G Protein-coupled Receptor 40 (GPR40) and Peroxisome Proliferator-activated Receptor γ (PPARγ)

AN INTEGRATED TWO-RECEPTOR SIGNALING PATHWAY

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Peroxisome proliferator-activated receptor γ (PPARγ) ligands have been widely used to treat type 2 diabetes mellitus. However, knowledge of PPARγ signaling remains incomplete. In addition to PPARγ, these drugs also activate G protein-coupled receptor 40 (GPR40), a Gαq-coupled free fatty acid receptor linked to MAPK networks and glucose homeostasis. Notably, p38 MAPK activation has been implicated in PPARγ signaling. Here, rosiglitazone (RGZ) activation of GPR40 and p38 MAPK was found to boost PPARγ-induced gene transcription in human endothelium. Inhibition or knockdown of p38 MAPK or expression of a dominant negative (DN) p38 MAPK or GPR40 abolished the ability of RGZ to induce phosphorylation and expression of PGC1α in primary human pulmonary artery endothelial cells (PAECs) was suppressed by knockdown of either p38 MAPK or GPR40. GPR40/PPARγ signal transduction was dependent on p38 MAPK activation and induction of PPARγ co-activator-1 (PGC1α). Silencing of p38 MAPK or GPR40 abolished the ability of RGZ to induce phosphorylation and expression of PGC1α in PAECs. Knockdown of PGC1α, its essential activator SIRT1, or its binding partner/co-activator EP300 inhibited RGZ induction of PPARγ-regulated genes in PAECs. RGZ/GPR40/p38 MAPK signaling also led to EP300 phosphorylation, an event that enhances PPARγ target gene transcription. Thus, GPR40 and PPARγ can function as an integrated two-receptor signal transduction pathway, a finding with implications for rational drug development.

Obesity-associated type 2 diabetes mellitus has reached epidemic proportions in the United States and is a major risk factor for coronary artery disease and stroke (1). Thiazolidinediones (TZDs), synthetic insulin-sensitizing drugs that activate peroxisome proliferator-activated receptor γ (PPARγ), have been widely used to treat this disease (2, 3). In addition to lowering glucose levels (3), TZDs also lower blood pressure (4), improve lipid profiles (5), and reduce vascular inflammation (6). These effects in non-adipose tissues have raised the possibility that PPARγ ligands may be more broadly useful in vascular disorders, such as atherosclerosis (7), pulmonary arterial hypertension (8), and septic shock (9). However, side effects, including weight gain, fluid retention, congestive heart failure, and bone fractures, have been linked to TZDs (10). These adverse effects underscore our still incomplete understanding of PPARγ signaling and the need to develop safer, more effective PPARγ ligands (10).

The direct binding of TZDs and other ligands to PPARγ activates two major but distinct signal transduction pathways. Cis-activation drives transcription through ligand-dependent recruitment of co-activators, such as PPARγ co-activator-1α (PGC1α), PPARγ dimerization with the retinoid X receptor (11), and the binding of this complex to peroxisome proliferator response elements (PPREs) in the promoter region of target genes. Trans-repression of inflammatory response genes re-
quires the covalent modification of PPARγ at lysine 395 by the small ubiquitin-like modifier with subsequent tethering of PPARγ to nuclear receptor co-repressor and histone deacetylase complexes at NFκB and AP-1 sites (12).

Besides these primary ligand-dependent pathways, adjunctive post-translational modifications of PPARγ and its co-activators also regulate PPARγ signaling. ERK and JNK directly phosphorylate PPARγ on serine 112 and inhibit its transcriptional activation (13–16). Cyclin-dependent kinase 5 phosphorylates PPARγ on serine 273, modulating the PPARγ transcriptional program (17). Furthermore, p38 MAPK phosphorylation of PGC1α (18–21) and CBP/EP300 (22) facilitate chromatin remodeling and PPARγ-dependent transcription. PGC1α, a key regulator of PPARγ, is additionally acetylated by GCN5 (23) and deacetylated by SIRT1 (24, 25), reducing and enhancing its co-transcriptional activity, respectively.

Although the anti-diabetic activity of TZDs was first reported in 1982 (26), recognition as lipid/agonists of the orphan nuclear receptor PPARγ came more than a decade later (27, 28). More recently, TZDs were found to bind to and activate GCαγ protein-coupled receptor 40 (GPR40) (29–34), a cell membrane receptor associated with free fatty acid- and glucose-induced insulin secretion (35, 36), effects that overlap with those of PPARγ (37, 38). Importantly, GPR40 signaling causes rapid activation of ERK, p38 MAPK, and JNK (31, 33). Whereas NO activation of p38 MAPK initiated PPARγ signaling in human endothelial cells (39), others have also associated p38 MAPK with PPARγ-related effects in adipocytes (20, 40). Collectively, these results suggest that TZD signaling through GPR40 with subsequent activation of p38 MAPK might modulate PPARγ transcriptional activity in human endothelium.

This investigation sought to determine whether TZD activation of GPR40 and p38 MAPK influences PPARγ signaling in human endothelial cells and, if so, to explore the underlying mechanism. A two-receptor paradigm for PPARγ signal transduction is proposed with implications for the development of PPARγ therapeutics.

**Experimental Procedures**

**Reagents**—Rosiglitazone (RGZ), pioglitazone (PGZ), N-nitro-L-arginine methyl ester hydrochloride (L-NAME), and DETA NONOate (DTANO) were obtained from Cayman Chemical (Ann Arbor, MI). SB202190 (SB) was from EMD Chemicals (Gibbstown, NJ). GW1100 was purchased from OTAVA Ltd. (Ontario, Canada). PrePore reporter constructs containing three copies of PPRE upstream of the reporter gene 3-thymidine kinase-chloramphenicol acetyltransferase (PPRE-CAT) or 3-thymidine kinase-luciferase (PPRE-LUC), were kindly supplied by Dr. Ronald M. Evans (Salk Institute, La Jolla, CA) (41). Dr. Ae-Kyung Yi (University of Tennessee Health Science Center) provided the dominant negative p38 MAPK (DN-p38 MAPK) expression plasmid (42). The dominant-negative G-protein αq mutant (DNGαq; Q209L/D277N) expression plasmid was obtained from the UMR cDNA Resource Center (University of Missouri, Rolla, MO). This mutant has a lowered affinity for guanine nucleotides and an enhanced affinity for xanthine nucleotides, resulting in stable and specific complexes with cognate receptors that compete with endogenous wild-type G-proteins (43). Expression plasmids for PPARγ2, (catalogue no. 8895), EP300 (catalogue no. 23252), and PGC1α (catalogue no. 10974) were purchased from Addgene Inc. (Cambridge, MA). The GPR40 expression plasmid was from OriGene (Rockville, MD). FuGENE® 6 transfection reagent, plasmid pRL-TK expressing Renilla luciferase, used as an internal control for cell transfection, and the Dual-Luciferase® reporter assay system were from Promega (Madison, WI). Specific On-TARGETplus SMARTpool siRNA for p38α MAPK, GPR40, PGC1α, SIRT1, or EP300 and control siGENOME non-targeting siRNA were purchased from Dharmacon Inc. (Lafayette, CO). Specific GPR40 shRNA pool and its control plasmid were from Qiagen Inc. (Valencia, CA). Specific TaqMan® primers/probes were purchased from Applied Biosystems (Foster City, CA).

**Cell Culture**—EA.hy926 cells, a hybrid human endothelial cell line, were obtained from ATCC (Manassas, VA). A retrovirus-transfected HeLaS cell line, stably expressing FLAG-tagged PPARγ (HeLaS/F-PPARγ), was kindly provided by Dr. Kai Ge (National Institutes of Health, NIDDK, Bethesda, MD) (44). Both EA.hy926 and HeLaS/F-PPARγ cell lines were maintained in DMEM supplemented with 10% FBS, d-glucose (4.5 g/liter), l-glutamine (2 mmol/liter), sodium pyruvate (1 mmol/liter), penicillin (100 units/ml), and streptomycin (100 mg/ml). Primary human pulmonary artery endothelial cells (PAECs) were purchased from Lonza (Walkersville, MD) and used at passages 1–4. PAECs were cultured in endothelial growth medium 2 (EGM2™) supplemented with growth factors (EGM2™ SingleQuiot kit) from Lonza containing 2% FBS on flasks precoated with type 1 collagen (BD Biosciences). In experiments using TZDs, charcoal-stripped FBS was used instead of regular FBS. Phenol red-free DMEM was used in experiments using DTANO or L-NAME.

**Reporter Gene Assay**—EA.hy926 cells (2 x 10⁵/2 ml/well) were seeded in 6-well plates 16 h prior to transfection with 100 ng of PPRE reporter (PPRE-CAT or PPRE-LUC), 100 ng of internal control pRL-TK, and 50 ng of PPARγ2 expression plasmid in the presence or absence of additional expression plasmids, including DN-p38 MAPK, DN-Gαq, GPR40, PGC1α, and EP300, as indicated. FuGENE® 6 transfection reagent was utilized at a ratio of 3 μl/μg of DNA. Twenty-four hours after transfection, cells were treated for an additional 24 h as indicated in the corresponding figure legends. Chronamphenicol acetyltransferase and luciferase activities were then measured using the CAT ELISA (Roche Diagnostics) and the Dual-Luciferase® reporter assay system (Promega), respectively. In reporter experiments with gene knockdown, cells were co-transfected with siRNA, shRNA, or their controls for 48 h, followed by 24-h stimulation. Non-targeting control or p38α MAPK siRNA was transfected using Nucleofector kits (Amaxa, Gaithersburg, MD), as described previously (39). GPR40 shRNA pool or its control plasmid was transfected using FuGENE® 6.

**PAEC siRNA Silencing**—PAECs (2 x 10⁵/2 ml/well) were seeded in 6-well plates 16 h prior to transfection. GPR40, p38α MAPK, PGC1α, SIRT1, and EP300 siRNAs or non-targeting siRNA controls (30 nm) were transfected using DharmaFECT 1 (Dharmacon Inc.) in OPTI-MEM medium (Life Technologies,
Inc.). Eight hours post-transfection, cells were washed once with PBS and cultured for 48 h in EGM2™ medium supplemented with growth factors and charcoal-stripped fetal calf serum, followed by treatment with RGZ for 24 h before measurement of PPARγ target gene mRNA or protein.

Detection of PPARγ Binding to Specific DNA Sequence—EA.hy926 cells were treated for 1 h with RGZ (10 μM) or vehicle control with or without SB (1 μM) pretreatment for 40 min, as indicated. Nuclear extracts (3–4 μg) were then prepared for TransAM® PPARγ ELISA (Active Motif, Carlsbad, CA), which detects human PPARγ1/2 binding to PPRE consensus sequence and does not cross-react with PPARα or PPARδ.

Western Blotting and Quantitative Real-time TaqMan® PCR—For Western blotting, samples (30 μg of whole cell lysates) were applied to a 4–12% Novex® Tris-glycine gel (Invitrogen) or a 4–15% Mini-PROTEAN® TGX gel (Bio-Rad) and subjected to electrophoretic separation. Separated protein was electrically transferred to a nitrocellulose membrane. The blot was blocked with 5% nonfat dry milk in PBS with 1% Tween 20. Anti-phospho-p38 MAPK (p38, catalog no. 9212), anti-PPARγ (catalog no. 2430), anti-EP300 (catalog no. D9B6; all from Cell Signaling Technology Inc., Danvers, MA), anti-GPR40 (catalog no. 3393-1; Epitomics, Burlingame, CA), and anti-SIRT1 (catalog no. 07-131; Millipore, Billerica, MA) primary antibodies were all used at a 1:1000 dilution. Anti-PGC1α (catalog no. 101707; Cayman Chemical) and anti-phospho-EP300 primary antibodies (anti-Ser(P)-1834; catalog no. PA5-12735; Thermo Scientific, Rockford, IL) were used at 1:200 and 1:500 dilutions, respectively. A secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG, was used at a 1:10,000 dilution. All antibodies were diluted in 5% nonfat dry milk in PBS with 1% Tween 20. The protein bands were detected using SuperSignal® West Femto chemiluminescence substrate (Thermo Scientific). Densitometry analysis of blots was performed using Image Lab™ software (Bio-Rad).

Total RNA was extracted using the RNeasy kit (Qiagen), and cDNA was synthesized with iScript™ cDNA synthesis kits (Bio-Rad). TaqMan® PCR was performed with the Applied Biosystems Vii™ 7 instrument.

Immunoprecipitation—PAECs were transfected with various siRNAs, as indicated. Forty-eight hours post-transfection, cells were treated with RGZ (10 μM) or vehicle control for 1 and 4 h to examine effects on PGC1α phosphorylation and acetylation, respectively, before preparation of nuclear protein and whole cell lysates. Immunoprecipitation of nuclear protein (40 μg) was performed with anti-phosphoserine (catalog no. AB1603) and anti-phosphothreonine (catalog no. AB1607) antibodies from Millipore. Immunoprecipitation of whole cell lysates (500 μg) used anti-acetyl-lysine antibody (catalog no. 06-933; Millipore). After incubation with rotation at 4°C overnight, Dynabeads® protein G (1.5 mg; Invitrogen) was added. Immunoprecipitates were then subjected to Western blotting with anti-PGC1α antibody (catalog no. 101707; Cayman Chemical).

Chromatin Immunoprecipitation (ChIP) Assay—PAECs were pretreated with SB (1 μM), GW1100 (20 μM), or vehicle control (CTRL) for 40 min and then incubated with RGZ (10 μM) or vehicle CTRL for another 24 h. The ChIP assay was carried out using the EZ ChIP™ kit (Millipore) as instructed by the manufacturer. Briefly, cells were cross-linked with 1% formaldehyde and then fragmented using a Misonix sonicator with microtip probe 4418 at a power setting of 4 and a 30% duty cycle. Sonication was performed three times for 10 s with a 50-s cooling on ice between pulses, shearing chromatin into 200–1000-bp fragments. Precipitation was carried out overnight at 4°C with anti-PPARγ (catalog no. SC-7196; Santa Cruz Biotechnology, Inc., Dallas, TX), anti-EP300 (catalog no. PA1-848; Thermo Scientific), anti-PPARγ (catalog no. ab54481; Abcam Inc., Cambridge, MA), or control mouse IgG. Precipitated protein-DNA complexes were eluted, and cross-linkings were reversed for purification of DNA. PCR was performed using the primers 5’-TTTACTATTTCCACACCGGTC-3’ and 5’-TCCCTCACACAGCAATTTACTG-3’ specific for the −355/−136 region of the human CD36 promoter that contains three potential PPREs. The PCR products were analyzed by electrophoresis on 2% agarose gels (E-Gel®, Invitrogen), stained with ethidium bromide, and quantified by densitometry.

Statistical Analysis—Data are presented as means ± S.E. For real-time PCR results, geometric means ± S.E. are plotted. All statistical analyses were carried out on log-transformed or ∆ cycle threshold data (for real-time PCR) using JMP® version 11 (SAS® Institute Inc., Cary, NC). To analyze continuous dose and time effects, one-way analysis of variance models were used. To test the effects of nominal factors and their interactions, linear mixed models were used, which consider the correlation within each experiment. Non-significant factors were dropped from statistical models to calculate final p values. Post hoc contrasts were tested for our interested comparisons. All p values are two-sided and considered significant if p was < 0.05.

Results

Ligand Activation of PPARγ Signaling in EA.hy926 Human Endothelial Cells Is p38 MAPK-dependent—Both RGZ and PGZ (10 μM for each), two clinically relevant TZD PPARγ ligands, activated p38 MAPK in EA.hy926 human endothelial cells (p = 0.002 for both; Fig. 1, A and B, respectively). Phosphorylation of p38 MAPK occurred as early as 5 min with a maximal effect at 15 min. As expected, SB (1 μM), a specific p38 MAPK inhibitor, blocked RGZ-induced p38 MAPK phosphorylation (p < 0.001 for RGZ versus SB plus RGZ; p = 0.001 for an interaction between RGZ and SB; Fig. 1C). Therapeutic RGZ dosing in humans produces peak plasma concentrations of 1–2 μM (45). In kidney (HEK293T) cells, half-maximal stimulation of PPARγ-dependent transcription was calculated to occur at a RGZ concentration of 6.5 μM (46). Consistent with these results, dose-response testing in HeLaS/F-PPARγ cells found that 10 μM RGZ induced nearly maximal PPRE reporter activity (data not shown). Therefore, subsequent experiments were conducted with RGZ (10 μM) as a prototype for this class of PPARγ ligands. Endogenous PPARγ expression in EA.hy926 endothelial cells is relatively low; therefore, in all experiments directly assessing genomic PPARγ signaling, these cells were transfected with a PPARγ2 expression plasmid (Fig. 1D, inset). SB (1 μM), a specific p38 MAPK inhibitor that blocked RGZ-induced p38 MAPK phosphorylation (Fig. 1C), significantly
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FIGURE 1. PPARγ ligands induce PPRE-driven transcription through p38 MAPK. A and B, RGZ and pioglitazone increased p38 MAPK phosphorylation in EA.hy926 cells (p < 0.001 for RGZ effect in A; p = 0.002 for PGZ effect in B). C, RGZ-induced p38 MAPK phosphorylation was blocked by SB, a specific p38 MAPK inhibitor (p < 0.001 for RGZ versus SB plus RGZ; p = 0.001 for an interaction between RGZ and SB). Phosphorylated p38 MAPK (p38 MAPK) and total p38 MAPK (p38) were measured by Western blotting. Cells were treated with SB (1 μM) for 40 min and then incubated with RGZ (10 μM) or vehicle CTRL for another 30 min in C. Densitometric results for A–C of three independent experiments and representative blots are shown. D, SB blocked RGZ-induced PPARγ DNA binding, as measured by the TranAM ELISA (p = 0.009 for RGZ versus SB plus RGZ). EA.hy926 cells were first transfected with PPARγ expression plasmid (insert). At 48 h after transfection, cells were treated with SB (1 μM) for 40 min and then incubated with RGZ (10 μM) or vehicle CTRL for another 1 h. E, SB (1 μM added 40 min before RGZ or its control) reduced RGZ activation of a PPARγ-reporter gene (p = 0.04 for RGZ versus SB plus RGZ). F and G, siRNA knockdown of p38α MAPK (p < 0.001 for RGZ versus p38 siRNA plus RGZ; p = 0.03 for an interaction between RGZ and p38 siRNA) expression of a dominant negative p38 MAPK mutant (DN-p38 MAPK; p = 0.03 for RGZ versus DN-p38 plus RGZ; p = 0.04 for an interaction between RGZ and DN-p38). These results demonstrate that GPR40 is necessary for RGZ activation of p38 MAPK in a human endothelial line.

Previously, we found that NO activated p38 MAPK in EA.hy926 cells (39). Therefore, we next examined whether RGZ/GPR40/p38 MAPK signaling might be mediated through NO as a second messenger. L-NAME, a NO synthase inhibitor, had no effect on RGZ-induced p38 MAPK phosphorylation (p = 0.05 for the main effect of L-NAME; Fig. 2E). Conversely, NO activation of p38 MAPK, which is probably related to its free radical biology (47), was not dependent on GPR40 in EA.hy926 cells. DTANO, a NO donor, time-dependently induced p38 MAPK phosphorylation (p = 0.008; Fig. 2F); this phosphorylation was not altered by either GW1100, a specific GPR40 antagonist (p = 0.55 for DTANO versus GW1100 plus DTANO; Fig. 2G) or GPR40 knockdown (p = 0.61 for DTANO versus GPR40 siRNA plus DTANO; Fig. 2H).

RGZ Activation of GPR40 Enhances PPARγ/PPRE-driven Transcription in EA.hy926 Human Endothelial Cells—The above experiments in EA.hy926 human endothelial cells show blocked RGZ-induced PPARγ DNA binding (p = 0.009 for RGZ versus SB plus RGZ; Fig. 1D) and PPRE reporter gene activity (p = 0.04 for RGZ versus SB plus RGZ; Fig. 1E) in EA.hy926 cells. Furthermore, both p38α MAPK siRNA silencing (p < 0.001 for RGZ versus p38α siRNA plus RGZ; p = 0.03 for an interaction between RGZ and p38 siRNA; Fig. 1F) and overexpression of a DN-p38 MAPK mutant (p = 0.03 for RGZ versus DN-p38 plus RGZ; p = 0.04 for an interaction between RGZ and DN-p38; Fig. 1G) significantly repressed RGZ-induced PPRE reporter activity. The p38α MAPK siRNA used here was previously shown to effectively knockdown p38 MAPK protein in EA.hy926 cells (39).

GPR40 Mediates RGZ Activation of p38 MAPK in EA.hy926 Human Endothelial Cells—Like RGZ, other PPARγ ligands, such as pioglitazone, ciglitazone, and troglitazone, have been shown to bind to Gq-coupled GPR40 (30–32), resulting in rapid activation of p38 MAPK as well as other downstream signaling pathways (31, 33). Notably, GPR40, also known as free fatty acid receptor 1, has been implicated in fatty acid- and glucose-induced insulin secretion (35, 36), effects also regulated by PPARγ (37, 38). EA.hy926 endothelial cells expressed both GPR40 and PPARγ (Fig. 2A), but as noted above, endogenous PPARγ protein expression is relatively low. We next determined whether RGZ activates p38 MAPK via GPR40 in EA.hy926 cells.

GW1100, a specific GPR40 antagonist, inhibited RGZ-induced p38 MAPK phosphorylation (p < 0.001 for RGZ versus GW1100 plus RGZ; p = 0.04 for an interaction between GW1100 and RGZ; Fig. 2B). Likewise, shRNA-mediated GPR40 silencing significantly blocked RGZ-induced p38 phosphorylation (p = 0.006 for RGZ versus GPR40 shRNA plus RGZ; p = 0.004 for an interaction between GPR40 shRNA and RGZ; Fig. 2C); shRNA knockdown decreased GPR40 protein expression about 50% in EA.hy926 cells (p = 0.007 for the main effect of GPR40 shRNA; Fig. 2D). These results demonstrate that GPR40 is necessary for RGZ activation of p38 MAPK in a human endothelial line.

transfection, cells were treated with RGZ (10 μM) or CTRL for 24 h prior to the measurement of chloramphenicol acetyltransferase activity. Results are presented as means ± S.E. (error bars) of three independent experiments in E–G.
that 1) PPARγ ligands activate p38 MAPK, 2) p38 MAPK modulates PPARγ transcriptional activity, and 3) PPARγ ligand activation of p38 MAPK occurs via GPR40 and is independent of NO. Next, we tested more directly whether GPR40 signaling modulates ligand-activated PPARγ genomic signaling. Like p38 MAPK silencing and dominant negative mutant (Fig. 1, F and G), GW1100, a specific GPR40 antagonist, significantly repressed RGZ-induced PPRE reporter activity in EA.hy926 cells (p < 0.001 for RGZ versus GW1100 plus RGZ; p = 0.03 for an interaction between GW1100 and RGZ; Fig. 3A). In contrast, GPR40 knockdown reduced both basal and RGZ-stimulated PPRE reporter activity (p < 0.001 for GPR40 shRNA main effect; p = 0.32 for an interaction between RGZ and GPR40 shRNA; Fig. 3B). Overexpression of DN-Gαq, a mutant that competes with endogenous wild-type G-proteins for GPR40 coupling (43), also blocked both basal and RGZ-induced PPRE reporter activity (p < 0.001 for DN-Gαq mutant main effect; p = 0.88 for an interaction between RGZ and DN-Gαq; Fig. 3C).

In addition, GPR40 overexpression enhanced both basal and RGZ-induced PPARγ transcriptional activity (p < 0.001 for GPR40 plasmid main effect; p = 0.11 for an interaction between RGZ and GPR40 plasmid; Fig. 3D). Consistent with our findings for RGZ-induced p38 MAPK activation (Fig. 2E), L-NAME (0–1 mM), a NO synthase inhibitor, did not affect basal or RGZ-induced PPRE reporter activity (p = 0.25 for the main effect of L-NAME; Fig. 3E), further evidence that NO plays no role in RGZ/GPR40/p38 MAPK/PPARγ signaling in EA.hy926 cells.

**RGZ/GPR40/p38 MAPK Signaling Boosts the Transcription of PPARγ Target Genes in PAECs**—The RGZ/GPR40/p38 MAPK/PPARγ signaling transduction pathway was further explored in PAECs. Similar to EA.hy926 cells, a hybrid human endothelial line, RGZ increased p38 MAPK phosphorylation ∼2-fold in PAECs (p < 0.001 for RGZ versus control within the control siRNA condition; Fig. 4A), and siRNA silencing of p38α blocked this effect (p = 0.43 for RGZ versus control within the p38 MAPK siRNA condition; p = 0.009 for an interaction between p38 MAPK siRNA and RGZ; Fig. 4A). Knockdown of p38α MAPK decreased total p38 MAPK protein about 80% (p < 0.001 for the main effect of p38α MAPK siRNA; Fig. 4A). PAECs expressed PPARγ protein, and neither RGZ (p = 0.55 for the main effect of RGZ; Fig. 4A) nor p38α MAPK knockdown (p = 0.10 for the main effect of p38α MAPK siRNA; Fig. 4A) significantly altered PPARγ expression. Furthermore, p38α MAPK siRNA silencing significantly inhibited RGZ-induced expression of CD36, CYP1A1, and FABP4, three PPARγ target genes (p < 0.001 for all three genes, RGZ versus RGZ plus p38α MAPK siRNA; Fig. 4B). Similar to effects in a PPARγ reporter system (Fig. 1F), significant interactions between RGZ and p38 MAPK knockdown were seen for CD36 (p < 0.001), CYP1A1 (p = 0.04), and FABP4 (p = 0.02). Table 1 shows all interactions.


**PPARγ Activation through GPR40**

*(p values) between RGZ activation of PPARγ and siRNA silencing of GPR40 pathway components on the expression of these target genes in PAECs.

Like EA.hy926 cells, PAECs also express GPR40 as measured by Western blotting, and RGZ did not alter this expression (p = 0.30 for the main effect of RGZ; Fig. 4C). GPR40 siRNA significantly reduced GPR40 protein expression (p = 0.002 for the main effect of GPR40 siRNA; Fig. 4C) but had no effect on PPARγ expression in PAECs (p = 0.56 for the main effect of GPR40 siRNA; Fig. 4C). Similar to p38α MAPK knockdown, GPR40 siRNA significantly blocked the RGZ-induced PPARγ target genes CD36, CYP1A1, and FABP4 (p = 0.005 for all three genes, RGZ versus RGZ plus GPR40 siRNA; Fig. 4D). Again, RGZ and GPR40 siRNA silencing appeared to interact at endogenous PPARγ target genes (Fig. 4D and Table 1). This was in contrast to the additive effects of RGZ and GPR40, knockdown, DN-Gαq, mutant, or overexpression on a PPARγ reporter (Fig. 3). PAEC donor, passage number, and day of experiment varied between Fig. 4, B and D, possibly accounting for the observed variability in gene expression among the siRNA control conditions. These results further support the existence of a functional pathway in endothelial cells that connects GPR40 on the cell surface to nuclear PPARγ signaling, two receptors that can be activated by shared ligands.

**PGC1α Transduces RGZ/GPR40/p38 MAPK Signals to PPARγ in PAECs—PGC1α is an essential co-activator of PPARγ.** Activated p38 MAPK phosphorylates PGC1α and thereby activates and stabilizes PGC1α protein (18, 19, 48, 49). Therefore, we investigated the role of PGC1α in transducing GPR40/p38 MAPK signals to PPARγ in human PAECs. RGZ increased PGC1α phosphorylation (p = 0.001 for RGZ versus control within the control siRNA condition; Fig. 5A), and siRNA knockdown of either p38α MAPK (p = 0.45) or GPR40 abolished this effect (p = 0.60; Fig. 5A). RGZ also increased total PGC1α protein expression in PAECs (p = 0.01 within control siRNA; Fig. 5B), an effect that was similarly blocked by either p38α MAPK or GPR40 siRNA silencing (p ≥ 0.62 for both; Fig. 5B). Notably, RGZ increased total and phosphorylated PGC1α without altering their ratio (Fig. 5, A and B), and both effects may have contributed to PPARγ transcriptional activation.

In contrast to the activation of PGC1α by p38 MAPK-mediated phosphorylation, acetylation inhibits PGC1α activity and therefore also plays a key regulatory role in the co-transcriptional activity of PGC1α (24, 25). SIRT1 binds to and deacetylates PGC1α both in vivo and in vitro and appears to be required for PGC1α activation (24, 25). Therefore, we evaluated the role of SIRT1 in RGZ/GPR40/p38 MAPK/PGC1α PPARγ signal transduction. RGZ decreased PGC1α acetylation in PAECs (p < 0.001 for RGZ versus control within the control siRNA condition; Fig. 5C), whereas SIRT1-specific siRNA increased PGC1α acetylation (p < 0.001 for SIRT1 siRNA versus control siRNA in the absence and presence of RGZ; Fig. 5C) and abolished the decrease in PGC1α acetylation seen with RGZ (p = 0.37 for RGZ versus control within the SIRT1 siRNA condition; Fig. 5C).

As expected and consistent with the importance of PGC1α as a PPARγ transcriptional co-activator, siRNA silencing of either PGC1α or SIRT1 significantly inhibited RGZ-induced PPARγ target genes (Fig. 5D, left and right panels, respectively) including CD36, CYP1A1, and FABP4 (p < 0.001 for all three genes, RGZ versus RGZ plus PGC1α or SIRT1 siRNA; Fig. 5D). As seen for p38 MAPK and GPR40 knockdown, the silencing of either PGC1α or SIRT1 profoundly reduced the ability of RGZ to induce PPARγ target genes (Fig. 5D and Table 1). Specific siRNA for PGC1α and SIRT1 effectively reduced PGC1α (p = 0.001 for the main effect of PGC1α siRNA; Fig. 5E) and SIRT1 (p < 0.001 for the main effect of SIRT1 siRNA; Fig. 5F) protein
expression in PAECs, respectively. RGZ did not alter SIRT1 protein expression \((p = 0.009)\) for an interaction between P38 MAPK and RGZ) and decreased total P38 MAPK protein \((p < 0.001)\) for an interaction between P38 MAPK and RGZ) but did not alter PPARγ protein expression \((p = 0.10)\) for an interaction between P38 MAPK and RGZ). B, p38 MAPK siRNA silencing significantly inhibited RGZ-induced expression of PPARγ target genes \((p < 0.001)\) for an interaction between P38 MAPK and RGZ and decreased total P38 MAPK protein \((p < 0.001)\) for an interaction between P38 MAPK and RGZ). C, GPR40 siRNA knockdown reduced GPR40 protein expression \((p = 0.02)\) for an interaction between GPR40 siRNA and RGZ) without affecting PPARγ expression \((p = 0.56)\) for an interaction between GPR40 siRNA and RGZ). PAECs were transfected with specific siRNA or a scrambled CTRL siRNA. At 48 h after transfection, cells were treated with RGZ \((10 \mu M)\) or vehicle CTRL for 30 min prior to the measurement of phosphorylated p38 MAPK \((pp38)\), total p38 MAPK \((p38)\), and PPARγ in A and for 24 h prior to the measurement of GPR40 and PPARγ in C by Western blotting. In B and D, 48 h after siRNA transfection, cells were treated with RGZ \((10 \mu M)\) or vehicle CTRL for 24 h followed by measurement of PPARγ target genes by real-time PCR. Densitometry results of three independent experiments using different PAEC donors and a representative blot are shown in A and C. In B \((n = 5)\) and D \((n = 4)\), results are presented as geometric means \(\pm\) S.E. (error bars) of independent experiments using different PAEC donors.

TABLE 1

The PPARγ agonist RGZ and signaling through the GPR40 pathway function synergistically to induce PPARγ-regulated genes

| siRNA target | CD36 | CYP1A1 | FABP4 | All three genes combined |
|--------------|------|--------|-------|-------------------------|
| p38 MAPK     | <0.001 | 0.04   | 0.02  | <0.001                  |
| GPR40        | 0.04  | 0.02   | 0.01  | 0.03                    |
| PGC1α        | 0.005 | 0.18   | 0.006 | 0.03                    |
| SIRT1        | <0.001 | 0.09   | 0.06  | <0.001                  |
| EP300        | <0.001 | 0.007  | <0.001| 0.008                   |
| All siRNA silencing combined | <0.001 | 0.001  | <0.001| <0.001                  |

FIGURE 4. RGZ induces PPARγ target genes through GPR40 and the downstream activation of p38 MAPK in PAECs. A, RGZ-induced p38 MAPK phosphorylation \((p < 0.001)\) for RGZ versus CTRL within the CTRL siRNA condition). Knockdown of p38 MAPK blocked RGZ-induced p38 MAPK phosphorylation \((p = 0.43)\) for RGZ versus CTRL within the p38 MAPK siRNA condition; \(p = 0.009\) for an interaction between p38 MAPK and RGZ) and decreased total p38 MAPK protein \((p < 0.001)\) for an interaction between p38 MAPK and RGZ) but did not alter PPARγ protein expression \((p = 0.10)\) for an interaction between p38 MAPK and RGZ). B, p38 MAPK siRNA silencing significantly inhibited RGZ-induced expression of PPARγ target genes \((p < 0.001)\) for RGZ versus RGZ plus p38 MAPK siRNA for all three genes) and decreased total P38 MAPK protein \((p < 0.001)\) for an interaction between p38 MAPK and RGZ). C, GPR40 siRNA knockdown reduced GPR40 protein expression \((p = 0.02)\) for an interaction between GPR40 siRNA and RGZ) without affecting PPARγ expression \((p = 0.56)\) for an interaction between GPR40 siRNA and RGZ). PAECs were transfected with specific siRNA or a scrambled CTRL siRNA. At 48 h after transfection, cells were treated with RGZ \((10 \mu M)\) or vehicle CTRL for 30 min prior to the measurement of phosphorylated p38 MAPK \((pp38)\), total p38 MAPK \((p38)\), and PPARγ in A and for 24 h prior to the measurement of GPR40 and PPARγ in C by Western blotting. In B and D, 48 h after siRNA transfection, cells were treated with RGZ \((10 \mu M)\) or vehicle CTRL for 24 h followed by measurement of PPARγ target genes by real-time PCR. Densitometry results of three independent experiments using different PAEC donors and a representative blot are shown in A and C. In B \((n = 5)\) and D \((n = 4)\), results are presented as geometric means \(\pm\) S.E. (error bars) of independent experiments using different PAEC donors.

EP300 contributes to the RGZ/GPR40/p38 MAPK/PGC1α/PPARγ signaling pathway—EP300, a general transcriptional co-activator with intrinsic histone acetyltransferase and chromatin remodeling activity, has been shown to interact with PGC1α and PPARγ, thereby enhancing PPARγ transcriptional activity (50, 51). Importantly, p38 MAPK has been demonstrated to directly phosphorylate EP300, thereby potentiating its acetyltransferase activity (22, 52). Therefore, the role of EP300 in the pathway described here was investigated in human endothelial cells. Consistent with the role of PGC1α in PPARγ signaling, PGC1α overexpression in EA.hy926 cells increased PPRE reporter activity \((p < 0.001)\) for a plasmid dose effect in the absence of RGZ; Fig. 6A). A similar dose response was seen in the presence of RGZ, but the RGZ effect became smaller with increasing amounts of PGC1α plasmid \((p < 0.001)\) for a negative interaction between RGZ and PGC1α; Fig. 6A), possibly
because RGZ/p38 MAPK-dependent induction and phosphorylation of PGC1α was rendered less important by direct PGC1α overexpression.

EP300 overexpression also increased both basal and RGZ-induced PPRE reporter activity (p = 0.003 for EP300 main effect; p = 0.68 for an interaction between EP300 and RGZ; Fig. 6B). Although PGC1α overexpression had a stronger overall effect on PPRE reporter activity (p < 0.02 for PGC1α versus EP300 in both the absence and presence of RGZ; Fig. 6C), EP300 overexpression better preserved RGZ effect size and increased PPRE reporter activity additively with PGC1α in the presence of RGZ (p < 0.001 for PGC1α and EP300 main effects; p = 0.77 for an interaction between PGC1α and EP300; Fig. 6C). Notably, RGZ treatment of EA.hy926 cells increased EP300...
phosphorylation in EA.hy926 cells ($p = 0.01$ for RGZ versus control within the control condition; Fig. 6D), an activating event blocked by either p38 MAPK (SB) or GPR40 (GW1100) inhibition ($p \geq 0.19$ for RGZ versus control in the presence of SB or GW1100; Fig. 6D). Specific siRNA silencing of EP300 inhibited both basal and RGZ-induced PPRE reporter activity in EA.hy926 cells ($p < 0.001$ for EP300 siRNA main effect; $p = 0.53$ for an interaction between EP300 siRNA and RGZ; Fig. 6E). Likewise, siRNA knockdown of EP300 in PAECs significantly inhibited RGZ-induced PPARγ target genes, CD36, CYP1A1, and FABP4 ($p < 0.001$ for all three, RGZ versus RGZ plus EP300 siRNA; Fig. 6F). Similar to the knockdown of other GPR40 pathway components, EP300 silencing markedly interfered with the ability of RGZ/PPARγ signaling to induce CD36, CYP1A1, and FABP4 (Fig. 6F and Table 1). Efficient knockdown of EP300 mRNA expression was
achieved in both EA.hy926 cells and PAECs (p < 0.001 for both; Fig. 6G).

**PPARγ Activation through GPR40**

In addition to these findings, RGZ treatment of PAECs increased the phosphorylation and expression of PGC1α, a key co-activator of PPARγ (50). Knockdown of either p38α MAPK or GPR40 abolished these effects of RGZ on PGC1α. RGZ also modestly decreased PGC1α acetylation, an activating event; knockdown of SIRT1, a deacetylase, eliminated this effect and increased PGC1α acetylation. As expected, given its essential role in the formation of a PPARγ transcriptional activation complex (50), knockdown of PGC1α or its essential activator SIRT1 inhibited RGZ-induced PPARγ target gene expression in PAECs. Finally, EP300, an acetylase that docks with PGC1α and remodels chromatin to optimize the transcription of PPARγ target genes (50, 53), was also phosphorylated and activated by p38 MAPK. Like PGC1α, EP300 appeared to play an important downstream role in RGZ/GPR40/p38 MAPK modulation of PPARγ signaling. Collectively, these experiments demonstrate that p38 MAPK, PGC1α, and EP300 link GPR40 to downstream PPARγ genomic signaling. Binding to and activating both GPR40 and PPARγ appears to be a common feature of several PPARγ agonists (29–34). This direct connection between GPR40 signaling and PPARγ transcriptional activation argues that the effects of these ligands on human endothelium might be best understood as a cognate two-receptor system, integrated by p38 MAPK, PGC1α, and EP300.

Activation of p38 MAPK increases transcription of PPARγ-regulated genes in endothelial cells (39) and adipocytes (20, 21, 54). TZDs (20, 55–57) have been long known to activate p38 MAPK independent of PPARγ in various cell types, including adipocytes, astrocytes, and epithelial cells. However, the potential role of GPR40 was not appreciated at the time of these early studies, and the underlying mechanisms seemed to be cell type-dependent (20, 55–57). Reactive oxygen species were implicated in astrocytes (55) and adipocytes (20), whereas endoplasmic reticulum stress was implicated in liver epithelial cells (57). Here, RGZ activation of p38 MAPK was directly tied to GPR40 in human endothelium. Both the GPR40 antagonist GW1100 and GPR40 gene silencing significantly blocked RGZ-induced p38 MAPK phosphorylation. This finding is consistent with recent reports that TZDs bind to and activate GPR40 in bronchoconstrictor cells.

**Discussion**

Our results demonstrate that GPR40 and PPARγ can function together as an integrated two-receptor signal transduction pathway. Besides the direct activation of its canonical receptor PPARγ, RGZ also required GPR40 to optimally propagate a PPARγ nuclear signal in human endothelium (Fig. 7). GPR40 and PPARγ appeared to function at least additively and sometimes synergistically to initiate PPARγ genomic responses, depending on the transcriptional context. This conclusion is based on the following: 1) RGZ activated p38 MAPK; 2) PPARγ DNA binding and reporter activity was at least partially p38 MAPK-dependent; 3) GPR40 inhibition or knockdown blocked RGZ activation of p38 MAPK; 4) inhibition of GPR40 signaling, including use of an antagonist, GPR40 silencing, or expression of a DN-Gaα mutant, suppressed, whereas GPR40 overexpression further increased, RGZ-induced PPRe reporter activity; 5) RGZ activation of p38 MAPK and the PPRe reporter was independent of ‘NO synthase, and ‘NO activation of p38 MAPK was likewise GPR40-independent; and importantly, 6) in human primary PAECs, knockdown of p38 MAPK or GPR40 substantially reduced the ability of RGZ to induce PPARγ target genes.

**FIGURE 6. CBP/EP300 participation in RGZ/GPR40/p38 MAPK/PGC1α/PPARγ signaling.** A–C, overexpression of PGC1α, CBP/EP300, or both enhanced PPARγ reporter gene activity. In A, PGC1α overexpression increased PPRe reporter activity in the absence of RGZ (p < 0.001 for a plasmid dose effect); the PGC1α effect became smaller in the presence of RGZ (p < 0.001 for a negative interaction between RGZ and PGC1α). In B, EP300 overexpression also increased both basal and RGZ-induced PPRe reporter activity (p = 0.003 for the main effect; p = 0.68 for an interaction between RGZ and EP300 plasmid). In C, PGC1α and EP300 additively enhanced PPRe reporter activity in the absence and presence of RGZ (p < 0.001 for PGC1α and EP300 main effects; p = 0.77 for an interaction between PGC1α and EP300). EA.hy926 cells were co-transfected with PPRe-LUC reporter, a PPARγ expression plasmid, and either a PGC1α expression plasmid, an EP300 expression plasmid, or both, as indicated. DNA amounts were balanced with the empty vector pcDNA3.1 plasmid. At 24 h after transfection, cells were treated with RGZ (10 μM) or vehicle CTRL for 24 h prior to the measurement of luciferase activity. D, RGZ induced EP300 phosphorylation in EA.hy926 cells (p = 0.01 for RGZ versus CTRL within CTRL), an effect blocked by SB, a specific p38 MAPK inhibitor, or GW1100, a specific GPR40 antagonist (p = 0.19 for RGZ versus CTRL with SB or GW1100). Cells were treated with SB (10 μM), GW1100 (20 μM), or vehicle CTRL for 40 min and then incubated with RGZ (10 μM) or vehicle CTRL for another 15 min prior to the extraction of whole cell lysates for measurement of phosphorylated and total EP300 by Western blotting. Densitometry results of three independent experiments and a representative blot are shown. E, siRNA knockdown of EP300 inhibited PPARγ reporter gene activity (p < 0.001 for EP300 siRNA main effect; p = 0.53 for an interaction between EP300 siRNA and RGZ). EA.hy926 cells were first transfected with EP300 siRNA or scrambled CTRL siRNA for 48 h and then co-transfected with PPRe-LUC reporter and a PPARγ expression plasmid for 24 h, followed by an additional 24-h treatment of RGZ (10 μM) or vehicle CTRL prior to the measurement of luciferase activity. F, EP300 siRNA inhibited RGZ-induced PPARγ reporter target genes (p < 0.001 for RGZ versus RGZ plus EP300 siRNA for all three genes) in PAECs. G, EP300 siRNA reduced EP300 mRNA in EA.hy926 cells and PAECs (p < 0.001 for the main effect of EP300 siRNA in both cell types). Cells were transfected with EP300 siRNA or scrambled CTRL siRNA for 48 h and then treated with RGZ (10 μM) or vehicle CTRL for 24 h prior to isolation of total RNA for measurement of PPARγ target genes and EP300 mRNA by real-time PCR. H, RGZ increased the binding of PPARe (p < 0.001), PGC1α (p = 0.004), and EP300 (p = 0.04) to PPRe in the CD36 promoter (RGZ versus CTRL within the CTRL condition), an effect blocked by SB or GW1100 (p = 0.18, RGZ versus CTRL for both inhibitors and all three proteins). PAECs were treated with SB (10 μM), GW1100 (20 μM), or vehicle CTRL for 40 min and then incubated with RGZ (10 μM) or vehicle CTRL for another 24 h prior to ChIP assay with anti-PPArγ, anti-PGC1α, anti-EP300, or control IgG. Densitometry measurements were normalized to input DNA. Representative PCR results are shown for each pull-down. Panels represent the results of three (A–F) or four (G and H) independent experiments. For PAECs, different donors were used in performing replicates. Error bars, S.E.
**PPARγ Activation through GPR40**

In the classical PPARγ signaling pathway, RGZ binds directly to PPARγ, inducing a conformational change that results in its dissociation from co-repressors (not depicted), such as nuclear co-repressor and histone deacetylases, and the recruitment of co-activators, including PGC1α and EP300. However, as shown here, RGZ and other PPARγ ligands also bind to and activate GPR40 on the cell surface, resulting in p38 MAPK phosphorylation, which in turn phosphorylates and thereby activates both PGC1α and EP300. As noted, PGC1α deacetylation by SIRT1 is also essential for its activation. Phosphorylation releases PGC1α from its repressor p160MBP and leads to a conformational change, permitting PGC1α to dock with PPARγ and recruit newly activated EP300. EP300 is a histone acetyltransferase that remodels local chromatin and enhances gene transcription. The activated PPARγ complex thus heterodimerizes with retinoid X receptor, which binds to peroxisome proliferator response elements in accessible promoters, inducing the transcription of target genes.

As already noted, p38 MAPK has been shown to phosphorylate the PPARγ co-activator PGC1α (18–21). Phosphorylation disrupts PGC1α binding to its repressor, p160 Myb-binding protein (p160MBP), freeing it to dock with PPARγ (19, 53). Docking with PPARγ changes the conformation of PGC1α and allows binding of EP300, a histone acetyltransferase essential for PGC1α/PPARγ-dependent gene transcription (19, 50, 53, 59). Moreover, p38 MAPK phosphorylation of PGC1α increases its half-life (18). Furthermore, the p38 MAPK/ATF2 pathway induces PGC1α mRNA transcription (48, 49), increasing PGC1α protein expression and further enhancing PGC1α/PPARγ-mediated gene transcription. In addition to PGC1α, p38 MAPK also phosphorylates EP300, potentiating its histone acetyltransferase activity (22, 52), an additional mechanism by which p38 MAPK contributes to the transcriptional activation of PPARγ. Consistent with these previous findings, PGC1α and EP300 were shown here to connect RGZ/GPR40/p38 MAPK transmembrane signaling to the downstream transcriptional activation of RGZ/PPARγ in the cell nucleus.

Like phosphorylation, reversible acetylation is another key modulator of PGC1α (24, 25). So far, only two proteins have been unequivocally shown to regulate the reversible acetylation of PGC1α, the acetyltransferase GCN5 (23) and the NAD+-dependent deacetylase SIRT1 (24, 25). GCN5 acetylates and inhibits PGC1α activity (23), whereas SIRT1 deacylates and enhances PGC1α activity and in turn induces transcription of its target genes (24, 25). Overexpression of Sirt1 in the liver of mice induces gluconeogenic genes under the control of PGC1α, whereas Sirt1 knockdown attenuates this effect (60). Also, in skeletal muscle, Sirt1 is required for PGC1α-mediated induction of the fatty acid oxidation pathway (61). In the present study, RGZ was seen to modestly decrease PGC1α acetylation in endothelial cells; SIRT1 knockdown eliminated this effect, increasing PGC1α acetylation. These results suggest that PGC1α phosphorylation might facilitate its deacetylation by SIRT1. It was previously reported that JNK1 phosphorylates SIRT1 and promotes its enzymatic activity in HEK293T cells (62); p38 MAPK has been reported to increase SIRT1 expression in neurons (63) and decrease it in chondrocytes (64). In our study, RGZ did not affect either SIRT1 phosphorylation or expression in endothelial cells. However, consistent with its essential role in activating PGC1α via deacetylation, SIRT1 knockdown significantly inhibited RGZ-induced PPARγ target gene expression in endothelial cells.

Although the effects of RGZ/PPARγ and RGZ/GPR40 were mostly additive in PPRE reporter assays, the dual activation of both receptors appeared interdependent and synergistic at PPARγ-regulated target genes in the chromatin microenvironment (see Table 1 for a summary of interactions between RGZ and GPR40 pathway siRNA silencing). Across all three PPARγ-regulated genes combined, interaction testing between RGZ and each siRNA target indicated that RGZ activation of GPR40 and PPARγ function synergistically (Table 1). Likewise, across all siRNA silencing combined, each PPARγ-regulated gene, CD36 (p < 0.001), CYP1A1 (p = 0.001) and FABP4 (p < 0.001), demonstrated significant interactions between RGZ-induced PPARγ responses and signaling through the GPR40 pathway (Table 1). Largely additive effects on a PPRE reporter plasmid and evidence for GPR40 and PPARγ interdependence at endogenous genes might be explained by the ability of the proposed signaling cascade to actively remodel chromatin. Consistent with this concept, p38 MAPK or GPR40 inhibition both markedly blocked the RGZ-induced binding of PPARγ, PGC1α, and EP300 to a PPRE-rich site in the proximal promoter of CD36, a prototypic PPARγ target gene.

TZDs, synthetic ligands of PPARγ, including RGZ, ciglitazone, troglitazone, and pioglitazone, have all been shown to activate GPR40 with subsequent signal transduction through stress kinases (29–34). RGZ compared with pioglitazone (two...
TZDs used to treat type 2 diabetes mellitus) produces a more potent and prolonged activation of ERK1/2 (31), a stress kinase pathway associated with vascular inflammation (65). These differences in the potency and sustainability of ERK1/2 activation could potentially explain some of the efficacy and safety differences among existing synthetic PPARγ ligands. Different from TZDs, 15-deoxy-Δ12,14-prostaglandin J2, a natural PPARγ ligand (27), did not appear to activate PPARγ in bronchial epithelial cells (32). Conversely, agonists selective for PPARγ have been described with little or no effect on PPARγ activity (29, 66). Therefore, it may be possible to design drugs that activate PPARγ independently of PPARλ or selectively activate PPARγ/p38 MAPK while circumventing PPARγ/ERK activation. Such selective agents or biased ligands (67, 68) might arguably be less inflammatory and thus have better risk/benefit profiles in patients with vascular disease. In addition, unexplored effects through other unidentified cognate GPR and nuclear receptor pairs, as exemplified by PPARλ/PPARγ, could explain important safety and efficacy differences among nuclear receptor-directed drugs.

Author Contributions—S. W. and R. L. D. conceived the study, designed experiments, analyzed and interpreted data, and wrote the paper. S. W. performed transfections, Western blots, PPAR target gene real-time PCR experiments, and the chromatin immunoprecipitation assays. K. A. identified the role played by EP300, helped design key experiments related to acetylation, maintained the pulmonary artery endothelial cell cultures, and revised drafts of the manuscript. J. M. E., E. J. D., and G. A. F. contributed to the scientific concept, provided technical advice, raised critical questions, and revised drafts of the manuscript. E. J. D. also lent important expertise in nuclear receptor signaling. J. Y. W. performed the experiments shown in Fig. 2, B–D. A. P. started the study and performed the experiments shown in Fig. 1. R. C. and J. S. performed the statistical analyses. All authors read, edited, and approved the final version of the manuscript.

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