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Role of Peroxisome Proliferator-activated Receptor δ/β in Hepatic Metabolic Regulation*[S]

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Pharmacological activation of peroxisome proliferator-activated receptor δ/β (PPARδ/β) improves glucose handling and insulin sensitivity. The target tissues of drug actions remain unclear. We demonstrate here that adenovirus-mediated liver-restricted PPARδ activation reduces fasting glucose levels in chow- and high fat-fed mice. This effect is accompanied by hepatic glycogen and lipid deposition as well as up-regulation of glucose utilization and *de novo* lipogenesis pathways. Promoter analyses indicate that PPARδ regulates hepatic metabolic programs through both direct and indirect transcriptional mechanisms partly mediated by its co-activator, PPARγ co-activator-1β. Assessment of the lipid composition reveals that PPARδ increases the production of monounsaturated fatty acids, which are PPAR activators, and reduces that of saturated FAs. Despite the increased lipid accumulation, adeno-PPARδ-infected livers exhibit less damage and show a reduction in JNK stress signaling, suggesting that PPARδ-regulated lipogenic program may protect against lipotoxicity. The altered substrate utilization by PPARδ also results in a secondary effect on AMP-activated protein kinase activation, which likely contributes to the glucose-lowering activity. Collectively, our data suggest that PPARδ controls hepatic energy substrate homeostasis by coordinated regulation of glucose and fatty acid metabolism, which provide a molecular basis for developing PPARδ agonists to manage hyperglycemia and insulin resistance.

The prevalence of metabolic diseases has increased substantially, partly because of rising obesity caused by sedentary lifestyles and energy surplus. Insulin resistance is at the core of these disorders. Excess energy substrates beyond the catabolic or storage capacity of the body are believed to cause organelle dysfunction (1). Elevated nonesterified free fatty acid has been shown to activate inflammatory response through JNK, which suppresses insulin signaling (2–4), whereas partitioning fatty acid substrates for catabolism or triglyceride synthesis prevents high fat diet-induced insulin resistance (5, 6). Conversely, *de novo* synthesis of beneficial MUFAs[5] alleviates cellular stress and protects against detrimental effects of saturated fatty acids (7). Therefore, a key step toward the development of drugs to treat metabolic diseases is to understand the mechanisms controlling energy substrate metabolism. In this regard, the liver is one of the most important tissues for energy homeostasis known for its role in sustaining energy availability through anabolic and catabolic pathways. Hepatic insulin resistance results in overproduction of glucose and VLDLs, worsening the extent of glucotoxicity and lipotoxicity (1). Metformin is one of the commonly prescribed anti-diabetic drugs that target hepatic glucose output (8). This drug increases the activity of AMPK, an energy sensor that is activated by elevated intracellular AMP or AMP/ATP ratio. In the liver, AMPK reduces glucose production by suppressing the expression of gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase (9). AMPK also mediates the beneficial effects of adiponectin on glucose and lipid metabolism through adiponectin receptors (10, 11).

Although not a major site for glucose deposition, the liver also plays a role in compartmentalizing glucose during feeding (12). Postprandial hyperglycemia triggers insulin secretion, which in turn suppresses gluconeogenesis and at the same time induces hepatic glucokinase (GK) expression (13–15). Glucose transported into the liver through glucose transporter 2 (GLUT2) is phosphorylated by GK to generate glucose-6-phosphate, which enters metabolic pathways for glycogen synthesis, glycolysis, and lipogenesis. Genetic manipulations that sustain GK protein levels in the liver have

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[5] The abbreviations used are: MUFA, monounsaturated fatty acid; PPAR, peroxisome proliferator-activated receptor; AMPK, AMP-activated protein kinase; GK, glucokinase; GLUT, glucose transporter; GTT, glucose tolerance test; ITT, insulin tolerance test; RER, respiratory exchange ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; qPCR, quantitative PCR; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; SCD, stearoyl-CoA desaturase; SREBP, sterol-responsive element-binding protein; PGC, PPARγ co-activator; MCAD, medium chain acyl-CoA dehydrogenase.

been shown to lower blood glucose and improve insulin sensitivity (16–18). This pathway appears to be an alternative approach to control hyperglycemia. However, it is unclear whether this process can be pharmacologically activated.

The three peroxisome proliferator-activated receptors, PPARα, δ/β and γ, belong to the nuclear receptor family. They are activated by dietary fats and are important metabolic regulators (19, 20). PPARα and PPARγ mediate the lipid-lowering and insulin-sensitizing effects of fenofibrates and thiazolidinediones, respectively (21, 22). PPARα reduces circulating triglycerides by up-regulation of fatty acid catabolism in the liver, whereas PPARγ increases insulin sensitivity, in part, through directing fatty acid flux into storage in adipocytes. PPARδ also shows promise as a drug target to treat metabolic diseases (23). The reported effects of PPARδ activation by systemic ligand administration or by transgenic approaches in animal models include correction of dyslipidemia and hyperglycemia, prevention of diet-induced obesity, enhancement of insulin sensitivity, and modulation of muscle fiber type switching (24–29). Most of the observed beneficial effects are believed to be mediated by increasing fatty acid catabolism and mitochondria function in muscle and adipocytes. It is proposed that in muscle AMPK activates PPARδ to increase oxidative metabolism and running endurance (30). We and others have recently shown that PPARδ also plays an important role in macrophage alternative activation, which exhibits anti-inflammatory properties and, as such, counteracts the inhibitory effect of inflammatory signaling on insulin sensitivity (31, 32).

A previous study demonstrated that administration of a synthetic PPARδ agonist, GW501516, lowered hyperglycemia in db/db mice by reducing hepatic glucose production and increasing glucose disposal (28). Expression profiling analyses suggested that fatty acid oxidation genes were up-regulated in muscle, whereas several lipogenic genes were induced in the liver. Although the function of PPARδ in muscle fat burning is well documented, whether alteration in hepatic gene expression observed in systemic drug treatment is a primary or secondary effect has not been addressed. In this study, we sought to determine whether PPARδ has a direct role in hepatic metabolic regulation. Our results demonstrated that PPARδ regulates energy substrate utilization and limits lipotoxicity in the liver.

EXPERIMENTAL PROCEDURES

Animal Experiments—C57BL/6 mice (14 age-matched, 8-week-old males from the Jackson Laboratory) were challenged with a high fat, high carbohydrate diet (F3282; Bio-Serv, Frenchtown, NJ) for 10 weeks. They were then transduced with purified adenovirus via tail vein injection ($n = 7$ for both GFP and PPARδ adenovirus). Adenoviral expression cassettes were constructed in the pShuttle-IRES-hrGFP vector and amplified in AD293 cells (Stratagene, La Jolla, CA). 100 μl of 5×10^{10} plaque-forming unit/ml virus was injected into each mouse. Liver-specific PPARδ$^{-/-}$ mice (in C57BL/6 background) was generated by crossing PPARδ f/f mice to albumin-Cre transgenic mice. The mice were fasted overnight for serum collection, tissue harvesting, and glucose tolerance test (GTT). Insulin tolerance test (ITT) was performed after a 6-h fast. A similar metabolic phenotype was observed in two additional cohorts ($n = 5$), which were used for metabolic cage studies and to determine adenine nucleotide concentrations. The experiment was repeated in 3-month-old chow-fed mice to evaluate gene expression at the fed state ($n = 4$/group). Statistics analyses were performed using Student's t test (two-tailed), unless otherwise indicated. The values were presented as the means ± S.E. Significance was established at $p < 0.05$. The animal studies were approved by the Harvard Medical Area Standing Committee on Animals.

Metabolic Studies—Metabolic cage studies were conducted in a Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). The mice were placed in metabolic cages for 2 days, and the data were collected at the beginning of the second dark (active) cycle for 24 h. The respiratory exchange ratio was determined by the ratio of CO_2 produced (VCO_2) over O_2 consumed (VO_2). The values of respiratory exchange ratio (RER) during the dark (active) and light (rest) cycles were averaged (supplemental Fig. S2). Because the mice were on high fat diet, RER was close to 0.7 throughout the day (RER = 0.7 for fatty acid usage; RER = 1 for glucose usage). For GTT, 1.5 mg of glucose/g of body weight was injected into the peritoneum. Blood glucose was measured before and after injection at the indicated time points using the OneTouch glucose monitoring system (Lifescan, Milpitas, CA). ITT was conducted similarly (0.5 units of insulin/kg of body weight). To determine triglyceride production, the mice were injected with Triton WR1339 (500 μg/g of body weight), and blood was drawn via tail bleeding at different time points for triglyceride concentration measurement. Serum and hepatic triglyceride, nonesterified fatty acid, total cholesterol, as well as serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using commercial kits (Wako Chemicals and ThermoDMA). Hepatic glycogen was determined as described (33). Insulin and adiponectin were measured using ELISA kits (Linco, St. Charles, MO). Adenine nucleotides (ATP, ADP, and AMP) were determined in perchloric acid extracts of freeze clamped tissues and normalized by protein concentration as described previously (34). Hepatic fatty acid/triglyceride composition was determined by gas-liquid chromatography as described (35).

Histology, Gene Expression, and Signaling Analysis—Liver samples were either cryo-preserved for GFP detection or fixed in formalin for hematoxylin and eosin or periodic acid-Schiff staining. All of the histology work was performed in the Dana Farber Research Pathology Cores, which provided preliminary histological assessment by a pathologist. SYBR green-based real time quantitative PCR (qPCR) was conducted as described (28), using 36B4 levels as loading controls to obtain relative expression levels. For Western blot analyses, tissue or cell lysates were prepared in a buffer containing protease and phosphatase inhibitors. Antibodies against AMPK, Akt, Erk, and JNK were purchased from Cell Signaling, and PPARδ and actin antibodies were from Santa Cruz. For reporter assays, the 2- and 0.3-kb mouse GK (liver-specific) as well as the 3-kb mouse fatty acid synthase (FAS) promoter fragment were

cloned in the pGL3-basic vector (Promega). Human acetyl-CoA carboxylase 2 (ACC2) promoters I and II (all in pGL3-basic) were as described previously (28). The resulting reporter was co-transfected with expression vectors for PPARδ/RXRα, sterol-responsive element-binding protein 1c (SREBP-1c), PPARγ co-activator-1α (PGC-1α), and PGC-1β, all under the control of a CMV promoter, together with a β-galactosidase internal control in HepG2 cells. The cells were harvested 40–48 h after transfection, and GW501516 (0.1 μM) was treated for 24 h. For endogenous gene regulation by PPARδ, primary hepatocytes were cultured in Williams' E medium with 5% lipoprotein-deficient, dialyzed FBS supplemented with 100 nM insulin and treated with 0.1 μM GW501516 for 6 h.

In Vitro Functional Assays—Primary hepatocytes were isolated from 2–3-month-old male C57BL/6 mice through portal vein perfusion with Blendzyme 3 (Roche Applied Science) and cultured in Williams' E medium with 5% regular FBS. Hepatocytes were infected with GFP or PPARδ virus for 24 h. The cells were washed and incubated with DMEM (low glucose) for 2 h. To measure glucose flux to glycogen synthesis, lipogenesis, and oxidation, hepatocytes transduced with GFP or PPARδ virus were labeled with 1 μCi/ml D-[^{14}C (U)]glucose overnight with or without 100 nM insulin. Medium was collected, and the cells were lysed. For measuring glucose oxidation to CO_2, the medium was transferred to a 15-ml conical tube, and 100 μl of 70% perchloric acid was added. Filter paper presoaked in 1 M NaOH was then placed on the top of the tube to capture CO_2. The samples were incubated at 37 °C overnight, and the filters were placed in scintillation vials to count radioactivity. Fatty acid oxidation was conducted by loading cells with [^{3}H]palmitate (albumin bound). The rate of β-oxidation was determined by measuring ^3H_2O produced in the supernatant. For glycogen synthesis from labeled glucose, cellular glycogen was isolated, and the radioactivity was determined. Glucose conversion to extractable lipids (fatty acid/triglyceride) was measured as described (28). For glucose production, hepatocytes were incubated for 2 h in glucose-free DMEM, containing 1 mM pyruvate and 10 mM lactate. Compound C (Calbiochem) and metformin (Sigma), an inhibitor and an activator of AMPK, respectively, were added at final concentrations of 20 μM and 2 mM, respectively. The glucose content in the supernatant was measured using a glucose oxidase kit (Trinity Biotech). All of the values were normalized by protein contents. Statistical analysis for glucose production was performed using one-way analysis of variance.

RESULTS

Liver-restricted PPARδ Expression Improves Glucose Homeostasis—To assess potential roles of hepatic PPARδ in the regulation of glucose homeostasis, we utilized adenovirus mediated gene delivery to increase PPARδ expression/activity in the liver. Previous studies have demonstrated that the overexpressed PPARδ is active *in vivo* (36). A cohort of wild type C57BL/6 male mice were fed a high fat diet for 10 weeks to induce insulin resistance, followed by injection with adenoviral GFP (control) or PPARδ (adPPARδ) through the tail vein. Adenovirus delivered through tail vein is known to concentrate in the liver, which is used commonly to achieve liver-restricted expression. Examination of liver sections showed that ~70% of hepatocytes were infected as determined by GFP expression, resulting in a 4–5-fold increase in the PPARδ protein level (supplemental Fig. S1*A*). A series of metabolic studies were conducted within a week following the injection. These mice were first placed in metabolic cages, and the RER was examined to determine whether increased hepatic PPARδ altered fuel substrate usage. We found a moderate but significant increase in the RER at the resting period in adPPARδ mice (Fig. 1*A* and supplemental Fig. S2), indicating that PPARδ may increase glucose utilization in the liver. In line with this, adPPARδ mice had a lower fasting glucose level compared with control animals at the basal state (GFP, 131 ± 7.13; PPARδ, 109.5 ± 3.15, $p < 0.05$) and throughout the course of GTT (Fig. 1*B*). Insulin levels measured during GTT showed no significant difference between the two groups (data not shown). Insulin tolerance tests demonstrated that adPPARδ mice had improved insulin sensitivity, supporting the notion that hepatic PPARδ overexpression enhances glucose handling (Fig. 1*C*).

To determine how hepatic PPARδ regulates glucose metabolism, liver samples were collected for histological and gene expression studies. Interestingly, hematoxylin and eosin staining of liver sections revealed signs of glycogen and lipid deposition in adenoviral PPARδ-infected livers (Fig. 1*D*). Glycogen and lipids accumulation were further determined by periodic acid-Schiff staining and oil red O staining. After an overnight fast, the livers of control mice contained minimal glycogen. In contrast, adenoviral PPARδ-infected livers showed a substantial increase in glycogen-positive staining (Fig. 1*D*). Similarly, adPPARδ-infected livers had elevated neutral lipid stains (Fig. 1*D*). Quantitative analyses demonstrated increased glycogen and triglyceride content in livers of adPPARδ mice, whereas fatty acid and cholesterol concentrations remained similar (Fig. 1*D*). We did not observe significant differences in white adipose tissue histology, body weight, the ratio of liver or white adipose tissue weight to body weight and levels of fasting free fatty acid, triglyceride, and cholesterol between the two groups, indicating that the effects of hepatic PPARδ activation on glucose homeostasis were not secondary to changes in other metabolic parameters (supplemental Table S1). Gene expression analysis determined by real time qPCR demonstrated that genes involved in glucose uptake and utilization, such as GLUT2, GK, and pyruvate kinase, were increased in the livers of adPPARδ mice compared with control animals (Fig. 1*E*). Lipogenic genes, including FAS, ACC1, ACC2, and stearoyl-CoA desaturase 1 (SCD1), were up-regulated, and most have been shown to be induced by systemic ligand treatment in livers of db/db mice (28). SREBP-1c and PGC-1β, which has been shown to regulate FAS through co-activation of SREBP-1c, were also induced (37). In contrast, gluconeogenic genes, including phosphoenolpyruvate carboxykinase and HNF4α, were suppressed in PPARδ virus-infected livers (Fig. 1*E*). Levels of PPARα and its targets genes, acyl-CoA oxidase, carnitine palmitoyl-coA transferase 1, and medium chain acyl-CoA dehydrogenase (MCAD) were unaffected, implicating that PPARδ overexpression did not cause nonspe-

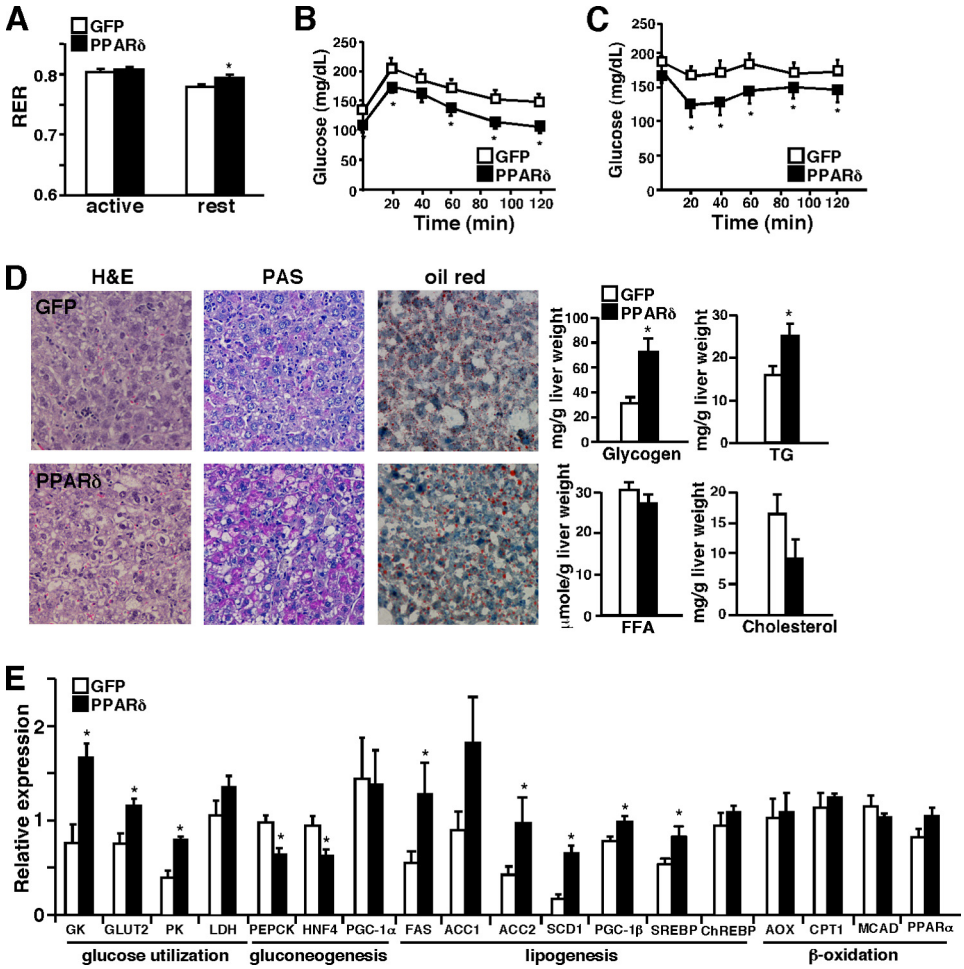

FIGURE 1. **Liver-restricted PPARδ expression improves glucose homeostasis in mice fed a high fat diet.** *A*, adenovirus mediated hepatic PPARδ expression increases the respiratory exchange ratio at the resting state. High fat-fed C57BL/6 male mice were injected with adenoviral GFP or PPARδ through the tail vein. Three days after viral injection, the mice ($n = 5$) were placed in metabolic cages to determine the RER. The mice were 20 weeks old and had been on a high fat diet for 10 weeks. *active*, average RER during the dark cycle; *rest*, average RER during the light cycle. *B* and *C*, GTT (*B*) and ITT (*C*) showing improved glucose handling and insulin sensitivity in adenoviral PPARδ-infected mice compared with control animals ($n = 7$). GTT (overnight fasted) and ITT (6 h fasted) were performed 4 and 5 days after virus injection, respectively. GFP and PPARδ indicate mice receiving adenoviral GFP and PPARδ, respectively. *D*, histological analyses of liver sections (200×) from GFP and PPARδ adenovirus-injected mice. Liver samples were collected 7 days following virus injection after an overnight fast. Hematoxylin and eosin (*H&E*) staining was conducted for morphological assessment, and periodic acid-Schiff (*PAS*) staining (counterstained with hematoxylin) was performed to identify glycogen, which stained *purple*. Hepatic glycogen and lipid contents were quantified by enzymatic assays. *TG*, triglyceride; *FFA*, free fatty acid. *E*, PPARδ regulates the expression of genes in glucose and lipid metabolism. Liver samples were harvested from control (GFP) or adPPARδ (PPARδ) mice after an overnight fast, and gene expression was determined by real time qPCR. *LDH*, lactate dehydrogenase; *ChREBP*, carbohydrate response element-binding protein; *AOX*, acyl-CoA oxidase; *CPT1*, carnitine palmitoyl-coA transferase 1; *PEPCK*, phosphoenolpyruvate carboxykinase. *, $p < 0.05$.

cific, cross-regulation of PPARα pathways. In addition, the expression of PPARδ and its target genes was unchanged in other tissues such as muscle and white adipose tissue (supplemental Fig. S1*B*). These data suggest that increased hepatic PPARδ activity lowers glucose levels in high fat-fed mice and implicate a role for PPARδ in hepatic metabolic regulation.

PPARδ Regulates Hepatic Glucose Utilization—The liver utilizes excess glucose for glycogen and lipid synthesis during feeding. To further probe the function of PPARδ activation in the liver at the fed state without the effects contributed by the high fat diet, adenovirus mediated PPARδ expression was conducted in a cohort of 3-month-old, lean C57BL/6 mice, and liver samples were collected under *ad libitum* feeding conditions. Histological and quantitative studies demonstrated that increased glycogen and triglyceride contents were also evident in livers of chow-fed adPPARδ mice (Fig. 2*A*). In concert, we found elevated protein levels of glycogen synthase and ACC (Fig. 2*B* and supplemental Fig. S3*A* for quantification). Under *ad libitum* feeding, only pyruvate kinase, ACC1, and SCD1 were significantly induced in adPPARδ livers (Fig. 2*C*), which was not unexpected, because genes such as GK and phosphoenolpyruvate carboxykinase are also regulated by insulin at the fed state. Hepatic PPARδ expression also reduced fasting glucose levels in these animals (GFP, 117 ± 3.34; PPARδ, 91.75 ± 9.39, $p < 0.05$, supplemental Table S1). However, chow-fed control and adPPARδ mice performed similarly in GTT and ITT, and there was no statistical difference in feeding glucose or triglyceride concentrations (data not shown). To determine whether the modulation of hepatic glucose metabolism is cell autonomous, we performed metabolic tracer studies in isolated primary hepatocytes. GFP or PPARδ virus-infected hepatocytes were labeled with

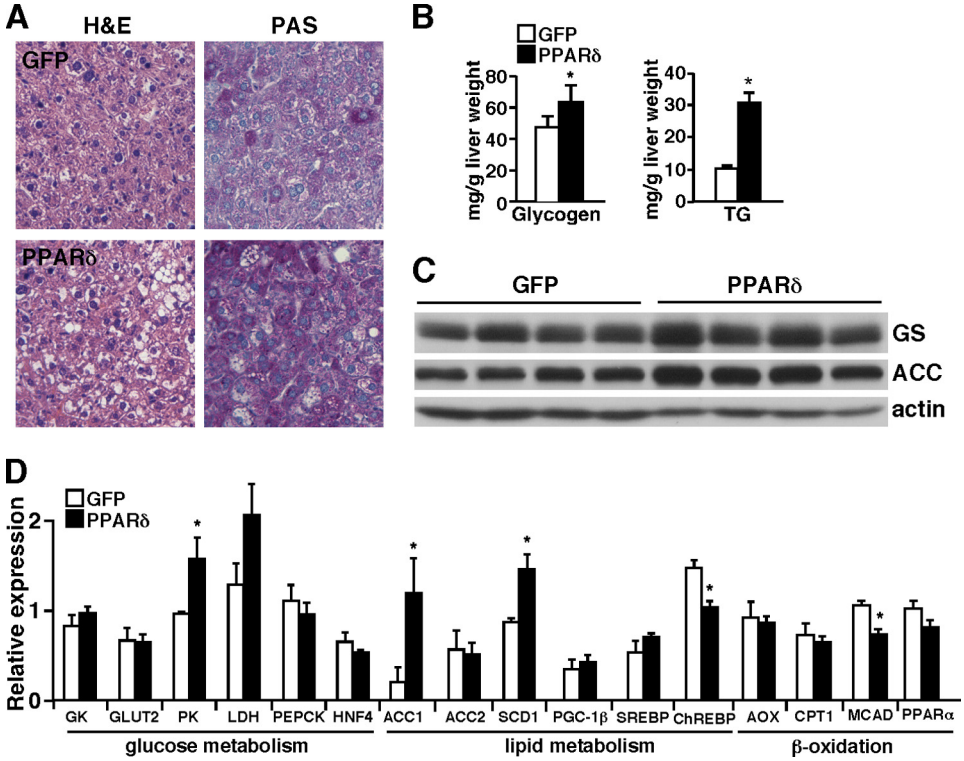

FIGURE 2. **Assessment of the effect of hepatic PPARδ expression on glycogen synthesis and lipogenesis in chow-fed mice.** *A* and *B*, histological, glycogen, and lipid analyses of liver samples from GFP and PPARδ adenovirus-injected mice on a chow diet. Liver samples were collected from *ad libitum* fed animals 2 weeks following virus injection. Hematoxylin and eosin (*H&E*) and periodic acid-Schiff (*PAS*) (counterstained with hematoxylin) staining (*A*) as well as enzymatic assays (*B*) were conducted to determine glycogen and triglyceride content. *C*, levels of liver glycogen synthase (*GS*) and ACC determined by Western blotting. The samples were collected from four individual animals from GFP and PPARδ adenovirus-injected mice. Actin was included as the loading control. *D*, hepatic gene expression determined by real time qPCR. Liver samples were harvested from control (GFP) or adPPARδ (PPARδ) mice under *ad libitum* feeding condition. *, $p < 0.05$.

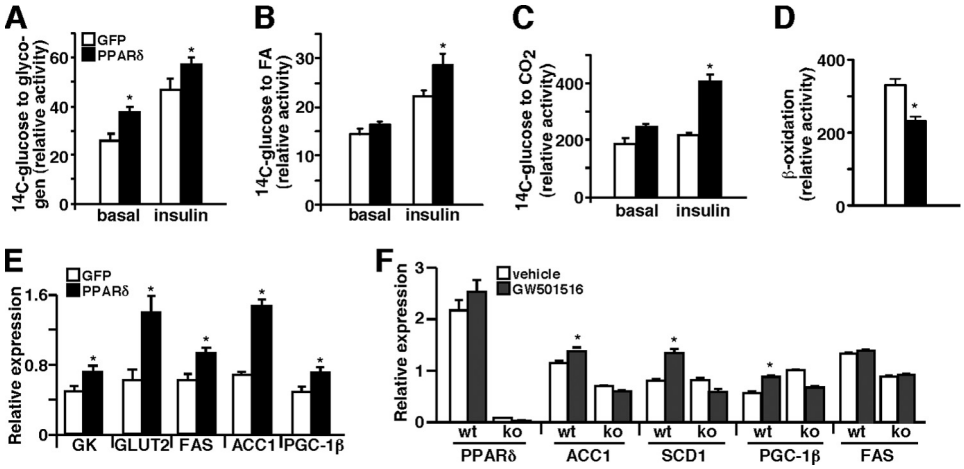

FIGURE 3. **PPARδ increases glucose utilization in primary hepatocytes.** *A–C*, PPARδ increases glucose flux to glycogen synthesis, lipogenesis, and glycolysis determined by radioactive tracers. Hepatocytes infected with GFP or PPARδ virus were labeled with [^{14}C]glucose without or with 100 nM insulin. The conversion of radioactive glucose to glycogen, fatty acid, and CO_2 (to estimate glycolysis) was determined and normalized to protein content. *D*, fatty acid β-oxidation assay determined by [^{3}H]palmitate. *E*, the expression of glucokinase (GK), GLUT2, and lipogenic genes is up-regulated in hepatocytes infected with adenoviral PPARδ. Gene expression was determined by real time qPCR 48 h post-infection. *F*, assessment of target gene regulation by endogenous PPARδ. Primary hepatocytes from wild type (*wt*) and liver-specific PPARδ$^{-/-}$ (*ko*) mice were given 0.1 μM GW501516 for 6 h, and gene expression was examined by real time qPCR. *, $p < 0.05$.

[^{14}C]glucose to trace glucose utilization for glycogen synthesis and oxidation as well as lipogenesis without or with insulin stimulation. Insulin-stimulated [^{14}C]glucose incorporation into glycogen (Fig. 3*A*) and fatty acids (Fig. 3*B*) were increased in adenoviral PPARδ-infected hepatocytes. In addition, insulin-stimulated glucose oxidation determined by $^{14}CO_2$ production was also enhanced in these cells (Fig. 3*C*), whereas basal fatty acid β-oxidation was reduced (Fig. 3*D*). The increased glucose oxidation and decreased fatty acid catabolism are consistent with the RER result (Fig. 1*A*). PPARδ overexpression in hepatocytes increased the expression of GK, GLUT2, FAS, ACC1, and PGC-1β (Fig. 3*E*). To validate gene

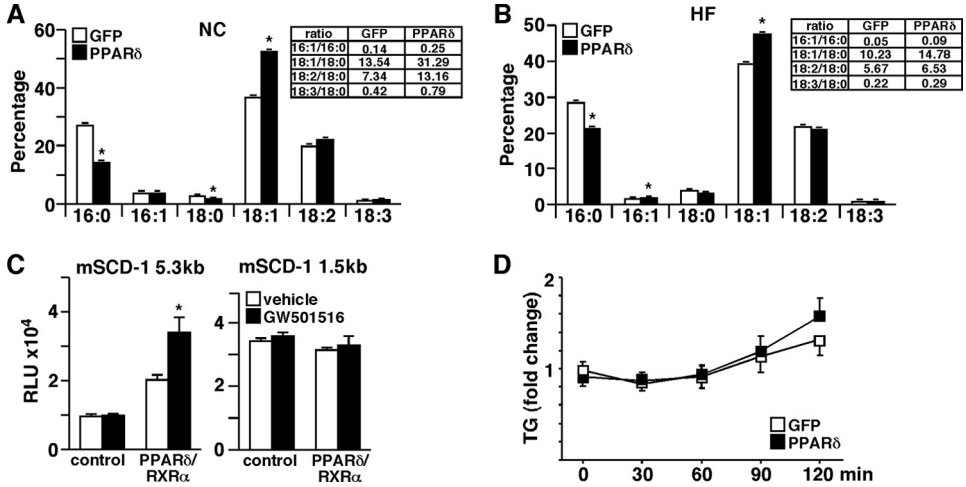

FIGURE 4. **Increased monounsaturated to saturated fatty acid ratios in adPPARδ livers.** *A* and *B*, triglycerides were isolated from GFP or PPARδ adenovirus-infected livers of normal chow-fed (*NC*) or high fat diet-fed (*HF*) mice. Fatty acid compositions in triglycerides were determined by gas-liquid chromatography. The ratios of monounsaturated to saturated fatty acids were shown in the tables. *C*, PPARδ regulates SCD1 promoter. Luciferase reporters driven by 5.3- or 1.5-kb mouse SCD1 promoter were co-transfected with expression vectors for PPARδ/RXRα into HepG2 cells, together with a β-galactosidase reporter internal control ± GW501516 (0.1 μM, PPARδ agonist) for 24 h. The reporter luciferase activity was normalized to β-galactosidase activity to obtain relative luciferase unit (*RLU*). *D*, triglyceride (*TG*) production determined by administration of a lipoprotein lipase inhibitor, Triton WR1339. Serum triglyceride concentrations were measured at the indicated time course after Triton injection. *, $p < 0.05$.

regulation by endogenous PPARδ and determine immediate targets, we treated primary hepatocytes from wild type or PPARδ$^{-/-}$ livers with a PPARδ ligand, GW501516, for 6 h and found that ACC1, SCD1, and PGC-1β were up-regulated in a PPARδ-dependent manner, whereas GK, GLUT2, and FAS were unchanged (Fig. 3*F* and data not shown). These data suggest that PPARδ overexpression is sufficient to drive target gene expression, likely because of the presence of endogenous ligands. In addition, PPARδ activation enhances hepatic glucose utilization through direct and indirect transcriptional regulation.

PPARδ Increases Monounsaturated Fatty Acid Pools—Fatty acids have been shown to serve as signaling molecules, which could exert beneficial (*e.g.* lipokines) or detrimental (*e.g.* lipotoxicity) metabolic outcomes (1, 7). To examine the effect of PPARδ-regulated lipogenic program on lipid compositions, hepatic fatty acids/triglycerides were analyzed. adPPARδ livers contained less saturated fatty acids, notably C16:0, in both normal chow and high fat-fed cohorts (Fig. 4, *A* and *B*). In contrast, the concentration of C18:1 (oleic acid) was increased. In addition, the ratios of MUFAs to saturated fatty acids were increased in livers expressing PPARδ. Previous work has demonstrated that C18 MUFAs are strong activators of PPARδ (38). Indeed, lipid extracts from adPPARδ livers exerted a stronger PPARδ-activating activity than control lipids (supplemental Fig. S4*A*). SCD1 catalyzes the conversion of saturated fatty acids to unsaturated fatty acids. We found that the activity of a 5.3-kb mouse SCD1 promoter could be induced by PPARδ activation, and this effect was lost in the proximal 1.5-kb promoter region (Fig. 4*C*). This result was consistent with the up-regulation of SCD1 in adPPARδ livers. To determine whether the enhanced lipogenesis led to an increase in VLDL production, circulating triglyceride concentrations were determined after administration of a lipoprotein lipase inhibitor, Triton WR1339, in control and adPPARδ mice. There was no difference in the rate of TG release by the liver between the two groups (Fig. 4*D*), indicating that PPARδ does not affect the steady state VLDL triglyceride production.

Transcriptional Regulation of Hepatic Gene Expression by PPARδ—PGC-1β has been shown to be induced by fatty acids and regulate certain lipogenic genes by serving as a co-activator for SREBP-1c (37). Up-regulation of PGC-1β in adPPARδ livers is expected to increase lipid synthesis. To investigate the molecular mechanism through which PPARδ regulates hepatic gene expression and the potential involvement of PGC-1β in this process, reporters driven by promoters of potential target genes were constructed, and their activities were examined in HepG2 cells by transient transfection assays. The activities of both 2- and 0.3-kb mouse GK promoters could be induced by PPARδ and RXRα co-transfection, which were further enhanced by PGC-1β (Fig. 5, *A* and *B*). Ligand activation had additional effects only in the presence of PGC-1β. PPARδ/RXRα up-regulated human ACC2 promoter I in a ligand-dependent manner, as described previously (28) (Fig. 5*E*). Similarly, this ligand activity was substantially amplified by PGC-1β co-activation. In contrast, PPARδ had no effect on 1.3-kb human ACC2 promoter II and 3-kb mouse FAS promoter, both of which are known SREBP-1c targets (39). PGC-1β was able to increase SREBP-1c activities on these gene promoters (Fig. 5, *C* and *D*). PGC-1α has also been shown to co-activate PPARδ, particularly in muscle. Unexpectedly, PGC-1α co-transfection reduced PPARδ effects on ACC2 promoter I (Fig. 5*E*, *left panel*). In contrast, it strongly potentiated PPARα activation of MCAD promoter (Fig. 5*E*, *right panel*). The preferential functional interaction of PPARδ/PGC-1β and PPARα/PGC-1α could also be observed using a reporter containing three copies of acyl-CoA oxidase PPRE (supplemental Fig. S4*B*). Collectively, these data suggest that PGC-1β is a co-activator of PPARδ in the liver and support the notion that PPARδ regulates hepatic gene expression through direct and indirect mechanisms.

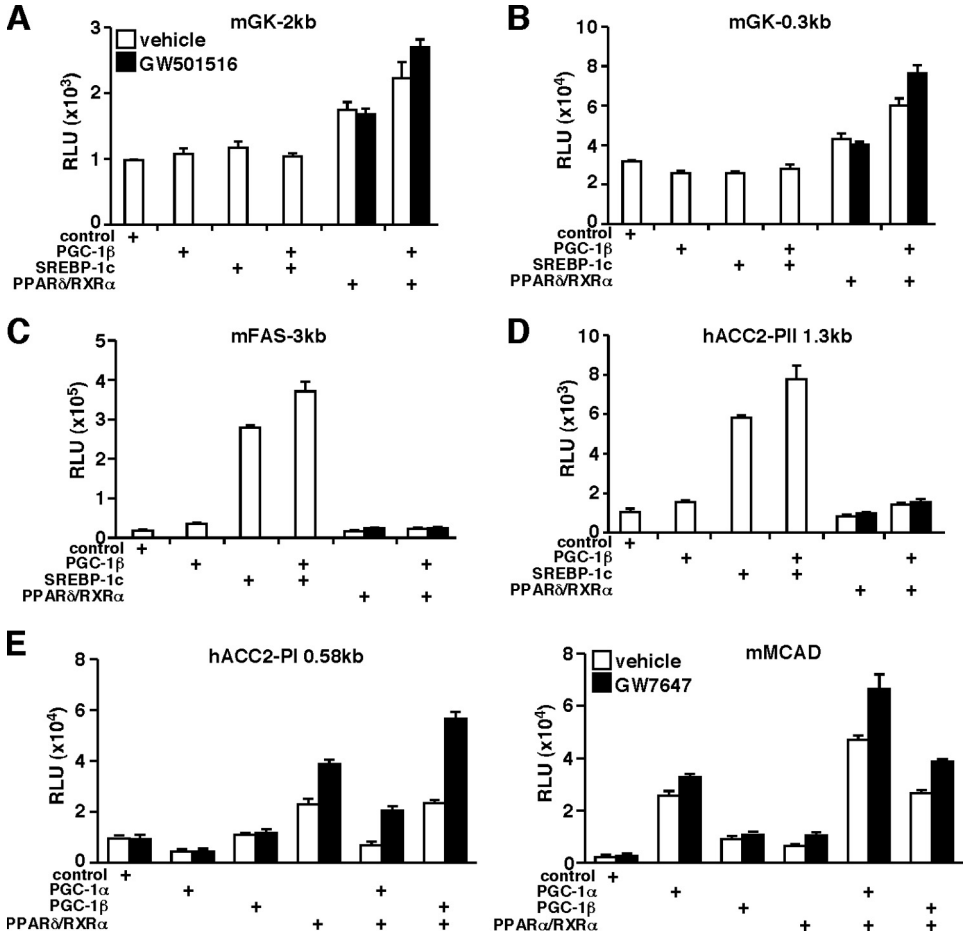

FIGURE 5. **Direct and indirect transcriptional mechanisms by PPARδ in the control of hepatic gene expression.** *A–E*, promoter analyses to determine PPARδ direct target genes. Promoter regions of potential target genes were cloned into a luciferase reporter. The resulting constructs were co-transfected with combinations of expression vectors for PPARδ/RXRα, SREBP-1c, PGC-1β, and PGC-1α (for *E* only) into HepG2 cells, together with a β-galactosidase reporter internal control. PPARδ/RXRα-transfected cells were cultured in the presence or absence of GW501516 (0.1 μM, PPARδ agonist) for 24 h. The reporter luciferase activity was normalized to β-galactosidase activity to obtain relative luciferase unit (*RLU*). *mGK-2kb*, mouse GK 2 kb promoter; *hACC2-PI* and *-PII*, human ACC2 promoter I and II; *mFAS*, mouse FAS promoter; *mMCAD*, mouse MCAD promoter.

adPPARδ Mice Are Protected from Lipotoxicity—The induced lipogenic program in adPPARδ mice raised the concern regarding whether increased lipid deposition caused hepatic pathology. Liver damage was assessed by serum levels of liver ALT and AST, which leak out to the circulation with liver injury. Intriguingly, both ALT and AST were reduced in adPPARδ mice (Fig. 6*A*). Consistent with this finding, the activity of the stress signaling JNK, determined by the level of phospho-JNK, was reduced in adPPARδ livers, whereas that of phospho-Erk, another member of the mitogen-activated protein kinase, was not affected (Fig. 6*B* and supplemental Fig. S3*B* for quantification). These results indicate that PPARδ may reduce lipotoxicity, thereby improving metabolic homeostasis. In fact, when treated with albumin-bound palmitic acid (C16:0), PPARδ adenovirus-infected hepatocytes had lower JNK phosphorylation and higher insulin-stimulated Akt phosphorylation, compared with control cells (Fig. 6*C*). There was an increase in triglyceride accumulation in adPPARδ hepatocytes (Fig. 6*C*). Free fatty acids have also been shown to induce chronic inflammation (2). We therefore examined the expression of genes in inflammatory response and found that pro-inflammatory cytokines/chemokines, including IL-1β, TNFα, IFNγ, and monocyte chemoattractant protein-1 were all down-regulated in adPPARδ livers, compared with GFP-infected livers from chow-fed mice (Fig. 6*D*). The expression of F4/80, a pan-macrophage marker, was also reduced. In contrast, markers for anti-inflammatory, alternative macrophage activation (40), such as Mgl1 and MRC1, were up-regulated in adPPARδ livers. The difference in inflammatory gene expression was less evident in the high fat-fed cohort, although there was a trend toward a reduction in TNFα ($p = 0.08$) and IFNγ in adPPARδ livers. These results indicate that PPARδ-controlled lipogenic program may protect the liver against lipotoxicity.

PPARδ Activates AMPK in the Liver—As mentioned earlier, AMPK plays a major role in reducing glucose production and has been linked to PPARδ activity (30). Expression analyses showed that PPARδ suppressed genes encoding gluconeogenesis (Fig. 1*E*). We sought to determine whether the activity of PPARδ in increasing glycogen storage (which decreases energy substrate availability) and lipogenesis (which consumes energy) might alter the energetic status, thereby exerting a secondary effect on AMPK activation. Western blot analyses demonstrated that levels of phospho-AMPK, which are indic-

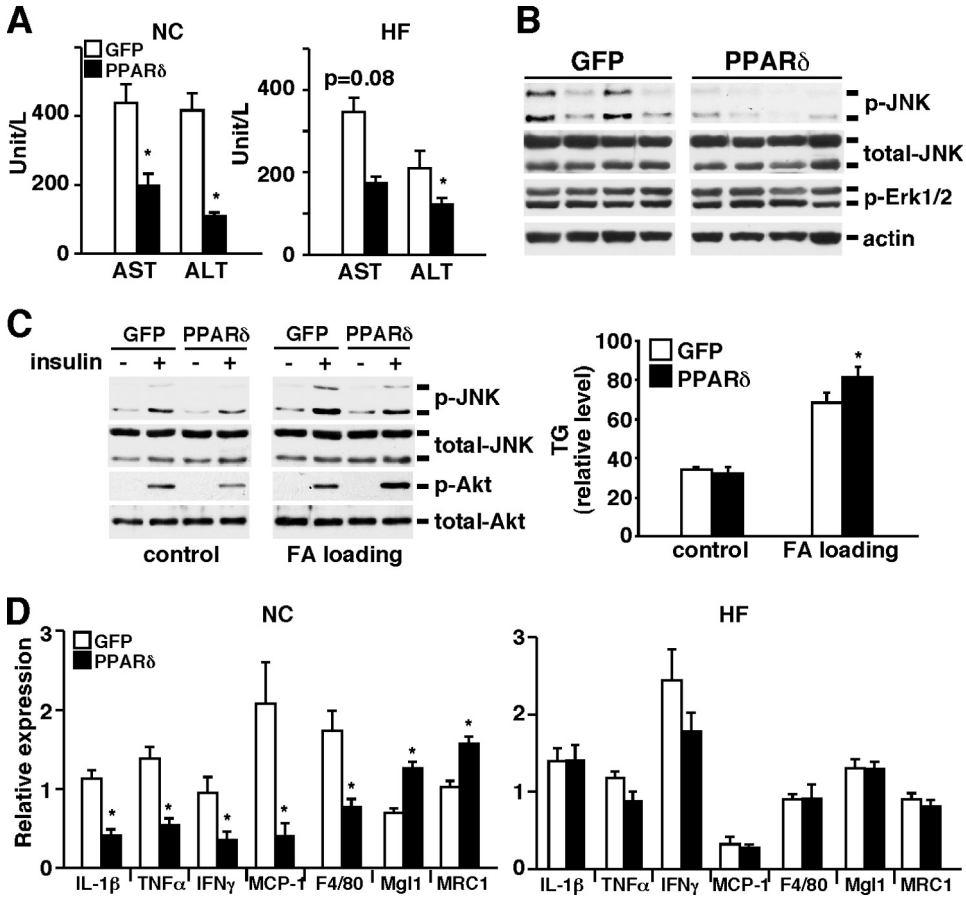

FIGURE 6. **Reduced stress signaling and inflammatory gene expression in the liver of adPPARδ mice.** *A*, assessment of liver damage in GFP or PPARδ adenovirus-infected mice on normal chow (*NC*) or high fat diet (*HF*) diets by serum AST and ALT activities. *B*, Western blot analyses demonstrating decreased JNK activity in livers of adPPARδ mice. Liver lysates were harvested from four individual mice/group of the high fat-fed cohort. *p-JNK*, phospho-JNK; *t-JNK*, total JNK; *p-Erk1/2*, phospho-Erk1/2. *C*, PPARδ inhibits phospho-JNK and increases insulin-stimulated phospho-Akt in primary hepatocytes. Hepatocytes were infected with GFP or PPARδ virus for 24 h in William's E, 5% FBS. The cells were washed and maintained in the same medium ± 100 μM palmitate (albumin-bound) overnight. Hepatocytes were serum-starved for 2 h, followed by insulin stimulation (100 nM) for 30 min. *Left panel*, JNK and Akt signaling was determined by Western blotting in GFP or PPARδ adenovirus-infected hepatocytes without (control) or with fatty acid treatment (*FA loading*). *Right panel*, normalized cellular triglyceride content. *D*, PPARδ suppresses the expression of pro-inflammatory genes. Liver samples were harvested from control (GFP) or adPPARδ (PPARδ) mice on normal chow (*NC*) or high fat (*HF*) diets, and gene expression was determined by real time qPCR. *, $p < 0.05$.

ative of AMPK activity, were higher in liver lysates of adPPARδ mice (Fig. 7*A* and supplemental Fig. S3*C* for quantification). It is known that AMPK can be activated by raising AMP coupled with falling ATP or by adiponectin signaling. To determine whether the increased AMPK activation was accompanied by changes in AMP and/or ATP levels, liver adenine nucleotide concentrations were measured by HPLC (Fig. 7*B*). Consistent with the increase in AMPK activity, levels of ATP were decreased ($p < 0.05$), and AMP were increased ($p = 0.08$) in livers of adPPARδ mice compared with those of control animals. ADP and total adenine nucleotide remained unchanged. Interestingly, we also found that adPPARδ livers expressed higher levels of adiponectin receptor 2, which activates AMPK through the adiponectin signaling pathway (10, 11) (Fig. 7*C*). We did not detect any difference in circulating adiponectin concentrations (Fig. 7*D*), suggesting that PPARδ may increase the response to adiponectin through up-regulation of adiponectin receptor 2 in the liver. To further demonstrate that the increased AMPK activity was mediated by hepatic PPARδ expression, AMPK phosphorylation was examined in primary hepatocytes infected with GFP or PPARδ adenovirus. The level of phospho-AMPK was higher in adenoviral PPARδ-infected hepatocytes (Fig. 7*E*). Furthermore, metformin-induced AMPK activation was further enhanced in these cells, compared with GFP-infected hepatocytes (Fig. 7*F*). To probe whether PPARδ-mediated AMPK activation modulates glucose metabolism, glucose production was assessed in isolated hepatocytes. The basal glucose production rate was lower in adenoviral PPARδ-infected hepatocytes compared with GFP-infected cells (Fig. 7*G*). A similar suppressive effect of adPPARδ was observed in glucagon-stimulated gluconeogenesis (supplemental Fig. S1*D*). The ability of adPPARδ to inhibit basal glucose production was abolished by the addition of compound C, an AMPK inhibitor (Fig. 7*G*), supporting the hypothesis that PPARδ could indirectly activate AMPK through limiting substrate availability, which contributes to the glucose lowering effect of PPARδ.

DISCUSSION

PPARδ is known for its role in regulating oxidative metabolism, particularly in muscle (27, 30). Previous studies have demonstrated that pharmacological activation of PPARδ low-

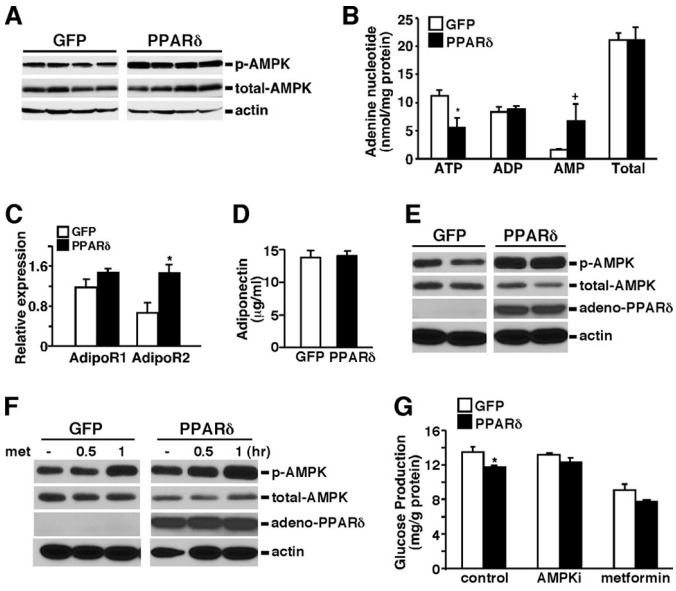

FIGURE 7. **Increased hepatic AMPK activity in adPPARδ mice.** *A*, Western blot analyses showing increased phospho-AMPK (p-AMPK, Thr-172) in adPPARδ livers. Liver lysates were collected from four individual GFP or adPPARδ mice. *B*, adenine nucleotide concentrations of liver lysates from control or adPPARδ mice ($n = 4$) determined by HPLC assays. *, $p < 0.05$; +, $p = 0.08$. *C*, real time qPCR analyses demonstrating up-regulation of adiponectin receptor 2 (*AdipoR2*) in adPPARδ livers. The difference in adiponectin receptor 1 (*AdipoR1*) expression was not significant. *D*, circulating adiponectin concentrations in control (GFP) and adPPARδ mice determined by ELISA. *E* and *F*, PPARδ expression increases AMPK phosphorylation. Hepatocytes were infected with GFP or PPARδ virus for 24 h in William's E, 5% FBS. The cells were washed and cultured in DMEM for 2 h. In *E*, hepatocytes were incubated in DMEM for 4 more hours before harvesting. The results from two representative samples were shown. In *F*, hepatocytes were treated with metformin (*met*, 2 mM) and harvested at different time points. The basal phospho-AMPK was higher at 6 h (*E*) than at 3 h (*F*, minus metformin) after replacing medium to DMEM in PPARδ-expressing hepatocytes. *G*, PPARδ reduces glucose production through AMPK activation in primary hepatocytes. Hepatocytes were treated as described above. The cells were then cultured in glucose free DMEM containing 1 mM pyruvate and 10 mM lactate, without or with 20 μM compound C (AMPK inhibitor, *AMPKi*) or 2 mM metformin (AMPK activator) for 2 h. Supernatant was collected to determine glucose concentration. Metformin was included as a control for AMPK-mediated suppression of glucose production. *, $p < 0.05$.

ered the glucose level and reduced hepatic glucose production (25, 28). However, whether the liver is a major site of PPARδ action has not been explored. In this study, we employ adenovirus mediated gene delivery to target PPARδ to the liver and show that PPARδ regulates glucose utilization for glycogen synthesis and lipogenesis, resulting in a secondary effect of AMPK activation. The combined actions effectively lower glucose levels in both chow- and high fat-fed mice. The lipogenic activity of PPARδ increases the production of MUFAs, which are activators of PPARδ and may protect the liver from free fatty acid-mediated lipotoxicity and inflammatory response. The current work unveils a function for PPARδ in the control of hepatic energy substrate homeostasis.

In response to substrate abundance, such as at the fed state, glucose is stored as glycogen and to a lesser extent, used for fatty acid synthesis in the liver. GK plays an important role in this process, because glucose entering the liver through GLUT2 is first phosphorylated by GK. The resulting product, glucose 6-phosphate, can then be utilized for glycogen synthesis, glycolysis, and lipogenesis (13). The level of GK is normally low during fasting and induced by feeding. Previous studies showed that hepatic GK overexpression increased glucose flux into glycogen synthesis, glucose oxidation, and lipogenesis, resulting in lowered glucose levels (16–18). This suggests that in addition to regulating glucose production, the liver has the capacity to modulate glycemia through glucose utilization mechanisms. Interestingly, many of the effects observed in adPPARδ mice mimic adenoviral GK overexpression (18), including reduced fasting glucose concentrations and increased hepatic glucose utilization (glycogen storage, glycolysis, and lipogenesis). Therefore, the glucose-lowering effect of adPPARδ is in part driven by increased glucose usage through GK up-regulation and *de novo* lipogenesis independent of insulin concentrations, although the insulin action on glucose utilization is likely amplified in adPPARδ mice. Our data show that increased hepatic PPARδ expression sustains GK levels leading to glycogen accumulation even after an overnight fast. PPARδ also up-regulates the fatty acid synthesis program as well as the lipogenic transcription factor and co-activator, SREBP-1c and PGC-1β, resulting in increased lipid content. There is no significant difference in the expression of GK and some lipogenic genes between *ad libitum* fed adPPARδ and control mice. Of note, the gene expression pattern at the fed state could be confounded by the timing of eating of individual animals before tissue collection. Nevertheless, glucose tracer experiments in primary hepatocytes support the hypothesis that PPARδ regulates glucose utilization, as evident from increased radioactive tracers in glycogen, fatty acid, and CO_2, the product of glycolysis. This functional outcome is mediated by direct and indirect transcriptional mechanisms. Promoter analyses suggest that PGC-1β co-activates PPARδ to increase ACC2 promoter I activity, whereas PGC-1β/SREBP-1c up-regulates the activities of reports driven by FAS and ACC promoter II. The regulation of GK is more complex. PPARδ expression up-regulated GK in a ligand-independent manner. However, PGC-1β is able to increase PPARδ-controlled GK promoter activity in the absence and presence of ligand. It is unclear how PPARδ induces PGC-1β and SREBP-1c. The increased fatty acid production may lead to PGC-1β up-regulation (37). Although PGC-1α has also been shown to co-activate PPARδ, our data suggest a preferential interaction between PPARδ/PGC-1β and PPARα/PGC-1α in the liver, which may explain the functional difference in fatty acid synthesis and oxidation, respectively. Previous work has demonstrated that the expression of PPARδ is up-regulated at the dark cycle, whereas PPARα is induced at the light cycle (41). It appears that the specificity of these two closely related receptors is determined by their temporal expression and co-factor interaction.

The lipogenic activity of PPARδ raises the concern of whether PPARδ activation is associated with steatosis or steatohepatitis. Interestingly, adPPARδ mice either on a normal chow or a high fat diet seem to have improved liver integrity determined by serum ALT and AST assays. The stress signaling JNK and inflammatory markers are also suppressed in adPPARδ livers. Free fatty acids are known to cause lipotoxicity, including induction of inflammatory response (2). It is possible that by partitioning fatty acids for triglyceride syn-

thesis, PPARδ activation protects the liver from free fatty acid-mediated damage. In fact, adenovirus mediated PPARδ expression in primary hepatocytes suppresses fatty acid-induced JNK activation and at the same time increases insulin-stimulated Akt phosphorylation, which is consistent with the improved ITT in high fat-fed adPPARδ mice. In addition, certain MUFAs, such as C16:1n7 (palmitoleate) and C18:1n7, have been shown to alleviate endoplasmic reticulum stress induced by saturated fatty acids and improve metabolic homeostasis (7, 42). These MUFAs are immediate products of SCD1 (35, 43). We find that adPPARδ livers contain more MUFAs and less saturated fatty acids on both chow and high fat diets, which are accompanied by increased SCD1 expression. Therefore, PPARδ may function to direct free fatty acid for storage and/or to convert toxic lipids to less toxic or even beneficial lipid species, thereby protecting livers from lipotoxicity. Additional work will be required to determine the role of SCD1 in mediating the protective effect.

PPARδ has been linked to AMPK activation (30). The underlying mechanism remains elusive. AMPK has been shown to suppress lipogenesis and glycogen synthesis (10, 44). At first glance, it seems paradoxical that PPARδ-expressing livers have more glycogen and lipid accumulation and at the same time show increased AMPK activity. Our data suggest that PPARδ limits substrate availability through the control of glucose utilization for glycogen store and lipogenesis, which consumes energy. Together with reduced β-oxidation, these changes lead to lowered ATP/increased AMP and a secondary effect of AMPK activation, which further contributes to the glucose lowering effect observed in adPPARδ mice. In support of this notion, PPARδ expression in primary hepatocytes increases the level of phospho-AMPK. Inhibition of AMPK activity reverts the effect of reduced basal glucose production in adenoviral PPARδ-infected hepatocytes. In addition, we observed increased adiponectin receptor 2 expression in adPPARδ livers, which could mediate adiponectin signaling, thereby increasing AMPK activity. Therefore, AMPK activation may serve as a feedback mechanism and explain why long term PPARδ ligand treatment does not cause severe hepatic lipid accumulation (25, 27). Of note, although adenovirus mediated overexpression has been useful for identifying hepatic functions for several metabolic regulators (45, 46), whether pharmacological activation of PPARδ could activate AMPK to the same extent as acute activation described in the current study remains to be determined.

Immune cells and inflammatory response have emerged as integral components of metabolic diseases (47). JNK, a major pro-inflammatory signaling molecule, phosphorylates insulin receptor substrate-1 and prevents insulin-mediated activation of phosphatidylinositol 3-kinase and its downstream effector Akt (2, 4). The current work demonstrates that PPARδ suppresses inflammation in the liver. It has been demonstrated that oleic acid (C18:1) or synthetic ligands activate macrophage PPARδ to turn on anti-inflammatory, alternative activation (31, 32). It is possible that hepatic PPARδ produces lipid ligands (MUFAs), which in turn activate macrophage (or Kupffer cells in the liver) PPARδ to modulate immune response. In fact, the expression of pro-inflammatory markers, such as TNFα and IFNγ, is down-regulated, whereas alternative activation markers, such as Mgl1 and MRC1, are induced in chow-fed adPPARδ livers. The reduction in pro-inflammatory gene expression is less evident on high fat diet, likely because high fat feeding also induces a strong inflammatory response in nonhepatic cells (*e.g.* immune cells) (46, 48). These observations indicate that PPARδ functions as a nuclear sensor of dietary fats capable of modulating immune response through regulation of metabolic programs. Despite the potential beneficial effects identified in this work, because fatty liver is often associated with type 2 diabetes, the use of PPARδ agonists to improve glucose handling may worsen the condition of steatosis. Nevertheless, results from the current study provide valuable information for designing drugs that target PPARδ for treating metabolic diseases.

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