Antihypertensive Effects of Peroxisome Proliferator-Activated Receptor-β Activation in Spontaneously Hypertensive Rats

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Abstract—Activation of nuclear hormone receptor peroxisome proliferator-activated receptor β/δ (PPARβ) has been shown to improve insulin resistance and plasma high-density lipoprotein levels, but nothing is known about its effects in genetic hypertension. We studied whether the PPARβ agonist GW0742 might exert antihypertensive effects in spontaneously hypertensive rats (SHRs). The rats were divided into 4 groups, Wistar Kyoto rat-control, Wistar Kyoto rat-treated (GW0742, 5 mg·kg⁻¹·day⁻¹ by oral gavage), SHR-control, and SHR-treated, and followed for 5 weeks. GW0742 induced a progressive reduction in systolic arterial blood pressure and heart rate in SHRs and reduced the mesenteric arterial remodeling, the increased aortic vasoconstriction to angiotensin II, and the endothelial dysfunction characteristic of SHRs. These effects were accompanied by a significant increase in endothelial NO synthase activity attributed to upregulated endothelial NO synthase and downregulated caveolin 1 protein expression. Moreover, GW0742 inhibited vascular superoxide production, downregulated p22phox and p47phox proteins, decreased both basal and angiotensin II–stimulated NADPH oxidase activity, inhibited extracellular-regulated kinase 1/2 activation, and reduced the expression of the proinflammatory and proatherogenic genes, interleukin 1β, interleukin 6, or intercellular adhesion molecule 1. None of these effects were observed in Wistar Kyoto rats. PPARβ activation, both in vitro and in vivo, increased the expression of the regulators of G protein–coupled signaling proteins RGS4 and RGS5, which negatively modulated the vascular actions of angiotensin II. PPARβ activation exerted antihypertensive effects, restored the vascular structure and function, and reduced the oxidative, proinflammatory, and proatherogenic status of SHRs. We propose PPARβ as a new therapeutic target in hypertension. (Hypertension. 2011;58:00-00.) • Online Data Supplement

Key Words: PPARβ/δ • spontaneously hypertensive rat • endothelial dysfunction • NADPH oxidase • regulators of G protein–coupled signaling proteins

The peroxisome proliferator-activated receptors (PPARs) PPARα, PPARβ/δ, and PPARγ are members of the nuclear hormone receptor superfamily. PPARs were initially believed to regulate genes involved only in lipid and glucose metabolism.¹ However, in recent years, evidence suggests that activation of PPARα or PPARγ may exert cardiovascular protection beyond their metabolic effects.² In fact, PPARα or PPARγ agonists exert antihypertensive effects in both human and animal models with or without metabolic disorders.³–⁶ The mechanisms underlying the beneficial effects of PPARs beyond glucose and lipid metabolism may relate to their anti-inflammatory and antioxidant actions.⁵ Thus, activation of both PPARα or PPARγ antagonizes angiotensin II (Ang II) actions, including the activation of NADPH oxidase and the generation of reactive oxygen species, as well as the increase in proinflammatory mediators and adhesion molecules in blood vessels.⁵,⁶ Activation of PPARβ/δ (PPARβ) also exhibits anti-inflammatory properties in the vessel wall by inhibiting the expression of vascular cell adhesion molecule 1 and monocyte chemotacttractant protein 1.⁷ Moreover, the oral administration of PPARβ agonist GW0742 reduces atherosclerosis in low-density lipoprotein receptor knockout (low-density lipoprotein receptor /− /−) mice⁸ and substantially attenuated Ang II–accelerated atherosclerosis and the associated arterial inflammatory and atherosclerotic gene expression.⁹ PPARβ
agonists also induce vasodilator effects in isolated aortas and mesenteric and pulmonary arteries.10,11 Furthermore, a PPARβ/γ agonist reduced the right heart hypertrophy and right ventricular systolic pressure in an experimental model of pulmonary arterial hypertension induced by chronic hypoxia.11 We hypothesized that the PPARβ agonist GW0742 would reduce blood pressure, improving vascular inflammation and endothelial function in the spontaneously hypertensive rat (SHR).

Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and our institutional guidelines for the ethical care of animals. Twelve-week-old, male SHRs and Wistar Kyoto rats (WKYs) were obtained from Harlan Laboratories (Barcelona, Spain). Forty WKYs and 40 SHRs were randomly assigned to a control group (vehicle, 1 mL of 1% methylcellulose orally by gavage) or a treated group (GW0742, 5 mg/kg per day, mixed in 1 mL of 1% methylcellulose) and followed for 5 weeks. Systolic blood pressure and heart rate were measured in conscious, prewarmed, restrained rats by tail-cuff plethysmography. Oral glucose tolerance was tested. Mesenteric arterial media thickness, lumen diameter, media cross-sectional area, and media:lumen ratio were measured in 5-μm cross-sections. The cardiac, left ventricular, and renal weight indices were calculated by dividing the heart, left ventricle, and kidney weight by the body weight. Histopathologic evaluation of kidneys and hearts was also performed. Insulin, tumor necrosis factor-α, and 8-iso-prostaglandin F₂α were quantified by enzyme immunoassay. Descending thoracic aortic rings were mounted in organ baths for isometric tension recording. Superoxide (O₂⁻) levels were estimated from the ratio of ethidium:4',6-diamidino-2-phenylindole fluorescence in sections of unfixed thoracic aortic rings incubated for 30 minutes with dihydroethidium and counterstained with the nuclear stain 4',6-diamidino-2-phenylindole. NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence assay stimulated by addition of NADPH in the absence or in the presence of Ang II. Protein and mRNA expression were measured by Western blotting analysis and RT-PCR analysis, respectively. An expanded Methods section is available in the online Data Supplement (please see http://hyper.ahajournals.org).

Results

Effects of GW0742 on Blood Pressure, Morphological Variables, and Plasma and Urinary Determinations

Long-term GW0742 administration induced a progressive reduction in systolic blood pressure (≈11% at the end of the 5 weeks; P<0.01 versus untreated SHR) and heart rate (≈8% at the end of the 5 weeks; P<0.05 versus untreated SHR) in SHRs but had no effect in WKYs (Figure 1).

Body weight (BW) increased in both WKY and SHR control groups after 5 weeks (10.1±0.6% and 8.4±0.7%, respectively). GW0742 treatment significantly decreased the gain in BW (6.8±0.4%) and the absolute visceral fat:BW at the end of treatment only in WKYs as compared with control-treated rats (Table 1). This effect seems to be unrelated to changes in food and water intake, which were unaffected by GW0742. Absolute left ventricle weight and left ventricle weight relative to heart weight and kidney weight relative to BW were higher in the SHR control group as compared with the WKY control group. GW0742 neither reduced left ventricle weight:heart weight nor kidney weight:BW in SHRs despite its antihypertensive effects (Table 1). Moreover, GW0742 increased kidney weight:BW

Table 1. Body and Organ Weights and Cardiac and Renal Indices

<table>
<thead>
<tr>
<th>Groups</th>
<th>BW Initial, g</th>
<th>BW Final, g</th>
<th>HW, mg</th>
<th>LWV, mg</th>
<th>KW, mg</th>
<th>VF, g</th>
<th>LVW/HW Ratio</th>
<th>LWV/BW Ratio</th>
<th>KW/BW Ratio</th>
<th>VF/BW Ratio</th>
</tr>
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<tbody>
<tr>
<td>WKY control</td>
<td>321±4</td>
<td>357±5</td>
<td>935±31</td>
<td>688±20</td>
<td>874±11</td>
<td>6.4±1</td>
<td>0.73±0.01</td>
<td>1.93±0.06</td>
<td>2.45±0.04</td>
<td>18.0±2.8</td>
</tr>
<tr>
<td>WKY treated</td>
<td>315±2</td>
<td>337±2†</td>
<td>924±29</td>
<td>674±22</td>
<td>912±17</td>
<td>4.0±0.3*</td>
<td>0.73±0.01</td>
<td>2.00±0.06</td>
<td>2.71±0.05†</td>
<td>11.8±1.0*</td>
</tr>
<tr>
<td>SHR control</td>
<td>295±5†</td>
<td>321±5†</td>
<td>1045±30*</td>
<td>812±26†</td>
<td>869±16</td>
<td>3.7±0.2*</td>
<td>0.78±0.01†</td>
<td>2.58±0.08†</td>
<td>2.71±0.05†</td>
<td>10.9±0.6*</td>
</tr>
<tr>
<td>SHR treated</td>
<td>280±6</td>
<td>307±6†</td>
<td>1055±19</td>
<td>827±17</td>
<td>867±17</td>
<td>3.9±0.2</td>
<td>0.78±0.01</td>
<td>2.70±0.04</td>
<td>2.83±0.02</td>
<td>12.0±0.8</td>
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</tbody>
</table>

BW indicates body weight; HW, heart weight; LWV, left ventricle weight; KW, kidney weight; VF, visceral fat; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat. Values are expressed as mean±SEM of 20 rats, except for VF (n=10).  
*P<0.05 vs WKY control.  
†P<0.01 vs WKY control.  
‡P<0.05 treated vs SHR control.
in the WKY strain, possibly as a result of reduced BW. Interestingly, histopathologic evaluation of the hearts showed that both cardiac fiber length (Figure S1A, available in the online Data Supplement) and calcineurin gene expression (Figure S1B), a marker of cardiac hypertrophy, were increased in SHRs and reduced by GW0742. Masson trichrome staining (collagen type I content; Figure S1C) was also increased in SHRs (area of fibrosis: 19 ± 2%) as compared with WKYs (5 ± 1%), which was reduced by GW0742 treatment (9 ± 2%), being without effect in WKYs (6 ± 2%). Histological analysis of the kidney showed no obvious abnormalities among groups (Figure S1D).

When compared with vehicle-treated WKYs, vehicle-treated SHRs showed structural alterations in small mesenteric arteries characterized by a significant increase in media cross-sectional area (89%) and media:lumen ratio (74%; Figure 2). Treatment with GW0742 had no effect on the morphological parameters of mesenteric vessels in WKYs but significantly reduced all of these structural alterations in SHRs.

Total cholesterol, triglycerides, free fatty acids, and plasma tumor necrosis factor-α concentrations were not elevated in SHRs and were unaffected by the GW0742 treatment (Table 2). Fasting insulin and glucose levels were increased significantly in SHRs but were unchanged by GW0742. However, impaired glucose tolerance in SHRs was improved by GW0742 (Figure S2). GW0742 also increased high-density lipoprotein levels in SHRs, which were decreased in SHR controls compared with the WKY control group.

Plasma malondialdehyde levels, a marker of lipid peroxidation, and 24-hour urinary iso-prostaglandin F\(_{2\alpha}\) excretion, a specific marker of systemic O\(_2^\cdot\) production, were higher in SHRs as compared with their normotensive counterparts. GW0742 treatment only significantly reduced these parameters in SHRs and was without effect in WKYs (Table 2).

**Effects of GW0742 on PPARβ and PPARβ Target Genes in Aortas**

The gene and protein expression of PPARβ was significantly increased in the aorta (Figure 3A and 3D) from SHRs as compared with WKYs. The mRNA levels of PPARβ were also increased in the kidney (Figure S3A) but reduced in the heart (Figure S3B) from SHRs. Chronic treatment with GW0742 restored these changes of PPARβ in SHRs to the levels found in WKY controls. In SHR control rings the

<table>
<thead>
<tr>
<th>Target</th>
<th>Group</th>
<th>WKY Control (n=10)</th>
<th>WKY Treated (n=10)</th>
<th>SHR Control (n=10)</th>
<th>SHR Treated (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol, mg/dL</td>
<td>53.5 ± 2.3</td>
<td>65.2 ± 5.6</td>
<td>48.7 ± 1.9</td>
<td>53.7 ± 1.6</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>38.3 ± 2.8</td>
<td>43.4 ± 5.7</td>
<td>32.4 ± 1.5</td>
<td>39.2 ± 2.5</td>
<td></td>
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<tr>
<td>HDL, mg/dL</td>
<td>51.7 ± 3.5</td>
<td>53.9 ± 3.6</td>
<td>41.7 ± 1.7&quot;</td>
<td>50.3 ± 1.8§</td>
<td></td>
</tr>
<tr>
<td>Fatty acids, mmol/L</td>
<td>0.59 ± 0.07</td>
<td>0.62 ± 0.03</td>
<td>0.57 ± 0.04</td>
<td>0.60 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Glucose (n=16), mg/dL</td>
<td>127.5 ± 7.4</td>
<td>130.7 ± 6.0</td>
<td>155.9 ± 5.6‡</td>
<td>146.1 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>Insulin (n=16), pg/mL</td>
<td>135 ± 21</td>
<td>133 ± 18</td>
<td>231 ± 20†</td>
<td>236 ± 35</td>
<td></td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>89.2 ± 16.2</td>
<td>90.4 ± 11.5</td>
<td>54.8 ± 11.6</td>
<td>74.6 ± 22.7</td>
<td></td>
</tr>
<tr>
<td>Malondialdehyde, μmol/L</td>
<td>14.2 ± 0.8</td>
<td>16.1 ± 0.8</td>
<td>25.0 ± 1.4†</td>
<td>18.8 ± 1.3§</td>
<td></td>
</tr>
<tr>
<td>Urinary iso-prostaglandin F(_{2\alpha}), ng/24 h per 100 g</td>
<td>1.18 ± 0.10</td>
<td>1.49 ± 0.28</td>
<td>2.78 ± 0.15†</td>
<td>2.03 ± 0.26‡</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM of 10 rats. HDL indicates high-density lipoprotein; TNF, tumor necrosis factor; WKY, Wistar-Kyoto rat; SHR, spontaneously hypertensive rat.

* \(P<0.05\) vs WKY control.
† \(P<0.01\) vs WKY control.
‡ \(P<0.05\) SHR treated vs SHR control.
§ \(P<0.01\) SHR treated vs SHR control.

Figure 2. Effects of GW0742 on morphological parameters of mesenteric arteries. Morphometric analysis of cross-sectional area (A) and media:lumen ratio (B). C. Representative histological sections of paraffin-embedded tissues stained with hematoxylin-eosin. Values are expressed as mean ± SEM (n=8 to 10 rats). # and # indicate \(P<0.05\) and \(P<0.01\) respectively, compared with WKY control group. * \(P<0.01\) as compared with spontaneously hypertensive rat (SHR) control group.
GW0742 Improves Endothelial Function in SHRs by Increasing Endothelial NO Synthase (eNOS Activity)

Aortae from control SHR rats showed significant reduced endothelium-dependent vasodilator responses to acetylcholine in arteries stimulated by phenylephrine as compared with aortae from control WKY (Emax = 37±4% versus 78±5%). GW0742 produced a significant increase in the relaxation induced by acetylcholine in SHR rats (Emax = 48±3%, P<0.05), being without effect in WKY (Figure 4A). In aortae from WKY and SHR rats treated with GW0742, no differences were observed in the endothelium-independent vasodilator responses to the NO donor sodium nitroprusside in vessels precontracted with phenylephrine or to the vasoconstrictor responses induced by phenylephrine as compared with their respective control WKY and SHR groups (Figure 4B).

No differences were found among all experimental groups in the concentration-contractile response induced by phenylephrine in intact aortic rings (Figure 4C). However, this response was significantly reduced in aortae from SHR as compared with WKY when the rings were incubated previously with the NO synthase inhibitor l-NAME, indicating a reduced basal NO formation in SHR. GW0742 increased this contractile response only in rings from SHR, suggesting a higher NO formation in these vessels (Figure 4D). The contraction induced by Ang II (Figure 4E) in aortic rings from SHR, both in the absence and in the presence of l-NAME, was greater than that found in WKY. GW0742 significantly reduced vasoconstriction to Ang II only in SHR.

GW0742 Reduces Vascular Reactive Oxygen Species Levels in SHRs by Reducing NADPH Oxidase Activity

Positive red nuclei could be observed in adventitial, medial, and endothelial cells from sections of aorta incubated with dihydroethidium (Figure 5A). Staining was almost abolished by the O2− scavenger tiron, indicating the specificity of this dye for O2− production under the experimental conditions. Nuclear red ethidium fluorescence was quantified and normalized to the corresponding α-actin. Results are shown as mean±SEM, derived from 4 to 6 separate experiments. #P<0.05 compared with WKY control group; * and ** indicate P<0.05 and P<0.01, respectively, compared with untreated rats.

GW0742 improved expression of PPARβ and its target genes, PDK4 (Figure 3B and 3E) and CPT-1 (Figure 3C and 3F), were similar to those found in WKYS. As expected, the PPARβ agonist significantly increased the mRNA of these genes, especially in SHRs. Hearts from SHRs showed reduced levels of PDK4 mRNA as compared with those in WKY controls (Figure S3C). GW0742 treatment also increased heart PDK4 expression in both rat strains.

GW0742 on peroxisome proliferator-activated receptor (PPAR)β target genes in aorta. mRNA levels and protein expression of PPARβ (A and D), pyruvate dehydrogenase kinase 4 (PDK4) (B and E), and carnitine palmitoyltransferase 1 (CPT-1) (C and F) in spontaneously hypertensive rat (SHR) and Wistar-Kyoto rat (WKY) aortas. Data presented as a ratio of arbitrary units of mRNA (2−ΔΔCt) or densitometric values protein band normalized to the corresponding α-actin. Results are shown as mean±SEM, derived from 4 to 6 separate experiments. #P<0.05 compared with WKY control group; * and ** indicate P<0.05 and P<0.01, respectively, compared with untreated rats.
creased staining in adventitial, medial, and endothelial cells as compared with WKYS, which was significantly reduced by GW0742 only in SHRs (Figure 5A and 5B). Aortic iso-prostaglandin F2\alpha content was greater in SHRs than in WKYS (Figure S5) and was normalized by GW0742.

NADPH oxidase activity, in both basal and Ang II–stimulated conditions, was increased in aortic rings from SHRs as compared with WKYS (Figure 5C and 5D). Chronic treatments with GW0742 reduced basal and Ang II–stimulated NADPH oxidase activity only in SHRs. Significant mRNA and protein overexpression of NADPH oxidase subunits, p47phox (Figure 5E and 5F) and p22phox (Figure 5G and 5H), were observed in aortic tissue from SHRs as compared with WKYS. Again, GW0742 treatment reduced gene and protein expression of both subunits in SHRs but not in WKYS.

GW0742 Reduces Vascular Inflammatory Response in SHR

The mRNA expression of proinflammatory cytokines interleukin 1β, interleukin 6, or tumor necrosis factor-α and
Figure 5. Effects of GW0742 on the superoxide (O$_2^-$) levels and NADPH oxidase pathway. A, Pictures show arteries incubated in the presence of dihydroethidium (DHE), which produces a red fluorescence when oxidized to ethidium by O$_2^-$, blue fluorescence of the nuclear stain 4',6-diamidino-2-phenylindole (DAPI; ×400 magnification), or merged images with green elastin autofluorescence. B, Averaged values, mean±SEM (n=5 to 7 rings from different rats) of the red ethidium fluorescence normalized to the blue DAPI fluorescence. NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence without (C) and with the presence of angiotensin II (Ang II; 10$^{-6}$mol/L; n=6 to 10; D), and expression of NADPH oxidase subunits p47(phox) (E) and p22(phox) (G) at the level of mRNA by RT-PCR and protein by Western blot (F and H) in Wistar-Kyoto rats (WKYs) and spontaneously hypertensive rats (SHRs). Panels show representative bands and histograms represent densitometric values normalized to the corresponding RT-PCR products of GADPH (D and F) or normalized to the corresponding α-actin (E and G; n=3 to 5). # and ## indicate P<0.05 and P<0.01, respectively, compared with the WKY control group. * and ** indicate P<0.05 and P<0.01, respectively, compared with the SHR control group.
GW0742 Increases Regulators of G-Protein Signaling Expression in Aortas

To determine whether PPARβ regulates components of vasoactive and chemokine receptor signaling, we examined the effects of GW0742 on aortic expression of 2 key regulators of G protein–coupled receptor signaling (RGSs), RGS4 and RGS5. Aortic rings from the SHR control group showed reduced expression of both RGS4 (Figure 7A) and RGS5 (Figure 7B) as compared with the normotensive counterparts. GW0742 treatment increased significantly the expression of both proteins in the 2 rat strains.

GW0742 Regulates Extracellular-Regulated Kinase 1/2 and AKT Phosphorylation in Aortas

The expressions of phospho-extracellular-regulated kinase (ERK) 1/2 and phospho-AKT proteins were increased and decreased, respectively, in aortas from the SHR control group as compared with WKY controls. Chronic stimulation of PPARβ with GW0742 reduced ERK1/2 phosphorylation and increased AKT phosphorylation (Figure S6) only in SHRs.

Effects of the PPARβ Ligand on Aortic Contractility In Vitro

To further analyze the mechanisms of the PPARβ activation on vascular function, a series of experiments was carried out in aortic rings incubated in vitro with GW0742 (1 µmol/L) for 6 hours (Figure 8). The PPARβ ligand had no effect on the contractile responses induced by KCl or phenylephrine. However, it produced a strong inhibitory effect on Ang II–induced contraction and a partial inhibition of endothelin (ET) 1–induced responses. Interestingly, these effects were abolished by the PPARβ antagonist GSK0660. As observed in vivo, GW0742 increased the aortic expression of RGS4 and RGS5, and this effect was prevented by GSK0660 (Figure 8G and 8H). No significant changes in mRNA Ang II type 1 receptor and ET₁ receptor were observed in all of the experimental groups (Figure 8E and 8F).

**Discussion**

Our experiments provide the first evidence that chronic treatment with the highly selective PPARβ agonist GW0742 reduces systolic blood pressure, mesenteric vascular hypertrophy, vascular inflammation, systemic and vascular oxidative stress, and endothelial dysfunction in SHRs. These effects seem to be related to a direct activation of PPARβ in the vascular wall. However, despite the fact that GW0742 did not modify the plasma levels of free fatty acid and glucose, well-established mediators involved in reactive oxygen species generation and endothelial dysfunction,12,13 the PPARβ agonist treatment increased high-density lipoprotein and improved glucose tolerance in SHRs, which might collaborate with the protective effects induced by GW0742 in the vascular wall. The inhibitory effects of the PPARβ agonist GSK0660 in the in vitro experiments support the role of PPARβ in the effects of GW0742. However, despite the selectivity of GW0742 for PPARβ, we cannot completely rule out that cross-activation of other PPARs might also be involved in the effects of this agonist. The expression of PPARβ in the aorta and in the kidney was increased in SHRs as compared with WKYs. Our results are in agreement with those found with PPARα expression in SHRs,14 suggesting that changes in PPAR expression may play a compensatory role.
role in blood vessels and kidney in SHRs, because chronic treatment with GW0742 reversed these changes. However, despite increased aortic or renal PPARβ, its target genes were decreased. This might be related to a limited uptake of PPARβ endogenous ligands (ie, fatty acids) in SHRs, which are genetically deficient in CD36, a membrane fatty acid transporter.15 By contrast, PPARβ mRNA and its target gene PDK4 were significantly decreased in the hearts from SHRs, which is in agreement with the reduction in fatty acid oxidation during the development of cardiac hypertrophy. GW0742 also reversed this change in the heart and increased PDK4 mRNA in both rat strains. The experiments on vascular protein expression and function were performed in the aorta, which, as a large vessel, may have some limitations concerning the mechanisms that lead to decreased blood pressure.

Sustained high blood pressure is one of the most powerful determinants of the development of cardiac and renal hypertrophy.16 In addition, resistance vessels from essential hypertensive patients and SHRs show inward eutrophic remodeling, that is, increased media:lumen ratio without changes in the amount of material.17,18 Chronic treatment with GW0742 strongly reduced the vascular remodeling in mesenteric arteries, suggesting that this may contribute to reduce blood pressure. The lack of inhibitory effects of GW0742 in cardiac hypertrophy despite the reduction of blood pressure may be explained by insufficient time of low systolic blood pressure to induce morphological regression and induction of rapid cardiac growth, as described previously in mice with either GW0742 or another PPARβ agonist GW501516,19 which counterbalances the effects of reduced blood pressure and Ang II pathway activity. However, in spite of the lack of macroscopic changes, GW0742 reduced both the increased cardiomyocyte diameter and collagen accumulation in SHRs, indicating that it prevents the histopathologic consequences of hypertension, which is in agreement with inhibition of Ang II–induced collagen synthesis in response to PPARβ activation.20,21 Moreover, chronic GW0742 reduced heart rate, an independent risk factor for cardiovascular morbidity and mortality in hypertensive patients.22

The fate of vascular smooth muscle cells, that is, the maintenance of a differentiated contractile phenotype or the dedifferentiation into a proliferative phenotype, may be determined by the balance between the AKT and the ERK pathways.23 We found an increased ERK1/2 activation and a decreased AKT phosphorylation in aortas from hypertensive rats. The activation of the phosphatidylinositol 3-kinase/AKT pathway by PPARβ activation has been reported previously in other cell types.10,24–26 We showed that GW0742 treatment increased AKT phosphorylation in SHR aortas. PPARβ also regulates the ERK1/2 pathway in keratinocytes,27 in
adipose tissue,\textsuperscript{28} in cardiac cells,\textsuperscript{29} and in vascular smooth muscle cells.\textsuperscript{9} In the present study, the increased ERK1/2 phosphorylation found in SHRs was reduced significantly after GW0742 treatment. The change in the balance between AKT/ERK pathways induced by this agent may participate in the regulation of vascular smooth muscle cell phenotypic change in SHRs, leading to reduced vascular remodeling. PPAR\textsubscript{\beta} inhibits vascular smooth muscle inflammation and proliferation through the induction of transforming growth factor-\beta1.\textsuperscript{30} In theory, such antiproliferative effects may contribute to the antihypertensive effects of GW0742 and explain its effects on resistance artery remodeling.

Increased superoxide anion production via NADPH oxidase is thought to contribute to hypertension and endothelial dysfunction in SHRs and in essential hypertension.\textsuperscript{31,32} As reported previously for PPAR\textsubscript{\alpha} and PPAR\textsubscript{\gamma} agonists,\textsuperscript{3–6} we found that chronic treatment with GW0742 decreases the systemic and vascular oxidative stress in SHRs, reduces basal and Ang II–stimulated NADPH oxidase activity, and downregulates the NADPH oxidase subunits p22\textsuperscript{phox} and p47\textsuperscript{phox}. Because p22\textsuperscript{phox} and p47\textsuperscript{phox} expression is regulated by ERK1/2 in vascular smooth muscle cells,\textsuperscript{33,34} it is likely that the inhibition of the ERK pathway induced by GW0742 is involved in the downregulation of these NADPH oxidase subunits.

In the rat aorta, endothelium-dependent vasodilatation relies almost entirely on the endothelial release of NO. GW0742 enhanced the endothelium-dependent vasodilator response to acetylcholine in SHRs without affecting the response to nitroglycerine. These data strongly suggest that GW0742 improves endothelial function in SHRs by increasing NO bioactivity without changes in the sensitivity to the NO-cGMP pathway. Several potential mechanisms may be involved in this effect, including an increase in the expression of eNOS, a decrease in its negative allosteric regulator Caveolin 1, and reduced vascular levels of O\textsubscript{2}– and, hence, reduced O\textsubscript{2}––driven NO inactivation. Moreover, the increased vasoconstriction induced by Ang II in SHRs was significantly reduced by PPAR\textsubscript{\beta} activation, possibly by restoring RGS expression (see below).

Vascular inflammation plays a crucial role in the progression of vascular damage associated with hypertension and has been proposed to be the link between elevated blood pressure and atherosclerosis. It is well established that ERK1/2 activation increases c-fos expression\textsuperscript{9} and also activates nuclear factor \textkappaB.\textsuperscript{9,28} Both c-fos binding to AP-1 and nuclear factor \textkappaB activation are known to enhance the expression of a variety of proinflammatory and proatherogenic genes, including tumor necrosis factor-\alpha, interleukin 1\beta, interleukin 6, and intercellular adhesion molecule 1. In fact, in agreement with an increased ERK1/2 activity in the aorta of SHRs, the expression of these genes was increased, which we found to be downregulated in the aorta of SHRs treated with GW0742. Thus, these results demonstrate that the PPAR\textsubscript{\beta} agonist effectively inhibits vascular inflammation and proatherogenic gene expression in the aorta of SHRs, confirming previous evidence in vivo in Ang II–accelerated atherosclerosis in low-density lipoprotein–/– mice fed a high-fat diet.\textsuperscript{9} The anti-inflammatory effects of PPAR-\beta could be related to the blood pressure–lowering effect. However, additional mechanisms must be involved, because antihypertensive drugs like hydralazine do not reduce proinflammatory cytokines,\textsuperscript{35} and PPAR\textsubscript{\beta} agonists also exert anti-inflammatory effects in vitro as well.\textsuperscript{29}

Vascular microarray profiling in 2 models of hypertension, including SHRs, identified caveolin 1, RGS2, and RGS5 as key antihypertensive targets.\textsuperscript{36} RGS proteins play important roles in the regulation of G protein–coupled receptor signaling by binding to the active G subunits and stimulating GTP hydrolysis, thus switching off G protein signaling.\textsuperscript{37} Thus, RGS downregulation potentiates the effect of vasoconstrictors like ET and Ang II,\textsuperscript{38} leading to ERK1/2 activation.\textsuperscript{39} In our experiment, mRNA expressions of both RGS4 and RGS5 were downregulated in SHRs as compared with control WKYs. The promoter regions of RGS4 and RGS5 contain potential peroxisome proliferator response element sequences, suggesting that RGSs are direct transcriptional targets of PPARs. Likewise, we found that PPAR\textsubscript{\beta} activation increased RGS4 and RGS5 expressions in aorta from SHRs, which would be involved in the reduced ERK1/2 phosphorylation found in SHRs treated with GW0742. In addition, PPAR\textsubscript{\beta} may attenuate chemokine receptor signaling by the induction of RGS proteins. All of these data suggest that upregulated RGS may be an essential step in the effects of the PPAR\textsubscript{\beta} agonist described herein, including its antihypertensive, antioxidant, and anti-inflammatory actions. To further support this hypothesis, in another set of in vitro experiments, we aimed to analyze the role of the PPAR\textsubscript{\beta} and RGSs on vascular activation mediated via G protein–dependent and –independent pathways. Incubation for 6 hours with GW0742 upregulated RGS4 and RGS5, and these effects were prevented by a selective PPAR\textsubscript{\beta} antagonist. Moreover, upregulated RGS was associated with strongly reduced contractions to Ang II, partially inhibited responses to ET-1, and unaltered responses to the \textalpha-adrenergic agonist phenylephrine and to the receptor-independent vasoconstrictor KCl. These reduced contractions were not associated with changes in Ang II type 1 and ET\textalpha receptor expression.

In conclusion, our results clearly demonstrated that PPAR\textsubscript{\beta} stimulation reduces the elevated blood pressure, the vascular remodeling, the endothelial dysfunction, and the vascular oxidative stress in this model of genetic hypertension. These effects seem to be related to the increased NO bioactivity, resulting from reduced NADPH oxidase–mediated O\textsubscript{2}– production. These protective effects in the vascular wall are related to the upregulation of eNOS and AKT pathways and downregulation of ERK1/2 activity through decreased expression of Caveolin 1 and upregulation of RGS4 and RGS5.

**Perspectives**

PPAR\textsubscript{\beta} ligands seem to be highly effective in regulating lipid metabolism and are currently in clinical trials for the treatment of dyslipidemia, aimed particularly at individuals with low high-density lipoprotein levels and metabolic syndrome. Our study describes for the first time the blood pressure–lowering effect of a PPAR\textsubscript{\beta} agonist, identifying a new target to treat hypertension. Moreover, the improvement of endo-
thelial function and the proinflammatory and proatherogenic status of the vascular wall confer interesting properties to these drugs, preventing the early pathophysiological features and independent predictors of poor prognosis in cardiovascular diseases. These results highlight a mutually antagonistic relationship between G protein–mediated signaling (Ang II and chemokines) and PPARβ/δ-RGS activation on vascular homeostasis and demonstrate the physiological relevance of these novel relationships in vivo.

Sources of Funding

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References


Antihypertensive Effects of Peroxisome Proliferator-Activated Receptor-β Activation in Spontaneously Hypertensive Rats

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Antihypertensive effects of PPARβ activation in spontaneously hypertensive rats.

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Short title: PPARβ agonist in hypertension.
Methods

Animals and experimental groups
Experiments followed our Institutional Guidelines for the ethical care of animals. Twelve-week old, male SHR and WKY rats were obtained from Harlan Laboratories (Barcelona, Spain).

All rats were maintained five per cage at a constant temperature (24 ± 1°C), with a 12-hour dark/light cycle and on standard rat chow. An adaptation period of two weeks for vehicle administration and blood pressure measurements was allowed before the initiation of the experimental protocols. Forty WKY and forty SHR were randomly assigned to a control group (vehicle, 1 ml of 1% methylcellulose) or a treated group (GW0742, 5 mg/kg per day, mixed in 1 ml of 1% methylcellulose). Rats were treated orally by gavage once a day for five weeks. GW0742 is a highly potent and selective PPARβ with an EC50 value of 50 nM for PPARβ. Plasma concentration of GW0742 was not determined in the present study, however mice treated with GW0742 with 1 and 10 mg/kg dose for 4 week showed plasma concentration of 440.7 nmol/L and 2,270 nmol/L, respectively [1], and LDLR-/- mice treated with 6 mg/kg for 16 weeks showed concentrations in the range of 805-1,250 nmol/L [2]. Notably, the plasma concentration of the ligand at the 5 mg/kg would be expected to specifically activate PPARβ without any cross-reactivity with other PPAR isoforms because the expected levels (< 2,210 nmol/L) are bellow the reported EC50 values for murine PPARα (8,900 nmol/L) and PPARγ (> 10,000 nmol/L) [2]. During the experimental periods rats had free access to tap water and chow. Body weight was measured every week. The last day of the experimental period the animals were placed on metabolic cages to collect urine.

Blood pressure measurements
Systolic blood pressure (SBP) and heart rate (HR) was measured weekly, at 18-20 hours after the administration of the drugs in conscious, pre-warmed for 10-15 minutes at 35°C, restrained rats by tail-cuff plethysmography, starting at 8 AM. At least seven determinations were made in every session and the mean of the lowest 3 values within 5 mmHg was taken as the SBP level [3].

Oral glucose tolerance test
Six 18 h fasting rats from each group were administered a 50% glucose solution in water by gavage at a dose of 1.75 g/kg body weight. Blood samples (0.4 ml) were collected in conscious rats from the previously cannulated carotid artery into heparinized capillary tubes at 0, 30, 60 and 120 min. The blood samples were chilled on ice and centrifuged for 20 min at 5000 g at 4 ºC, and the plasma frozen at −70 ºC.

Cardiac and renal weight indices and histological analysis
At the end of the experimental period, 18 h fasting animals were anaesthetized with 2.5 mL/kg equitensin (i.p.) and blood was collected from the abdominal aorta. A sample of the mesenteric vessels corresponding to the second branch was obtained from each rat by dissection, immersed in free-calcium Krebs solution for 30 min and then in 10% formol. Samples were then dehydrated in graded ethanol solutions and embedded in paraffin. In each artery a series of four 5 µm cross sections were made on a precision microtome and stained with hematoxylin-eosin. Arterial media thickness (MT), lumen diameter (LD), media cross-sectional area (MCSA) and media-lumen ratio (M/L) were measured using a computer equipped with a Leica Q500MC image analyser connected to a video camera of a Leica Leitz DMRB microscope. The heart, kidneys and visceral fat were excised, cleaned and weighed. The atria and the right ventricle were then removed and the remaining left ventricle weighed. The cardiac, left ventricular and renal weight indices were calculated by dividing the heart, left ventricle and kidney weight by the body weight. Some left ventricles and kidneys from all experimental groups were fixed in 10% buffered formalin and embedded in paraffin wax. Two consecutive sections were cut at 4 µm and stained with haematoxylin and eosin and Masson’s Trichrome Stain (MTC).
MTC, the collagen type I fibers were stained green, the nuclei were stained black and the muscle fibers were stained red. Mean cardiomyocyte diameters were determined measuring at least 30 cells per heart. The degree of fibrosis was calculated measuring the area of interstitial conjunctive tissue (staining green with MTC) related to total area observed in representative fields of the myocardium. Microphotographs and histomorphometry were made using the computer software “Cell A” (Olympus).

**Plasma and urinary determinations**

Plasma glucose, triglycerides, HDL and total cholesterol concentrations were measured by colorimetric methods using Spinreact kits (Spinreact, S.A., Spain). Plasma free fatty acids concentration was determined using a Wako NEFA C test kit (Wako Chemicals, Richmond, VA). Plasma insulin concentration was quantified using a rat insulin enzymeimmunosassay system (Amersham Biosciences, Buckinghamshire, UK). Plasma tumor necrosis factor-α (TNFα) concentrations was determined using a ELISA kit (Dialclone, Inc., Besancon, France) specific for rat TNFα. Plasma malonyldialdehyde (MDA) content was evaluated as previously described [3]. Briefly, 100 µl of plasma reacted with a chromogenic reagent, 1-methyl-2-phenylindole (10.3 mmol/L) in acetonitrile and 37% aqueous HCl (10.4 M). After incubation of the reaction mixture for 40 minutes in a 45ºC water bath, the absorbance was measured at 586 nm in a GBC 920 spectrophotometer.

For total 8-iso-prostaglandin (iso-PGF)F$_2$α determination, 50 µl of urine was used for assay. The total iso-PGF$_2$α concentration was measured by competitive enzyme immunoassay kit (Cayman Chemical), and the results were expressed as nanograms excreted during 24 h per 100 g of body weight.

**Vascular reactivity studies**

Descending thoracic aortic rings (3 mm) were dissected and mounted in organ chambers filled with Krebs solution (composition in mmol/L: NaCl 118, KCl 4.75, NaHCO$_3$ 25, MgSO$_4$ 1.2, CaCl$_2$ 2, KH$_2$PO$_4$ 1.2 and glucose 11) at 37 ºC and gassed with 95% O$_2$ and 5% CO$_2$. Rings were stretched to 2 g of resting tension by means of two L-shaped stainless-steel wires inserted into the lumen and attached to the chamber and to an isometric force-displacement transducer (Letigraph 2000), respectively, as previously described [3]. The concentration-relaxation response curves to acetylcholine (ACh) (10$^{-9}$mol/L-10$^{-5}$ mol/L) were performed in rings pre-contracted by 10$^{-6}$ mol/L phenylephrine. The concentration-relaxation response curves to nitroprusside (10$^{-9}$ mol/L -10$^{-5}$ mol/L) were performed in the dark in rings without endothelium pre-contracted by 10$^{-6}$ mol/L phenylephrine.

To evaluate the formation of basal NO, the contraction induced phenylephrine (10$^{-9}$ mol/L -10$^{-5}$ mol/L) was measured 30 minutes after aortic incubation with the NOS inhibitor N$^6$-nitro-L-arginine methyl ester (L-NAME, 10$^{-4}$ mol/L) [3]. The contraction induced by angiotensin II (AngII, 10$^{-9}$ mol/L -10$^{-6}$ mol/L) was also analyzed in the absence and in presence of L-NAME (10$^{-4}$ mol/L).

To evaluate the role of PPARβ in the response to vasoconstrictors in in vitro conditions, endothelium-denuded rat aortic rings, isolated from male Wistar rats and mounted in organ chambers, were incubated for 6 hours with or without GW0742 (10$^{-6}$ mol/L) and with or without prior (1 hour) incubation with the PPARβ antagonist, GSK0660 (10$^{-6}$ mol/L). After washing, concentration-response curves to KCl, phenylephrine, Ang II and endothelin-1 were constructed in individual rings, by adding cumulative concentrations of each drug. Then, these rings were frozen (-80º C) until quantitative RT-PCR analysis. Contractile responses were expressed as percentage of control KCl (80 mmol/L) response obtained prior incubation with the PPARβ agonist.

**In situ detection of vascular superoxide anion (O$_2$) production**

Unfixed thoracic aortic rings were cryopreserved (phosphate buffer solution 0.1 mol/L, PBS, plus 30% sucrose for 1-2h), included in OCT, frozen (-80ºC), and 10 µm cross sections were obtained in a cryostat (Microm International Model HM500 OM). Sections were incubated for 30 min in Hepes buffered solution containing dihydroethidium (DHE, 10$^{-5}$ mol/L), counterstained with the nuclear stain DAPI and in the following 24 h examined on a
fluorescence microscope (Leica DM IRB, Wetzlar, Germany). Sections were photographed and ethidium and DAPI fluorescence were quantified using ImageJ (version 1.32j, NIH, http://rsb.info.nih/ij/). O$_2^-$ production was estimated from the ratio of ethidium/DAPI fluorescence [3]. In preliminary experiments, DHE fluorescence was almost abolished by the O$_2^-$ scavenger tiron, indicating the specificity of this reaction.

The total iso-PGF2α concentration was also measured in aortic tissue by competitive enzyme immunoassay kit.

**NADPH oxidase activity**
The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase activity in intact aortic rings, as previously described [4]. Aortic rings from all experimental groups were incubated for 30 minutes at 37 ºC in HEPES-containing physiological salt solution (pH 7.4) of the following composition (in mmol/L): NaCl 119, HEPES 20, KCl 4.6, MgSO4 1, Na2HPO4 0.15, KH2PO4 0.4, NaHCO3 1, CaCl2 1.2 and glucose 5.5. Aortic production of O$_2^-$ was stimulated by addition of NADPH (100 µmol/L). Rings were then placed in tubes containing physiological salt solution, with or without NADPH, and with and without AngII (10⁻⁶ mol/L) and lucigenin was injected automatically at a final concentration of 5 µmol/L to avoid known artifacts when used a higher concentrations. NADPH oxidase activity were determined by measuring luminescence over 200 s in a scintillation counter (Lumat LB 9507, Berthold, Germany) in 5-s intervals and was calculated by subtracting the basal values from those in the presence of NADPH. Vessels were then dried, and dry weight was determined. NADPH oxidase activity is expressed as relative luminescence units (RLU)/min/mg dry aortic tissue.

**Western blotting analysis**
Aortic homogenates were run on a sodium dodecyl sulphate (SDS)-polyacrilamide electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (PVDF), incubated with primary monoclonal mouse anti-eNOS and anti-caveolin-1 antibodies (Transduction Laboratories, San Diego, California, USA), polyclonal goat anti-p22phox, rabbit polyclonal anti-PPARβ, goat polyclonal anti- pyruvate deshydrogenase kinase 4 (PDK4), goat polyclonal anti-carnitine palmitoiltransferase 1 (CPT-1) (SantaCruz Biotechnology, Santa Cruz, California, USA), polyclonal rabbit anti-p47phox (Upstate Cell Signaling) overnight and with the correspondent secondary peroxidase conjugated antibodies. Phosphorylated protein kinase B (Akt), Akt, phospho-ERK1/2, and ERK1/2, were detected after the membranes were incubated with the respective primary antibodies (Rabbit anti-phospho-Akt-ser-473, rabbit anti-Akt, rabbit anti-ERK1/2, Cell Signalling Technology, MA, USA, or mouse anti-phospho-ERK1/2-Thr183 and Tyr185, Sigma-Aldrich, all at 1:1000 dilution). Antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, Amersham, UK) and densitometric analysis was performed using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com) [5]. Samples were re-probed for expression of smooth muscle α-actin.

**Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis**
For Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis, total RNA was extracted from aorta by homogenization and converted to cDNA by standard methods. Polymerase chain reaction was performed with a Techne Techegene thermocycler (Techne, Cambridge, UK). A quantitative real-time RT-PCR technique was used to analyze mRNA expression of PPARβ and PPARβ-target genes, such as PDK4 and CPT-1, caveolin-1, eNOS, p22phox, p47phox, AT$_1$ receptor, ET$_1$ receptor, regulator G-protein signalling (RGS)4 and RGS5, intercellular adhesion molecule (ICAM)-1, TNF-α, interleukin (IL)-1β, IL-6 and calcineurin. The sequences of the sense and antisense primers used for amplification are described in supplementary Table S1. Preliminary experiments were carried out with various amounts of cDNA to determine nonsaturating conditions of PCR amplification for all the genes studied. Therefore, under these conditions, relative quantification of mRNA was assessed by the RT-PCR method used in this study [6]. The efficiency of the PCR reaction was determined using a
dilution series of a standard vascular, heart or kidney samples. Quantification was performed using the ∆∆Ct method. The housekeeping gene β-actin was used for internal normalization.

**Drugs**

GW0742 was purchased by Tocris Bioscience (Bristol, UK). All other drugs used were obtained from Sigma (Alcobendas, Madrid, Spain). All drugs and chemicals were dissolved in distilled deionized water.

**Statistical analysis.**

Results are expressed as means ± s.e. means of measurements. The evolution of tail SBP with time was compared using the nested design, with treatment and days as fixed factors and the rat as random factor. When the overall difference was significant comparisons were made using Bonferroni’s method with an appropriate error. Analysis of the nested design was also carried out with groups and concentrations to compare the concentration-response curves to ACh. The remaining variables were compared using a two way factor design, where group and treatment were fixed effect factors with unequal sample sizes in the different groups. When interaction was significant Bonferroni’s method was used for pairwise comparisons. P < 0.05 was considered statistically significant.

**References**


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Table S1. Oligonucleotides for real-time RT-PCR
Figure S1. Histological analysis of the effects of GW0742 in heart and kidney. Magnifications of heart sections stained with haematoxylin and eosin (A, left) or MTC (C) and kidney sections stained with haematoxylin and eosin (D) from all experimental groups. (A, right) Mean values ± SEM of cardiomyocyte diameters. (B) mRNA levels of calcineurin in the heart. Data presented as a ratio of arbitrary units of mRNA \(2^{-\Delta\Delta Ct}\). Results are shown as mean ± SEM, derived from 9-10 separate experiments. ### indicate P<0.01 compared to the WKY control group. * and ** indicate P<0.05 and P<0.01, respectively, compared to the SHR control group.
**Figure S2.** Effects of GW0742 in oral glucose tolerance test. Rats were fasted for 18 h prior administration of a 50% glucose solution in water by gavage at a dose of 1.75 g/kg body weight. AUC: area under curve. Values are expressed as mean ± SEM (n = 6). # and ## indicate P<0.05 and P<0.01, respectively, compared to the WKY control group. * indicate P<0.05 compared to the SHR control group.
Figure S3. Effects of GW0742 on mRNA levels of PPARβ in kidney (A) and heart (B), and pyruvate deshydrogenase kinase 4 (PDK4) in heart (C) from SHR and WKY. Data presented as a ratio of arbitrary units of mRNA ($2^{-\Delta\Delta C_t}$). Results are shown as mean ± SEM, derived from 6-10 separate experiments. # indicate P<0.05 compared to the WKY control group. * and ** indicate P<0.05 and P< 0.01, respectively, compared to the SHR control group.
**Figure S4.** Effects of GW0742 on contractile responses induced by angiotensin II (Ang II) in the absence (A) and presence (B) of L-NAME (10^{-4}mol/L) in control and treated WKY and SHR rats. Values are expressed as mean ± SEM (n= 7-9 rings from different rats). # and ## indicate P<0.05 and P<0.01, respectively, compared to the WKY control group. * and ** indicate P<0.05 and P< 0.01, respectively, compared to the SHR control group.
Figure S5. Effects of GW0742 on aortic content of iso-PGF$_{2\alpha}$. Values are expressed as mean ± SEM (n= 7-9 rings from different rats). # indicate P<0.05 compared to the WKY control group. * indicate P<0.05 compared to the SHR control group.
Figure S6. Effects of GW0742 on ERK and AKT pathways. Panels (A and B) show bands and histograms represent phospho-ERK1/2 and phospho-AKT relative to total ERK1/2 and AKT protein levels (n = 3-5). # indicate P<0.05 compared to the WKY control group. * indicate P<0.05 compared to the SHR control group.