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Estrogen-related Receptor γ (ERRγ) Is a Novel Transcriptional Regulator of Phosphatidic Acid Phosphatase, LIPIN1, and Inhibits Hepatic Insulin Signaling*

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Background: The PAP function of LIPINs is involved in the regulation of intracellular lipid levels and hepatic insulin receptor signaling.
Results: ERRγ-mediated induction of LIPIN1 results in the perturbation of hepatic insulin signaling through DAG-mediated activation of PKCε.
Conclusion: ERRγ is a novel transcriptional regulator of LIPIN1.
Significance: An ERRγ inverse agonist could ameliorate LIPIN1-mediated perturbation of hepatic insulin signaling.

LIPINs have been reported to perform important roles in the regulation of intracellular lipid levels. Their mutations induce lipodystrophy, myoglobinuria, and inflammatory disorders. Recently, the phosphatidic acid phosphatase function of LIPINs has been associated with the perturbation of hepatic insulin receptor signaling via the diacylglycerol-mediated stimulation of PKCε activity. Here, we report that nuclear estrogen-related receptor (ERR) γ is a novel transcriptional regulator of LIPIN1. Overexpression of ERRγ significantly increased *LIPIN1* expression in primary hepatocytes, whereas the abolition of ERRγ gene expression attenuated the expression of *LIPIN1*. Deletion and mutation analyses of the *LIPIN1* promoter showed that ERRγ exerts its effect on the transcriptional regulation of *LIPIN1* via ERRE1 of the *LIPIN1* promoter, as confirmed by ChIP assay. We also determined that the gene transcription of *LIPIN1* by ERRγ is controlled by the competition between PGC-1α and small heterodimer partner. Additionally, ERRγ leads to the induction of hepatic LIPIN1 expression and diacylglycerol production *in vivo*. Finally, an inverse agonist of ERRγ, GSK5182, restores the impaired insulin signaling induced by LIPIN1-mediated PKCε activation. Our findings indicate that the selective control of ERRγ transcriptional activity by its specific inverse agonist could provide a novel therapeutic approach to the amelioration of impaired hepatic insulin signaling induced by LIPIN1-mediated PKCε activation.

LIPIN1 was originally identified via positional cloning as the mutated gene in fatty liver dystrophy (*fld*) mice, a mouse model of lipodystrophy (1, 2). LIPIN1 was later shown to be involved in lipid and glucose metabolism in a variety of tissues, including adipose tissue, liver, and skeletal muscle (3–6). *LIPIN1* mutations in *fld* mice lead to significant reductions in body fat, insulin resistance, and increased susceptibility to atherosclerosis (7). Conversely, high levels of LIPIN1 in adipose tissue or skeletal muscle of transgenic mice result in the obesity phenotype (6).

Early biochemical studies have shown that LIPIN1 indicates soluble Mg^{2+}-dependent phosphatidic acid phosphatase type 1 (PAP1) activity. PAP1 has been shown to perform a key function in the catalysis of PA[2] conversion to DAG, a direct precursor of triglycerides and phospholipids (8, 9). It has been reported that lipid infusions designed to increase plasma fatty acid in skeletal muscles and liver can result in the accumulation of fatty acid metabolites, such as DAGs, which function as second messengers for signaling cascades (10). DAG, in particular, has been shown to be a major activator of protein kinase Cε (PKCε), which accounts for the link between lipid accumulation and insulin signaling in the liver (11, 12). Recently, the PAP function of hepatic LIPIN1 has been reported to disrupt insulin

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[2] The abbreviations used are: PA, phosphatidic acid; ERR, estrogen-related receptor, DAG, diacylglycerol; PAP, phosphatidic acid phosphatase; SHP, small heterodimer partner; ERRE, ERR response element.

receptor signaling via the DAG-mediated induction of PKCε activity in mice (4). In addition, LIPIN1 has been reported to be induced by PGC-1α, which in turn functions as a transcriptional coactivator of the hepatic PGC-1α/peroxisome proliferator-activated receptor α regulatory pathway via its direct interaction (3).

The estrogen receptor-related receptor subfamily consists of three members, ERRα, -β, and -γ (NR3B1–3), which bind as either monomers or dimers (13). In adult mice, both ERRα and ERRγ are ubiquitously expressed, whereas ERRβ expression is restricted to the brain, kidney, and heart (14). The results of structural studies have indicated that the ERRs are constitutively active without endogenous ligands (15, 16), whereas several synthetic ligands that could either stimulate or repress the transcriptional activity of the ERRs by recruiting or disrupting the ERRs-coactivator interactions have been also identified (15). The ligand-independent transcriptional activities of ERRα and -γ depend on nuclear receptor coregulators, such as steroid receptor coactivator 2 (SRC-2), PGC-1α, receptor interacting protein 140 (RIP140), and small heterodimer partner (SHP), all of which are known regulators relevant to various metabolic programs (15, 17–26). Interestingly, ERRs are regulated by the peripheral circadian clock in key metabolic tissues, such as white and brown adipose tissues, muscle, and liver, thereby suggesting that these receptors may function as molecular links between the circadian oscillator and energy metabolism (27). Recently, ChIP-on-chip and expression analysis studies have demonstrated that ERRα and ERRγ are involved in the regulation of mitochondrial programs in cardiomyocytes (28). In particular, ERRγ is known to regulate a nuclearly encoded mitochondrial genetic network that coordinates postnatal metabolic transition in the heart (29). However, the role of ERRγ in the liver remains to be clearly determined.

In this study, we demonstrated that ERRγ is a previously unrecognized transcriptional regulator of hepatic LIPIN1 that leads to the induction of DAG and PKCε activation, which inhibits insulin signaling. The control of ERRγ transactivity by an inverse agonist could provide an effective approach to the LIPIN1-mediated regulation of DAG generation, which induces impaired insulin signaling.

EXPERIMENTAL PROCEDURES

Plasmid and Recombinant Adenoviruses—The reporter plasmids for *mLIPIN1*-luc (−1153/+56) and its serial deletion constructs were kindly provided by Dr. Seung-Hoi Koo in Sungkyunkwan University. Expression vectors for FLAG-ERRα, HA-ERRβ, and FLAG-ERRγ were described previously (30). The ERR response element (ERRE)-mutated *mLIPIN1* promoter was generated via site-directed mutagenesis (Stratagene), and the construct was confirmed by DNA sequencing. Ad-GFP, Ad-FLAG-ERRγ, Ad-PGC-1α, Ad-SHP, Ad-US, and Ad-shERRγ were described previously (31–33). All viruses were purified using $CsCl_2$ or an Adeno-X maxi purification kit (Clontech).

Cell Culture and Transient Transfection Assay—Maintenance of HepG2, AML12, and 293T cells was carried out as mentioned previously (34). In brief, the cells were maintained in DMEM (Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone) and antibiotics (Hyclone) in a humidified atmosphere containing 5% CO_2 at 37 °C. Transient transfections were conducted as described previously (34). In brief, cells were transfected with the indicated reporter plasmids together with expression vectors encoding for various genes using Lipofectamine 2000™ (Invitrogen), in accordance with the manufacturer's instructions. Total cDNA used for each transfection was adjusted to 1 μg/well via the addition of appropriate amounts of empty vector and cytomegalovirus (CMV)-β-galactosidase plasmids as an internal control. The cells were harvested at 40–48 h post-transfection for luciferase and β-galactosidase assays. The luciferase activity was normalized to β-galactosidase activity.

Culture of Primary Hepatocytes—Primary hepatocytes were isolated from Sprague-Dawley rats (male, 8 weeks) via the collagenase perfusion method as described previously (4). Cells were maintained in M199 media (Cellgro) overnight for attachment and adenoviral infection, or chemical treatments were carried out as described in the figure legends.

Animal Experiments—Male 8-week-old C57BL/6J mice (The Jackson Laboratory) were used for this study. All mice were acclimatized to a 12-h light/dark cycle at 22 ± 2 °C with free access to food and water in a specific pathogen-free facility. Ad-GFP and Ad-FLAG-ERRγ were injected into the tail veins of mice, and the mice were sacrificed on day 3 after the injection. All animal experiments were approved and performed by the Institutional Animal Use and Care Committee of the Korea Research Institute of Bioscience and Biotechnology.

RNA Isolation and Analysis—Total RNAs were isolated from cell lines or primary hepatocytes using TRIzol reagent (Invitrogen), in accordance with the manufacturer's instructions, and semi-quantitative and real time quantitative PCR analysis were conducted using primers for ERRα, ERRγ, LIPIN1, LIPIN2, SHP, PGC-1α and PDK4 as described previously (4, 30, 32). All data were normalized to ribosomal L32 expression.

Western Blot Analysis—50–100 μg of proteins from whole cell lysates or liver tissue by RIPA buffer were separated via 8–10% SDS-PAGE and then transferred to nitrocellulose membranes as described (34). The following primary antibodies were used for the immunoblotting assay: ERRγ (R&D Systems, PPMX), M2 for FLAG (Stratagene), LIPIN1 (Novus), PGC-1α (Santa Cruz Biotechnology), AKT (Cell Signaling), phospho-Ser-473 AKT (Cell Signaling), PKC (Santa Cruz Biotechnology), phospho-Ser-729 PKC (Santa Cruz Biotechnology), α-tubulin (AbFrontier), and β-tubulin (Cell Signaling).

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was carried out in accordance with the manufacturer's protocol (Upstate). In brief, AML12 cells were infected with Ad-ERRγ, Ad-PGC-1α, or Ad-SHP for 48 h, fixed in 1% formaldehyde, and then harvested. Soluble chromatin was immunoprecipitated using an antibody against ERRγ or PGC-1α. After recovering the DNA, PCR was carried out using primers encompassing the mouse *LIPIN1* promoter (−531/−256) forward 5′-CCTTGTCTCCTAGTAGTCTCGT-3′ and reverse 5′-TAGGCAGCCAAAGTCTTCTA-3′ and (−1010/−840) forward 5′-CACAAGCCACTCCTCCCTGG-3′ and reverse 5′-AGAGAGCCCAAGTATAATGT-3′.

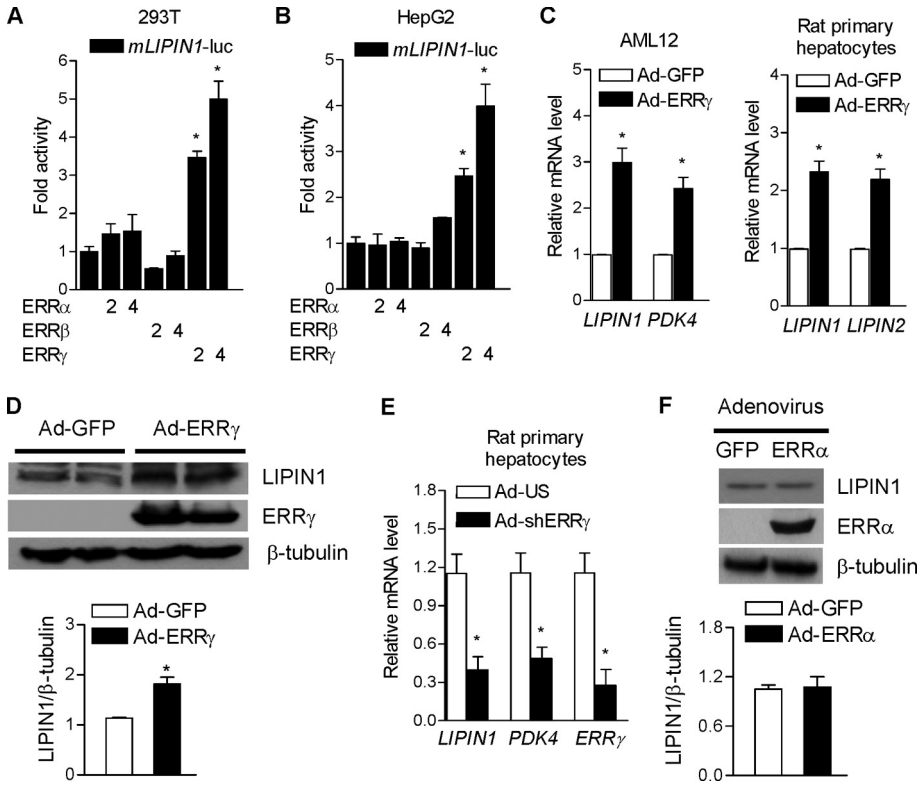

FIGURE 1. **ERRγ is a positive regulator of *LIPIN1* gene expression.** *A* and *B*, effect of the ERR subfamily on *mLIPIN1* promoter activity. Transient transfections were conducted using the indicated plasmids (2200 and 4400 ng) in 293T (*A*) and HepG2 (*B*). *C*, effect of Ad-GFP or Ad-ERRγ on mRNA levels for *LIPIN1* in AML12 cells (*left*) and rat primary hepatocytes (*right*). *D*, Western blot analysis showing the protein levels of LIPIN1 in AML12 cells infected with Ad-GFP and Ad-ERRγ (*top panel*). Quantitation of LIPIN1 protein levels is shown in the *bottom panel*. *E*, effect of knockdown of ERRγ in rat primary hepatocytes. Rat primary hepatocytes were infected with Ad-US or Ad-ERRγ, and real time quantitative PCR was conducted using isolated total RNA. *F*, Western blot analysis showing the effect of ERRα on LIPIN1 protein levels (*top panel*). AML12 cells were infected with Ad-GFP or Ad-FLAG-ERRα and Western blot analysis was carried out. Quantitation of LIPIN1 protein levels is shown in the *bottom panel*. *Error bars* in *A*--*F* represent mean ± S.E. (*, $p < 0.05$, *t* test).

Measurement of DAGs—DAGs were extracted from cell pellets or liver tissues with chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. A known amount of 1,2-dinonadecanoin (19:0 DAG) was added as an internal standard. After vortexing and centrifugation, the lower organic phase was collected. Samples in organic phase were evaporated and dissolved in hexane/methylene chloride/methyl *tert*-butyl ether for loading onto the diol-bonded solid phase extraction column (Waters, Inc., Milford, MA) under vacuum. DAGs were eluted as described previously by Pacheco *et al.* (35). Extracted lipids were dried under nitrogen gas and redissolved in methanol. Then the contents of DAGs among extracted lipids were measured via liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a method described previously (36) with some modifications, employing a bench-top tandem mass spectrometer, API 4000 Q-trap (Applied Biosystem, Framingham, MA) interfaced with an atmospheric pressure chemical ionization source and an Agilent series 1200 micro-pump equipped with an autosampler. Five selected DAG molecular species (di-16:0, di-18:1, 16:0–18:1, 18:0–18:2, and 18:0–20:4) were separated by a Gemini C6-phenyl column (50 × 2.0 mm inner diameter, 3 μm, Phenomenex, Torrance, CA) and ionized in positive atmospheric pressure chemical ionization mode. The mobile phase was 98% methanol with a flow rate of 0.25 ml/min, and 10 μl of sample was analyzed.

Statistical Analysis—Data were expressed as means ± S.E. Statistical analysis was conducted via Student's *t* test and one-way analysis of variance among groups. Differences were considered statistically significant at $p < 0.05$.

RESULTS

ERRγ Positively Regulates the Gene Expression of LIPIN1 in Hepatocytes—To assess the possibility that the expression of the *LIPIN1* gene is regulated by nuclear receptor ERRs, we carried out transient transfection with vectors expressing ERRs and the *mLIPIN1* promoter in 293T and HepG2 cells. Interestingly, ERRγ, but not ERRα and ERRβ, significantly increased *mLIPIN1* promoter activity in both 293T and HepG2 cells (Fig. 1, *A* and *B*). In an effort to further examine the transcriptional regulation of *LIPIN1* by ERRγ, we conducted adenoviral overexpression of GFP (Ad-GFP) or ERRγ (Ad-ERRγ) in a nontransformed mouse liver (AML12) cell line and rat primary hepatocytes. As anticipated, Ad-ERRγ elicited a marked increase in *LIPIN1* mRNA and protein levels (Fig. 1, *C* and *D*). Expression of pyruvate dehydrogenase 4 (PDK4), a known ERRγ target gene, was also higher in Ad-ERRγ-infected AML12 cells compared with controls. Interestingly, *LIPIN2*, an isoform of LIPIN, also evidenced a 2-fold induction by Ad-ERRγ in rat primary hepatocytes. Conversely, the reduction of endogenous ERRγ via the adenoviral overexpression of short hairpin RNA for ERRγ

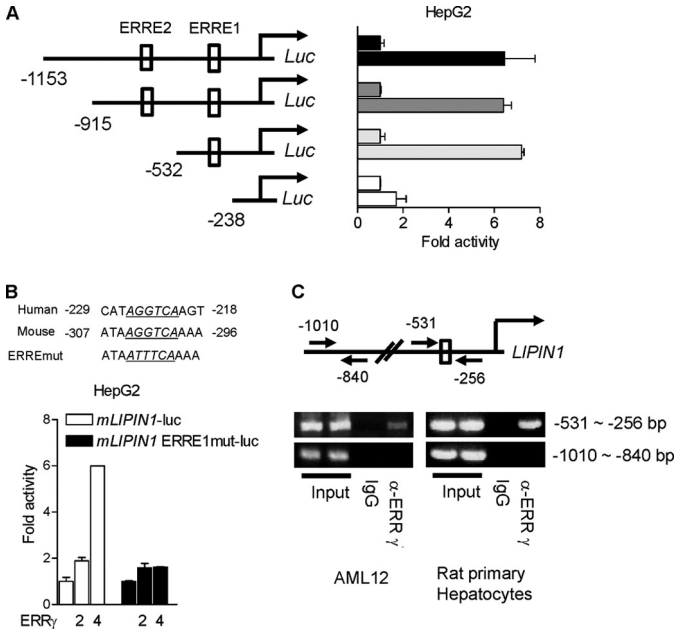

FIGURE 2. **LIPIN1 is directly regulated by ERRγ.** *A*, mapping of *mLIPIN1* promoter. HepG2 cells were transfected with serial deletion constructs of the *mLIPIN1* promoter and pcDNA3-FLAG-ERRγ (200 ng). *B*, ERRE-dependent activation of the *mLIPIN1* promoter. Alignment of potential ERRE in human and mouse *LIPIN1* promoter is shown in the *top panel*. HepG2 cells were transfected with the indicated plasmids (2200 and 4400 ng) (*bottom panel*). *C*, ChIP assay shows the occupancy of ERRγ on the ERRE of the *mLIPIN1* promoter. Ad-ERRγ was infected into AML12 cells or rat primary hepatocytes, and soluble chromatin was immunoprecipitated with ERRγ antibody or IgG. 10% of the soluble chromatin was used as an input. *Error bars* in *A* and *B* represent mean ± S.E.

(Ad-shERRγ) in rat primary hepatocytes resulted in a considerable reduction of basal *LIPIN1* expression (Fig. 1*E*). However, Ad-ERRα had no measurable effect on LIPIN1 expression (Fig. 1*F*). We conclude that ERRγ is a transcriptional inducer of hepatic LIPIN1 expression.

LIPIN1 Is a Direct Target of ERRγ—Next, the molecular mechanisms by which ERRγ regulates *LIPIN1* gene transcription were explored. Close investigation of the *LIPIN1* promoter revealed two putative ERRE motifs (ERRE1 and ERRE2) conserved in both humans and mice. To determine whether ERRγ could directly regulate *LIPIN1* transcription through these putative ERREs, we generated serial deletion constructs of *LIPIN1* promoter containing either ERRE1 or ERRE2 and performed transient transfection in HepG2 cells. As is shown in Fig. 2*A*, the activity of the ERRE2-deleted *LIPIN1* promoter still evidenced a 7-fold induction by cotransfection with ERRγ, whereas that of the ERRE1-deleted *LIPIN1* promoter was profoundly reduced. Moreover, the dose-dependent activation of wild-type *LIPIN1* promoter by ERRγ was almost wholly blocked in the ERRE1-mutated *LIPIN1* promoter (*mLIPIN1* ERREmut-luc) (Fig. 2*B*). Finally, a ChIP assay in AML12 and rat primary hepatocytes demonstrated that ERRγ was strongly recruited to the ERRE1 region of the *LIPIN1* promoter but not to the upstream region (Fig. 2*C*). These findings strongly indicate that ERRγ is a direct transcriptional regulator of LIPIN1.

Transcriptional Activity of ERRγ on LIPIN1 Is Controlled by Coregulators—The transcriptional activity of ERRγ is known to be regulated by coregulators such as PGC-1α and SHP (18, 21), suggesting that these coregulators could be involved in the regulation of ERRγ-mediated LIPIN1 expression. As anticipated, PGC-1α potentiated and SHP inhibited the ERRγ-mediated induction of *LIPIN1* promoter activity in a dose-dependent manner (Fig. 3*A*). Moreover, SHP significantly attenuated the potentiation of PGC-1α for the ERRγ-mediated activation of *LIPIN1* promoter. Consistent with the observed promoter activity, the ERRγ-mediated induction of *LIPIN1* mRNA was increased further by PGC-1α in rat primary hepatocytes, which was largely inhibited by SHP (Fig. 3*B*). To further evaluate the involvement of these coregulators in ERRγ-mediated LIPIN1 transcription, we conducted a ChIP assay using AML12 cells infected with either Ad-SHP or Ad-PGC-1α. As anticipated, the recruitment of ERRγ and PGC-1α on the ERRE1 of the *LIPIN1* promoter was increased significantly, whereas Ad-SHP reduced the PGC-1α occupancy on ERRE1 without changing ERRγ occupancy (Fig. 3*C*). We subsequently evaluated the role of ERRγ in the PGC-1α-mediated induction of LIPIN1. The ablation of endogenous ERRγ significantly reduced PGC-1α-induced *LIPIN1* mRNA levels in rat primary hepatocytes (Fig. 3*D*). Additionally, the knockdown of endogenous ERRγ significantly reduced both basal and PGC-1α-induced LIPIN1 protein levels in AML12 cells (Fig. 3*E*). These results demonstrate that the transcriptional regulation of *LIPIN1* by ERRγ depends on the coregulators.

Inverse Agonist of ERRγ Inhibits LIPIN1-mediated DAG Production—Based on the molecular mechanism by which ERRγ regulates *LIPIN1* gene expression, we also attempted to determine whether ERRγ results in the induction of hepatic LIPIN1 expression and DAG production *in vivo*, because LIPIN1 has a PAP1 function to catalyze the conversion of PA to DAG, which is a precursor of triglyceride (8, 9). Consistent with the *in vitro* results, the hepatic overexpression of ERRγ via the tail vein injection of Ad-ERRγ resulted in a marked induction of LIPIN1 compared with controls (Fig. 4*A*). Additionally, hepatic DAG levels were also significantly increased by overexpression (Fig. 4*B*), thereby suggesting that ERRγ does indeed regulate hepatic LIPIN1 expression, resulting in DAG production both *in vitro* and *in vivo*.

GSK5182, a 4-hydroxytamoxifen analog, is an inverse agonist that selectively inhibits the transactivity of ERRγ relative to estrogen receptor α due to additional noncovalent interaction with Tyr-326 and Asn-346 (37). Based on the ability of ERRγ to stimulate LIPIN1 expression, we subsequently evaluated the effects of GSK5182 on the regulation of LIPIN1 by ERRγ in HepG2 cells. As shown in Fig. 4*C*, GSK5182 resulted in a marked reduction of ERRγ-induced *LIPIN1* promoter activity in a dose-dependent manner. Additionally, the ERRγ-mediated induction of *LIPIN1* mRNA was significantly reduced by GSK5182 treatment in rat primary hepatocytes but not that of ERRγ Y326A, which cannot interact with GSK5182; this suggests that the inverse agonist selectively inhibits the transcriptional activity of ERRγ (Fig. 4*D*).

Next, we attempted to determine whether the inverse agonist of ERRγ could inhibit LIPIN1-mediated DAG production. As shown in Fig. 4*E*, the adenoviral overexpression of ERRγ in AML12 cells significantly induced cellular DAG levels as compared with controls, which was blocked by Ad-

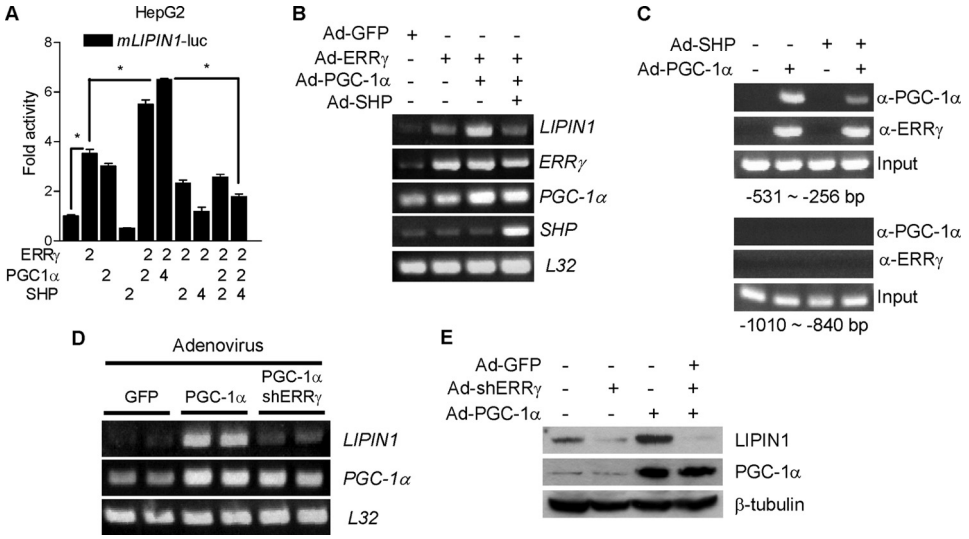

FIGURE 3. **Transcriptional activity of ERRγ for *LIPIN1* transcription is regulated via competition between PGC-1α and SHP.** *A*, transient transfection assay showing competition between PGC-1α and SHP on ERRγ-induced *mLIPIN1* promoter activity. HepG2 cells were transfected with indicated plasmids (2200 and 4400 ng). *B*, effects of PGC-1α and SHP on ERRγ-induced *mLIPIN1* gene expression in rat primary hepatocytes. *C*, ChIP assay showing the competition between PGC-1α and SHP on ERRE of *mLIPIN1* promoter. Ad-PGC-1α and Ad-SHP were infected into AML12 cells, and soluble chromatin was immunoprecipitated with α-PGC-1α, α-ERRγ, or IgG. 10% of the soluble chromatin was used as input. *D*, effect of knockdown of ERRγ on PGC-1α-induced *LIPIN1* gene expression. Ad-PGC-1α and Ad-shERRγ were infected in rat primary hepatocytes, and RT-PCR was carried out using isolated total RNA. *E*, Western blot analysis showing the protein levels of LIPIN1 in AML12 cells infected with Ad-PGC-1α or Ad-shERRγ. *Error bars* in *A* represent mean ± S.E. (*, $p < 0.05$, *t* test).

shLIPIN1, thereby indicating that LIPIN1 is a downstream mediator of ERRγ in DAG generation. Furthermore, the ERRγ-mediated induction of DAG was largely attenuated by GSK5182 treatment, thus suggesting that the inhibition of transcriptional activity of ERRγ by GSK5182 could be an attractive means for the suppression of LIPIN1-mediated DAG production.

GSK5182 Restores Impaired Insulin Signaling via LIPIN1-mediated PKCε Activation—Abundant evidence indicates that increased production is associated with obesity-related insulin resistance via the activation of PKC in peripheral tissues (10–12). Recently, it has been reported that the induction of LIPIN1 by TORC2 stimulates DAG production through its PAP activity and disrupts hepatic insulin receptor signaling via the DAG-mediated stimulation of PKCε activity in the livers of mice (4). Therefore, we subsequently attempted to determine whether the ERRγ-mediated induction of LIPIN1 expression interrupts insulin signaling via the stimulation of PKCε activity. AML12 cells were infected with Ad-GFP and Ad-ERRγ in the absence or presence of GSK5282. As expected, the adenoviral overexpression of ERRγ significantly increased LIPIN1 expression and the phosphorylation of PKCε (Ser-729) as compared with GFP, which was dramatically reduced by GSK5182 treatment (Fig. 5, *A* and *B*). Considering the inhibitory effect of GSK5182 on PKCε activation, we next attempted to determine whether the inverse agonist of ERRγ restores the inhibition of insulin-mediated AKT phosphorylation (Ser-473) via the ERRγ-dependent induction of LIPIN1 expression and PKCε activation. Surprisingly, the insulin-mediated phosphorylation of AKT was significantly diminished by the ERRγ-mediated increase of LIPIN1 expression and PKCε phosphorylation, which was completely restored by GSK5182 treatment (Fig. 5*B*). These results indicate that the inactivation of ERRγ by its specific inverse agonist could ameliorate the LIPIN1-mediated dysregulation of hepatic insulin receptor signaling.

DISCUSSION

In this study, we demonstrated that the nuclear receptor ERRγ is a previously unrecognized transcriptional regulator of hepatic LIPIN1, which is known to have a PAP function, catalyzing the conversion of PA to DAG. PA and DAG, major precursors of phospholipid biosynthesis, perform a pivotal role in cellular signaling cascades, energy storage, and lipid biosynthetic pathways. Recently, DAG, as a second messenger for signaling cascades, has been shown to activate PKCθ and PKCε in muscle and liver, respectively, thereby inhibiting insulin receptor signaling, indicating that DAG functions as a link between lipid accumulation and insulin signaling (11, 12). Consistent with these results, it has also been reported that the induction of LIPIN1 by TORC2 results in the perturbation of hepatic insulin receptor signaling via the induction of DAG and PKCε activity in the livers of mice, thereby suggesting that the control of hepatic LIPIN1 expression is important for the amelioration of impaired insulin receptor signaling via DAG-mediated PKCε activation (4). Therefore, based on our findings, the inhibition of transcriptional activity of ERRγ by its inverse agonist could constitute an attractive means for ameliorating the impaired insulin receptor signaling induced by LIPIN1-mediated DAG production.

In addition to the cytosolic function of LIPIN1 as a PAP, it evidences a putative nuclear function as a transcriptional coactivator. Finck *et al.* (3) have demonstrated that hepatic LIPIN1 expression is induced by PGC-1α under fasting conditions and functions as an amplifier of nuclear receptors peroxisome proliferator-activated receptor α and PGC-1α in a complex that regulates fatty acid oxidation gene expression. However, the molecular mechanism by which PGC-1α induces

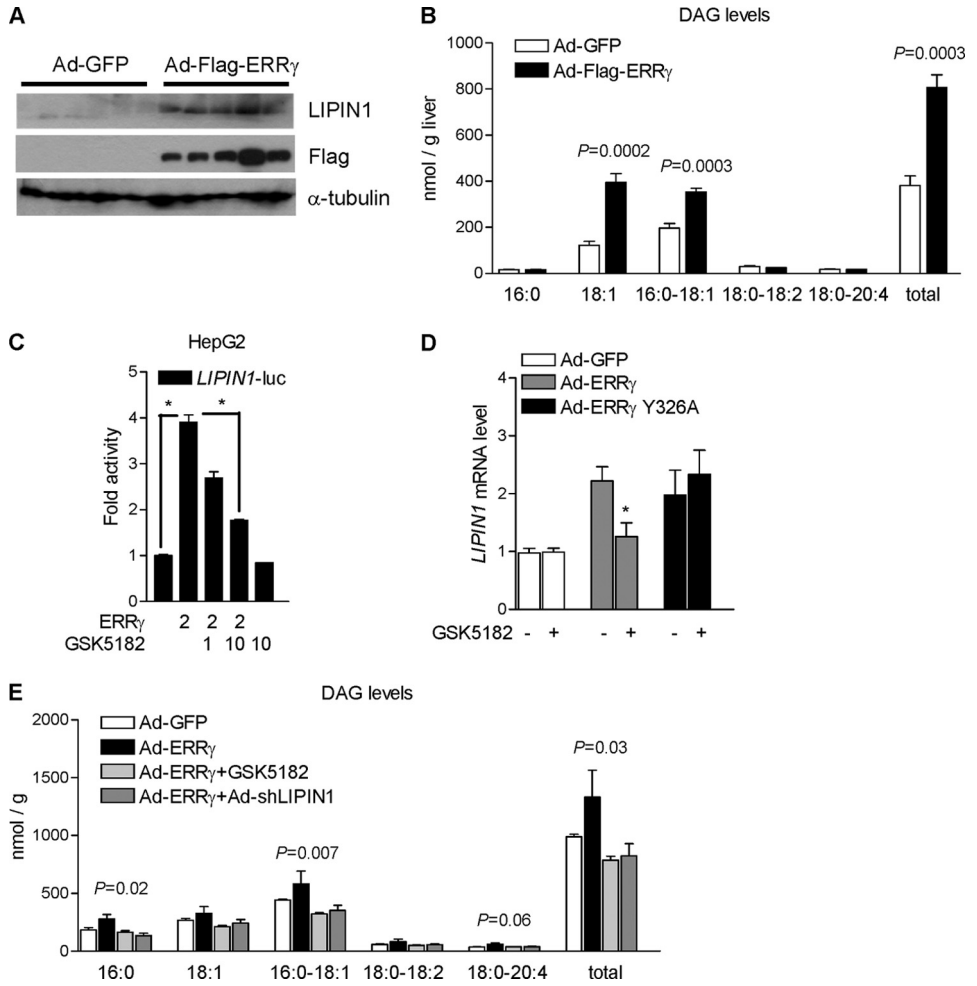

FIGURE 4. **Inverse agonist of ERRγ inhibits LIPIN1-mediated induction of DAG.** *A* and *B*, effect of ERRγ overexpression on LIPIN1-mediated induction of DAG in mouse livers. Western blot analysis shows ERRγ and LIPIN1 expression (*A*) and DAG levels (*B*) in the livers of C57BL/6 mice injected with Ad-GFP and Ad-FLAG-ERRγ ($n = 5$ per group). *C*, effect of GSK5182 on ERRγ-mediated *LIPIN1* promoter activity. HepG2 cells were transfected with the indicated plasmids (2200 ng) and treated with GSK5182 (*1*, 1 μM; *10*, 10 μM) for the final 24 h. *D*, effect of GSK5182 on ERRγ-mediated induction of *LIPIN1* in rat primary hepatocytes. Ad-GFP, Ad-ERRγ, or Ad-ERRγ Y326A was infected in rat primary hepatocytes and treated with GSK5182 (10 μM) for the final 24 h. *E*, DAG levels. AML12 cells were infected with Ad-GFP, Ad-ERRγ, and Ad-ERRγ plus Ad-shLIPIN1 and treated with GSK5182 (10 μM) for 24 h (one-way analysis of variance). *Error bars* in *B–E* represent mean ± S.E. (*, $p < 0.05$, *t* test).

LIPIN1 expression remains unclear. Here, we have demonstrated that the transcriptional regulation of PGC-1α for LIPIN1 is achieved through the ERRγ-binding site on the *LIPIN1* promoter (Fig. 4*C*) and that the induction of LIPIN1 by PGC-1α was almost completely blocked by the knockdown of ERRγ (Fig. 4, *D* and *E*), thereby suggesting that ERRγ is a key transcriptional mediator of the PGC-1α-mediated induction of LIPIN1.

However, unlike the function of LIPIN1 as a coactivator in the nucleus, our data indicate that hepatic LIPIN1 has a cytosolic function, activating DAG production. These results are consistent with previous findings showing that hepatic LIPIN1 functions as a cytosolic PAP in the regulation of the insulin signaling cascade (4). Recent studies also demonstrated that hepatic LIPIN1 is associated with triglyceride synthesis and secretion, although the results between *in vitro* and *in vivo* conditions are somewhat inconsistent (38, 39), thereby suggesting that the role of hepatic LIPIN1 appears to involve a cytosolic function related to *de novo* lipogenesis rather than a nuclear function related to fatty acid oxidation. However, functional differences in hepatic LIPIN1 according to its localization will require further characterization.

Studies of the molecular mechanism underlying the transcriptional regulation of LIPIN1 expression have progressed greatly in recent years. It is known to be induced by glucocorticoids in adipose tissues and the liver and by β-adrenergic signaling in skeletal muscle through the glucocorticoid receptor and the orphan nuclear receptor NOR-1 (40, 41). It has also been reported that LIPIN1 is a target of SREBP-1 (sterol regulatory element-binding protein 1) and nuclear factor Y in hepatoblastoma cells (42). While this study was being carried out, one report was published regarding the role of LIPIN1 in cardiac myocytes. In a study conducted using cultured ventricular myocytes and cardiac tissue, *LIPIN1* gene expression and its PAP activity were shown to be up-regulated by the PGC-1α-dependent activation of ERRα or ERRγ via the ERR-response elements within the first intron of the *LIPIN1* gene (43). These results are similar to those of our study conducted in hepatocytes, with the exception of the ERRγ-specific regulation of hepatic *LIPIN1* transcription among the ERR subfamily via

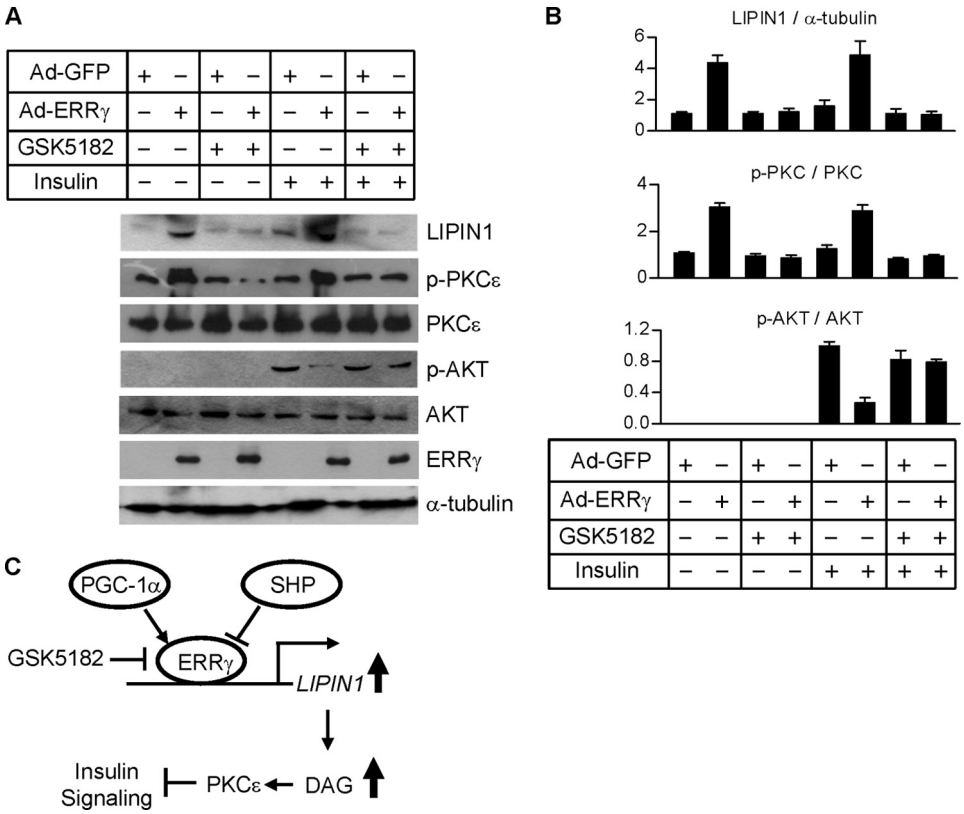

FIGURE 5. **Inactivation of ERRγ by GSK5182 leads to improved insulin signaling via the inhibition of LIPIN1-induced PKCε activation.** *A*, Western blot analysis showing the effect of GSK5182 on the phosphorylation of AKT and PKCε. AML12 cells were infected with Ad-GFP or Ad-FLAG-ERRγ and then treated with GSK5182 (10 μM) for the final 24 h. Insulin (100 nM) stimulation was carried out for the final 10 min before cell harvest. *B*, quantitation of LIPIN1, p-PKC, and p-AKT protein levels. *C*, schematic diagram of regulation of hepatic LIPIN1 by nuclear receptor ERRγ. ERRγ regulates *LIPIN1* gene expression leading to DAG production, which is regulated by PGC-1α and SHP. Subsequently, DAG increases PKCε activation, inducing the inhibition of insulin signaling. The inverse agonist of ERRγ and GSK5182 restores the inhibition of insulin signaling by LIPIN1-mediated DAG production. *Error bars* in *B* represent mean ± S.E.

ERRE1 in the *LIPIN1* promoter. However, the SHP promoter is activated by ERRγ but not ERRα or ERRβ (18), and Dufour *et al.* (28) demonstrated that ERRα and ERRγ could regulate the same direct target genes, thereby suggesting the complexity of their function in terms of the transcriptional output of each ERR.

In this study, we have revealed a molecular mechanism by which the nuclear receptor ERRγ regulates hepatic LIPIN1 expression, resulting in the production of DAG and the activation of PKCε (Fig. 5*C*). Interestingly, the regulation of hepatic LIPIN1 by ERRs is ERRγ-specific. The overexpression of ERRγ resulted in the induction of LIPIN1 expression and promoter activity in hepatocytes. We also demonstrated that the ERRγ-mediated transcriptional regulation of LIPIN1 is achieved via direct binding of the *LIPIN1* promoter; competition between PGC-1α and SHP is also involved in the regulation of LIPIN1 by ERRγ. Additionally, we determined that GSK5182, the inverse agonist of ERRγ, resulted in a marked reduction of ERRγ-induced LIPIN1 expression and DAG production via the inhibition of ERRγ transactivity. Finally, we have demonstrated here that the inverse agonist attenuates LIPIN1-mediated DAG production, leading to the inhibition of insulin receptor signaling, and it also restores the PKCε-mediated perturbation of insulin signaling. These findings indicate that the control of ERRγ by its inverse agonist might provide an attractive means for the regulation of LIPIN1-mediated DAG generation and PKCε activation, thereby disrupting hepatic insulin receptor signaling.

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