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Effects of peroxisome proliferator-activated receptor-β activation in endothelin-dependent hypertension

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Aims We analysed the chronic effects of the peroxisome proliferator-activated receptor β/δ (PPAR-β) agonist GW0742 on the renin-independent hypertension induced by deoxycorticosterone acetate (DOCA)-salt.

Methods and results Rats were treated for 5 weeks with: control-vehicle, control-GW0742 (5 or 20 mg kg^{-1} day^{-1}), DOCA-vehicle, DOCA-GW0742 (5 or 20 mg kg^{-1} day^{-1}), DOCA-GSK0660 (1 mg kg^{-1} day^{-1}), and DOCA-GSK0660-GW0742. Rats receiving DOCA-vehicle showed increased systolic blood pressure, left ventricular and kidney weight indices, endothelin-1 (ET-1), and malondialdehyde plasma levels, urinary iso-PGF2α excretion, impaired endothelium-dependent relaxation to acetylcholine, and contraction to ET-1 when compared with controls. Aortic reactive oxygen species content, NADPH oxidase activity, and $p47^{phox}$, $p22^{phox}$, NOX-4, glutathione peroxidase 1, hemeoxygenase-1, and preproET-1 expression were increased, whereas catalase and regulators of G protein-coupled signalling proteins (RGS)5 expression were decreased in the DOCA-vehicle group. GW0742 prevented the development of hypertension in a dose-dependent manner but the reduction of renal and cardiac hypertrophy, systemic and vascular oxidative stress markers, and improvement of endothelial dysfunction were only observed after the higher dose. GW0742, at 20 mg kg^{-1} day^{-1}, attenuated ET-1 contraction by increasing RGS5 expression and restored the intracellular redox balance by reducing NADPH-oxidase activity, and by increasing the antioxidant genes expression. The PPAR-β antagonist GSK0660 prevented all vascular changes induced by GW0742 but not its antihypertensive effects.

Conclusion Vascular protective effects of GW0742 operate via PPAR-β by interference with the ET-1 signalling as a result of increased expression of RGS5 and up-regulation of antioxidant genes and via PPAR-β-independent mechanisms to decrease blood pressure.

Keywords PPARβ/δ • DOCA-salt hypertension • Endothelial dysfunction • Endothelin-1 • Regulators of G protein-coupled signalling proteins

1. Introduction

Hypertension is a well-established risk factor for the development of atherosclerosis. The inactivation of nitric oxide (NO) by vascular superoxide anion ($O_2^{\bullet -}$) plays a critical role in the pathogenesis of cardiovascular diseases, including hypertension.[1] Arterial $O_2^{\bullet -}$ is elevated in angiotensin II (Ang II)-induced hypertension, attributable to a large extent to nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation by Ang II.[2,3] However, an excess of vascular $O_2^{\bullet -}$ production has also been found in deoxycorticosterone acetate (DOCA)-salt hypertension,[4–6] a model with a markedly depressed renin–Ang system because of sodium retention.[7] Patients with low renin (i.e. salt-sensitive hypertension) represent ~30% of the essential hypertensives and show a poor therapeutic response to Ang-converting enzyme

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inhibitors and Ang receptor blockers. Endothelin-1 (ET-1) has been shown to contribute to the pathogenesis of salt-sensitive hypertension secondary to a low-renin state in animals and humans.[8,9] This is, at least in part, due to vascular $O_2^{\bullet -}$ production via the ET_A/NADPH oxidase pathway[10] leading to the endothelial dysfunction.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily, which heterodimerize with the retinoid X receptor to regulate the transcription of diverse genes.[11] There are three isotypes of PPARs: PPARα, PPARβ/δ (PPARβ), and PPARγ. Their role on lipid and glucose metabolism is well known.[12] In addition, PPARα and PPARγ activators prevent hypertension, cardiac fibrosis, vascular hypertrophic remodelling, endothelial dysfunction, and renal injury in DOCA-salt rats,[13–18] while the effects of PPARβ activators are unknown. These protective effects seem to be associated with a decreased ET-1 production,[13–16,18] by inhibiting the activator protein-1 signalling pathway.[19,20] Recently, it has been found that PPARα and PPARγ ligands also inhibit the ET-1 pathway to induce both vascular proinflammatory effects[21] and cardiac hypertrophy.[22,23] The activation of PPARβ also exhibits anti-inflammatory properties in the vessel wall.[24] Moreover, the oral administration of PPARβ agonist GW0742 reduces atherosclerosis in the LDL receptor knockout ($LDLR^{-/-}$) mice,[25] and substantially attenuates Ang II-accelerated atherosclerosis and the associated arterial inflammatory and atherosclerotic gene expression.[26] Recently, we found that GW0742 reduced blood pressure, improved the endothelial dysfunction, and reduced vascular proinflammatory and proaterogenic status in spontaneously hypertensive (SHR) by interfering with the Ang II signalling pathway, through up-regulation of the regulators of G-protein-coupled signalling proteins (RGS): RGS4 and RGS5.[27] RGS proteins play important roles in the regulation of G protein-coupled receptor signalling by binding to the active G subunits and stimulating GTP hydrolysis, thus switching off G protein signalling.[28] Moreover, up-regulated RGSs induced by PPARβ activation was associated with reduced contractions to ET-1.[27] In addition, RGS5 knockdown in intact mesenteric artery increased myogenic tone.[29] PPARβ activation also inhibits AP-1 signalling.[30,31] However, there is no information about the effects of PPARβ ligands on ET-1 production and the ET-1 pathway in the vascular system. We hypothesized that GW0742 affects the development of the ET-1-dependent and Ang II-independent hypertension induced by DOCA-salt by interfering with the production of ET-1 and/or its signalling. Therefore, the aim of the present study was to examine whether chronic intake of GW0742 prevents the DOCA-salt-induced hypertension and endothelial dysfunction and, if so, to determine the underlying mechanism, focusing on the involvement of ET-1 and oxidative stress.

2. 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. Two experiments were performed. Experiment 1: rats were randomly divided into five groups: control-vehicle, control-treated with 5 mg kg^{-1} day^{-1}, DOCA-salt-vehicle, DOCA-salt-treated with 5 mg kg^{-1} day^{-1}, and DOCA-salt-treated with 20 mg kg^{-1} day^{-1}. Experiment 2: rats were randomly divided into six groups: control-vehicle, control-treated with 20 mg kg^{-1} day^{-1} GW0742, DOCA-salt-vehicle, DOCA-salt-treated with the PPARβ antagonist GSK0660 at 1 mg kg^{-1} day^{-1} intraperitoneally, DOCA-salt-treated with 20 mg kg^{-1} day^{-1} GW0742, and DOCA-salt-treated with GSK0660 and 20 mg kg^{-1} day^{-1} GW0742. DOCA-salt hypertension was induced as previously described.[6] GW0742 was given by intragastric gavage at doses of 5 or 20 mg kg^{-1} day^{-1}, mixed in 1 mL of 1% methylcellulose. The vehicle groups only received methylcellulose. Systolic blood pressure (SBP) and heart rate (HR) were measured in conscious, pre-warmed, restrained rats by tail-cuff plethysmography. The cardiac, left ventricular, and renal weight indices were calculated by dividing the heart, left ventricle, and kidney weight by the body weight. Plasma ET-1 levels were determined with the use of a commercially available enzyme-linked immunosorbent assay kit. Descending thoracic aortic rings were mounted in organ baths for isometric tension recording. Reactive oxygen species (ROS) levels were estimated from the ratio of 2′,7′-dichlorofluorescein (DCF)/4′,6-diamidino-2-phenylindole (DAPI) and ethidium/DAPI fluorescence in sections of unfixed thoracic aortic rings incubated for 30 min with 2,7′-dichlorofluorescein diacetate or dihydroethidium (DHE) and counterstained with the nuclear stain DAPI. NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence assay stimulated by addition of NADPH. Protein and mRNA expression was measured by western blotting analysis and reverse transcriptase-polymerase chain reaction (RT–PCR) analysis, respectively. An expanded Methods section is available in the Supplementary material online.

3. Results

3.1 Effects of GW0742 on blood pressure, morphological variables, and plasma and urinary determinations

Rats receiving DOCA-salt showed a progressive increase in SBP when compared with animals in the control group (*Figure 1A*). This increase was significant ($P < 0.05$) from the first week, reaching a difference of ∼44 mmHg at the end of the treatment. Chronic treatment with 5 and 20 mg kg^{-1} GW0742 prevented, in a dose-dependent manner, the increase in SBP by ∼61 and 100%, respectively, in DOCA-salt-treated rats ($P < 0.01$), being without effect in control animals. At the end of the experiment, reduced HR (∼9%, $P < 0.05$ vs. untreated control rats) was also observed in the DOCA-salt group when compared with the control group (*Figure 1B*). This effect was not modified by GW0742.

The increase in body weight (BW) was higher in control than in the DOCA-salt group after 5 weeks (144 ± 6 and 100 ± 6%, respectively, $P < 0.01$). This increase was also accompanied by a higher visceral fat weight either in absolute terms or relative to BW. GW0742-treatment did not modify the gain in BW or the visceral fat weight either in the control or in the DOCA-salt group (*Table 1*). These effects of DOCA-salt seem to be related to a lower food intake (26 ± 2 and 22 ± 2 g day^{-1}, in the control and DOCA-salt groups, respectively, $P < 0.05$), which were also unaffected by GW0742. Absolute left ventricle weight (LVW) and LVW relative to BW or to heart weight (HW) and kidney weight (KW) relative to BW were higher in DOCA-salts group when compared with the control group. GW0742 at 20, but not at 5 mg kg^{-1}, significantly reduced cardiac hypertrophy parameters while KW/BW was unchanged by the drug (*Table 1*).

The plasma levels of MDA (Supplementary material online, *Figure S1A*), a marker of lipid peroxidation induced by ROS, and the 24 h urinary iso-$PGF_{2\alpha}$ excretion (Supplementary material online, *Figure S1B*), a more specific marker for lipid peroxidation, were increased in DOCA-salt-treated animals when compared with the control group. GW0742 treatment, at 20 mg kg^{-1}, reduced both MDA concentrations and iso-$PGF_{2\alpha}$ excretion in DOCA-salt rats.

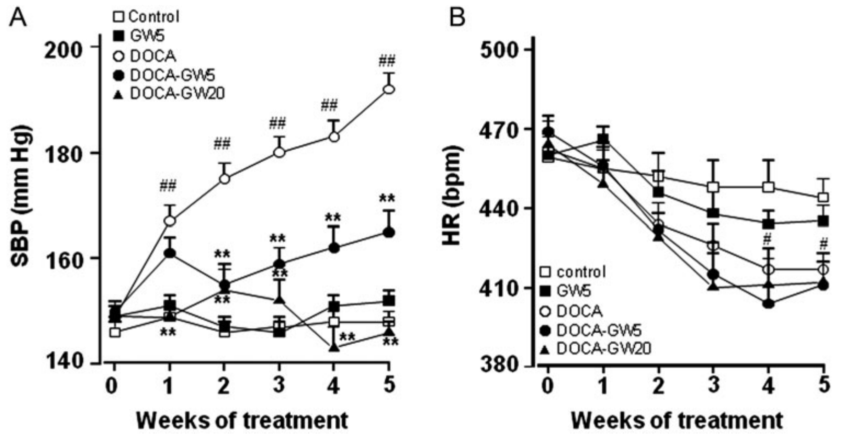

Figure 1 Effects of chronic GW0742 treatment on SBP (*A*) and HR (*B*) as measured by tail-cuff plethysmography in DOCA-salt-induced hypertension. Experimental groups: control-vehicle (open square) ($n = 20$), DOCA-vehicle (open circle) ($n = 18$), GW0742 (5 mg kg^{-1}, closed square) ($n = 19$), DOCA-GW0742 (5 mg kg^{-1}, closed circle) ($n = 20$), and DOCA-GW0742 (20 mg kg^{-1}, closed triangle) ($n = 9$). Values are expressed as mean ± SEM. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ when compared with the control group, respectively. $^{**}P < 0.01$ when compared with the DOCA group.

3.2 Effects of GW0742 on PPARβ and PPARβ-target genes in aorta

The gene and protein expression of PPARβ was significantly increased in the aorta (Supplementary material online, *Figure S2A* and *S2C*) from the DOCA-salt group when compared with control rats. Chronic treatment with GW0742 at both doses restored these changes of PPARβ in DOCA-salt to the levels found in the control group. In DOCA-salt aorta the mRNA levels and protein expression of a well-known PPARβ-target gene, PDK4 (Supplementary material online, *Figure S2B* and *S2D*) were similar to those found in control rats. As expected, the PPARβ agonist significantly increased the mRNA of PDK4 in a dose-dependent manner. Moreover, GW0742 also increased the expression of the fatty acid translocase CD36 dose dependently (Supplementary material online, *Figure S3*).

3.3 GW0742 improves the endothelial function in DOCA-salt rats

Aortic rings from DOCA-salt-treated animals showed reduced endothelium-dependent vasodilator responses to acetylcholine in arteries stimulated by phenylephrine when compared with the control aortic rings ($E_{max} = 52 \pm 6$ vs. $80 \pm 5\%$, respectively, $P < 0.01$) (*Figure 2A*). GW0742, at 5 mg kg^{-1}, did not produce a significant increase in the relaxation induced by acetylcholine neither in DOCA-salt rats ($E_{max} = 52 \pm 7\%$) nor in control rats ($E_{max} = 72 \pm 5\%$). However, at 20 mg kg^{-1} GW0742 restored the maximal relaxation to acetylcholine ($E_{max} = 81 \pm 4\%$) in DOCA-salt to values found in control rats. No differences were observed among groups in the endothelium-independent vasodilator response to the NO donor sodium nitroprusside in vessels pre-contracted with phenylephrine (*Figure 2B*). Only GW0742, at 20 mg kg^{-1}, tended to increase the sensitivity to nitroprusside in DOCA-salt rats ($pD_2 = 8.26 \pm 0.10$ vs. 8.00 ± 0.11, treated and control, respectively, $P > 0.05$).

Endothelial nitric oxide synthase (eNOS) gene expression in the aorta was similar in all experimental groups (*Figure 2C*). The gene and protein expression of caveolin-1, an allosteric negative regulator of eNOS, was unaltered in DOCA-salt when compared with control rats, but GW0742 at 20 mg kg^{-1} markedly reduced the expression of caveolin-1 (*Figure 2D* and *E*).

3.4 GW0742 reduces vascular ROS levels in DOCA-salt rats by reducing NADPH oxidase activity and by up-regulation of antioxidant genes

Positive red nuclei could be observed in adventitial, medial, and endothelial cells from sections of aorta incubated with DHE (*Figure 3A*). Staining was almost abolished by the O_2^- scavenger tiron (10 mM), an intracellular O_2^- scavenger (data not shown), indicating the specificity of this dye for O_2^- production under the experimental conditions. Nuclear red ethidium fluorescence was quantified and normalized to the blue fluorescence of the nuclear stain DAPI, allowing comparisons between different sections. Rings from DOCA-salt rats showed a marked increased staining in adventitial, medial, and endothelial cells when compared with control rats which was prevented by GW0742 at 20 mg kg^{-1} (*Figure 3A* and *B*). When we analysed the DCF fluorescence, a peroxide-sensitive dye that increased green fluorescence was also observed in aortic rings from DOCA-salt animals. Moreover, both doses of GW0742 reduced DCF staining (*Figure 3C* and *D*).

NADPH increased lucigenin luminescence in normal aortic rings, which was strongly inhibited ($85 \pm 4\%$) by previous incubation for 30 min with the flavoprotein inhibitor DPI (10 μM) indicating that external NADPH increased NADPH oxidase activity in vascular tissue. NADPH oxidase activity was increased in aortic rings from DOCA-salt rats when compared with control rats (*Figure 4A*). Chronic treatment with GW0742, at 20 mg kg^{-1}, reduced significantly this activity in DOCA salt rats, being without effect at 5 mg kg^{-1}. Significant mRNA and protein up-regulation of NADPH oxidase subunits, NOX4 (*Figure 4B* and *C*), $p47^{phox}$ (*Figure 4E*), and $p22^{phox}$ (*Figure 4F*) were observed in aortic tissue from DOCA-salt rats when compared with control rats, without changes in NOX1 (*Figure 4D*). Again, only GW0742 treatment at 20 mg kg^{-1} reduced gene and protein (*Figure 4B* and *C*) up-regulation of NOX4 subunit in DOCA-salt rats, being without effects in the rest of NADPH oxidase subunits.

Table 1 Body and organ weights and cardiac and renal indices

Groups	BW (g)	HW (mg)	LVW (mg)	KW (mg)	VF (g)	HW/BW	LVW/BW	LVW/HW	KW/BW	VF/BW
Control ($n = 20$)	361 ± 8	912 ± 19	665 ± 14	924 ± 14	5.4 ± 0.4	2.53 ± 0.05	1.85 ± 0.05	0.73 ± 0.01	2.57 ± 0.05	14.7 ± 1.0
GW5 ($n = 19$)	360 ± 8	935 ± 19	685 ± 16	969 ± 23	5.4 ± 0.4	2.61 ± 0.05	1.91 ± 0.04	0.73 ± 0.01	2.70 ± 0.05	14.8 ± 0.9
DOCA ($n = 18$)	310 ± 10†	1080 ± 35†	837 ± 31†	1675 ± 81†	2.4 ± 0.3†	3.50 ± 0.10†	2.71 ± 0.08†	0.77 ± 0.01†	5.41 ± 0.21†	7.4 ± 0.7*
DOCA-GW5 ($n = 20$)	291 ± 9†	1035 ± 39†	789 ± 32†	1602 ± 46†	2.4 ± 0.3†	3.58 ± 0.11†	2.73 ± 0.10†	0.76 ± 0.01*	5.53 ± 0.11†	7.7 ± 0.9*
DOCA-GW20 ($n = 9$)	331 ± 12†	975 ± 35	724 ± 26‡	1627 ± 23†	2.6 ± 0.4*	2.96 ± 0.10§	2.19 ± 0.07§	0.74 ± 0.01§	4.96 ± 0.17†	7.8 ± 1.2*

BW indicates body weight; HW, heart weight; LVW, left ventricle weight; KW, kidney weight; VF, visceral fat. Values are expressed as mean ± SEM.
*$P < 0.05$ vs. control group.
†$P < 0.01$ vs. control group.
‡$P < 0.05$ vs. DOCA group.
§$P < 0.01$ vs. DOCA group.

The aortic mRNA levels of antioxidant genes are shown in Supplementary material online, *Figure S4*. Cu/Zn-SOD and Mn-SOD were unchanged and catalase was reduced, while GPx1 and HO-1 were increased in DOCA-salt rats when compared with control. The lower dose of GW0742 significantly increased the gene expression of GPx1 and HO-1 in control animals and Mn-SOD and catalase in DOCA-salt rats, whereas the higher dose increased the expression of all antioxidant genes analysed.

3.5 Effects of GW0742 on ET-1 synthesis and ET-1 contractile response

ET-1 plays an important role in the endothelial dysfunction seen in mineralocorticoid hypertension, and PPARα and γ agonists normalize this dysfunction by improving the abnormal ET-1 system.[13,14] In order to determine whether there is a relationship between PPARβ/δ and ET-1 in the vascular wall, we investigated the expression of ppET-1, endothelin-converting enzyme (ECE) 1 and ET_A receptor mRNA after GW0742 treatment (*Figure 5*). As expected, the aortic expression of ppET-1 mRNA was increased in DOCA-salt rats when compared with control rats, without changes in ECE1 or ET_A receptor expression. This raise in vascular ppET-1 was accompanied with increased ET-1 plasma levels (*Figure 5C*). Chronic GW0742 treatment, at 5 mg kg^{-1}, did not modify aortic ppET-1, ECE1, and ET_A mRNA, and the ET-1 concentration in plasma in both DOCA-salts and control rats. However, the higher GW0742 dose increases ppET-1 mRNA and reduced ECE1 mRNA in DOCA-salt, without affecting the ET_A receptor expression and plasma ET-1 levels.

ET-1 induced sustained contractions in concentration-dependent manners in both the control and DOCA-salt hypertensive rats (*Figure 5E*). The response to ET-1 was significantly decreased in DOCA-salt when compared with control rats. Chronic GW0742 treatment, at 5 mg kg^{-1}, did not modify this contractile response neither in control nor in DOCA-salt rats, but 20 mg kg^{-1} reduced this contraction in DOCA-salt rats.

3.6 GW0742 increases regulators of G-protein signalling (RGS)-5 expression in aorta

To determine whether PPARβ regulates components of vasoactive (such as ET-1) and chemokine receptor signalling we examined the effects of GW0742 on aortic expression of a key regulator of G-protein-coupled receptor signalling, RGS5. Aortic rings from the DOCA-salt group showed a reduced expression of RGS5 (*Figure 5F*), when compared with the normotensive counterparts. GW0742 treatment, at 20 mg kg^{-1}, increased significantly the expression of RGS5 in the DOCA-salt rats.

3.7 GW0742 improves endothelial function and vascular oxidative stress by PPARβ activation, but prevents the increase in SBP independently of PPARβ

To determine whether PPARβ are involved in the effects induced by GW0742 at 20 mg kg^{-1}, the PPARβ antagonist GSK0660 was administered in addition to GW0742. GSK0660 abolished the effects induced by GW0742 in DOCA-salt aorta on the mRNA levels of PPARβ (Supplementary material online, *Figure S5A*), and the well-known PPARβ-target genes, PDK4 (Supplementary material online, *Figure S5B*) and CD36 (Supplementary material online, *Figure S5C*), indicating that GSK0660

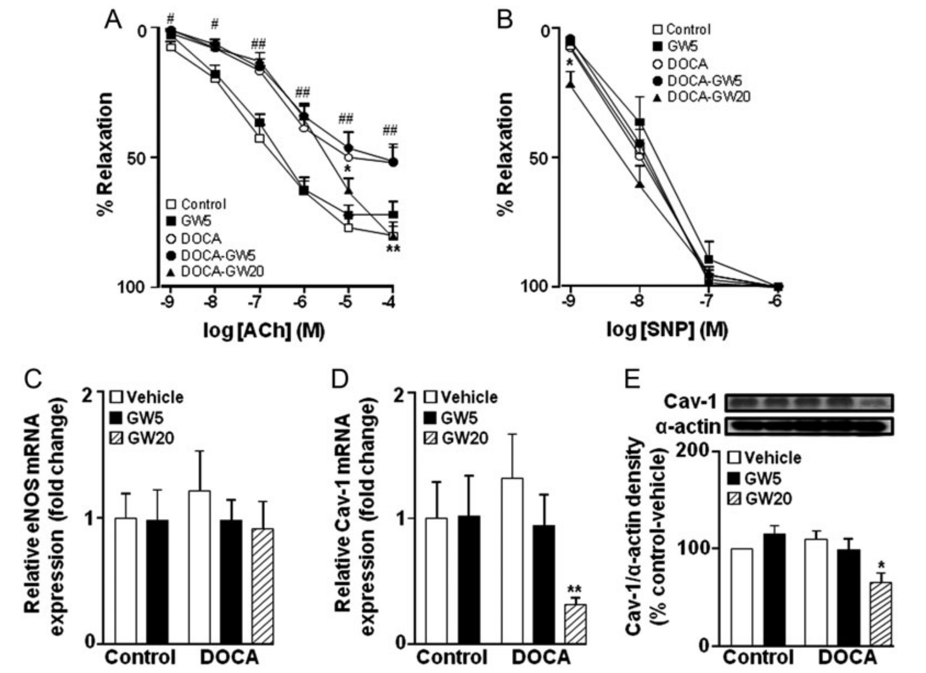

Figure 2 Effects of chronic GW0742 treatment on endothelial function and the NO pathway. Vascular relaxant responses induced by acetylcholine (Ach) (*A*) and sodium nitroprusside (SNP) (*B*) in aortae pre-contracted by 10^{-6} mol/L phenylephrine (Phe) during 30 min. Experimental groups: control-vehicle (open square) ($n = 20$), DOCA-vehicle (open circle) ($n = 18$), GW0742 (5 mg kg^{-1}, closed square) ($n = 19$), DOCA-GW0742 (5 mg kg^{-1}, closed circle) ($n = 20$), and DOCA-GW0742 (20 mg kg^{-1}, closed triangle) ($n = 9$). Values are expressed as mean ± SEM. Effects of GW0742 on the expression of eNOS (*C*) and caveolin-1 (*D* and *E*) at the level of mRNA by RT–PCR (*C* and *D*) and protein by western blot (*E*). Data presented as a ratio of arbitrary units of mRNA ($2^{-\Delta\Delta Ct}$) or densitometric values of protein band normalized to the corresponding α-actin, compared with the control-vehicle group. The results of western blots are shown as mean ± SEM, derived from four to six separate rings from different rats. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ when compared with the control group, respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared with the DOCA group, respectively.

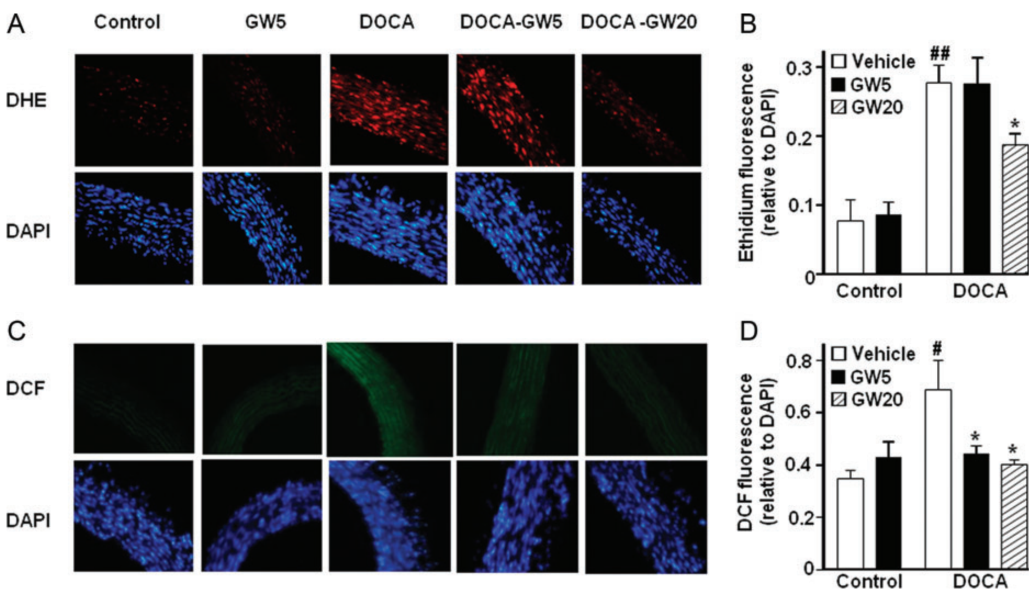

Figure 3 Effects of chronic GW0742 treatment in *in situ* localization of ROS content in aortic rings. Pictures show arteries incubated for 30 min in the presence of DHE (*A*), which produces a red fluorescence when oxidized to ethidium by $O_2^{\bullet -}$, or incubated for 30 min with DCFH (*C*) which acquires fluorescent properties on reaction with ROS (mainly H_2O_2) and yields the green fluorescent product DCF; blue fluorescence of the nuclear stain DAPI (×400 magnification). Averaged values, mean ± SEM ($n = 5–6$), of red ethidium fluorescence (*B*) or green DCF fluorescence (*D*) normalized to blue DAPI. Experimental groups: control-vehicle, DOCA-vehicle, GW0742 (5 mg kg^{-1}), DOCA-GW0742 (5 mg kg^{-1}), and DOCA-GW0742 (20 mg kg^{-1}). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ when compared with the control group. $^{*}P < 0.05$ when compared with the DOCA group.

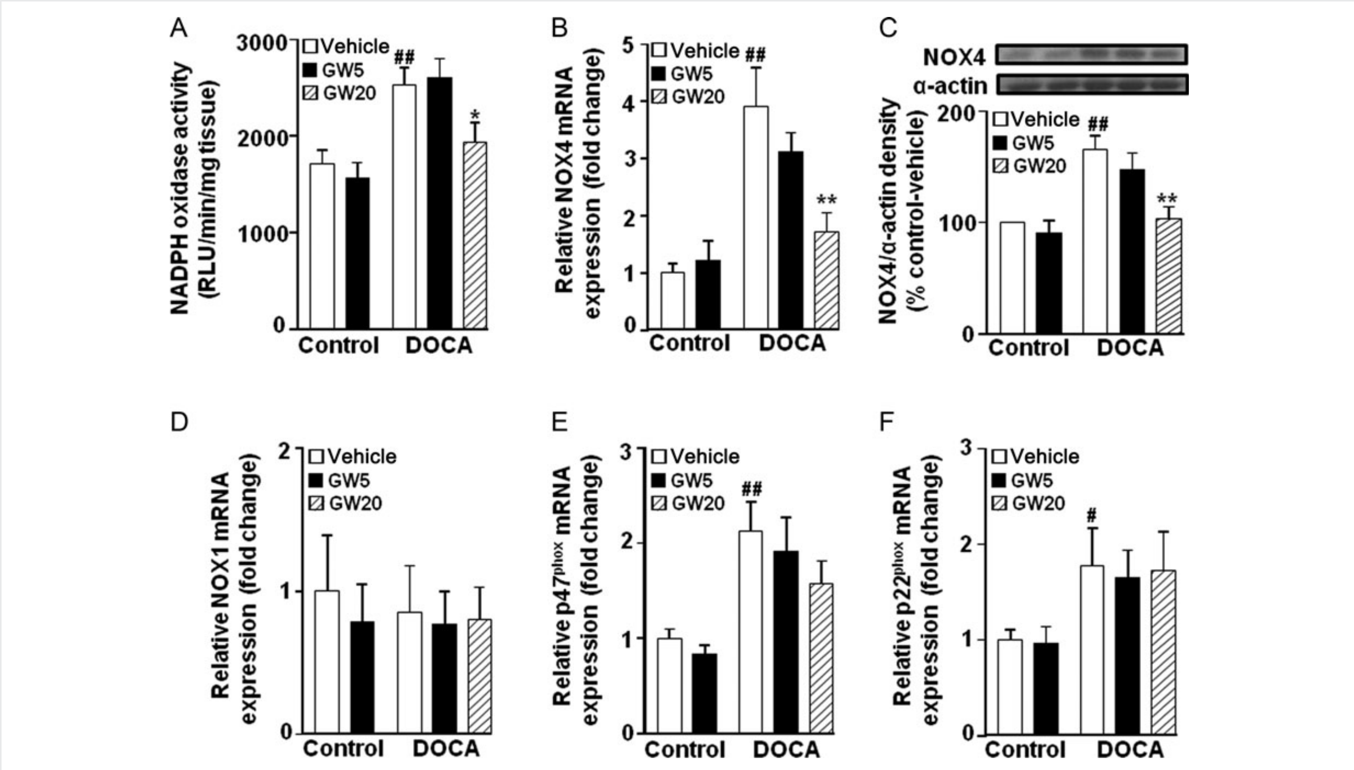

Figure 4 Effects of chronic GW0742 treatment on the NADPH oxidase pathway. The NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence (*A*), and expression of NADPH oxidase subunits NOX-4 (*B* and *C*), NOX-1 (*D*), p47^{phox} (*E*) and p22^{phox} (*F*) at the level of mRNA by RT−PCR (*B*, *D*, *E* and *F*) and protein by western blot (*C*) in aortic rings. Experimental groups: control-vehicle, DOCA-vehicle, GW0742 (5 mg kg^{-1}, GW5), DOCA-GW0742 (5 mg kg^{-1}, DOCA-GW5), and DOCA-GW0742 (20 mg kg^{-1}, DOCA-GW20). Data presented as a ratio of arbitrary units of mRNA ($2^{-\Delta\Delta Ct}$) or densitometric values of protein band normalized to the corresponding α-actin, compared with the control-vehicle group. The results of western blots are shown as mean ± SEM, derived from four to six separate rings from different rats. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ when compared with the control group, respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared with the DOCA group, respectively.

administered intraperitoneally effectively blunted PPARβ in the vasculature. However, GSK0660 did neither modify the development of hypertension induced by DOCA-salt administration nor the effect of GW0742 preventing the increase in SBP (*Figure 6A*), and reducing cardiac hypertrophy parameters (Supplementary material online, *Table S2*) in DOCA-salt rats. In contrast, the increased relaxation to acetylcholine induced by GW0742 in DOCA-salt rats was abolished by GSK0660 (*Figure 6B*). The plasma NOx levels were lower in DOCA-salt when compared with control rats (Supplementary material online, *Figure S5D*). GW0742 significantly increased NOx levels in DOCA-salt rats, which was abolished by GSK0660. Furthermore, PPARβ blockade suppressed the reduction induced by GW0742 in aortic DHE staining (*Figure 6D*), NADPH oxidase activity (*Figure 6C*), and NOX4 mRNA levels (*Figure 6E*), and the increase induced by GW0742 in the antioxidant genes, Cu/Zn-SOD (Supplementary material online, *Figure S5E*), Mn-SOD (Supplementary material online, *Figure S5F*), catalase (Supplementary material online, *Figure S5G*), GPx1 (Supplementary material online, *Figure S5H*), and HO-1 (Supplementary material online, *Figure S5I*) expression. GSK0660 did not modify all these parameters in DOCA-salt rats when administered alone.

The aortic expression of ppET-1 mRNA, which was increased by GW0742 in DOCA-salt rats, was unaltered by co-administration of GSK0660 (*Figure 6F*). However, the increase in RGS5 mRNA induced by GW0742 was abolished by PPARβ blockade (*Figure 6G*). In addition, the contractile response induced by ET-1 was inhibited by GW0742 in both the control and DOCA-salt groups (*Figure 6H*). This inhibitory effect was also suppressed by GSK0660 in DOCA-salt animals.

3.8 GW0742 induces relaxant responses in mesenteric arteries *in vitro*

GW0742 causes, in a concentration-dependent manner (1, 10, 30 μM), a progressive downward shift of the concentration-contractile response curve to phenylephrine (Supplementary material online, *Figure S6A*). Cumulative increases in the concentration of GW0742 (10^{-7}–10^{-4} M) in small mesenteric arteries previously contracted with phenylephrine also resulted in a concentration-dependent relaxation (IC_{50} 4.3 ± 1.0 μM, Supplementary material online, *Figure S6B*). This relaxation was not altered neither by eNOS inhibition with L-NAME nor by incubation with the PPARβ antagonist GSK0660.

4. Discussion

Our experiments provide the first evidence that chronic treatment with the highly selective PPARβ agonist GW0742 prevents the increase in SBP, the systemic and vascular oxidative stress, and endothelial dysfunction in salt-sensitive hypertension. These effects, except the changes in SBP, seem to be related to a direct activation of PPARβ in the vascular

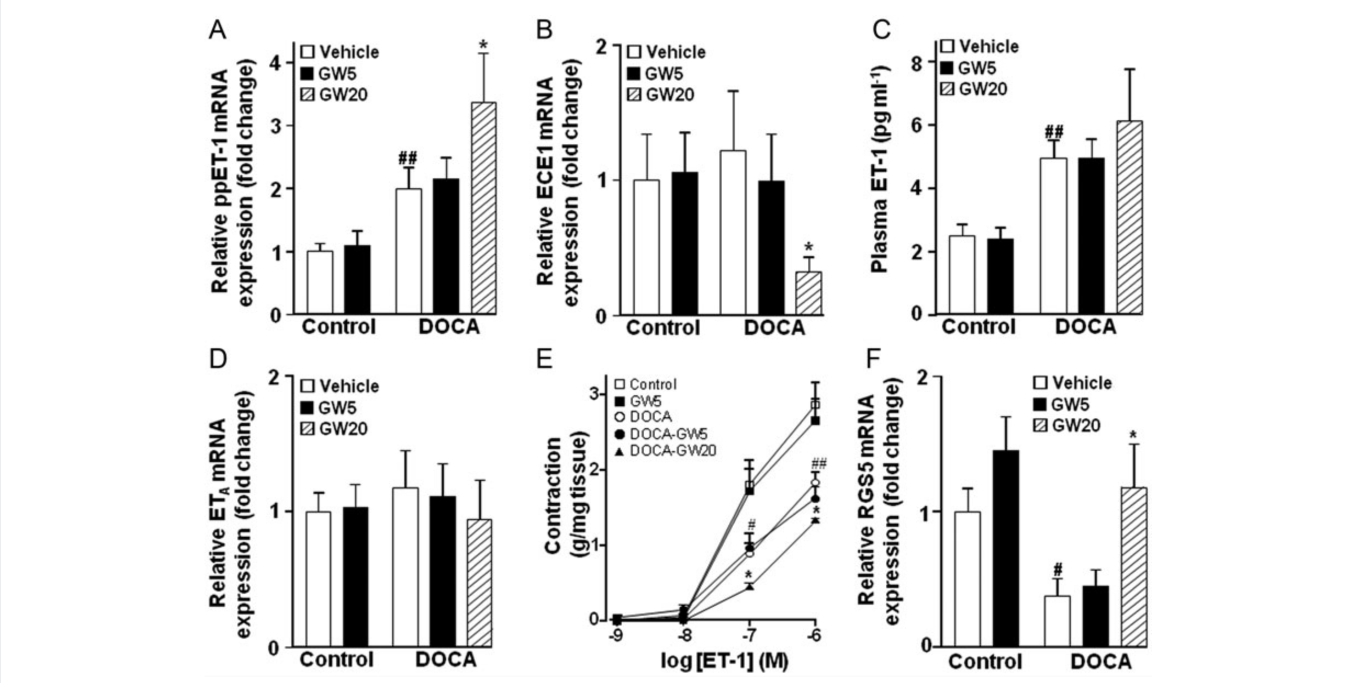

Figure 5 The effect of GW0742 on the ET-1 pathway. mRNA levels of ppET-1 (*A*), ECE1 (*B*), and ET_A (*D*) in aorta, ET-1 plasma levels (*C*), RGS5 (F), and ET-1 induced aortic contractile response (*E*). Experimental groups: control-vehicle, DOCA-vehicle, GW0742 (5 mg kg^{-1}), DOCA-GW0742 (5 mg kg^{-1}), and DOCA-GW0742 (20 mg kg^{-1}). Expressional data are presented as a ratio of arbitrary units of mRNA ($2^{-\Delta\Delta Ct}$) compared with the control-vehicle group. The results are shown as mean ± SEM, ($n = 9-20$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ when compared with the control group. $^{*}P < 0.05$ when compared with the DOCA group.

wall, which increased RGS5 expression, with the subsequent interference with ET-1 signalling, leading to reduced contractile response to ET-1 and intracellular ROS generation. Moreover, the activation of PPARβ up-regulated antioxidant genes, including Cu/Zn-SOD, Mn-SOD, catalase, GPx1, and HO-1.

Our previous study demonstrates that GW0742, at 5 mg kg^{-1} exerted antihypertensive effects, partly restored the vascular structure and function, and reduced the oxidative, proinflammatory, and proatherogenic status of SHR. These protective effects seem to be related to increased expression of RGS4 and RGS5, which negatively modulated the vascular actions of Ang II.[27] In the present study we also show a clear dissociation between high blood pressure and endothelial function, since GW0742 at 5 mg kg^{-1} prevented the raise in SBP (∼61%) but had no effect on the endothelial function. Similar results have been previously described for the PPARα agonist fenofibrate.[14] Moreover, the antihypertensive effect induced by GW0742 seems to be independent of PPARβ activation, since it was unaffected by the PPARβ antagonist GSK0660.

PPARα and PPARγ activators prevent the raise in SBP in DOCA-salt rats by inhibition of ppET-1 mRNA production.[14] This agrees with the blood pressure-lowering effects of selective ET receptor blockers and with the notion that ET-1 participates in the pathophysiology of this model of hypertension.[32] However, GW0742 was unable to reduce vascular ppET-1 mRNA and plasma ET-1 levels. Paradoxically, GW0742 at 20 mg kg^{-1} increased ppET-1 mRNA in DOCA-salt rats, but reduced the expression of ECE1, the enzyme converting pro-ET-1 into ET-1, which could collaborate to maintain similar ET-1 plasma levels. The raise in ppET-1 mRNA induced by GW0742 was unaltered by PPARβ blockade, showing that the expression of this gene is PPARβ independent.

The expression of PPAR in the aorta was increased in DOCA-salt rats (present results) and in SHR[27] when compared with control rats, and chronic treatment with GW0742 reversed these changes. The expression of PPARβ is modulated by oxidative stress and inflammation. Jiang *et al.*[33] have shown that in human umbilical vein endothelial cells, repetitive low grade of H_2O_2 stress enhances PPARβ expression while Tan *et al.*[34] found increased PPARβ in keratinocytes by another pro-oxidant stimulus TNFα. Therefore, we speculate that in the DOCA salt rats the up-regulation of aortic PPARβ may be due to the increased H_2O_2 content. Conversely, GW0742 via a reduction in the vascular H_2O_2 content in the DOCA-salt animals may restore the expression of PPARβ. As expected, GW0742 induced, in a concentration-dependent manner, an increased expression of PPARβ-target gene PDK4 in the aorta, which was abolished by GSK0660, confirming that chronic GW0742-activated PPARβ in the vasculature. Moreover, the PPARβ agonist increased CD36 expression which is expected to increase the uptake of the endogenous PPARβ ligands, e.g. fatty acids.

DOCA-salt rat showed a reduced contractile response to ET-1, which may act as a compensatory mechanism to the increased vascular resistance and blood pressure during DOCA-salt hypertension. This reduced response to ET-1 has been explained in the literature by (i) decreased density of ET_A receptors, (ii) reduced increment of intracellular calcium concentration to ET-1, and (iii) reduced activation of p38 MAPK to ET-1 with the subsequent diminished caldesmon phosphorylation.[35] In our experiments, the mRNA level of ET_A receptor was similar in all experimental groups suggesting downstream changes to explain the reduced response to ET-1 found in DOCA-salt rats. RGS proteins play important roles in the regulation of G protein-coupled receptor signalling by binding to the active G subunits and stimulating GTP hydrolysis,

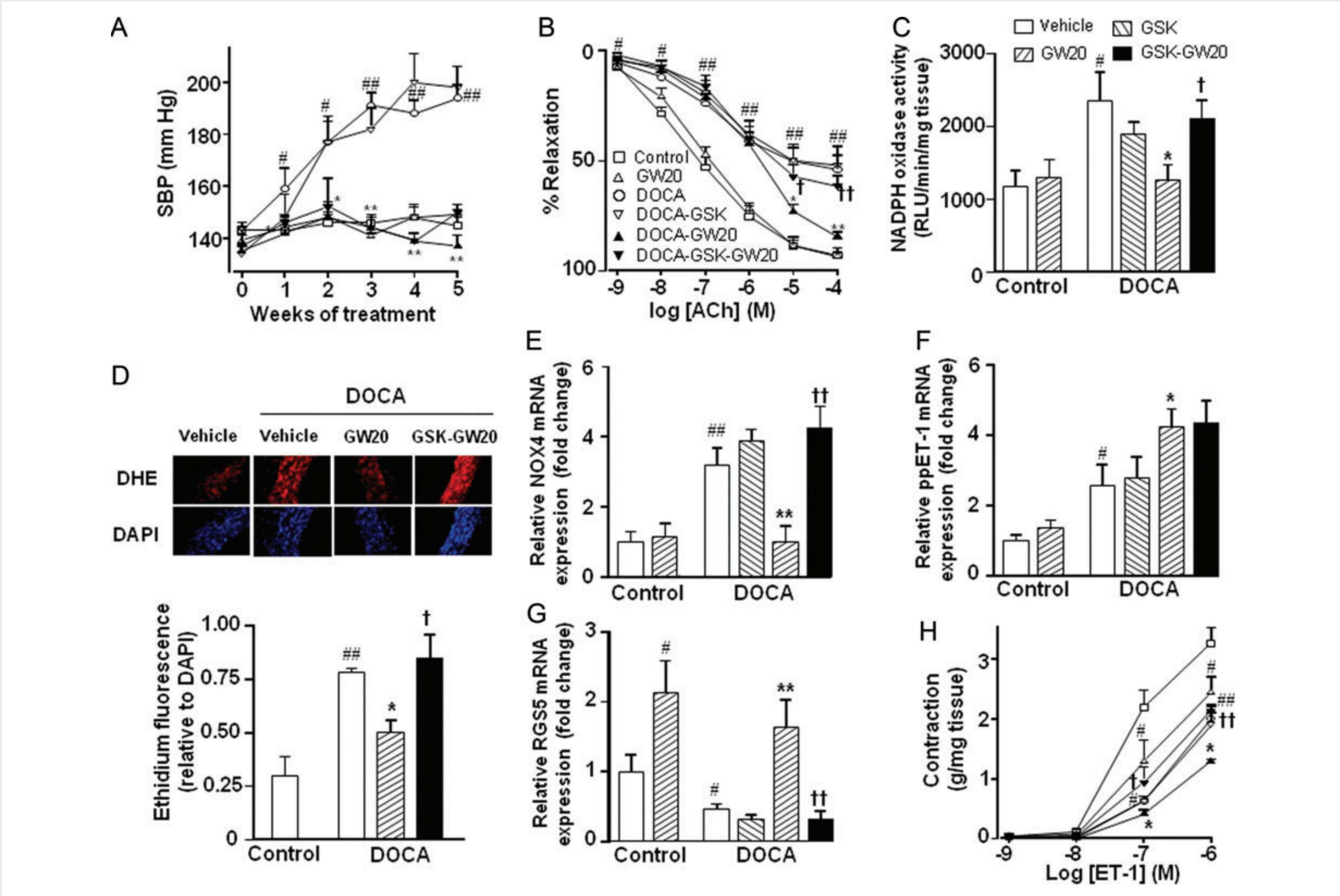

Figure 6 Effects of the PPARβ antagonist GSK0660 (GSK) on the changes in the blood pressure, endothelial function, NADPH oxidase and ET-1 pathways induced by chronic GW0742 treatment in DOCA-salt rats. SBP (*A*) as measured by tail-cuff plethysmography in DOCA-salt-induced hypertension. Vascular relaxant responses induced by acetylcholine (ACh) (*B*) in aortae pre-contracted by 10^{-6} mol/L phenylephrine for 30 min. NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence (*C*), ROS content measured by ethidium fluorescence (*D*), and the expression of NOX-4 (*E*), ppET-1 (*F*), and RGS5 (*G*) at the level of mRNA by RT–PCR in aortic rings. ET-1 induced aortic contractile response (*E*). Experimental groups (Experiment 2): control-vehicle (open square) ($n = 6$), DOCA-vehicle (open circle) ($n = 8$), GW0742 (20 mg kg^{-1}) (open triangle) ($n = 7$), DOCA-GSK (1 mg kg^{-1}) (inverted open triangle) ($n = 6$), DOCA-GW0742 (20 mg kg^{-1}) (closed triangle) ($n = 8$), and DOCA-GSK (1 mg kg^{-1})-GW0742 (20 mg kg^{-1}) (inverted closed triangle) ($n = 7$). Data are presented as a ratio of arbitrary units of mRNA ($2^{-\Delta\Delta Ct}$) compared with the control-vehicle group. The results are shown as mean ± SEM. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ when compared with the control-vehicle group. $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared with the DOCA group. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ when compared with the DOCA-GW20 group.

thus switching off G protein signalling.[28] Thus, RGS down-regulation potentiates the effect of vasoconstrictors like endothelin and Ang II.[36] RGS5 exhibits a striking expression pattern in vascular smooth muscle cells and is a potent GTPase-activating factor for a number of Giα- and Gqα-mediated pathways, including those of Ang II and endothelin 1, suggesting a role in blood pressure regulation.[37] Genome-wide linkage and candidate gene-based association studies have identified the human RGS5 gene as one of the three genes that contribute to elevated blood pressure in human.[38] Very recently, down-regulation of RGS5 was sufficient to cause enhanced myogenic constriction in intact resistance arteries, which is essential in the regulation of blood flow and provides the basal tone in resistance arteries.[29] Similarly, overexpression studies in the aorta showed that RGS5 inhibits contractions via Gqα.[27,39] Paradoxically, in our study, the mRNA expression of RGS5 was down-regulated in DOCA-salt rats. Therefore, reduced contractile responses to ET-1 cannot be attributed to changes in RGS5. RGS5 is a direct gene target of PPARβ.[29] Likewise, we found that GW0742 at 20 mg kg^{-1} induced an increase in RGS5 expression in the aorta from the control and DOCA-salt rats, which was suppressed by PPARβ blockade, without changes in the ET_A receptor expression. This increase in RGS5 would reduce ET_A receptor signalling and hence ET-1-induced vasoconstriction. In fact, GW0742 at 20 mg kg^{-1} reduced the ET-1 contraction in both control and DOCA-salt rats, whereas GW0742 at 5 mg kg^{-1} had no effect on either RGS5 expression or ET-1 contractility.

ET-1 has been involved in the development of vascular oxidative stress and endothelial dysfunction in DOCA-salt rats since ET_A receptor blockade reduced arterial $O_2^{\bullet-}$ levels with a concomitant improvement in endothelium-dependent relaxation.[10] In DOCA-salt rats, we also found impaired endothelium-dependent vasodilator responses, decreased plasma levels of NOx, increased plasma levels of ET-1 and MDA, increased urinary isoprostanes excretion, and a higher content of aortic ROS. It is well established that ET-1 activates NADPH oxidase to produce vascular $O_2^{\bullet-}$ generation in DOCA-salt hypertensive rats via up-regulation of the NADPH oxidase subunits[5,6,10,40] (present study). In this paper, GW0742 at 20 mg kg^{-1} reduced the increased aortic intracellular ROS content, NADPH oxidase activity, up-regulation of NOX4, and the impaired acetylcholine-induced relaxation. We also found that the main H_2O_2 detoxifying enzyme catalase was significantly down-regulated in the aorta from DOCA-salt rats, which may also account for the higher staining of the peroxide-sensitive dye DCF. The chronic treatment of the control rats with GW0742-induced transcriptional up-regulation of antioxidant genes HO-1 and GPx1, as previously reported by PPARβ activation in cultured vascular smooth muscle cell.[41] Moreover, GW0742 also up-regulated other PPARβ-sensitive antioxidant genes, such as Cu/Zn-SOD,[42] Mn-SOD,[41] and catalase[42] in DOCA-salt rats, contributing to detoxify intracellular ROS. Collectively, all these data indicate that the improvement of endothelial dysfunction induced by PPARβ activation seems to be related to increased NO bioactivity by reducing NADPH oxidase-driven $O_2^{\bullet -}$ production stimulated by ET-1 in aortic rings and by increasing antioxidant defences. Other mechanisms might also contribute, including a possible increased NO synthesis as a result of a decrease in the negative allosteric regulator of eNOS, caveolin-1, and an increased sensitivity, to the NO-cGMP pathway, as demonstrated by small increase in nitroprusside relaxation induced by GW0742 in the DOCA-salt rats.

All these data suggest that up-regulation of RGS5 may be an essential step in the effects of the PPARβ agonist described herein including its antihypertensive, antioxidant actions, and protective effect in the endothelial function. However, the dose of 5 mg kg^{-1} GW0742 was unable to up-regulate RGS5 in the DOCA-salt rats, which suggests the involvement of additional mechanisms independent of ET-1 synthesis and signalling. Acute non-genomic vasodilator effects of GW0742 have also been described in the rat aorta[43] and in mice vessels.[44] The vasodilator response in the rat aorta was partially prevented by the PPARβ antagonist GSK0660[43] and was reduced in the aorta but not in pulmonary or mesenteric vessels from PPARβ −/− when compared with wild-type mice.[44] Herein we also found that in rat small mesenteric arteries, as in mice mesenteric arteries, GW0742 induced a concentration-dependent relaxant response which was independent of PPARβ activation, because it was unaffected by the PPARβ antagonist GSK0660. This effect, which was also independent of endothelial NO release, since it was unaltered by eNOS inhibition, may also collaborate to reduce SBP.

In conclusion, our results clearly demonstrate that the PPARβ agonist GW0742 reduces the increase in blood pressure, and at the higher dose of 20 mg kg^{-1} improves the cardiac hypertrophy, the endothelial dysfunction, and the vascular oxidative stress in this model of mineralocorticoid-induced hypertension. These effects on the endothelial function seem to be related to PPARβ activation via reduced NADPH-oxidase-mediated $O_2^{\bullet -}$ production stimulated by ET-1, up-regulation of RGS5, and increased antioxidant enzymatic defences, finally resulting in increased NO bioactivity.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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