Peroxisome proliferator–activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor superfamily. Three PPAR isoforms have been cloned: PPAR-α, PPAR-β/δ, and PPAR-γ. PPARs bind to PPAR-response elements and regulate many genes with effects on lipid metabolism and carbohydrate metabolism, endothelial function, inflammation, and thrombosis. Insulin-sensitizing thiazolidinediones (TZDs) or glitazones are PPAR-γ synthetic ligands and have been proposed as therapeutic candidates in cardiovascular disease. They are potent inhibitors of monocyte chemotaxis, cell growth, and migration of vascular smooth muscle cells (VSMCs) and reduce vascular inflammation and vasoconstriction, thereby modulating vascular structure and function.

Vascular remodeling in hypertension, which involves a pathophysiological adaptation of the vascular wall, comprises several processes, including VSMC contraction and migration, growth and apoptosis, extracellular matrix deposition, and inflammation. Angiotensin (Ang) II plays a role in vascular remodeling through the G-protein–coupled Ang type 1 (AT₁) and AT₂ receptors. Multiple growth-promoting signaling pathways are activated by Ang II through AT₁ receptors, such as phosphatidylinositol 3-kinase (PI3K/p85α) and extracellular signal–regulated kinase (ERK) 1/2.

P/EPI3K regulates PI3K activation and its downstream protein Akt in rat aorta and mesenteric arteries. Key Words: aorta ■ mesenteric arteries ■ hypertension, experimental ■ signal transduction ■ phosphatases

Abstract—Angiotensin (Ang) II is implicated in hypertension, vascular remodeling, and insulin resistance. Peroxisome proliferator–activated receptor (PPAR) γ activators increase insulin sensitivity and improve Ang II–induced vascular remodeling. We evaluated the effects of the PPAR-γ activator rosiglitazone on Ang II signaling in aorta and mesenteric arteries. Rats received Ang II by subcutaneous infusion and/or rosiglitazone per os for 7 days. Blood pressure rise in Ang II–infused rats was attenuated by rosiglitazone. Ang II significantly increased Ang II type 1 receptor expression in the mesenteric arteries (P<0.001), whereas that of the aorta was decreased (P<0.05), changes which were reversed by rosiglitazone. Akt activity was increased by Ang II and returned to basal levels under rosiglitazone in both vascular beds. However, Ang II–induced extracellular signal–regulated kinase 1/2 activity increased in aorta but not in mesenteric vessels (P<0.001), where 4E-binding protein 1 activity was significantly increased by Ang II and inhibited by PPAR-γ activation. In response to Ang II, Src homology (SH) 2–containing inositol phosphatase 2 activity was increased (P<0.05) in both vascular beds. In conclusion, PPAR-γ activator rosiglitazone attenuated Ang II–induced blood pressure elevation and intracellular signaling on aorta and mesenteric vessels. There was differential inhibition of Ang II type 1 receptor receptors/phosphatidylinositol 3-kinase/Akt and extracellular signal–regulated kinase 1/2 in both vessels. Effects of PPAR-γ activators on these pathways could contribute to regression of vascular remodeling in models of hypertension and diabetes and, accordingly, in hypertensive diabetic patients. (Hypertension. 2006;47:102-108.)

Key Words: aorta ■ mesenteric arteries ■ hypertension, experimental ■ signal transduction ■ phosphatases
Ile5-Ang II (Calbiochem, 120 ng/kg per minute SC) via osmotic pump and hydralazine. Ang II animals were infused with rosiglitazone, and hydralazine. Ang II receptors in aortic VSMCs.4,23–25 We demonstrated previously that PPAR-γ activators decreased blood pressure (BP) and cell growth and improved endothelial dysfunction in mesenteric resistance arteries from Ang II-infused rats,4 thus suggesting that PPAR-γ could contribute to the regulation of different vascular genes in hypertension. The in vivo mechanisms whereby PPAR-γ activators affect Ang II signaling in the vasculature remain unclear. Here, we questioned whether Ang II-induced PI3K and MAPK activation and some of their distal signaling pathways in aorta and mesenteric arteries could be inhibited in vivo by the PPAR-γ activator rosiglitazone.

Methods

Animal Experiments
The study was conducted according to recommendations from the Animal Care Committee of the Clinical Research Institute of Montreal and the Canadian Council of Animal Care. Male Sprague-Dawley rats (250 g, Charles River), were divided in 6 groups: control, Ang II, Ang II+rosiglitazone, Ang II+hydralazine, rosiglitazone, and hydralazine. Ang II animals were infused with Ile'-Ang II (Calbiochem, 120 ng/kg per minute SC) via osmotic minipumps for 7 days. Rosiglitazone (5 mg/kg day) was mixed with food and hydralazine, used to evaluate the effect of BP lowering, was given in drinking water (25 mg/kg day). Before the end of the study, systolic BP (SBP) was measured by the tail-cuff method. Rats were killed humanely. Defatted aorta and mesenteric arteries were snap frozen and stored at −80°C until analysis.

Electrophoretic Mobility Shift Assay for PPAR-γ
Frozen tissues were homogenized, suspended in a PBS buffer (containing Na2VO4, 1 mM NaCl, pepstatin A 1 μg/mL, leupeptin 1 μg/mL, aprotonin 1 μg/mL, and PMSF 1 mM) and centrifuged (4000 g, 4 minutes at 4°C) as described previously.4 The pellet was resuspended and lysed into cytoplasmic and nuclear fractions using a Pierce NE-PER kit. Protein concentration was determined with a BioRad Dc kit. VSMC PPAR-γ activity was detected using the Nushift kit (Active Motif). Wild-type oligonucleotides 5'-GGAACTAGGTCAAAGGTCATCCCCT-3' and mutant oligonucleotides 5'-GGAACTAGGTCAAAGGTCATCCTCCCT-3' were used in a competition assay, whereas supershift was performed using PPAR-γ antibody from Active Motif.

Western Blot Analysis
Extracted protein was loaded on 10% SDS-PAGE and transferred onto nitrocellulose membranes, which were then blocked and incubated with specific antibodies: AT1, PPAR-γ (Santa Cruz Biotechnology), PI3K-p85α (UpState), phospho- (Thr308 and Ser473) and nonphospho-specific Akt, 4E-BP1, and ERK1/2 (Cell Signaling). Membranes were subsequently washed, incubated with specific secondary horseradish peroxidase–conjugated antibodies, and revealed with BM chemiluminescence blotting substrate (Roche Diagnostics). Thereafter, band intensity was measured by ImageQuant 5.0 software (Molecular Dynamics).

Immunoprecipitation
Vessels were lysed, and 500 μg of total protein extract was immunoprecipitated with SHIP2 (UpState) antibody and then incubated with protein G Plus-Agarose (Calbiochem). Thereafter, Western blotting was carried out as mentioned above, and membranes were incubated with the anti-phosphotyrosine (PY20, BD Transduction Laboratories) antibody for activity measurement and then stripped and reblotted with SHIP2 antibody for total protein.

Data Analysis
Values are mean±SEM. Differences were evaluated by 1-way ANOVA, followed by Tukey-Kramer’s post-hoc test. P<0.05 was considered significant.

Results

Physiological Parameters
Ang II significantly increased SBP (P<0.001 versus controls), which was significantly attenuated by both rosiglitazone and hydralazine (P<0.001). Neither rosiglitazone nor hydralazine alone affected SBP. The body weight of rats was unaltered during the study (Table I, available online at http://hyper.ahajournals.org).

Vascular PPAR-γ Expression and Activity
Rosiglitazone treatment significantly increased mesenteric artery PPAR-γ expression (P<0.01 versus controls) and activity (P<0.05) in Ang II–infused rats, whereas rosiglitazone alone increased PPAR-γ expression only (P<0.05; Figure 1A). Ang II elicited a trend to a reduction of PPAR-γ expression and activity in mesenteric arteries, although this did not achieve statistical significance. Hydralazine did not affect either of these parameters (Figure 1B).

Vascular Effects of Rosiglitazone on AT1 Expression and Signaling
Ang II induced a 7-fold increase in AT1 expression of mesenteric arteries (P<0.001), which was significantly downregulated by rosiglitazone (Figure 2A). In aorta, Ang II downregulated AT1 expression (P<0.05), which was upregulated 4-fold (P<0.01) by rosiglitazone cotreatment (Figure 2B). Ang II increased Akt activity in both mesenteric arteries and aorta 10-fold (P<0.001) and 3-fold (P<0.01), respectively. Both changes were inhibited by rosiglitazone cotreatment, whereas rosiglitazone alone induced a 5-fold increase in mesenteric artery but not aortic Akt activity (Figure 3). Neither Ang II nor rosiglitazone altered aortic or mesenteric artery basal expression of Akt or p85α (Figure 3). Ang II–induced Akt activity was unaffected by hydralazine. In mesenteric arteries, but not in aorta, Ang II induced a 2-fold increase (P<0.01) in 4E-BP1 activity, which was significantly inhibited (P<0.01) by cotreatment with rosiglitazone (Figure 4). Neither Ang II nor rosiglitazone altered vascular 4E-BP1 expression. In both vascular beds, Ang II induced a 2-fold increase (P<0.01) in SHIP2 activity, which was unaffected by rosiglitazone (Figure 5). Neither Ang II nor rosiglitazone altered SHIP2 expression in aorta or mesenteric arteries.

ERK1/2 expression increased (P<0.05) in mesenteric arteries after Ang II infusion and was reduced by rosiglitazone cotreatment (3-fold; P<0.05; Figure 6A), but ERK1/2 activ-
ity was unaffected (Figure 6B). Neither Ang II nor rosiglitazone altered ERK1/2 expression in aorta (Figure 6C), whereas ERK 1/2 activity was significantly increased by Ang II (P<0.001) and significantly inhibited by rosiglitazone cotreatment (P<0.01). Rosiglitazone administered alone increased (P<0.001) ERK1/2 activity in aorta 10-fold (Figure 6D), whereas hydralazine decreased Ang II–induced ERK1/2 phosphorylation (P<0.05).

Figure 1. Rosiglitazone effect on vascular PPAR-γ expression and activity. (A) Top: representative immunoblots of total PPAR-γ expression in mesenteric arteries and Ponceau S staining. Bottom: results are mean±SEM (n=5), expressed as arbitrary units (A.U.), which represent the ratio of the protein of interest/Ponceau S. (B) Left: representative PPAR-γ activation by electrophoretic mobility shift assay in mesenteric arteries, where SS represents supershift. Right: results are mean±SEM (n=5), Control (white), Ang II (black), Ang II + rosiglitazone (Rosi, hatched), Ang II + hydralazine (Hyd, vertical), rosiglitazone (checkered), hydralazine (dotted). *P<0.001 vs Ang II, †P<0.01 vs Ctrl, ‡P<0.01 vs Ctrl and Ang II.

Figure 2. Effect of rosiglitazone on vascular AT1 receptor expression. (A and B) Top: representative immunoblots of total mesenteric artery and aorta AT1 expression. Bottom: results are mean±SEM (n=5), expressed as arbitrary units (A.U.), which represent ratio of the protein of interest/Ponceau S. Control (white), Ang II (black), Ang II + rosiglitazone (Rosi, hatched), Ang II + hydralazine (Hyd, vertical), rosiglitazone (checkered), hydralazine (dotted). *P<0.05 vs Ang II, †P<0.05 vs Ang II + Rosi, ‡P<0.01 vs Ctrl, §P<0.01 vs Ang II.
The present study demonstrates for the first time that rosiglitazone treatment in Ang II–infused rats differentially modulates PI3K and MAPK signaling in a conduit vessel, aorta, and in mesenteric vessels. We and others have shown previously that in models of experimental hypertension, TZDs are antihypertensive and reverse structural, functional, and molecular changes in blood vessels. The mechanisms of these potentially beneficial cardiovascular effects are in part clarified in the present study. PPAR-γ activation negatively modulated Ang II–induced growth–promoting intracellular signaling pathways, such as MAPK and PI3K, and their target protein, such as 4E-BP1. Furthermore, we demonstrated that chronic Ang II treatment increased the activity of a phosphatase linked to Akt activation, SHIP2, in both mesenteric vessels and aorta.

Although rosiglitazone alone did not alter AT1 receptor expression, rosiglitazone cotreatment in Ang II–infused rats returned AT1 receptor expression to basal levels in both aorta and mesenteric vessels. This suggests that PPAR-γ activation regulates AT1 receptor expression. Our findings extend those of Takeda et al and Sugawara et al, who demonstrated that 24-hour treatment with rosiglitazone returned VSMC AT1 receptor expression back to basal levels compared with 6 to 12 hours of rosiglitazone treatment. Similarly, we observed that 24-hour PPAR-γ activation in vitro did not alter VSMC AT1 receptor expression. Nonetheless, we have demonstrated differential AT1 expression in aorta compared with mesenteric vessels from Ang II–infused rats. Consequently, this may result in a differential regulation of Ang II signaling pathways in these vascular beds. Rosiglitazone-induced mesenteric artery AT1 receptor downregulation may represent a mechanism whereby PPAR-γ activation may inhibit vascular remodeling induced by Ang II. This could support previously documented beneficial effects of PPAR-γ on vascular remodeling. Conversely, rosiglitazone-induced upregulation of AT1 in aorta may be a compensatory mechanism to the effects of Ang II on AT1 expression.

On binding to the AT1 receptor, Ang II activates multiple signaling pathways, such as PI3K and MAPK, that participate in cell growth and vascular remodeling. We demonstrated that 7-day Ang II infusion induced DNA synthesis in mesenteric arteries that were inhibited by PPAR-γ activators, whereas others have shown that infusion of Ang II for 24

![Image of Figure 3: Vascular effect of rosiglitazone on PI3K/p85α/Akt signaling.](image)

![Image of Figure 4: Vascular effect of rosiglitazone on 4E-BP1 activation.](image)
hours induced protein synthesis in both aorta and mesenteric arteries through activation of PI3K/p85α and MAPK.31 We also demonstrated that p85α subunit expression in the vasculature was unaltered after 7-day Ang II or rosiglitazone treatment, in contrast to reports in human adipocytes that PPAR-γ activation increased p85α protein expression.32 Because blood vessels and adipose tissue have different metabolic activities, differential PI3K protein regulation may occur.

Figure 5. Vascular effect of rosiglitazone on SHIP2 activation. (A and B) Top: representative immunoblots of mesenteric artery and aorta SHIP2 phosphorylation (p-SHIP2) and expression, where IP and WB represent immunoprecipitation and Western blotting, respectively. Bottom: results are mean±SEM (n=4), expressed as arbitrary units (A.U.), which represent ratio of phosphorylated/nonphosphorylated protein. Control (Ctrl, white), Ang II (black), Ang II+rosiglitazone (Rosi, hatched), Ang II+hydralazine (Hyd, vertical), rosiglitazone (checkered), hydralazine (dotted). *P<0.05 vs Ctrl.

Figure 6. Effect of rosiglitazone on MAPK signaling pathway. Top: representative immunoblots of ERK 1/2 expression in mesenteric artery (A) and aorta (C) and activity (p-ERK 1/2) in mesentry (B) and aorta (D). Bottom (A and B): results are mean±SEM (n=5), expressed as arbitrary units (A.U.), 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 (~37 kDa) was used for ratio calculation. Bottom (C and D): results are mean±SEM (n=5), expressed in arbitrary units (A.U.) as ratio of phosphorylated/nonphosphorylated protein. Control (Ctrl, white), Ang II (black), Ang II+rosiglitazone (Rosi, hatched), Ang II+hydralazine (Hyd, vertical), rosiglitazone (checkered), hydralazine (dotted). *P<0.05 vs Ctrl and Ang II+Rosi. †P<0.05 vs Ang II+Rosi and Ang II+Hyd, ‡P<0.001 vs Ctrl, §P<0.01 vs Ctrl.
Ang II–increased vascular Akt activation was inhibited by rosiglitazone treatment, similar to our in vitro studies.\textsuperscript{29} Neither Ang II nor rosiglitazone affected vascular Akt expression, whereas rosiglitazone treatment alone increased Akt phosphorylation in mesenteric arteries but not in aorta. Because PPAR-\(\gamma\) activation has direct effects on adipocyte differentiation and lipid storage,\textsuperscript{33} underlying mesenteric fat could activate different tyrosine kinase receptors, thereby increasing PI3K/Akt signaling. Indeed, recent evidence suggests that the periodontal fat may regulate vascular function in a paracrine fashion.\textsuperscript{34}

Regulation of the MAPK signaling pathway was differentially modulated by Ang II and PPAR-\(\gamma\) activation in mesenteric vessels and aorta. Chronic Ang II infusion increased ERK 1/2 activity in the aorta but not in mesenteric arteries, which extends previous observations by Daigle et al\textsuperscript{31} However, others found that ERK 1/2 activity increased after acute ex vivo stimulation of Ang II in pressurized mesenteric resistance vessels.\textsuperscript{35} Basal ERK 1/2 activity was greater in mesenteric vessels compared with that of aorta, which may be an expression of the heterogeneity of the 2 vascular territories. It is noteworthy that the combined effects of BP elevation and Ang II increased ERK 1/2 activity in aorta to the level found in mesenteric arteries. Based on the different functions of these vessels (conductance versus resistance), Ang II may regulate ERK 1/2 differentially.

Cotreatment with hydralazine partially inhibited Ang II–induced ERK 1/2 activation in the aorta, suggesting that this effect may be BP dependent. Alternatively, this finding could be attributed to a direct effect of hydralazine, for example, as an antioxidant,\textsuperscript{36} which has been shown to inhibit MAPK activation.\textsuperscript{37} In support of this, we reported previously that hydralazine decreased ROS generation in the aorta but not in mesenteric arteries.\textsuperscript{38} We also found that rosiglitazone administration alone tended to increase ERK 1/2 activity in conduit vessels, while at the same time it abrogated Ang II–induced ERK 1/2 in mesenteric arteries, again indicative of differential regulation in these 2 vascular beds. Mesenteric artery VSMC growth in response to Ang II treatment appears to be regulated through an ERK 1/2-independent pathway.\textsuperscript{35} Because different transcription factors, such as nuclear factor \(\kappa B\), AP-1, and Sp1, can be activated by Ang II,\textsuperscript{4,28,39} it is possible that chronic Ang II treatment may increase these transcription factors, which are implicated by ERK 1/2 gene expression regulation without altering ERK 1/2 activity. Moreover, increased activity of SHIP2 phosphatase in mesenteric arteries occurred after Ang II and rosiglitazone treatment. In support of this, we demonstrated in vitro that both Ang II and rosiglitazone regulate phosphatase activity in VSMCs derived from mesenteric arteries.\textsuperscript{29}

We have shown here for the first time that the phosphorylation of 4E-BP1, which is an important cell growth regulator, was increased by Ang II in mesenteric arteries but not in aorta and that this increase was prevented by rosiglitazone. This extends previous in vitro observations that acute stimulation of aortic VSMC with Ang II increased activation of 4E-BP1, associated with cell growth,\textsuperscript{40} found also in mesenteric VSMC.\textsuperscript{29} The beneficial effects of PPAR-\(\gamma\) activation on Ang II–induced vascular remodeling could be partially mediated through inhibition of 4E-BP1. Furthermore, the activation of 4E-BP1 in mesenteric arteries could be associated with vascular remodeling, which is not observed in aorta.\textsuperscript{41} This suggests that PPAR-\(\gamma\) can exert an inhibitory effect on vascular remodeling in type 2 diabetic and hypertensive patients, because type 2 diabetic patients exhibit significant remodeling of small arteries compared with nondiabetic normotensive patients.\textsuperscript{42}

It is well known that cell growth is under the control of many kinases and phosphatases. Here we demonstrated for the first time that chronic Ang II–increased SHIP2 activity was associated with vascular Akt activation. This extends in an in vivo paradigm our previous in vitro findings showing that acute Ang II stimulation of mesenteric VSMC increased SHIP2/Akt activation and that both could be inhibited by rosiglitazone cotreatment.\textsuperscript{29} Although an acute stimulation with Ang II increased SHIP2 activity, chronic cotreatment with rosiglitazone did not reduce this effect, which suggests that the inhibitory effect of rosiglitazone on Ang II–induced cell growth and vascular remodeling through SHIP2 may be important in the acute stimulatory phase.

In conclusion, although the precise mechanism of action of the PPAR-\(\gamma\) activator rosiglitazone on chronic Ang II–induced PI3K and MAPK signaling remains elusive, we have demonstrated that this agent has potent modulatory effects on aorta and mesenteric vessels by the inhibition of PI3K/Akt/4E-BP1 and ERK1/2 signaling pathways. These data provide new insights into potential beneficial effects of TZDs in the prevention or treatment of cardiovascular disease and hypertension.

**Perspectives**

Although additional investigation is required to elucidate the effects of rosiglitazone on vascular AT, receptor regulation under chronic Ang II treatment, PPAR-\(\gamma\) regulation of PI3K and MAPK may represent an important link between molecular dysfunction and hypertensive and diabetic vascular disease. Consequently, regulation of these pathways by PPAR-\(\gamma\) activators could contribute to regression of vascular remodeling in hypertensive and diabetic models, and, accordingly, in diabetic hypertensive patients.

**Acknowledgments**

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Peroxisome Proliferator–Activated Receptor γ Regulates Angiotensin II–Stimulated Phosphatidylinositol 3-Kinase and Mitogen-Activated Protein Kinase in Blood Vessels In Vivo

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Table I. Body weight and systolic blood pressure.

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<th>Parameter</th>
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<th>Ang II</th>
<th>Ang II +</th>
<th>Ang II + Rosiglitazone</th>
<th>Hydralazine</th>
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<td>Body weight (g)</td>
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Results are mean±SEM (n=6).*P<0.001 vs. controls, †P<0.001 vs. controls and Ang II, ‡P<0.001 vs. Ang II.