PPARδ siRNA or scramble siRNA. The total protein was extracted and blotted with PPARδ or actin antibodies. (B) Lipid droplet accumulation was visualized by oil red O staining. Original magnification is ×200. (C) Cells were labeled with 200 nM BODIPY 505/515 for 30 min, and quantification of lipid accumulation was determined by flow cytometry analysis. Values represent means±S.E.M. *P<.05, **P<.01, ***P<.001 as compared with the control group.

concentrations of the inhibitors were discussed in previous reports [36,37].

3.3. Deletion of PPARδ-augmented steatosis in HepG2 cells

To explore the role of PPARδ in OA-induced steatosis, HepG2 cells were transfected with PPARδ-specific siRNA (Fig. 3A). OA induced lipid accumulation in HepG2 cells determined by oil red O staining (Fig. 3B) and BODIPY505/515 staining (Fig. 3C). PPARδ deletion augmented OA-induced lipid accumulation, whereas nonspecific siRNA had no effect (Fig. 3B and C).

3.4. Deletion of PPARδ-induced IR through a PTEN-dependent mechanism

After 6 h of incubation, OA induced a significant decrement in PTEN expression. PPARδ deletion reversed OA-induced PTEN decrement, whereas nonspecific siRNA had no effect (Fig. 4A). We then investigated whether OA modulates PTEN expression to increase insulin sensitivity through PPARδ. The phosphorylation of insulin receptor and Akt in response to insulin was increased in OA-treated cells (Fig. 4B) as compared with the control group. PPARδ-specific siRNA decreased insulin-induced Akt phosphorylation in OA-treated cells (Fig. 4B and C). However, phosphorylation of insulin receptor was not modified in OA-treated cells with PPARδ-specific siRNA (Fig. 4B and C). As a functional consequence, insulin-induced glycogen synthesis was measured in OA-treated cells with PPARδ deletion. PPARδ-specific siRNA inhibited the insulin-induced glycogen synthesis in OA-treated cells, whereas nonspecific siRNA had no effect (Fig. 4D).

4. Discussion

It was widely believed that the development of IR is associated with hepatic steatosis; however, this concept is controversial. Recent studies indicated that hepatic steatosis is not always associated with IR and that IR is not a critical factor in the development of hepatic steatosis [38–40]. In the present study, we demonstrated that OA increased the expression of PPARδ in HepG2 cells through a GPR40-PLC-calcium pathway. We also clarified the possible mechanism of OA-induced PPARδ expression in the regulation of insulin sensitivity in steatotis status. Treatment of OA in HepG2 cells induced steatosis with increased insulin sensitivity in consistency with a previous study [16]. PPARδ deletion not only augmented the lipid accumulation in HepG2 cells but also impaired insulin signaling. Our results indicated that OA increased PPARδ expression to regulate PTEN and further improved IR in hepatic steatosis. It was reported that activation of PPARδ increases the production of UFA and reduces that of SFA [41]. In the present study, we found that treatment of OA at 0.5 mM for 6 h significantly increased the expression of PPARδ (Fig. 1). It has been reported that OA at high concentrations (0.6 mM) induces apoptosis in HepG2 cells [27]. Therefore, the OA-induced PPARδ expression being more significant at 0.5 mM than that at 1.0 mM might be related to the cytotoxicity of OA at 1.0 mM (Fig. 1A). Although UFA increases the expression of PPARδ in hepatocytes,

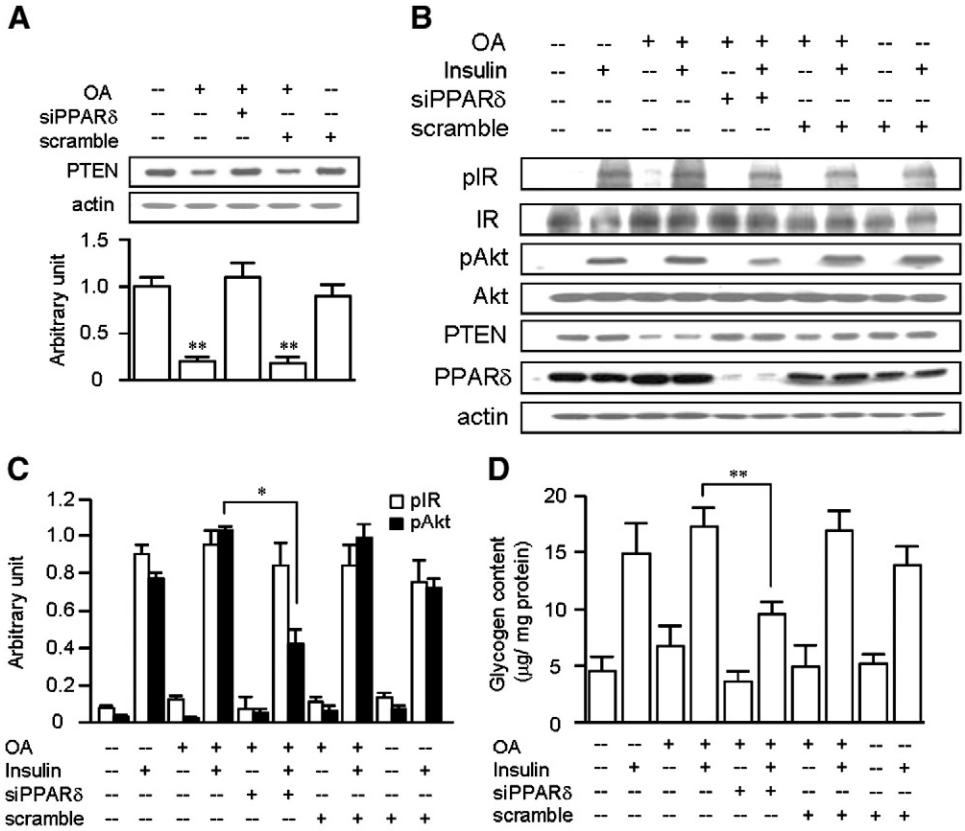

Fig. 4. PPARδ deletion impaired insulin signaling in OA-induced steatosis. (A) Cells were transfected for 48 h with 100 pmol PPARδ siRNA or scramble siRNA before OA treatment for 6 h. The total protein was extracted and blotted with PTEN or actin antibodies. (B) Cells were exposed or not to 0.1 μM insulin. Equal amounts of the total lysates of each group were immunoblotted with anti-phospho-Akt (pAkt) and anti-insulin receptor antibodies (pIR). (C) The glycogen content of each group was determined after normalization with the total protein of the cells. Values represent means±S.E.M. of three independent experiments. *P<.05, **P<.01 as compared with the control group.

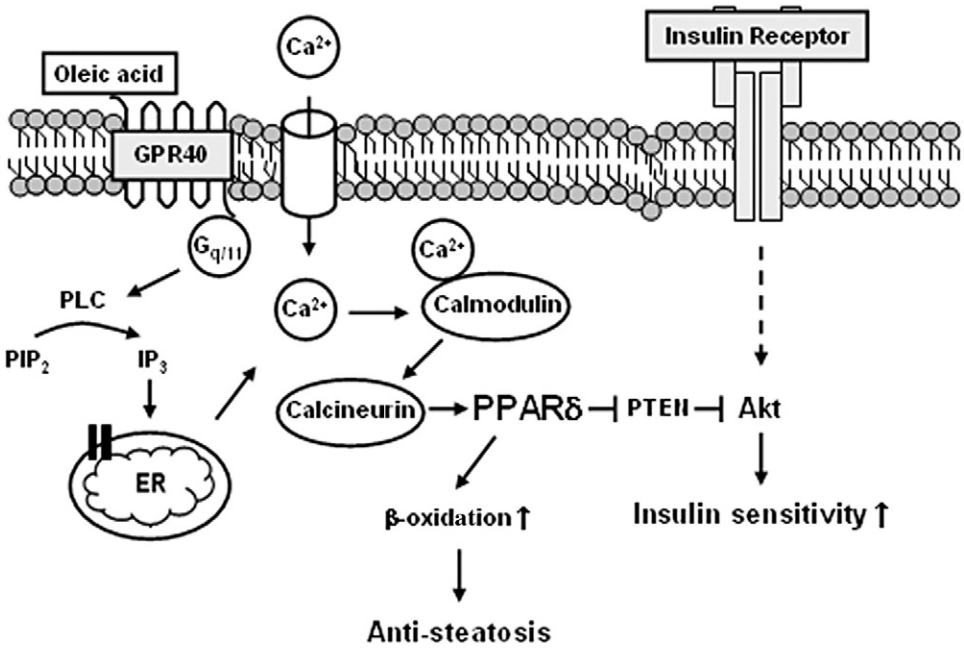

Fig. 5. Hypothetical scheme of the role of PPARδ in hepatic steatosis. In this study, we provide evidence that pharmacological activation of PPARδ not only improved hepatic steatosis but also regulated insulin sensitivity. Treatment of OA in HepG2 cells increased PPARδ via a GPR40-mediated and calcium-dependent mechanism. Moreover, elevated expression of PPARδ by OA negatively regulates PTEN and increased insulin sensitivity to facilitate glycogen synthesis.

the precise signalling pathways are unclear [26]. GPR40 is a G-protein-coupled receptor and highly expressed in brain and pancreatic islet [42] and a large range of both medium- to long-chain SFA and UFA are agonists for GPR40 [43]. In consistency with a previous study [26], GPR40 was not only minimally expressed in chicken hepatocytes but also expressed in HepG2 cells (Fig. 2A). OA significantly increased the expression of GPR40 (Fig. 2A), whereas GPR40 knockdown partially reversed the effect of OA on PPARδ expression (Fig. 2B and C), implying that the primary role of GPR40 may therefore be partially involved in the increased PPARδ expression by OA (Fig. 2C). Moreover, we found that OA increased PPARδ expression through a calcium-dependent pathway (Fig. 2D). Chelating of calcium by BAPTA-AM and blockade of calmodulin and calcineurin by KN93 or CsA significantly inhibited OA-induced PPARδ expression (Fig. 2E). In consistency with previous studies, intracellular Ca^{2+} levels and calcium-related kinases regulate the expression of PPARδ [44,45]. These results suggest that OA-induced PPARδ expression requires the influx of Ca^{2+} and stimulation of calcium-dependent kinases.

Overexpression of hepatic PPARδ improves hepatic steatosis in diabetic db/db mice [25]. In consistency with a previous study [25], we found that PPARδ deletion in HepG2 cells augmented OA-induced lipid accumulation (Fig. 3). Fatty acid composition is altered in patients with hepatitis C and steatosis, and the higher OA level is detected in hepatic total lipids [46]. However, the IR in patients with hepatitis C is predominantly correlated with muscle and is independent of the liver [47]. Therefore, we further investigated the mechanism of OA-improved insulin sensitivity in hepatic steatosis. Activation of PPARδ increased glucose utilization and insulin sensitivity in diabetic mice [25,48]. In consistency with previous studies [25,48], PPARδ deletion in HepG2 cells significantly developed IR in OA-induced steatosis (Fig. 4) and we further clarified the possible mechanism.

PTEN is a phosphoinositide phosphatase, which dephosphorylates the PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 second messengers on the 3′-position of the inositol ring. PTEN thus antagonizes PI3K activation and inhibits insulin activity [49]. It has been reported that OA down-regulates PTEN expression in HepG2 cells [50] and PPARδ regulates PTEN expression [51]. A specific PPARδ agonist, GW501516, induces the proliferation of non–small cell lung cancer via inhibition of PTEN expression through activation of PPARδ [51]. In the present study, we confirmed that PTEN expression is down-regulated by OA and also evaluated the role of PPARδ in the down-regulation of PTEN and insulin sensitivity (Fig. 4A). Because of the increased expression of PTEN by PPARδ deletion cells treated with OA, impaired insulin signaling was observed (Fig. 4B and C). In addition, the levels of glycogen content imply the level of insulin sensitivity. In parallel with the impaired insulin signaling induced by PPARδ deletion in cells treated with OA, the glycogen content was also decreased (Fig. 4D). Moreover, UFA decreases PTEN gene expression in hepatocytes through mechanisms unrelated to methylation of PTEN promoter, histone deacetylase activities or repression of the PTEN promoter activity. UFA up-regulates microRNA-21 to induce PTEN degradation, whereas SFA shows no effects on the levels of PTEN [50]. Thus, the interplay between PPARδ and miR-21 may exist, whereas this effect is still unknown. Although it was indicated that PPARδ activates AMP kinase to regulate blood glucose [41], our study provides a novel mechanism for the regulation of insulin sensitivity by PPARδ in the liver.

In conclusion, we provide evidence that OA increases the expression of PPARδ through a GPR40-PLC-calcium pathway, and the increase of PPARδ further regulates lipid metabolism and insulin sensitivity in steatotic status (Fig. 5).

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