PPAR-γ inhibits ANG II-induced cell growth via SHIP2 and 4E-BP1

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Benkirane, Karim, Farhad Amiri, Quy N. Diep, Mohammed El Mabrouk, and Ernesto L. Schiffrin. PPAR-γ inhibits ANG II-induced cell growth via SHIP2 and 4E-BP1. Am J Physiol Heart Circ Physiol 290: H390–H397, 2006. First published September 9, 2005; doi:10.1152/ajpheart.00662.2005.—The present study evaluated the effects of peroxisome proliferator-activated receptor (PPAR)-γ activators on ANG II-induced signaling pathways and cell growth. Vascular smooth muscle cells (VSMC) derived from rat mesenteric arteries were treated with ANG II, with/without the AT1 receptor blocker valsartan or the AT2 receptor blocker PD-123319, after pretreatment for 24 h with the PPAR-γ activators 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2) or rosiglitazone. Both 15d-PGJ2 and rosiglitazone decreased ANG II-induced DNA synthesis. Rosiglitazone treatment increased nuclear PPAR-γ expression and activity in VSMC. However, rosiglitazone did not alter expression of PPAR-α/β, ERK 1/2, Akt, or ANG II receptors. 15d-PGJ2 and rosiglitazone decreased ERK 1/2 and Akt peak activity, both of which were induced by ANG II via the AT1 receptor. Rosiglitazone inhibited ANG II-enhanced phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), as well as Src homology (SH) 2-containing inositol phosphatase 2 (SHIP2). PPAR-γ activation reduced ANG II-induced growth associated with inhibition of ERK 1/2, Akt, 4E-BP1, and SHIP2. Modulation of these pathways by PPAR-γ activators may contribute to regression of vascular remodeling in hypertension.

vascular smooth muscle cell; phosphatidylinositol 3-kinase; mitogenactivated protein kinase; 4E-binding protein 1; Src homology 2-containing inositol phosphatase 2; angiotensin II; peroxisome proliferatoractivated receptor-γ

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS (PPARs) are transcription factors of the nuclear hormone receptor family that bind to the PPAR-response element (PPRE) (39). Three isoforms, encoded by separate genes, have been demonstrated. PPAR-α is predominantly expressed in tissues exhibiting high fatty acid catabolism such as liver, heart, kidney, and skeletal muscle, whereas PPAR-γ is most abundantly expressed in adipose tissue, large intestine, and cells of the monocye lineage. PPAR-γ is implicated in adipocyte differentiation, cellular energy homeostasis and regulation of genes that affect insulin activity (1, 22, 29, 39). PPAR-β/δ, which is expressed ubiquitously, is involved in fatty acid oxidation (13). PPARs are activated by natural ligands such as fatty acids and eicosanoids. The lipid-lowering fibrates and the insulin-sensitizing thiazolidinediones (TZDs) or glitazones are synthetic ligands for PPAR-α and PPAR-γ, respectively. The role of PPARs in gene regulation has been studied primarily in liver and adipose tissue, and they have been implicated in metabolic diseases such as obesity, diabetes, and atherosclerosis (35). However, PPAR-γ is also present in vascular smooth muscle cells (VSMCs), but its function in resistance arteries, which are important in blood pressure (BP) elevation in hypertension, remains to be elucidated (7, 29, 34).

Vascular remodeling, which is the pathophysiological adaptation of the vascular wall in hypertension, involves several processes including VSMC growth, apoptosis, and collagen deposition. ANG II plays a detrimental role in the pathogenesis of hypertension through VSMC growth by activation of ANG II type 1 (AT1) receptors (9, 36). The AT1 receptor activates phosphatidylinositol 3-kinase (PI3K) and MAPK pathways, both of which are involved in growth-promoting processes (23, 37). We have shown that both PPAR-α and PPAR-γ are expressed at significantly greater levels in blood vessels of spontaneously hypertensive rats than of normotensive Wistar-Kyoto rats, suggesting PPARs may contribute to regulation of different genes of the vasculature in hypertension (7, 20). In addition to the known inhibitory effects of TZDs on insulin-induced VSMC proliferation in vitro (14), we previously showed that TZDs have antihypertensive effects in ANG II-induced hypertension by exerting direct effects on the vascular wall, independent of their role on lipid metabolism (6).

In VSMCs, insulin exerts a mitogenic effect mediated through the MAPK pathway. Insulin-mediated signaling pathways may be inhibited by ANG II in VSMCs (31), mainly occurring through two signaling mechanisms. The PI3K pathway mediates insulin-sensitive metabolic processes, including stimulation of glucose transport and activation of the p70 S6 kinase, Akt/protein kinase B, and eukaryotic initiation factor (eIF) 4E-binding protein 1 (4E-BP1), thus subsequently stimulating glycogen and protein synthesis (21). Moreover, tyrosine-phosphorylated Src homology (SH) 2-containing inositol phosphatase 2 (SHIP2) has been demonstrated to regulate PI3K/Akt signaling pathway by altering PI3K enzymatic product and play an important role in insulin resistance (27). The second mechanism is the MAPK pathway that mediates insulin growth-promoting effects. It has been shown that TZDs troglitazone and pioglitazone decreased BP in insulin-resistant rats and in type 2 diabetic patients (19, 24). Furthermore, rosiglitazone, another TZD, enhanced the ability of insulin to transport glucose into skeletal muscle, thus lowering circulating insulin levels (26). However, the mechanisms by which PPAR-γ activators affect ANG II signaling pathways remain unclear. Thus we hypothesized that PPAR-γ activators inhibit ANG II-induced cell growth by interfering with the PI3K/Akt signaling pathways in VSMCs from resistance arteries. We questioned whether this occurs through inhibition of SHIP2 activity, which would reduce the bioactive phospholipids pro-

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Fig. 1. Peroxisome proliferator-activated receptor (PPAR)-γ activators inhibit vascular smooth muscle cell (VSMC) growth. A–E: VSMC growth after 24 h control (Ctrl; open bars), ANG II (black bars), rosiglitazone (Rosi) or 15d-PGJ₂ (light gray bars), or cotreatment of ANG II + rosiglitazone or ANG II + 15-deoxy-Δ₁₂,₁₄-prostaglandin J₂ (15d-PGJ₂; dark gray bars). Results are means ± SE (n = 3–8). *p < 0.001; †p < 0.01; ‡p < 0.001; §p < 0.05. Results are expressed as arbitrary units (AU), which represent relative counts/min of [³H]thymidine or [³H]leucine incorporation.
Fig. 2. Effect of ANG II on PPAR-γ expression and activity. A, top: immunoblots of nuclear PPAR-γ expression in VSMCs after 24 h in control (left) and after treatment with rosiglitazone (right). A, bottom: results are means ± SE (n = 5). B: representative PPAR-γ activation by electrophoretic mobility shift assay (EMSA) in VSMCs treated for 24 h with rosiglitazone. Results are means ± SE (n = 5). *P < 0.01 vs. control. Results are expressed as AU, which represent the ratio of the protein of interest/Ponceau S (PS) to correct for sample loading. The most intense band stained by Ponceau S in each lane (~50 kDa) was used for ratio calculation. PPRE, PPAR-response element.

Fig. 3. Effect of PPAR-γ activators on ANG II signaling pathways. Top: immunoblots of ERK 1/2 phosphorylation (p-ERK 1/2) and expression (ERK 1/2) (A) and Akt phosphorylation (p-Akt), and Akt expression (B). Bottom: results are ERK 1/2 phosphorylation (A) or p-Akt phosphorylation (B) and represent means ± SE (n = 5). ●, VSMCs treated with ANG II; ○, VSMCs treated with ANG II + rosiglitazone; ▲, VSMCs treated with ANG II ± 15d-PGJ2. *P < 0.05, †P < 0.01 vs. ANG II. Results are expressed as AU, which represent the ratio of the protein phosphorylation/protein expression.
duced by PI3K that stimulate Akt, or whether it results from inhibition of 4E-BP1, which would have an effect further downstream to inhibit protein synthesis and cell growth.

**MATERIALS AND METHODS**

*Cell culture.* VSMCs were obtained from mesenteric arteries of male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada) as previously described (10). Cells from passages 3–6 were grown in DMEM with 10% calf serum (Invitrogen, Grand Island, NY). All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

[^3H]thymidine and [^3H]leucine incorporation and cell proliferation. Subconfluent cells (85–90%) were serum deprived for 24 h before experiments to render them quiescent. DNA and protein synthesis, markers of hyperplasia and hypertrophy, respectively, were evaluated by [^3H]thymidine and [^3H]leucine incorporation as previously described (36), while cell proliferation was determined with light microscopy. Briefly, cells were stimulated for 24 h with ANG II (10–7 mol/l, Peptide International, Louisville, KY) without or with increasing concentrations of rosiglitazone (10–7–10–5 mol/l, Cayman Chemical, Ann Arbor, MI) or the putative endogenous PPAR-γ activator 15d-PGJ2 (10–5–10–7 mol/l, Cayman Chemical). Cells exposed to [^3H]thymidine or [^3H]leucine were then incubated with trichloroacetic acid (0.75 mol/l, 45 min) and then with NaOH (1 mol/l, 1 h at 37°C) and were analyzed by liquid scintillation counting.

Electrophoretic mobility shift assay. Quiescent cells were stimulated with rosiglitazone (10–7 mol/l) for 24 h, and nuclear extract was obtained by using Pierce NE-Per kit (Rockford, IL). Protein concentration was determined with Bio-Rad Dc kit (BioRad Laboratories, Mississauga, Canada) as previously described (36). Cells from male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada) were used as a positive control (UpState). Western blot analysis. Cells, prestimulated with or without rosiglitazone (10–7 mol/l) or 15d-PGJ2 (5 × 10–6 mol/l) for 24 h, were stimulated for various times with or without ANG II (10–7 mol/l) in the presence/absence of valsartan (10–5 mol/l, gift from Norvatis) or PD-123319 (10–6 mol/l, Tocris, Ellisville, MO). After stimulation, lysis buffer [1 mmol/l NaF, 5 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l NP40 (Roche Diagnostics, Indianapolis, IN), 10 mmol/l HEPES, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mmol/l sodium orthovanadate, 1 mmol/l PMSF] was added to cells, and total protein was extracted as previously described (36). Samples were analyzed by Western blotting using specific antibodies: PPAR-γ (Santa Cruz Biotechnology, Santa Cruz, CA) or phospho- and non-phospho-specific Akt, 4E-BP1, and ERK 1/2 (Cell Signaling, Beverly, MA). Band intensity was measured by the ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

*Cell growth.* ANG II induced a fourfold increase in [^3H]thymidine DNA incorporation, which was significantly decreased (P < 0.001) by 24 h treatment with 15d-PGJ2 (5 μmol/l) or rosiglitazone (10 μmol/l) (Fig. 1, A and B). Similarly, ANG II

![Fig. 4](http://ajpheart.physiology.org/)

Fig. 4. Acute and chronic effect of rosiglitazone on 4E-binding protein 1 (4E-BP1). A and B: immunoblots of 4E-BP1 phosphorylation (p-4E-BP1) and 4E-BP1 expression. Lanes for p-4E-BP1 correspond to bars below (left to right): 4E-BP1 phosphorylation (control; open bars) treated for 5 min (A) or 24 h (B) with ANG II without (black bars) or with rosiglitazone (light gray bars) or treated with rosiglitazone alone (dark gray bars). Results are means ± SE (n = 4). *P < 0.001; †P < 0.01; ‡P < 0.001.
induced a significant increase in [3H]leucine incorporation ($P < 0.01$), whereas neither 15d-PGJ$_2$ nor rosiglitazone alone had any effects on both thymidine and leucine incorporations. Twenty-four-hour treatment with 15d-PGJ$_2$ (5 μmol/l) or rosiglitazone (10 μmol/l) significantly decreased ANG II-induced [3H]leucine incorporation ($P < 0.001$) and ($P < 0.05$), respectively (Fig. 1, C and D). Accordingly, ANG II-induced increase in cell number was prevented by pretreatment with rosiglitazone and 15d-PGJ$_2$, respectively (Fig. 1E).

Effect of rosiglitazone on PPAR-γ expression and activation. Rosiglitazone treatment for 24 h significantly increased PPAR-γ1 nuclear expression but had no effect on PPAR-γ2 (Fig. 2A). As assessed by electrophoretic mobility shift assay, rosiglitazone induced a twofold increase in PPAR-γ activation (Fig. 2B).

Effects of PPAR-γ activators on ANG II receptors and signaling. ANG II induced a time-dependent tyrosine/threonine phosphorylation of ERK 1/2, which was reduced, at 5 min, by 55% by 15d-PGJ$_2$ and by 59% by rosiglitazone (Fig. 3A), whereas neither agent affected ERK 1/2 expression. These results demonstrate that 15d-PGJ$_2$ treatment inhibits ANG II-induced ERK 1/2 phosphorylation more rapidly (2.5 min) than rosiglitazone treatment (5 min). Similarly to ERK 1/2, Akt serine phosphorylation induced by ANG II was partially inhibited by rosiglitazone and 15d-PGJ$_2$ (71% and 58%, respectively) while Akt expression remained unaltered compared

![Fig. 5. Rosiglitazone effect on AT$_1$ receptor signaling. Immunoblots of p-ERK 1/2 and ERK 1/2 (A) or p-Akt and Akt (B). Immunoblot lanes for p-ERK 1/2 (A) or p-Akt (B), respectively, correspond to bars below (left to right): ERK 1/2 or Akt phosphorylation (open bars); 5 min ANG II treated without (black bars) or with valsartan or PD-123319 (light gray bars); 24-h rosiglitazone-pretreated cells with ANG II (dark gray bars) with valsartan or PD-123319 (horizontally hatched bars); ANG II-treated cells with valsartan and PD-123319 (cross-hatched bars). Results are means ± SE (n = 3). *P < 0.05; †P < 0.001; ‡P < 0.01.](http://ajpheart.physiology.org/)

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with controls (Fig. 3B). ANG II significantly increased 4E-BP1 phosphorylation, which was significantly inhibited by rosiglitazone ($P < 0.01$, Fig. 4A). Long-term treatment with ANG II (24 h) also significantly increased 4E-BP1 activity. 4E-BP1 was completely inhibited with rosiglitazone cotreatment ($P < 0.001$, Fig. 4B), while rosiglitazone alone did not significantly alter 4E-BP1 serine phosphorylation. VSMC AT$_1$ or AT$_2$ expression was unaltered by 24 h rosiglitazone treatment (data not shown). The AT$_1$ antagonist valsartan significantly ($P < 0.01$) inhibited ANG II-induced ERK 1/2 and Akt phosphorylation, by 88% and 62%, respectively, whereas the AT$_2$ antagonist PD-123319 had no effect (Fig. 5). Rosiglitazone did not have any additional effect on AT$_1$ or AT$_2$ blockade, suggesting that its inhibitory effects on ANG II stimulatory events were downstream of the AT$_1$ receptor (Fig. 5). Stimulation with ANG II significantly increased SHIP2 activity ($P < 0.01$), which was inhibited by rosiglitazone ($P < 0.001$) (Fig. 6). Rosiglitazone alone decreased SHIP2 activity by 81% ($P < 0.01$). However, SHIP2 protein expression was unaltered under the different experimental conditions.

**DISCUSSION**

ANG II mediates its growth-promoting effects through different signaling pathways (36). In the present study we targeted two of these signaling pathways, MAPK and PI3K. Specific inhibitors of PI3K and MEK inhibited ANG II-induced DNA incorporation in VSMCs derived from mesenteric resistance vessels (data not shown). Similar effects were previously observed in aortic VSMCs from normotensive and hypertensive rats and on human resistance and conduit vessels (10, 12, 36, 40). In this study, a putative endogenous (15d-PGJ$_2$) and an exogenous PPAR-γ activator (rosiglitazone) significantly reduced ANG II effects on DNA and protein synthesis, and cell proliferation. PPAR-γ activation negatively modulated ANG II stimulatory effects on PI3K/Akt signaling pathways through inhibition of SHIP2 activity. Furthermore, these events were associated with inhibition of the translational modulator 4E-BP1, resulting in reduced cell growth.

Law et al. (20) demonstrated nuclear PPAR-γ1 and PPAR-γ2 expression in rat and human aortic VSMC and that PPAR-γ1 expression was greater than PPAR-γ2 in nonadipose tissues, such as VSMCs. In the present study, nuclear PPAR-γ expression increased under rosiglitazone, whereas the PPAR-γ2 expression was unaltered, while the active form of PPAR-γ in the nucleus of VSMCs after rosiglitazone treatment was increased. The specificity of the interaction of PPAR-γ with its PPRE was confirmed by supershift, mutant competition, and cold competition assays. VSMC PPAR-α and PPAR-β expression and nuclear binding to the PPRE domain was unaltered by rosiglitazone treatment (data not shown), demonstrating that rosiglitazone acts as a specific PPAR-γ activator, as also demonstrated elsewhere (5).

Growth factor-mediated activation of PI3K leads to the generation of phosphatidylinositol 3,4-bisphosphate [PI(3,4)P$_2$] and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P$_3$], which recruits inactive Akt from the cytosol to the plasma membrane via pleckstrin homology domain binding. Akt is then sequentially phosphorylated at Thr308 and Ser473 by 3-phosphoinositide-

![Fig. 6. Rosiglitazone effect on SHIP2. Top: immunoblots of Src homology 2-containing inositol phosphatase 2 (SHIP2) phosphorylation and expression. Bottom: SHIP2 phosphorylation (p-SHIP2) (control; open bar) with 5 min ANG II (black bar); 24-h rosiglitazone treated cells (light gray bar) with ANG II (dark gray bar). Results are means ± SE ($n = 4$). *$P < 0.01$; †$P < 0.001$. Unstimulated Jurkat cell lysate was used as a positive control. WB, Western blot; IP, immunoprecipitate; PY20, anti-phosphotyrosine.](http://ajpheart.physiology.org/)
dependent kinase-1 and -2, respectively, which activate the kinase (3, 25, 33). Activated Akt is proposed to regulate many downstream proteins such as insulin receptor substrate 1, the mammalian target of rapamycin (mTOR) and 4E-BP1. Akt is also implicated in cell metabolism, survival, and growth and thus could potentially play a crucial role in vascular remodeling. Dugourd et al. (8) demonstrated that ANG II-induced proliferation was mediated by activation of PI3K and its downstream protein Akt in rat aortic VSMC. Similarly, we demonstrated that ANG II induced Akt activation without change in protein expression in mesenteric VSMC, which was significantly inhibited by 24-h treatment with 15d-PGJ2 or rosiglitazone.

4E-BP1 is an important translation and cell growth regulator. In the nonphosphorylated form, it binds and segregates eIF4E, the mRNA 5’ cap-binding protein, which plays a role in the recruitment of ribosomal subunits and other complexes to initiate proteins synthesis (21). Insulin and ANG II (via the AT1 receptor) induce activation of the PI3K/Akt/mTOR pathway and phosphorylate 4E-BP1, which, in turn, dissociates from eIF4E (2, 16). Here we demonstrate for the first time that rosiglitazone inhibits acute stimulatory effects on 4E-BP1 in VSMCs from resistance arteries, thus blocking translation initiation. Furthermore, 24-h ANG II treatment maintains 4E-BP1 activation and translational activity in VSMC that was inhibited by PPAR-γ activator. These data suggest important antihypertrophic effects of rosiglitazone by altering protein synthesis through 4E-BP1, which correlate with the reduced Akt activity, thus demonstrating an important pathway by which PPAR-γ activator may reduce ANG II-induced cell growth effects.

The AT1 receptor can activate many signaling pathways in different cell types, including MAPK. Once ERK 1/2 are activated, they translocate into the nucleus where they regulate several transcription factors involved in cellular growth and differentiation (30). In conduit vessels, the PPAR-γ activator troglitazone reduced ANG II-induced ERK 1/2 activation (12, 15). In the present study we found that PPAR-γ activation exerted similar effects on VSMCs derived from resistance arteries. Although 15d-PGJ2 treatment inhibited ANG II-induced ERK 1/2 tyrosine/threonine phosphorylation more rapidly than rosiglitazone treatment, both these agonists blocked cell growth. Furthermore, it is possible that 15d-PGJ2 interferes with other signaling pathways that are linked to ERK 1/2 activation, thus causing a more rapid alteration in ANG II stimulatory effects.

Most of the known functional effects of ANG II on VSMCs are mediated by AT1 receptors (9, 36). Takeda et al. (34) showed a significant decrease of AT1 mRNA after 6 h treatment with PPAR-γ activators troglitazone and pioglitazone, with return to basal levels after 12–24 h in VSMCs (34). In resistance artery VSMCs, the present study showed that 24 h rosiglitazone treatment had no effect on ANG II receptor expression, whereas AT1-dependent MAPK and PI3K/Akt/4E-BP1 activation were downregulated. ANG II-activated signaling molecules are regulated by various kinases and phosphatases. We demonstrated for the first time that the lipid phosphatase SHIP2 is activated by ANG II. Activation of SHIP2 hydrolyzes the PI3K product PI(3,4,5)P3 to PI(3,4)P2, which is the main lipid necessary for Akt activation and recruitment to the cell membrane (11, 18, 28). Here we show that ANG II stimulation significantly increased SHIP2 phosphorylation, an effect that was prevented by rosiglitazone. Furthermore, rosiglitazone alone significantly reduced SHIP2 activity, suggesting an ANG II-independent inhibitory effect. This raises the possibility that rosiglitazone may interfere directly with SHIP2 activity. However, it is noteworthy that 10 μmol/l rosiglitazone alone did not decrease cell growth, even if SHIP2 activity was reduced. It has been demonstrated that crosstalk between ANG II and insulin inhibits signaling pathways, leading to insulin resistance and vascular pathologies such as atherosclerosis (4, 32, 38). Furthermore, Hori et al. (17) demonstrated that SHIP2 expression was increased in insulin-resistant diabetic db/db mice compared with controls. Taken together, this suggests that rosiglitazone could exert its effect on insulin sensitivity in part through the inhibition of ANG II-induced SHIP2 activity.

In conclusion, although the precise mechanism of action of PPAR-γ activators on ANG II-induced signaling is not yet completely clear, we demonstrate that PPAR-γ activators have potent negative effects on ANG II-mediated cell growth by interfering with PI3K/Akt signaling pathway. This occurs through inhibition of SHIP2 activity, which would reduce the bioactive phospholipids produced by PI3K that stimulate Akt, and by the inhibition of 4E-BP1, which would have an effect further downstream to inhibit protein synthesis and cell growth. These data provide new insights into potential beneficial effects of PPAR-γ activators in the prevention or treatment of cardiovascular disease and hypertension. Although further experiments are needed to clarify rosiglitazone inhibitory effects on ANG II-induced SHIP2 activation and its regulatory effects on PI3K phospholipid synthesis, downregulation of these pathways by PPAR-γ activators could contribute to prevention or regression of vascular remodeling in hypertensive and diabetic patients.

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