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Aldose Reductase Regulates Hepatic Peroxisome Proliferator-activated Receptor α Phosphorylation and Activity to Impact Lipid Homeostasis*

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Aldose reductase (AR) is implicated in the development of a number of diabetic complications, but the underlying mechanisms remain to be fully elucidated. We performed this study to determine whether and how AR might influence hepatic peroxisome proliferator-activated receptor α (PPARα) activity and lipid metabolism. Our results in mouse hepatocyte AML12 cells show that AR overexpression caused strong suppression of PPARα/δ activity (74%, $p < 0.001$) together with significant down-regulation of mRNA expression for acetyl-CoA oxidase and carnitine palmitoyltransferase-1. These suppressive effects were attenuated by the selective AR inhibitor zopolrestat. Furthermore, AR overexpression greatly increased the levels of phosphorylated PPARα and ERK1/2. Moreover, AR-induced suppression of PPARα activity was attenuated by treatment with an inhibitor for ERK1/2 but not that for phosphoinositide 3-kinase, p38, or JNK. Importantly, similar effects were observed for cells exposed to 25 mM glucose. In streptozotocin-diabetic mice, AR inhibitor treatment or genetic deficiency of AR resulted in significant dephosphorylation of both PPARα and ERK1/2. With the dephosphorylation of PPARα, hepatic acetyl-CoA oxidase and apolipoprotein C-III mRNA expression was greatly affected and that was associated with substantial reductions in blood triglyceride and nonesterified fatty acid levels. These data indicate that AR plays an important role in the regulation of hepatic PPARα phosphorylation and activity and lipid homeostasis. A significant portion of the AR-induced modulation is achieved through ERK1/2 signaling.

The polyol pathway is a glucose metabolic shunt that is defined by two enzymatic reactions catalyzed by aldose reductase (AR,[4] AKR1B1, EC 1.1.1.21) and sorbitol dehydrogenase (SDH, EC 1.1.1.14), respectively (1). AR catalyzes the rate-limiting reduction of glucose to sorbitol with the aid of co-factor NADPH. SDH converts sorbitol to fructose using NAD^+. The apparent K_m value of AR for glucose is 30–80 mM (the K_m value for the open chain conformation of glucose, the true substrate, is in the low micromolar range) (2). At normal physiological glucose, about 5 mM glucose extracellularly, intracellular glucose is typically directed largely to glycolysis by hexokinase. However, at higher glucose concentrations, glycolysis becomes inhibited by citrate and ATP, and glucose can more readily enter other pathways, such as glycogenesis or the polyol pathway. Under normoglycemic conditions, about 1–3% of glucose is channeled through the polyol pathway in erythrocytes *in vitro* (3). By contrast, in 12–36 mM extracellular glucose, 30–50% of the lens glucose is metabolized by the polyol pathway to fructose (4). The function of this glucose shunt remains unclear in many tissues because fructose and its metabolites re-enter the glycolytic pathway.

Dyslipidemia is a major risk factor contributing to atherosclerotic cardiovascular diseases, diabetes, obesity, and liver diseases. Symptoms of dyslipidemia in diabetic patients include elevated blood triglycerides (TG) and nonesterified fatty acids (NEFA) and altered distribution of very low density lipoprotein and high density lipoprotein. Interestingly, it has been shown that in these patients there are significant increases in plasma or serum and urinary sorbitol and fructose (5–7), indicating increased flux in the polyol pathway concomitant with hyperglycemia. In addition, significant positive correlations were recently reported in human subjects with diabetes between

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[4] The abbreviations used are: AR, aldose reductase; ACO, acetyl CoA oxidase; APOA5, apolipoprotein A-V; APOC3, apolipoprotein C-III; ARI, aldose reductase inhibitor; CPT1, carnitine palmitoyltransferase-1; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NEFA, nonesterified fatty acids; PPARα/γ/δ, peroxisome proliferator-activated receptor α/γ/δ; PPRE, peroxisome proliferator-response element; p38, p38 MAPK; PI3K, phosphoinositide 3-kinase; RT, reverse transcription; SDH, sorbitol dehydrogenase; STZ, streptozotocin; TG, triglyceride; zopol, zopolrestat; siRNA, small interfering RNA; PKC, protein kinase C; RLU, relative luciferase unit.

monocyte AR and serum TG ($r = 0.41$, $p < 0.011$ for AR) and SDH and TG ($r = 0.48$, $p < 0.002$) (8). Moreover, ponalrestat, an ARI, was previously shown *in vivo* in mouse adipose tissue to be able to prevent a reduction in lipoprotein lipase activity, a central enzyme in TG catabolism (9). Furthermore, pharmacological administration of several other ARIs, including zopolrestat (zopol), were shown to reduce blood TG in rats (10), tumor bearing mice (9), and diabetic human patients (11), respectively. Together these data suggest a possible link between the activation/deactivation of AR/the polyol pathway and altered regulation in lipid metabolism.

The liver is a major organ for glucose and lipid homeostasis. Depending on species and relative to a related enzyme, aldehyde reductase, the liver was found to have low-to-moderate levels of AR (12–14) and a high level of SDH (15) under normal physiological conditions. Besides aldehyde reductase and AR, the liver contains several other AR-like proteins that possess AR-like activities (16–18). In human, AR has been successfully purified from dissected liver tissues (19). Of great interest is that hepatic AR has been shown to be greatly induced under certain conditions (20–22). Collectively, the above data raised the question of whether the changes in substrate flux through AR or activation/deactivation of AR/the polyol pathway in the liver might affect hepatic or whole body lipid metabolism.

A recent study by Roglans *et al.* (23) demonstrated that fructose feeding in rats impairs the activation of hepatic transcriptional factor Stat3 and the activity of PPARα; the latter is a major lipid sensor and a transcriptional regulator for lipid metabolic enzymes in the liver (24). Prompted by this, we conducted this study to determine the effects of activation/deactivation of AR on the phosphorylation and activity of PPARα, in cultured mouse hepatocyte AML12 cells, and in diabetic mice. We also attempted to identify the possible kinase signaling pathways involved by using a number of protein kinase inhibitors in cell culture experiments. We further investigated the effects of up-regulation and inhibition of the activities of AR on several lipid metabolism end points in streptozotocin (STZ)-induced diabetic mice. Our data suggest that AR/the polyol pathway is indeed involved in the hepatic modulation of lipid metabolism, and it does so by influencing the activities of transcriptional factors and enzymes linked to lipid metabolism, in particular PPARα. This modulation is mainly achieved through the phosphorylation/dephosphorylation of hepatic PPARα via an AR-linked ERK1/2 pathway.

EXPERIMENTAL PROCEDURES

Antibodies, Inhibitors, and Plasmids—Antibodies were obtained from the following vendors, respectively: ERK1/2 and phospho-ERK1/2 (catalog number 9100) Cell Signaling (Beverly, MA); PPARα (catalog number sc9000) and AR (catalog number sc17735), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); phospho-Ser-12 PPARα (catalog number ab3484) and phospho-Ser-21 PPARα (catalog number ab3485), Abcam (Cambridge, UK); β-actin (catalog number A1978) and anti-FLAG M2 (catalog number F1804), Sigma. PD98059, SB203580, SP600125, LY694002, and GW3335 were of analytical grade quality and from Sigma. Zopol was synthesized by the Department of Medicine Chemistry, Pfizer Global Research and Development (Groton, CT).

Mouse AR cDNA was PCR-amplified from pBluescript-mAR, a plasmid containing a cDNA fragment for mouse AR, using the following primers: ATATGCGGCCGCGATGGCCAGCCATCTGGAA and TGCTCTAGATCAGACTTCGGCGTGGAA, carrying a NotI site and an XbaI site, respectively (underlined). The PCR products were cut with NotI and XbaI and cloned into the corresponding sites of pFLAG-CMV2 (Sigma) to obtain plasmid pFLAG-mAR. PPRE-tk-luc with three PPAR-response elements, as described previously (25), was obtained from the Addgene. Plasmid pSV-β-galactosidase was purchased from Promega.

Cell Culture—AML12 mouse hepatocytes cells were obtained from ATCC and cultured as instructed.

PPAR Transcriptional Activities—AML12 cells were plated on 24-well plates at the cell density of ~2.2×10^5/well. The next day, cells were co-transfected with pFLAG-mAR or pFLAG-CMV, the PPRE-tk-Luc plasmid, and pSV-β-galactosidase at a ratio of 1:3:1 using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Six hours after transfection, cells were fed with fresh media with or without 10 μM PPARγ antagonist GW3335 (Sigma) and 100 μM zopol together with other treatments. Cells were further incubated for an additional 48 h prior to luciferase reporter assays. Luciferase reporter activities were determined using a luciferase reporter gene assay system (Promega) as instructed. PPAR activities were expressed as relative luciferase activity corrected for transfection efficiency (β-galactosidase activity) and normalized to the controls (normalized RLU).

Semi-quantitative Analyses of mRNA Expression by RT-PCR—Total RNA was isolated from cultured AML12 cells and liver tissues using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RT-PCR was performed to determine the levels of acetyl-CoA oxidase (ACO), carnitine palmitoyltransferase-1(CPT1), apolipoprotein C-III (APOC3), and apolipoprotein A-V (APOA5) mRNAs. The primers used were as follows: 5′-CCGCCACCTTCAATCCAGAGTTA-3′ and 5′-TCACAGTTGGGCTGTTGAGAATG-3′ (ACO); 5′-GGACGAATCGGAACAGGGATA-3′ and 5′-CCTTGTAATGTGCGAGCTGCA-3′ (CPT1); 5′-CCTCTTGGCTCTCCTGGCATCT-3′ and 5′-TGCTCCAGTAGCCTTTCAGGG-3′ (APOC3); 5′-GTGGGAGAAGACACCAAGGCTC-3′ and 5′-GGTCAATGGCCTGAGTAAATGC-3′ (APOA5); and 5′-CGAGACCCCACTAACATCAAA-3′ and 5′-AGTCTTCTGGGTGGCAGTGAT-3′ (glyceraldehyde-3-phosphate dehydrogenase). DNA amplification was carried out using a High Fidelity primeScript™ RT-PCR Kit (Takara). The PCR products were electrophoresed on 2% agarose gels and visualized by staining with ethidium bromide. The integrated density values of the bands representing amplified products were acquired and analyzed by Image-Pro Plus software (Media Cybernetics).

Kinase Inhibitors and ERK1/2 and PPARα Activities and Phosphorylation—AML12 cells were prepared and transfected as described above. Six hours after transfection, cells were fed with fresh media containing 10 μM GW3335 together with either vehicle or one of the four kinase inhibitors, which

included 25 μM PI3K inhibitor LY294002 (Sigma), 100 μM ERK1/2 inhibitor PD98059 (Sigma), 10 μM SB203580 (JNK), and 10 μM SP600125 (p38). Cells were further incubated for an additional 36 h prior to luciferase reporter assays.

Time Course Effects of 25 mM Glucose on AR, PPARα, and ERK1/2 Expression and Phosphorylation—AML12 cells were prepared as stated previously and incubated with 5.5 mM glucose Dulbecco's modified Eagle's media for 24 h and then replaced with fresh 25 mM glucose Dulbecco's modified Eagle's medium. Cells were harvested at 0, 3, 6, 12, 24, and 48 h, respectively, starting from 25 mM glucose incubation. Cells lysates were prepared as stated below for Western blot analyses.

Effects of High Glucose and Fructose on PPAR Activities—AML12 cells were prepared as stated previously but transfected with PPRE-tk-Luc and pSV-β-galactosidase at the ratio of 2:1. 18 h after transfection, cells were fed with fresh Dulbecco's modified Eagle's media containing either 5.5 or 25 mM glucose or 5.5 mM glucose plus 22.5 mM fructose together with 10 μM GW3335 and with or without 100 μM zopol. The cells were further incubated for an additional 12 h prior to luciferase reporter assays.

Effects of AR siRNA Knocking Down on PPARα and ERK1/2 Expression and Phosphorylation—AML12 cells were prepared as stated above and were transfected with chemically synthesized AR siRNA with the sense sequence being 5′-CGGUGUCUCCAACUUCAATT-3′ as described previously (26), and control siRNA being 5′-UUCUCCGAACGUGUCACGUTT-3′ (GenePharma, China). The transfections were performed with the siPORT™ NeoFX™ transfection reagent (Ambion) according to the manufacturer's instruction. After siRNA transfection, the cells were initially incubated in 5.5 mM glucose media for 24 h and then replaced with 25 mM glucose media and the incubation was continued for another 24 h before harvesting for Western blot analyses.

Animals—Mice carrying $AR^{-/-}$ mutations were backcrossed with C57BL/6 mice for five consecutive generations before use as described previously (27, 28). Mice carrying a mutation in *SDH* (C57BL/LiA) and their controls (C57BL/10N) were derived from stock strains as described (29). The mice were maintained as described previously unless otherwise stated (28). Animal care and experimental procedures were in accordance with the guidelines of the Xiamen University Institutional Animal Care and Use Committee and the University of Hong Kong Committee on the Use of Live Animals in Research and Teaching. AR null allele and the SDH mutant alleles as well as their wild type counterparts were determined as described previously by Yang *et al.* (28) and Lee *et al.* (29) respectively.

PPARα and ERK1/2 Expression and Phosphorylation in Diabetic C57BL/6 Mice—7-Week-old male mice were injected with a single dose of 200 mg/kg STZ for diabetes induction. Two days after successful diabetes induction (blood glucose $>=$ 16 mM), one group of mice were treated with 50 mg/kg zopol daily, and the other groups received vehicle (saline) for 7 days. After the ARI treatment, liver tissues were dissected from these mice and analyzed by Western blots as described below to determine the *in vivo* PPARα and ERK1/2 expression and phosphorylation under hyperglycemia.

Western Blot Analyses—AML12 cells were prepared and transfected as described above. Whole cell extracts were prepared by dissolution of cell pellets in ice-cold buffer (1% Triton X-100, 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerin, 10 mM $Na_4P_2O_7$, 20 mM glycerophosphate, 10 mM NaF, 10 mM sodium orthovanadate and proteinase inhibitor mixture) until the cells were completely lysed and stored at −80 °C before use. Mice liver tissues were dissected and immediately frozen in liquid N_2 and stored at −80 °C before homogenization. Liver tissues were homogenized with Polytron in the same buffer as above. The protein concentrations of the extracts were measured using a bicinchoninic acid protein assay kit (Pierce). 40 μg protein of each sample was loaded and separated on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore). Blotted membranes were then incubated by either anti-ERK or anti-phospho-ERK (1:1000) or anti-PPARα (1:200) or anti-phospho-PPARα (1:1000) or anti-AR (1:200) in TBS, 0.1% Tween 20 with 5% nonfat milk at 4 °C overnight. After several washes, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-goat IgG (1:2000) in TBS, 0.1% Tween 20 with 5% nonfat milk. The detection was achieved using the SuperSignal chemiluminescent substrate kit (Pierce).

Blood Sample Analyses—Tail blood TG levels were assayed according to the manufacturer's instructions with a Roche Applied Science Accutrend® GCT system. Blood glucose was measured with a Bayer Glucometer Elite (model 3049C) as instructed. Plasma NEFA levels were determined by NEFA C kits (Wako Chemicals, Japan).

Lipid Metabolism in STZ-induced Diabetic Mice—Diabetes induction was achieved by a single intraperitoneal injection of STZ at a dosage of 200 mg/kg body weight immediately after grouping of mice at the age of 4 weeks. Mice to be treated with ARI were injected intraperitoneally once daily with zopol at 50 mg/kg body weight. ARI treatment was initiated at the age of 30-day and continued for 2 weeks. Blood and plasma samples were collected by heart puncture of Hypnorm-Dormicum-anesthetized mice at the age of 44-day, *i.e.* 2 weeks post-ARI treatment.

Statistical Analysis—All data were processed and analyzed by GraphPad software (Prism 4.0) and expressed as mean ± S.E. Student's *t* test was used for pairwise comparisons and one-way analysis of variance with Bonferroni's multiple comparison test for multiple group analyses. Probability values less than 0.05 (*) were considered to be statistically significant; those less than 0.01 (**) more so.

RESULTS

AR Up-regulation Caused Suppression in PPARα/δ Transcriptional Activity and AR Inhibition Attenuated the Suppression—To investigate the effects of hepatic activation of AR/polyol pathway on lipid metabolism, we first examined the effects of AR overexpression on the transcriptional activity of PPARs, nuclear receptors that serve as important lipid and glucose sensors and transcription factors and that control a large group of lipid metabolic enzymes, in particular the β-oxidation enzymes (24). To this end, we PCR-amplified a mouse AR cDNA fragment and cloned it into the expression vector

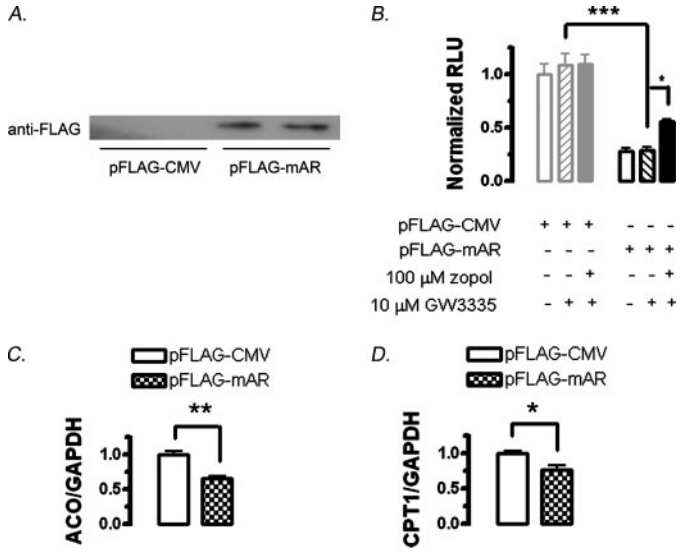

FIGURE 1. **AR modulates PPARα/δ activities and the mRNA expression of ACO and CPT1.** Values are expressed as the mean ± S.E. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. *A,* typical Western blot demonstrating greatly increased AR protein levels in AML12 cells transfected with pFLAG-mAR for 48 h and detected by anti-FLAG antibody. This result had been reconfirmed with both anti-FLAG and anti-mAR antibodies. *B,* AR up-regulation caused suppression in PPARα/δ activity (normalized RLU), whereas AR inhibition attenuated the suppression. AML12 cells were transfected with pFLAG-CMV or pFLAG-mAR in the presence or absence of 100 μM zopol and 10 μM GW3335 and harvested after 48 h of incubation. Experiments were performed with at least three separate samples ($n = 3$) analyzed in triplicate. *C,* AR overexpression down-regulates ACO mRNA expression. Total RNA were extracted 48 h after AML12 cells were transfected with pFLAG-CMV or pFLAG-mAR and analyzed by RT-PCR ($n = 3$). *D,* AR overexpression down-regulates CPT1 mRNA expression. Total RNAs were extracted 48 h after AML12 cells were transfected with pFLAG-CMV or pFLAG-mAR and analyzed by RT-PCR ($n = 3$). *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

pFLAG-CMV2. The resultant vector pFLAG-mAR was used to transfect mouse hepatocyte AML12 cells whose endogenous AR expression is relatively low under normal circumstances. Our transfection studies indicated that transfection with pFLAG-mAR in AML12 cells greatly increased AR protein levels as determined by Western blots using an anti-FLAG antibody (Fig. 1*A*) and confirmed with an anti-AR antibody (data not shown). The PPAR-mediated transcriptional activity was assayed with the luciferase reporter assays. Because liver cells contain all three known isotypes of PPARs, *i.e.* PPARα, PPARγ, and PPARδ, PPARγ antagonist GW3335 was used in the reporter assay to diminish the activity of PPARγ. The relative luciferase activities (normalized RLU) therefore represented all PPAR activities for those without the GW3335 treatment but PPARα/δ activities for those that were GW3335-treated. As shown in Fig. 1*B*, increased AR expression reduced PPARα/PPARδ transcriptional activities by about 74% ($p < 0.001$). Of note is that there was virtually no difference in PPAR activities between AML12 cells transfected with both pFLAG-CMV or pFLAG-mAR and with or without PPARγ antagonist GW3335. This suggests that the endogenous hepatic PPARγ activity is negligible, and AR overexpression did not significantly affect its activity. Meanwhile, treatment of cells with 100 μM zopol significantly attenuated, *i.e.* tended to normalize, the AR-induced suppression ($p < 0.05$) of PPARα/PPARδ activity, although not completely to the control levels. One of the possible reasons for the incomplete restoration of PPARα/PPARδ activity might be the incomplete inhibition of AR. Together these results indicated that increased activity of AR caused suppression in PPARα/PPARδ activity, whereas the inhibition of AR tended to normalize PPARα/δ activity.

AR-induced Reductions in PPARα/δ Activities Were Associated with Down-regulated ACO and CPT1 mRNA Expression in AML12 Cells—ACO and CPT1 are two major enzymes critical for peroxisomal and mitochondrial fatty acid oxidation, respectively. These enzymes are known to be tightly regulated by hepatic PPARα in liver cells. We analyzed the mRNA expression of ACO and CPT1 in AR overexpressing AML12 cells by semi-quantitative RT-PCR and found that, as a consequence of AR overexpression, ACO mRNA expression was down-regulated by 34.3% ($p < 0.01$) and CPT1 by 23.3% ($p < 0.05$), respectively (Fig. 1, *C* and *D*), in comparison with the controls.

AR-induced PPARα/δ Suppression Is Associated with Increased Phosphorylation of PPARα and ERK1/2—PPARα is a phosphoprotein, and phosphorylation is one of the most rapid and efficient mechanisms whereby its activity can be modulated (30, 31). To determine whether PPARα phosphorylation contributes to the AR-induced activity suppression, we performed Western blots with lysates of AML12 cells transfected with pFLAG-mAR and control plasmids using antibodies that recognize phosphoserine PPARα at either Ser-12 or Ser-21 and antibodies that detect phospho-ERK1/2. As shown in Fig. 2*A*, AR overexpression greatly increased phosphorylated PPARα levels, at both Ser-12 (2.2-fold, $p < 0.001$) and Ser-21 (2.1-fold, $p < 0.05$) (Fig. 2, *A–C*), two major phosphorylation sites located at the A/B domain (AF-1) of PPARα. Paralleling the increase in PPARα phosphorylation, there was a greatly increased phosphorylation of both ERK1 (14-fold, $p < 0.001$) and ERK2 (4.1-fold, $p < 0.01$) (Fig. 2, *A*, *D* and *E*), suggesting that the ERK-MAPK signaling pathway contributes to the increased PPARα phosphorylation. Both Ser-21 and Ser-12 were previously demonstrated to be the ERK-mediated phosphorylation sites on PPARα in cardiac myocytes, and inhibition of their phosphorylation by PD98059 caused increased transcription of CPT1 (32). Furthermore, PPARα phosphorylation at both Ser-21 and Ser-12 has recently been demonstrated to decrease the transcriptional activity of PPARα, whereas dephosphorylation at both positions significantly increased the activity in human hepatoma HepG2 cells (33). The AR-induced hepatic phosphorylation of PPARα at both Ser-21 and Ser-12 thus appeared to explain the reductions of PPARα transcriptional activity in AR overexpressing mouse liver cells.

Inhibition of ERK, but Not PI3K, JNK, or p38 Significantly Attenuated the AR-induced Suppression of PPARα/δ Activity—To further confirm the contribution of ERK1/2 to the AR-induced suppression of PPARα/δ, similar transfection studies were performed in the presence of one of the four inhibitors for kinase signaling pathways: LY294002 (PI3K), SP600125 (JNK), or SB203580 (p38). As shown in Fig. 3, treatment with inhibitor for PI3K, JNK, and p38 did not significantly change the activity of PPARα/δ, excluding their involvement in PPARα phosphorylation. In contrast, treatment with ERK1/2 inhibitor PD98059 in pFLAG-mAR transfected cells significantly restored the activity of PPARα (to 78% of the control level), confirming significant contribution of ERK1/2 to the AR-induced PPARα

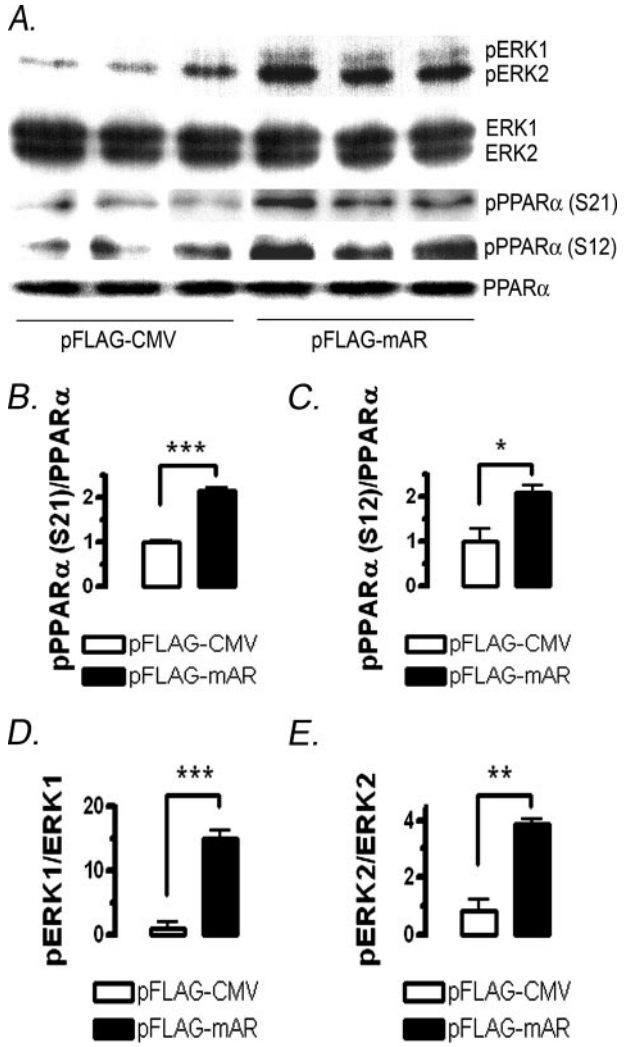

FIGURE 2. **Increased AR activity is associated with increased PPARα and ERK1/2 phosphorylation.** *A,* effects of AR overexpression on PPARα and ERK1/2 expression and phosphorylation in AML12 cells. Western blots were performed as described under "Experimental Procedures" ($n = 3$). *pERK1/2*, phospho-ERK1/2; *pPPARα(S12)*, phosphoserine 12 PPARα; *pPPARα(S21)*, phosphoserine 21 PPARα. *B–E,* Statistical analysis of the Western blots. Values are expressed as the mean ± S.E. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

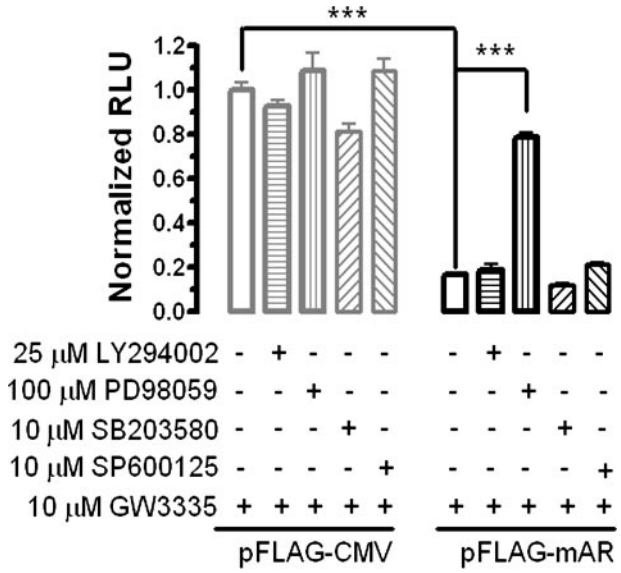

FIGURE 3. **Treatment of ERK inhibitor but not that for JNK, PI3K, or p38 significantly attenuated the AR-induced suppression of PPARα/δ.** AML12 cells were transfected with pFLAG-mAR or pFLAG-CMV and in the absence or presence of kinase inhibitors. PPARα/δ activity (normalized RLU) was assayed as described in the presence of 10 μM PPARγ antagonist GW3335 ($n = 4$). Values are expressed as the mean ± S.E. ***, $p < 0.001$.

phosphorylation and reduction in transcriptional activity. The restoration, however, was not complete, suggesting either incomplete ERK1/2 inhibition or the involvement of other protein kinases or regulatory mechanisms. Despite this, these data indicated that the great majority of the AR-induced PPARα phosphorylation and activity reduction was reversibly mediated by ERK1/2.

High Glucose Time-dependently Stimulated Hepatic AR Expression and Increased Phosphorylation of ERK1/2 and PPARα—As described previously, the hepatic basal expression for AR is low. We therefore wanted to determine whether hepatic AR is significantly up-regulated under high glucose conditions. As shown in Fig. 4*A*, AR was indeed significantly up-regulated by 25 mM glucose in a time-dependent manner. The AR band became clearly detectable 3 h after the glucose exposure and reached a very high level at 48 h. In association with this, there appeared to have been a time-dependent increase in the phosphorylation of PPARα at both Ser-21 and Ser-12, although there simultaneously appeared to have been a small decrease in total PPARα protein level. Meanwhile, ERK1/2 phosphorylation was also significantly increased as the exposure time increased, with no apparent change in total ERK1/2 protein level (Fig. 4*A*). Moreover, high glucose was shown to lead to a reduction in PPARα activity (35%, $p < 0.01$) that was alleviated by co-treatment of 100 μM zopol ($p < 0.01$) or 50 μM PD98059 ($p < 0.05$), suggesting inhibition of either AR or ERK1/2 led to significant restoration of PPARα activity (Fig. 4*B*). Of particular note is that 5.5 mM glucose plus 22.5 mM fructose also caused a strong reduction in PPARα activity (43%, $p < 0.001$). This result is consistent with dietary fructose-induced derangements of hepatic PPARα expression and activities in rats (23, 34), suggesting *in vivo* activation of AR/the polyol pathway at least in this regard might be physiologically equivalent to dietary fructose ingestion. It also further suggested that the polyol pathway, not AR only, modulates hepatic PPARα activities (Fig. 4*B*). The effects of AR inhibition on PPARα and ERK phosphorylation under high glucose, on the other hand, were further investigated by knocking down AR with specific siRNAs. As shown in Fig. 4*C*, siRNA knocking down of AR significantly reduced the levels of both phosphorylated PPARα and ERK1/2 induced by 25 mM glucose, further confirming the role of AR in the regulation of phosphorylation of PPARα and ERK1/2.

AR Deficiency or AR Inhibition Significantly Decreased Hyperglycemia-induced Hepatic Phosphorylation of ERK1/2 and PPARα in Vivo in Diabetic Mice That Are Associated with Altered Expression of Hepatic APOC3 and ACO—To determine the effects of AR deficiency or AR inhibition on hepatic PPARα and ERK1/2 *in vivo* and under hyperglycemia, we examined the hepatic expression and phosphorylation of these two proteins with Western blots using liver tissues from STZ-induced diabetic mice and their controls. No significant difference in blood

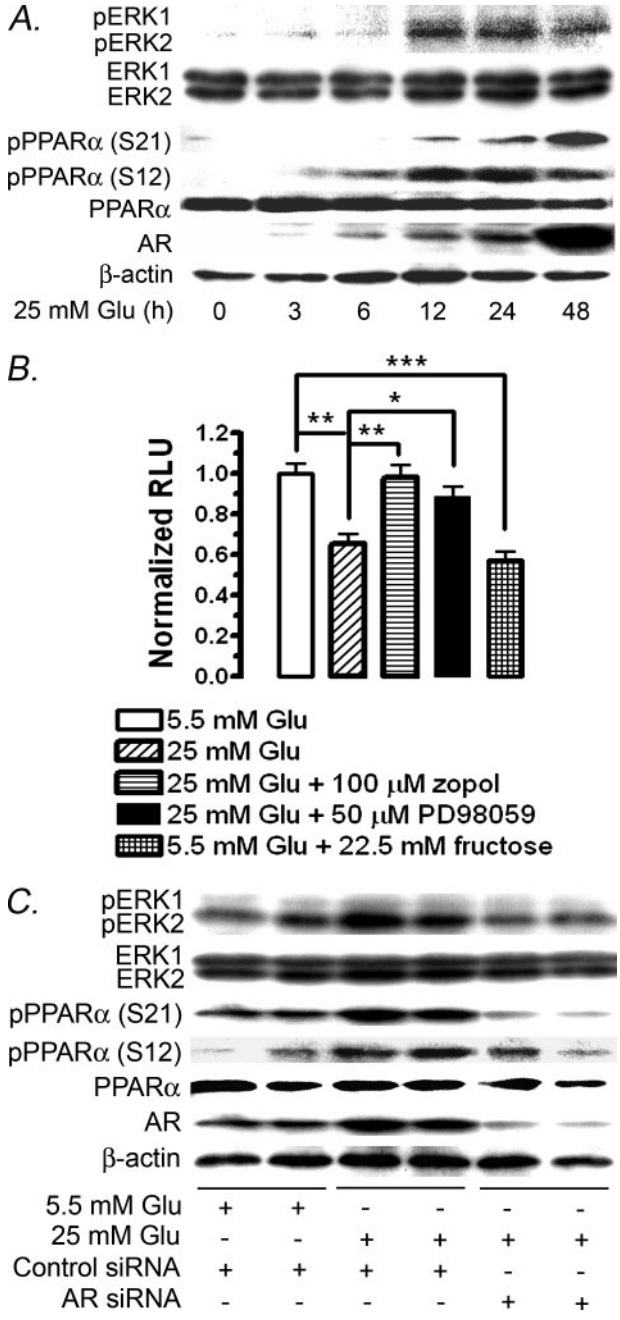

FIGURE 4. **The effects of high glucose and fructose on the activity of PPARα/δ or the phosphorylation status of PPARα and ERK1/2 in AML12 cells.** *Glu*, glucose; *pERK1/2*, phospho-ERK1/2; *pPPARα*(*S12*), phosphoserine 12 PPARα; *pPPARα*(*S21*), phosphoserine 21 PPARα. *A*, 25 mM glucose time-dependently up-regulated AR and enhanced PPARα and ERK1/2 phosphorylation. *B*, both 25 mM glucose or 5.5 mM glucose plus 22.5 mM fructose exposures for 12 h led to significant suppression on PPARα/δ activity, and the 25 mM glucose-induced suppression can be attenuated by co-treatments with 100 μM zopol or 50 μM PD98059 ($n = 3$). PPARα/δ activity was assayed as described in the presence of 10 μM PPARγ antagonist GW3335. Values are expressed as the mean ± S.E. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. *C*, pretreatment of AR siRNA 24 h greatly reduced phosphorylated PPARα and ERK1/2 levels induced by 25 mM glucose exposure for 24 h.

glucose levels was observed among control groups and among STZ-treated wild type mice, wild type mice treated with zopol, or AR-deficient mice, although there appeared to have been a slight elevation in the latter two groups (Fig. 5*B*). As shown in Fig. 5*A*, STZ-induced diabetes greatly stimulated ERK1/2 and PPARα phosphorylation, and this was significantly diminished in both AR-deficient mice and mice that were zopol-treated at a dosage of 50 mg/kg/day for 7 days, suggesting AR mediated the stimulation of ERK-MAPK and the phosphorylation of PPARα *in vivo* and under hyperglycemic conditions and that AR deficiency or inhibition caused significant dephosphorylation of ERK1/2 and PPARα. To determine the effects of AR deficiency or inhibition-induced PPARα dephosphorylation on its target genes, we analyzed hepatic ACO, APOC3, and APOA5 mRNA expression by semi-quantitative RT-PCR for STZ-diabetic mice and its controls. As shown in Fig. 5, *C* and *D*, diabetes down-regulated hepatic ACO but up-regulated APOC3 significantly, and the latter is a protein capable of inhibiting TG hydrolysis by lipoprotein lipase. In zopol-treated diabetic mice, however, ACO was up-regulated and APOC3 was down-regulated significantly. This suggested that hepatic PPARα activity is up-regulated, which is consistent with the greatly reduced PPARα phosphorylation. Similar effects were observed for mice deficient in AR. In contrast to both ACO and APOC3, only slight and not significant alterations were observed for APOA5 (Fig. 5*E*).

AR Deficiency or AR Inhibition Had Significant Impact on Blood Lipid Profiles in STZ-induced Diabetic Mice—To determine the effects of AR deficiency or inhibition on the overall lipid metabolism in the diabetic animals, we examined the levels of blood TG and plasma NEFA in STZ-treated mice that had maintained the diabetic status with or without similar ARI treatment for a period of 2 weeks. As shown in Fig. 6, *A* and *B*, three treatment groups of normoglycemic mice had only small but not significant differences in blood TG and NEFA levels. In contrast, zopol-treated diabetic mice had significantly lower blood TG (113 ± 12 mg/dl for $AR^{+/+}$ (STZ/ARI) *versus* 237 ± 29 mg/dl for $AR^{+/+}$ (STZ), $p < 0.05$) (Fig. 6*A*) and plasma NEFA (0.36 ± 0.06 meq/liter for $AR^{+/+}$ (STZ/ARI) *versus* 0.79 ± 0.11 meq/liter for $AR^{+/+}$ (STZ), $p < 0.05$) (Fig. 6*B*) in comparison with the wild type diabetic mice. Diabetic mice deficient in AR also had reduced TG and NEFA, but the magnitude of the changes were between the diabetic wild type and the ARI-treated diabetic wild type and not statistically different from either.

In contrast to TG and NEFA, there were small and not significant disturbances in blood cholesterols in AR-deficient mice and ARI-treated mice (data not shown). Importantly, no apparent difference in food consumption was observed in both the normoglycemic groups or in the diabetic groups (data not shown). The effect of blocking the polyol pathway on blood TG levels was further investigated in mice deficient in SDH, the second enzyme of the polyol pathway. As shown in Fig. 6*C*, the level of blood TG in SDH-deficient ($SDH^{-/-}$) mice was close to that of the wild type control mice under normoglycemic condition (99 mg/dl for $SDH^{-/-}$ mice *versus* 95 mg/dl for $SDH^{+/+}$ mice). In diabetic animals, however, the level of blood TG in $SDH^{-/-}$ mice was greatly reduced in comparison with mice that have normal SDH and polyol pathway activities (159 mg/dl for $SDH^{-/-}$ mice *versus* 261 mg/dl for $SDH^{+/+}$ mice, $p < 0.05$). Again, no difference in food consumption was evident for these groups of mice (data not shown). Because blocking the polyol pathway by inhibition of either AR or SDH led to similar reduc-

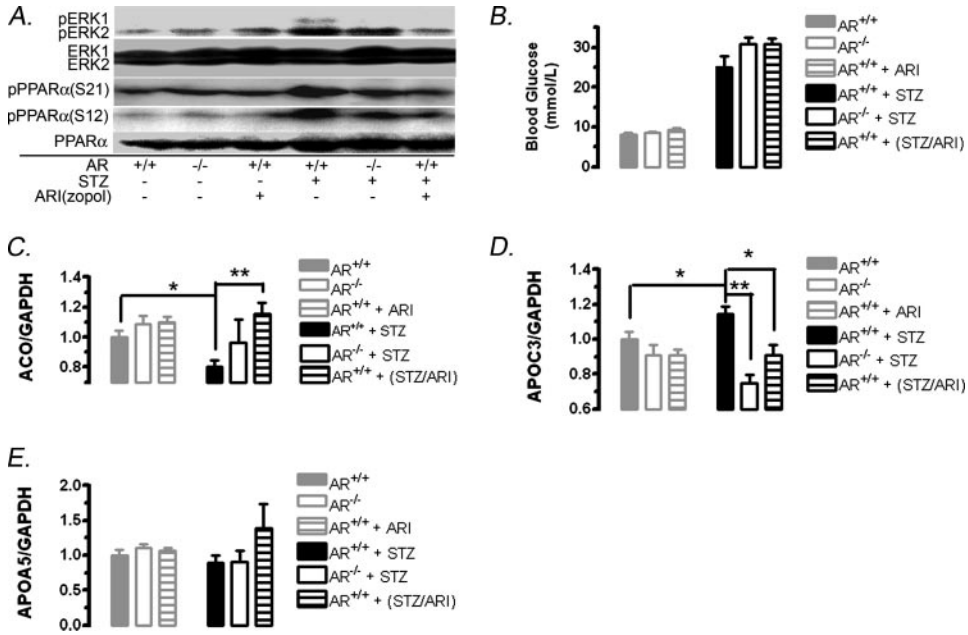

FIGURE 5. **The *in vivo* effects of AR/the polyol pathway on PPARα and ERK1/2 phosphorylation.** *A,* representative Western blot for three separate experiments demonstrating that hyperglycemia-induced phosphorylation of ERK1/2 and PPARα in mouse liver was greatly diminished in diabetic mice that are AR-deficient or ARI-treated. Diabetes was induced with a single injection of STZ at the dosage of 200 mg/kg to 7-week-old C57BL/6 mice with or without the daily co-treatment of 50 mg/kg zopol starting from 2 days after STZ diabetes induction. Liver tissues were dissected and analyzed 7 days after successful STZ diabetes induction. *B,* blood glucose levels of STZ-treated and control mice ($n = 4$). *C–E,* $n = 4$. Hepatic mRNA expression of ACO, APOC3, and APOA5 analyzed by semi-quantitative RT-PCR. Values are expressed as the mean ± S.E. *, $p < 0.05$, **, $p < 0.01$.

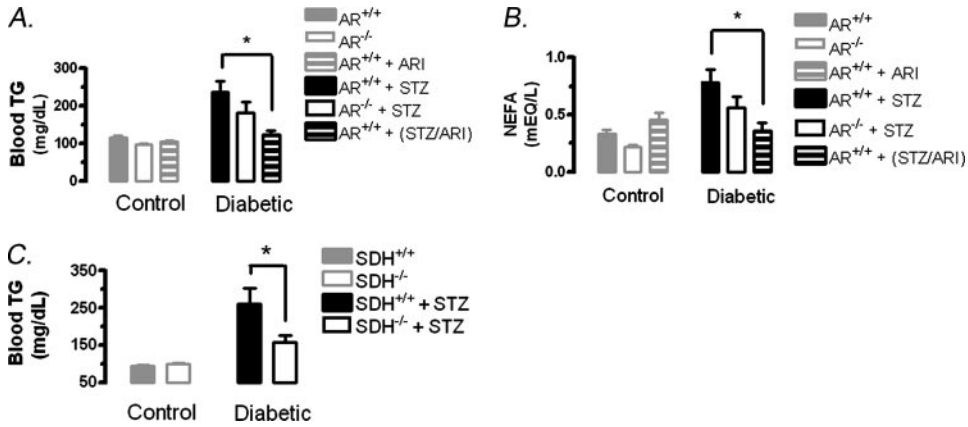

FIGURE 6. **Blood lipid profile in STZ-diabetic C57BL/6 mice.** *A* and *B,* effects of AR deficiency/ARI treatment on NEFA/TG in normoglycemic and diabetic C57BL/6 mice. Normoglycemic mouse groups are $AR^{+/+}$ ($n = 10$), $AR^{-/-}$ ($n = 8$), $AR^{+/+}$ (ARI) ($n = 8$); diabetic mouse groups are $AR^{+/+}$ (STZ) ($n = 11$), $AR^{-/-}$ (STZ) ($n = 11$), and $AR^{+/+}$ (STZ/ARI) ($n = 7$), respectively. Values are expressed as the mean ± S.E. *, $p < 0.05$. *C,* effects of SDH null mutation on normoglycemic and diabetic C57BL/10 mice. Normoglycemic mouse groups are $SDH^{+/+}$ ($n = 10$) and $SDH^{-/-}$ ($n = 11$); diabetic mouse groups are $SDH^{+/+}$ (STZ) ($n = 8$) and $SDH^{-/-}$ (STZ) ($n = 13$), respectively. Values are expressed as the mean ± S.E. *, $p < 0.05$.

tions in blood TG, these results therefore reinforce the concept that it is the polyol pathway, not just AR activity alone, that contributes to altered phosphorylation of ERK1/2 and PPARα that cause changes in TG and NEFA metabolism.

DISCUSSION

Our data that TG and NEFA levels for diabetic mice deficient in AR were between the diabetic wild type and the ARI-treated diabetic wild type and not statistically different from either is surprising, as a genetic ablation will result in a complete loss of the activity (Fig. 6, *A* and *B*). In this regard there appeared to have been two possible reasons to explain the discrepancy between AR deficiency and ARI treatment. On one hand, the AR null mutation was created by deleting part of the coding region of the AR gene, neither the expression nor the function of other genes besides AR is expected to be reduced or down-regulated. It is unknown whether any of a number of AR-related enzymes such as mouse vas deferens protein, mouse fibroblast growth factor-regulated protein, and AR-like-1 (16) might have been up-regulated to compensate for AR deficiency. These AR-like enzymes theoretically could contribute to the conversion of glucose to sorbitol *in vivo*. Indeed, a small amount of sorbitol was found in a few tissues in our AR knock-out mice, and this was not because of the reverse reaction catalyzed by SDH.[5] On the other hand, some ARIs might not be exclusively specific for AR, and they might have inhibitory effects toward other AR-like enzymes.

AR/the polyol pathway have been demonstrated to play important roles in the development and progression of diabetic complications in a number of tissues where insulin dependence is usually not very pronounced. These tissues include kidney, retina, lens, and peripheral neuron tissues (2, 36–39). Little data, however, are available regarding the role of AR/the polyol pathway in the liver. In this study, we first demonstrated that like in other tissues vulnerable to diabetic complications, AR can be greatly induced by either high glucose in cultured hepatocytes or by diabetes *in vivo* in mouse liver, indicating hepatic activation of the polyol pathway under glucose abundance or hyperglycemia. We further demonstrated that AR overexpression in liver cells led to decreased PPARα activity, and AR inhibition significantly restored the activity. Furthermore, alterations in hepatic PPARα activities were shown to be associated with changes in the status of PPARα phosphorylation that positively correlated with the status of ERK1/2 phos-

[5] J. Y. Yang, unpublished data.

phorylation. Moreover, the effectiveness of PD98059 in significantly restoring the activity of PPARα implicated ERK1/2 as being upstream of PPARα. Finally, we demonstrated that blocking of the polyol pathway by either pharmacological inhibition of AR or genetic ablation of AR or SDH all caused consistent and significant alterations in lipid homeostasis that were associated with significant dephosphorylation of both ERK/1/2 and PPARα phosphorylation. Together these data clearly indicate that diabetes or high glucose can greatly activate flux through hepatic AR/the polyol pathway that is linked to regulation of PPARα phosphorylation and activity that in turn affect cellular lipid homeostasis.

PPARα is an important metabolic nuclear receptor that regulates lipid metabolism as well as glucose homeostasis in the liver, heart, and skeletal muscles, etc. It influences intracellular lipid and carbohydrate metabolism through direct transcriptional control of genes involved in peroxisomal and mitochondrial β-oxidation pathways, fatty acid uptake, and TG catabolism, etc. (40). Indeed, mice lacking PPARα are unable to oxidize fatty acids, which results in elevated TG and free fatty acid levels and fatty livers (41), indicating the liver is particularly vulnerable to suppression of PPARα activity. Conversely, hepatic activation of PPARα by its agonists such as WY 14,643 decreases blood TG levels by up-regulating the expression of lipoprotein lipase and apolipoprotein A5 (35) and down-regulating apolipoprotein C3, etc. (42). These data and others (24) established that hepatic PPARα plays an obligatory role in regulating extracellular and intracellular fatty acid metabolism. Our demonstration of the correlations between AR activity, PPARα phosphorylation, and the mRNA expression of ACO and APOC3 *in vivo* provided further evidence for the possible connections between them.

There are a few mechanisms whereby the transcriptional activity of PPARα can be regulated, which include post-translational modifications, heterodimer formation with retinoic X receptor, PPARα, and retinoic X receptor ligand binding, recruitment/dissociation of co-activators and co-repressors etc. Post-translational modification by phosphorylation is one of the most important mechanisms whereby PPARα is modulated (30, 43). Multiple phosphorylation sites have been identified on different domains on mouse PPARα, which include the A/B domain, the DNA binding domain, and the ligand binding domain. Furthermore, PPARα phosphorylation was shown to be catalyzed by a diverse group of kinases, including protein kinase A/protein kinase C (PKC), ERK1/2, glycogen synthase kinase, etc. Depending on the types of cells and stimuli, phosphorylation can either lead to activation or inactivation of PPARα (32, 33, 43). In this study, we demonstrated that AR-induced PPARα phosphorylation at Ser-12 and Ser-21 contributed to the suppression in transcriptional activity in hepatocyte cells. Although we clearly showed that AR-induced PPARα phosphorylation *in vitro* and *in vivo* was largely mediated by ERK1/2, it is not clear how AR activates the ERK-MAPK signaling pathway to phosphorylate PPARα. PKC has long been known to be strongly activated by hyperglycemia and AR (44–46). On the other hand, PKC activation was also shown to affect the expression or activities of PPARα in various cells (47–49). One possibility therefore might be that AR activates PKC and then PKC either directly or indirectly leads to the activation of ERK1/2. This, however, remains to be proved in future investigations.

In times of starvation the liver has to switch from glucose to fatty acid for energy needs, and in the meantime it will turn itself into a glucose-producing organ, providing fuel for the brain and other tissues. Because PPARα-deficient mice are unable to accomplish the substrate switch because of their inability to activate the transcription of PPARα-controlled lipid catabolic enzymes, short term starvation caused hepatic steatosis, myocardial lipid accumulation, and death, etc., suggesting PPARα is required for cellular response to fasting (50). Although these results clearly illustrate the essential roles of PPARα under low glucose or fasting in the liver and other tissues, there are little data regarding whether PPARα plays any role in cellular response to the opposite of starvation, *i.e.* glucose abundance or hyperglycemia. In this regard, the AR/the polyol pathway-induced suppression of hepatic PPARα to impact on lipid homeostasis might reflect an important regulatory mechanism for animals to respond to glucose abundance or hyperglycemia. Because under high glucose conditions the liver does not have to rely on fatty acids for energy needs, fatty acid oxidation might have to be down-regulated or even turned off. As we demonstrated here, this probably is achieved at least in part through activating hepatic AR/the polyol pathway to suppress the PPARα activity. In this scenario, AR/the polyol pathway senses the levels of intracellular glucose and then adjusts hepatic PPARα activity accordingly via phosphorylation/dephosphorylation. When glucose is high, increased metabolic flux through AR deactivates PPARα through ERK1/2-mediated phosphorylation on its activation function domain. When glucose level is low, AR/the polyol pathway becomes less active with subsequent PPARα reactivation via dephosphorylation. AR/the polyol pathway thus might represent yet another "endogenous modulator" for PPARα.

Fibrates such as bezafibrate, gemfibrozil, clofibric acid, ciprofibrate, and fenofibrate or its analog WY 14,643 are all ligands for PPARα. These chemicals have been widely used clinically as hypolipidemic drugs for the effective treatment of hyperlipidemia for years. Interestingly, there are recent reports demonstrating that a number of these PPARα agonists possess AR inhibitory activities in addition to their PPARα agonist activities (51, 52). Based on their *in vitro* studies, Balendiran *et al.* (51) suggested that AR, AR-like proteins, and other aldo-keto reductase members, might be involved in mechanisms of action for PPARα agonists such as WY 14,643 or fibrates. Specifically, the suggestion is that a significant portion of the biological effects of PPARα agonists, such as lipid lowering and inflammatory response, might in principle be contributed by inhibition of AR/the polyol pathway, at least in certain tissues or cells. In the liver, for example, fibrates or WY 14,643 on one hand might serve as ligands to bind to and stimulate PPARα, and on the other hand by inhibiting AR, they might act to dephosphorylate PPARα. Further studies at physiologically and pharmacologically relevant fibrate drug levels are warranted to further explore this intriguing hypothesis.

Our present demonstration of the regulatory role of AR/the polyol pathway for hepatic PPARα and lipid metabolism links

for the first time the activity of this glucose shunt with the activity of an important glucose and lipid metabolic nuclear receptor, PPARα. It also provides a possible mechanism for the TG-lowering effects of the ARIs. In addition, it is conceivable that these novel findings will help us better understand the development of lipid metabolism disorders in diabetic as well as over-nourished mammals. Our results also suggest that the polyol pathway enzymes AR and SDH are potential targets for the treatment and prevention of dyslipidemia and other related metabolic diseases.

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