Pioglitazone reduces angiotensin II-induced COX-2 expression through inhibition of ROS production and ET-1 transcription in vascular cells from spontaneously hypertensive rats

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Pioglitazone reduces angiotensin II-induced COX-2 expression through inhibition of ROS production and ET-1 transcription in vascular cells from spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol 306: H1582–H1593, 2014. First published April 11, 2014; doi:10.1152/ajpheart.00924.2013.—Glitazones have anti-inflammatory properties by interfering with the transcription of proinflammatory genes, such as cyclooxygenase (COX)-2, and with ROS production, which are increased in hypertension. This study analyzed whether pioglitazone modulates COX-2 expression in hypertension by interfering with ROS and endothelin (ET)-1. In vivo, pioglitazone (2.5 mg·kg−1·day−1, 28 days) reduced the greater levels of COX-2, pre-pro-ET-1, and NADPH oxidase (NOX) expression and activity as well as O2·− production found in aortas from spontaneously hypertensive rats (SHRs). ANG II increased COX-2 and pre-pro-ET-1 levels more in cultured vascular smooth muscle cells from hypertensive rats compared with normotensive rats. The ET(A) receptor antagonist BQ-123 reduced ANG II-induced COX-2 expression in SHR cells. ANG II also increased NOX-1 expression, NOX activity, and superoxide production in SHR cells; the selective NOX-1 inhibitor ML-171 and catalase reduced ANG II-induced COX-2 and ET-1 transcription. ANG II also increased c-Jun transcription and phospho-c-Jun and p65 NF-κB expression and activation observed in this strain. Furthermore, pioglitazone reduced the effects of ANG II on NOX activity, NOX-1, pre-pro-ET-1, COX-2, and c-Jun mRNA levels, JNK activation, and nuclear phospho-c-Jun and p65 expression. In conclusion, ROS production and ET-1 are involved in ANG II-induced COX-2 expression in SHRs, explaining the greater COX-2 expression observed in this strain. Furthermore, pioglitazone inhibits ANG II-induced COX-2 expression likely by interfering with NF-κB and activator protein-1 proinflammatory pathways and downregulating ROS production and ET-1 transcription, thus contributing to the anti-inflammatory properties of glitazones.

angiotensin II; endothelin-1; reactive oxygen species; cyclooxygenase-2; hypertension; peroxisome proliferator-activated receptor-γ

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR)-γ, a member of the nuclear receptor superfamily, is expressed in the vascular wall, where it exerts a role in vascular biology (53). PPARs have cardiovascular protective effects resulting from their anti-inflammatory and antioxidant actions (53). These protective effects have been associated with inhibition of expression of proinflammatory enzymes, such as the inducible isoform of cyclooxygenase (COX-2) (50, 52), with inhibition of expression of NADPH oxidase (NOX) components and subsequent ROS production (22, 34) and with the ability to antagonize the vascular damage and functional alterations induced by ANG II (15, 48, 57). In addition to the reported antioxidant properties, the anti-inflammatory mechanisms proposed for PPAR-γ ligands comprise interference with MAPK activation and/or with downstream activator protein (AP)-1 and NF-κB transactivation (42).

ANG II, the main effector peptide of the renin-angiotensin system (RAS), is increased in tissues and plasma of different hypertension models, where it actively contributes to the proinflammatory state observed in this pathology (46). Thus, ANG II induces cytokines and ROS production and the expression of adhesion molecules and proinflammatory enzymes such as COX-2 (4, 6, 16, 46). Increased COX-2 expression and activity could contribute to the vascular alterations found in hypertension. In this sense, we have described increased production of COX-2-derived contractile prostanoids and its participation in vasoconstrictor responses in vessels from hypertensive rats, which was reduced by treatment with ANG II type 1 (AT1) receptor antagonists (4). COX-2 expression is highly regulated at both transcriptional and posttranscriptional levels. Among the signaling pathways involved in COX-2 regulation, ROS, several transcription factors, such as NF-κB or AP-1, or different kinases involved in cell transduction signaling seem to play an important role (4, 6, 12).

Endothelin (ET)-1, a peptide of 21 amino acids, constitutes the most abundant and important member of the ET family (5, 43). Although ET-1 is primarily produced by endothelial cells (5, 43), its expression may also be induced in vascular smooth muscle cells (VSMCs) by proinflammatory mediators such as ANG II (23, 29) through AP-1 and NF-κB activation (10, 23, 24). In addition to its role on vascular tone regulation, ET-1 also acts as a mediator of the vascular inflammatory response by inducing the expression of proinflammatory enzymes, including COX-2 (14, 37), thereby contributing to the pathophysiological alterations observed in different vascular inflammatory disorders, such as hypertension (5, 41, 43). In addition, ET-1 has been proposed to mediate some of the deleterious cardiovascular effects of ANG II, such as renal effects, fibrosis, or cardiac hypertrophy (1, 7, 36, 40, 44).

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Our hypothesis is that the reduction in the increased COX-2 expression observed in hypertension, associated with the augmented RAS activity, contributes to the described anti-inflammatory effects of glitazones. Among the mechanisms involved, a decrease in oxidative stress and in ET-1 transcription might contribute to this effect. Therefore, the aim of the present study was to investigate whether PPAR-γ agonists attenuate the increased COX-2 expression observed in hypertension by interfering with ROS production and ET-1 transcription as well as to determine the possible molecular mechanisms involved. The specific objectives were to analyze 1) the in vivo effect of pioglitazone in oxidative stress, ET-1 mRNA levels, and COX-2 expression in the aorta from spontaneously hypertensive rats (SHRs); 2) the effect of pioglitazone on ANG II-induced COX-2 expression and the involvement of oxidative stress and ET-1 in such effects in VSMCs from SHRs; and 3) the contribution of interference with the NF-κB and AP-1 signaling pathways in the pioglitazone effect.

MATERIALS AND METHODS

All experiments were approved by the Ethical Committee of Research of the Universidad Autónoma de Madrid, Spain (CEI-UAM 31-759). All animal care and experimental procedures conformed with current Spanish and European laws on the use of animals (RD 223/88 MAPA and 609/86).

For experiments, aortas from 6-mo-old normotensive [Wistar Kyoto (WKY)] rats and SHRs untreated or treated with the PPAR-γ agonist pioglitazone (2.5 mg·kg \(^{-1}\)·day \(^{-1}\)) for 28 days suspended in 0.5% methylcellulose and administered in drinking water were used. This treatment did not modify blood pressure, as previously described (22). For some experiments, we also used SHRs treated with the AT\(_1\) receptor antagonist losartan (15 mg·kg \(^{-1}\)·day \(^{-1}\)), the COX-2 expression in the aorta from spontaneously hypertensive rats (SHRs); 2) the effect of pioglitazone on ANG II-induced COX-2 expression and the involvement of oxidative stress and ET-1 in such effects in VSMCs from SHRs; and 3) the contribution of interference with the NF-κB and AP-1 signaling pathways in the pioglitazone effect.

For determination in aortic segments, frozen segments were cut into 14-μm-thick sections and placed on a glass slide. Serial aortic segments/cells or untreated samples as calibrators. mRNA levels of the housekeeping genes were not modified by any of the treatments used. NOX activity. A lucigenin-enhanced chemiluminescence assay was used to determine NOX activity. Aortas or VSMCs were homogenized in lucigenin assay buffer (50 mM KH\(_2\)PO\(_4\), 1 mM EGTA, and 200 μg/ml streptomycin (Sigma Chemical, St. Louis, MO). After the adventitia had been carefully removed, VSMCs were obtained by the explant method (4, 32). Cells were identified as VSMCs by the typical spindle shape, by the typical “hills and valleys” distribution, and by positive immunocytochemical staining with specific monoclonal anti-α-actin antibody (Sigma Chemical). For experiments, cells from passages 3–8 were made quiescent by incubation in DMEM containing 0.2% FBS for 24 h. Cells were stimulated with ANG II (for the times indicated in the results) and with ET-1 (1 h) with or without pretreatment for 18 h with the PPAR-γ agonist pioglitazone or for 45 min with the ETA receptor antagonist BQ-123, the ET\(_A\) receptor antagonist BQ-788, the AT\(_1\) receptor antagonist losartan, the presumed NOX inhibitor apocynin, the NOX-1 inhibitor ML-171, the H\(_2\)O\(_2\) scavenger catalase, the JNK inhibitor SP-600125, or the pro-inflammatory effects of glitazones. Among the mechanisms involved, a decrease in oxidative stress and in ET-1 transcription might contribute to this effect. Therefore, the aim of the present study was to investigate whether PPAR-γ agonists attenuate the increased COX-2 expression observed in hypertension by interfering with ROS production and ET-1 transcription as well as to determine the possible molecular mechanisms involved. The specific objectives were to analyze 1) the in vivo effect of pioglitazone in oxidative stress, ET-1 mRNA levels, and COX-2 expression in the aorta from spontaneously hypertensive rats (SHRs); 2) the effect of pioglitazone on ANG II-induced COX-2 expression and the involvement of oxidative stress and ET-1 in such effects in VSMCs from SHRs; and 3) the contribution of interference with the NF-κB and AP-1 signaling pathways in the pioglitazone effect.

Western blot analysis. Protein expression was determined in whole cell lysates (20–25 μg protein) or nuclear extracts (15 μg protein) by Western blot analysis, as previously described (32). Proteins were separated by 10% SDS-PAGE and then transferred to polyvinyl difluoride membranes overnight. Membranes were incubated with rabbit polyclonal antibodies for COX-2 (1:250, Cayman Chemical; Ann Arbor, MI), 65 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated (p-ε-Jun (1:500, Santa Cruz Biotechnology), and p-JNK1/2 (1:2,000, Cell Signaling Technology, Danvers, MA). Immunoreactive bands were visualized using fluorescent secondary antibodies from the corresponding host (1:5,000, Bio-Rad Laborator-
each experimental condition were analyzed with MetaMorph image-analysis software (Molecular Devices, Downingtown, PA). The integrated optical densities in the target region were calculated.

VSMCs were plated onto glass coverslips inserted into six-well plates and cultured as described above. Subconfluent cells were stimulated with ANG II for 2 h in the absence and presence of pioglitazone or apocynin that were respectively added 18 h or 45 min before ANG II. Afterwards, cells were loaded with DHE (10 μM) in serum-free DMEM with 0.1% BSA for 30 min at 37°C. Nonstimulated VSMCs were imaged daily in parallel using the same image settings. Images were captured with a fluorescent laser scanning confocal microscope (Leica TCS SP2). The fluorescence intensity values of 10–20 nuclei per experiment were measured using the Metamorph Image Analysis Software.

Immunofluorescence. VSMCs were seeded in a 24-well culture plate with coverglasses in the bottom surface. After reaching 60% confluence, cultures were starved in DMEM with 0.2% FBS for 24 h. Cultures were stimulated with ANG II after treatment or not with pioglitazone. At the end of the treatment, cells were washed and fixed in 4% paraformaldehyde diluted in PBS and permeabilized in PBS containing 0.2% BSA and 0.5% Triton X-100. The rabbit polyclonal primary antibody against p65 was added to cells at 1:200 dilution and incubated overnight at 4°C. After cells had been washed, green FITC-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Life Technologies, Paisley, UK) was added at 1:2000 dilution in the dark. In control experiments, the primary antibody was omitted. After cells were washed and incubated for 15 min with 4',6-diamidino-2-phenylindole (1:10,000, Invitrogen Life Technologies) to stain nuclei, ProLong Gold antifade mounting reagent (Invitrogen Life Technologies) was added to the microscope slides. Cells fixed and treated in the coverglasses were placed and sealed in the slides and allowed to dry completely in the dark until the next day. Immunofluorescence-stained cells were observed under a laser scanning confocal microscope (Nikon, C1 plus, Nikon Instruments, Melville, NY) and analyzed using ImageJ software (http://rsb.info.nih.gov/ij). The fluorescence intensity of cells was measured at four preset areas per sample, and at least three independent experiments were performed.

Additional reagents. ANG II, ET-1, BQ-123, BQ-788, ML-171, apocynin, catalase, and lactacystin were obtained from Sigma Chemical. SP-600125 and losartan were purchased from Calbiochem (Darmstadt, Germany). DHE was from Molecular Probes (Invitrogen Life Technologies). Pioglitazone was generously supplied by Takeda-Lilly.

Statistical analysis. Results are expressed as means ± SE. n denotes the number of animals used or the number of different cultures (each obtained from three different animals). Statistical analysis was performed using a paired Student’s t-test and one-way ANOVA followed by the Bonferroni test or the Mann-Whitney nonparametric test using Graph Pad Prism software. P values of ≤0.05 were considered to indicate statistically significant differences.

RESULTS

In vivo treatment with pioglitazone reduced the increased COX-2 and ET-1 transcription as well as oxidative stress observed in aortas from hypertensive rats. We (2, 4) have previously described that COX-2 protein expression and mRNA levels were greater in aortic segments from SHRs compared with WKY rats. Now, we confirmed these results and also observed increased pre-pro-ET-1 mRNA levels in segments from SHRs (Fig. 1, A and B). Treatment of hypertensive rats with pioglitazone (2.5 mg·kg⁻¹·day⁻¹, 28 days) reduced the greater mRNA levels of both COX-2 and ET-1 observed in aortic segments (Fig. 1, A and B).

In aortas from SHRs, mRNA levels of the NOX catalytic subunits NOX-1 and NOX-4, NOX activity, and vascular O₂⁻ production were greater compared with WKY segments (Fig. 1, C–E), as previously described (3, 4, 33). We also observed increased mRNA levels of the NOX regulatory subunit p47phox (Fig. 1C). Pioglitazone treatment normalized the increased NOX expression and activity as well as the greater O₂⁻ production observed in SHRs (Fig. 1, C–E).

We have previously described increased COX-2 expression and oxidative stress in ANG II-infused mice (33) and that losartan treatment reduced the increased vascular COX-2 expression and oxidative stress observed in SHRs (4). In addition, the greater mRNA levels of ET-1 found in aortic segments from SHRs compared with WKY rats (relative expression: 0.92 ± 0.12 in WKY rats, n = 6, compared with 1.99 ± 0.37 in SHRs, n = 6, P < 0.05) was reduced by losartan (0.94 ± 0.26, n = 6, P < 0.05), suggesting the involvement of ANG II in such increases. In an attempt to clarify the mechanisms by which pioglitazone reduces the increased COX-2 and ET-1 transcription as well as the oxidative stress observed in hypertensive rats, the following experiments were carried out using cultured VSMCs stimulated with ANG II.

Pioglitazone reduced ANG II-induced COX-2 expression in VSMCs. Basal mRNA levels of COX-2 were also greater in VSMCs from SHRs compared with WKY rats (data not shown), as previously described (32). ANG II (100 nM, 15 min to 3 h) time dependently increased COX-2 mRNA levels in VSMCs from WKY rats and SHRs (Fig. 2A). The increase in both COX-2 mRNA levels and protein expression after 2-h ANG II incubation was higher (P < 0.05) in VSMCs from SHRs compared with WKY rats (Fig. 2, A and B). Losartan treatment (10 μM) reduced the increase in COX-2 mRNA levels in both WKY rats (relative expression: 2.34 ± 0.45 for ANG II vs. 1.25 ± 0.30 for ANG II + losartan, n = 5, P < 0.05) and SHRs (relative expression: 6.27 ± 1.24 for ANG II vs. 2.15 ± 0.35 for ANG II + losartan, n = 5, P < 0.05), supporting the involvement of AT1 receptors in such induction.

Treatment of VSMCs with the PPAR-γ agonist pioglitazone (10 μM, 18 h) reduced ANG II-induced COX-2 protein and mRNA levels in cells from SHRs (Fig. 2, C and D) and in those from WKY rats (COX-2 mRNA levels, relative expression: 2.76 ± 0.50 for ANG II vs. 1.42 ± 0.25 for ANG II + pioglitazone, n = 6, P < 0.05)

Pioglitazone reduced ANG II-induced COX-2 expression by reducing ET-1 transcription in VSMCs from SHRs. Basal mRNA levels of pre-pro-ET-1 were greater in VSMCs from SHRs compared with WKY rats (Fig. 3A). ANG II (100 nM, 15 min to 3 h) did not modify pre-pro-ET-1 mRNA levels in cultures from normotensive rats; however, in cells from SHRs, ANG II increased those levels in a time-dependent manner, with a peak at 1 h (Fig. 3B). ANG II-induced pre-pro-ET-1 gene expression was dependent on AT1 receptor activation since it was abolished by 10 μM losartan (relative expression: 1.74 ± 0.06 for ANG II vs. 1.08 ± 0.10 for ANG II + losartan, n = 5, P < 0.05).

In VSMCs from SHRs, the ETA receptor antagonist BQ-123 (1 μM; Fig. 3C), but not the ETB receptor antagonist BQ-788 (1 μM; data not shown), reduced, but not abolished, the increased COX-2 protein and mRNA levels induced by ANG II (2 h). These data suggest that ET1, through ETA receptor activation, contributes, at least partially, to the ANG II-induced COX-2 expression. Accordingly, incubation of VSMCs from SHRs with ET-1 (100 nM, 1 h) increased both COX-2 protein
The effect of ET-1 on COX-2 mRNA levels was reduced by BQ-123 (Fig. 3D), suggesting the involvement of ETA receptors in such induction. As observed with COX-2 expression, treatment of VSMCs from SHRs with pioglitazone reduced ET-1 transcription induced by ANG II (1 h; Fig. 3E). Pioglitazone reduced ANG II-induced ET-1 and COX-2 expression by reducing oxidative stress in VSMCs from SHRs. The specific NOX-1 inhibitor ML-171 (0.5 μM) and the H₂O₂ scavenger catalase (1,000 U/ml) reduced the increase in COX-2 and pre-pro-ET-1 mRNA levels induced by ANG II in VSMCs from SHRs (Fig. 4, A and B), suggesting the involvement of oxidative stress in ANG II-induced COX-2 and ET-1 expression.

ANG II increased NOX-1 mRNA levels and NOX activity, and pioglitazone reduced these increases (Fig. 4, C and D). In keeping with this, pioglitazone also reduced the increase in O₂⁻ production observed after ANG II incubation (Fig. 4E). NOX seems to be the source of this superoxide production based on the reduction elicited by apocynin (30 μM; Fig. 4E).

Pioglitazone reduced ANG II-induced ET-1 and COX-2 expression by reducing AP-1 and NF-κB activation in VSMCs from SHRs. It has been previously described that ET-1 and COX-2 as well as NOX-1 genes have regulatory sites in their promoters to bind the proinflammatory transcription factor AP-1 (8, 12, 30, 43, 51). Therefore, we analyzed the involvement of AP-1 on NOX-1 and ET-1 expression and its contribution to the increased ANG II-induced COX-2 expression. Treatment of VSMCs from SHRs with the specific JNK inhibitor SP-600125 (20 μM) diminished both pre-pro-ET-1 and NOX-1 expression as well as NOX activity induced by ANG II (Fig. 5, B–D). In agreement with the above results indicating the role of ROS and ET-1 in ANG II-induced COX-2 expression, we found that this expression was also reduced by SP-600125 (Fig. 5A).

ANG II increased JNK1/2 phosphorylation at 5 and 10 min (Fig. 5, E and F) in VSMCs from SHRs. Accordingly, mRNA

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**Fig. 1.** A–E: cyclooxygenase (COX)-2, pre-pro-endothelin (ET)-1, NADPH oxidase (NOX)-1, NOX-4, and p47^phox^ mRNA levels (A–C), NOX activity (D), and representative confocal photomicrographs and quantitative analysis of vascular O₂⁻ production (E) in aortic segments from Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs) untreated and treated with pioglitazone (Pio; 2.5 mg·kg⁻¹·day⁻¹, 28 days). Results are expressed as relative values compared with WKY rats. *P < 0.05 vs. WKY rats; #P < 0.05 vs. SHRs by a Mann-Whitney nonparametric test or one-way ANOVA.
levels of c-Jun (Fig. 5G) and nuclear expression of p-c-Jun (Fig. 5H) were also increased in the presence of ANG II. It has been previously described that PPAR-γ activation interferes with the AP-1 pathway (42). We then analyzed whether the effect of pioglitazone on COX-2 expression could be associated with negative regulation of this proinflammatory transcription factor. Pioglitazone treatment reduced p-JNK1/2 expression (Fig. 5F), c-Jun mRNA levels (Fig. 5G), and p-c-Jun nuclear expression (Fig. 5H) induced by ANG II.

NF-κB is another proinflammatory transcription factor that may regulate COX-2, ET-1, and NOX-1 expression (12, 31, 43, 51). Treatment of VSMCs from SHRs with the proteasome inhibitor lactacystin (10 μM) reduced pre-pro-ET-1 and NOX-1 mRNA levels and NOX activity induced by ANG II (Fig. 6, B–D), and thus, the increased ANG II-induced COX-2 mRNA levels (Fig. 6A).

ANG II increased the nuclear expression of p65 (Fig. 6E). Immunofluorescence experiments confirmed the augmented localization of p65 in the nucleus (Fig. 6F). The interference by PPAR-γ activators of the NF-κB signaling pathway has also been suggested (42). Accordingly, pioglitazone reduced ANG II-induced p65 nuclear expression in VSMCs from SHRs (Fig. 6, E and F).

**DISCUSSION**

This study shows, in conductance vessels from hypertensive animals, that the PPAR-γ agonist pioglitazone reduces the
increased proinflammatory enzyme COX-2, which is associated with the increased RAS activity observed in this pathology. Among the mechanisms involved, we propose that the glitazone, by interfering with NF-κB and AP-1, reduces the ANG II-elicited ROS production and ET-1 transcription, which are involved in the increased COX-2 expression. These mechanisms could contribute to the well-documented anti-inflammatory properties of glitazones.

PPAR-γ has cardioprotective effects associated, among others, with inhibition of vascular expression of proinflamma-

Fig. 3. A: basal pre-pro-ET-1 mRNA levels in VSMCs from WKY rats and SHRs. B: time course of ANG II (100 nM)-induced pre-pro-ET-1 mRNA levels in VSMCs from WKY rats and SHRs. C: effect of ANG II (100 nM, 2 h) on COX-2 protein expression (top) and mRNA levels (bottom) in the absence and presence of BQ-123 (1 μM) in VSMCs from SHRs. D: effect of ET-1 (100 nM, 1 h) on COX-2 protein expression (top) and mRNA levels (bottom) in the absence and presence of BQ-123 in VSMCs from SHRs. E: effect of Pio (10 μM, 18 h) on ANG II-induced pre-pro-ET-1 mRNA levels in VSMCs from SHRs. Results are expressed as relative values compared with WKY cells or untreated cells (0' or Ctrl). $n = 4–7$. * $P < 0.05$ vs. WKY or untreated cells. # $P < 0.05$ vs. ANG II by one-way ANOVA or a Student’s $t$-test.
matory enzymes and improvement of vascular inflammation (53). Thus, PPAR-\(\gamma\) agonists reduce the increased COX-2 expression found in different inflammatory models (13, 49), and we (32) have previously described that pioglitazone also reduces the increased COX-2 induced by IL-1\(\beta\) in SHR cells. In keeping with this, we found that pioglitazone treatment reduced the greater COX-2 expression observed in aortic segments from hypertensive rats. Surprisingly, increased COX-2 expression was observed in rat mesenteric resistance arteries after pioglitazone treatment (22); we do not have explanation for such contradictory results, but differences in the compensatory mechanisms occurring in resistance versus conductance arteries could explain them. Furthermore, PPAR-\(\gamma\) agonists inhibit the expression of several components of NOX and the subsequent ROS production, therefore contributing to the anti-inflammatory and vascular protective effects of these drugs (22, 28, 32, 34). Accordingly, pioglitazone also reduced the increased mRNA levels of NOX-1, NOX-4, and \(p47^{phox}\), NOX activity, and vascular \(O_2^-\) production. These effects of pioglitazone could contribute to explain the improvement of endothelium-dependent relaxation induced by glitazones in different pathologies (15, 22, 28, 35).

ET-1 is a peptide originally isolated from the supernatants of cultured endothelial cells that exerts a wide variety of biological effects in different tissues, including the vascular wall. Thus, endothelial ET-1 can act in a paracrine manner on adjacent smooth muscle cells affecting vascular reactivity and mitogenesis (5, 43). However, ET-1 has also an autocrine role in vasculature regulation, as it has been previously described to be released by VSMCs in response to growth factors and/or vasoactive hormones (21, 23, 29). Increased ET-1 levels have been previously described in several models of hypertension (20, 38). We found greater pre-pro-ET-1 mRNA levels in aortic segments and VSMCs from SHRs compared with WKY rats. It has been proposed that PPAR-\(\gamma\) agonists inhibit ET-1 expression, thus contributing to the improvement of cardiovascular alterations observed not only in hypertension but also in other pathological processes such as diabetes, atherosclerosis, or cardiac hypertrophy (34, 47, 55). In agreement, in vivo pioglitazone treatment of SHRs reduced pre-pro-ET-1 mRNA levels in aortic seg-
ments. Nevertheless, despite the above-mentioned effects of pioglitazone on COX-2, ET-1, and oxidative stress, no changes were observed in blood pressure in SHRs. Glitazones have proven blood pressure-lowering effects in patients or animal models of diabetes and/or metabolic syndrome; however, in those conditions in which hypertension is not associated with diabetes or with other factors of metabolic syndrome, no changes in blood pressure have been generally described unless long-term treatment (28, 58) or high doses of PPAR-α agonists (9, 17, 28) were used.

It is possible that, although in our model of well-established hypertension pioglitazone did reduce some proinflammatory markers, these mechanisms are before the blood pressure-lowering effect, which is likely to occur with long-term treatment.

Apart from its well-known physiological role, ANG II has been implicated in the pathophysiological processes that occur in hypertension through its significant proinflammatory actions in the vascular wall (45). Using different models of hypertension, our group and others have previously described increased renal and vascular COX-2 expression, vascular O$_2$•− production, and plasmatic malondialdehyde levels (4, 26, 33), which were normalized by treatment with AT$_1$ receptor antagonists (4, 26), confirming the involvement of ANG II in these increases. Moreover, we (33) have recently shown increased COX-2 expression and oxidative stress in the hypertension model of ANG II-infused mice. Furthermore, it has been previously described that ANG II induces the release of ET-1 (25, 40) and that treatment with angiotensin-converting enzyme inhibitors normalized the increased ET-1 plasma levels found in hypertensive patients (54). The fact that losartan treatment reduced the increased levels of ET-1 found in aortas from SHRs confirms that the increased RAS activity described in hypertension contributes to the elevated ET-1 levels in this pathology. The ability of PPAR-α agonists to antagonize the vascular damage promoted by ANG II (15, 48, 58) could contribute to their beneficial effects. In VSMCs, we found...
that ANG II induced the proinflammatory enzyme COX-2, in a greater extent in cells from SHRs compared with WKY rats, and pre-pro-ET-1 expression only in cells from SHRs. In agreement with the in vivo data, in cells from SHRs, pioglitazone reduced the ANG II-induced expression of both COX-2 and ET-1. These results could contribute to the above-mentioned anti-inflammatory effect of glitazones.

It has been proposed that ANG II-induced ET-1 production is important to explain the cardiovascular damage induced by this peptide. Thus, hypertension, proliferation, and renal effects observed after ANG II infusion were attenuated by ET-1 antagonists (7, 36, 40). Moreover, ANG II-induced cardiac hypertrophy and fibrosis were reduced in endothelial cell-specific ET-1-deficient mice (1). We found that ET-1, acting on ETA receptors, contributes to the ANG II-induced COX-2 expression in VSMCs from SHRs based on the following results: 1) ET-1, via ETA receptors, induced COX-2 expression and 2) blockade of ETA receptors reduced the ANG II-induced COX-2 expression; however, the fact that this blocker did not completely abolished such expression suggests that additional mechanisms contribute to this increase. Similarly, ET-1 increases COX-2 expression in mesenteric resistance artery VSMCs from stroke-prone SHRs but not in those from WKY rats (37). The fact that ET-1 levels were lower in WKY rats compared with SHRs and that ANG II did not increase ET-1 expression in cells from normotensive rats could contribute to explain the lower COX-2 levels found in aortic segments and VSMCs from SHRs as well as the lower increase in ANG II-induced COX-2 expression found in VSMCs from this strain.

Furthermore, a relationship between ROS and COX-derived products has been previously described. Thus, ROS can activate COX-2 expression and/or activity (4, 18, 27, 32). Additionally, ROS are also involved in ET-1 gene induction (10), although it has also been previously described that ET-1 can act as a mediator of the vascular inflammatory response by increasing ROS production (29). The specific NOX-1 inhibitor ML-171 (19) and the H2O2 scavenger catalase prevented the ANG II-induced COX-2 expression.

Fig. 6. A–D: effect of lactacystin (Lacta; 10 μM) on ANG II-induced COX-2 protein expression (A, top) and mRNA levels (A, bottom), pre-pro-ET-1 (B) and NOX-1 mRNA levels (C), and NOX activity (D) in VSMCs from SHRs. E, bottom: effect of Pio (10 μM, 18 h) on nuclear p65 NF-κB protein expression induced by ANG II (100 nM, 40 min) in VSMCs from SHRs; a representative blot of the cytosolic (Cy) and nuclear (Nu) expression is also shown (top). TATA-binding protein (TBP) cytosolic and nuclear expressions are also shown to guarantee the successful cellular fractioning. F: representative photomicrographs of p65 NF-κB immunofluorescence (green) in VSMCs from SHRs in Ctrl and after incubation with ANG II (100 nM, 40 min) in the absence and the presence of pioglitazone. The insets in F are magnified images of the indicated areas (arrows). Results are expressed as relative values compared with untreated (Ctrl) cells; n =4–8. *P < 0.05 vs. untreated cells. #P < 0.05 vs. ANG II by one-way ANOVA.
and ET-1 gene expression, supporting the involvement of ROS in such inductions. As observed in our in vivo study, in SHR VSMCs, pioglitazone reduced the increase in NOX-1 mRNA levels, NOX activity, and O$_2^-$ production induced by ANG II, confirming the antioxidant properties of glitazones.

Among the mechanisms proposed to explain the anti-inflammatory effects of PPAR-$\gamma$ activation, transrepression mechanisms by interfering with AP-1 and NF-κB signaling pathways have been proposed (42). AP-1 activation plays a relevant role in ET-1 induction (43, 51). In fact, mutational analysis of the ET-1 gene promoter showed that the AP-1-binding site is an important element in the ET-1 upregulation by different stimuli, such as ANG II (11). Furthermore, the ET-1 promoter also contains at least one functional NF-κB-binding site (43, 51). In support of this, overexpression of the NF-κB p65 subunit leads to an increase in ET-1 transcription levels, whereas overexpression of inhibitor of NF-κB (IκBα) decreases ET-1 promoter activity (39). On the other hand, activation of both AP-1 and NF-κB by ANG II has been previously described in different cell types (46, 56, 57). In agreement, ANG II increased p-JNK activation, c-Jun mRNA levels, and nuclear p-c-Jun expression in VSMCs from SHRs. Additionally, ANG II also increases nuclear expression of the NF-κB p65 subunit. The fact that treatment with the JNK inhibitor SP-600125 and the proteasome inhibitor lactacystin significantly decreased pre-pro-ET-1 mRNA levels suggests its dependence on both signaling pathways. In addition, the NOX-1 promoter contains AP-1-binding motifs (8, 30), and NF-κB is an essential regulator of NOX-1 (31). Accordingly, NOX-1 mRNA levels as well as NOX activity were reduced using SP-600125 and lactacystin. In support of the role of ROS and ET-1 on ANG II-elicited COX-2 expression, both AP-1 and NF-κB inhibitors also decreased ANG II-induced COX-2 at both mRNA and protein levels. The effect of pioglitazone on ROS and ET-1 levels seems to be mediated by interference with AP-1 activation, based on the inhibitory effect of the glitazone on JNK phosphorylation, c-Jun transcription, and p-c-Jun nuclear expression; in addition, pioglitazone also interfered with the NF-κB pathway, as suggested by the reduction of p65 nuclear expression observed. Consequently, and considering that ROS and ET-1 participate in the increased ANG II-induced COX-2 expression in SHR VSMCs, we can conclude that the effect of pioglitazone on ANG II-induced COX-2 expression is due, at least in part, to interference with the AP-1 and NF-κB pathways.

One potential limitation of this study arises from the fact that due to the difficulty of analyzing in vivo the mechanisms involved in pioglitazone effects, the experiments were performed in cultured VSMCs. VSMCs in culture can dedifferentiate, and the results obtained might be not predictive of the mechanisms operating in VSMCs in vivo. However, despite the possible differences between the in vivo and in vitro situations, the effects of pioglitazone on COX-2, oxidative stress, and ET-1 were qualitatively similar, suggesting that this model of VSMC cultures mimics the in vivo situation; therefore, the results obtained would be similarly valid.

In summary, our study provides evidence that ROS-dependent mechanisms increase ET-1 and that this contributes, among others, to the greater ANG II-induced COX-2 expression observed in conductance vessels from hypertensive animals. In addition, we propose a new mechanism that would contribute to the cardioprotective effects of PPAR-$\gamma$ agonists and go further in knowledge about the involvement and relevance of PPAR-$\gamma$ in the improvement of vascular inflammation associated with hypertension. Thus, glitazones inhibit ANG II-induced COX-2 expression in cells from SHRs associated with downregulation of ROS production and ET-1 levels by interfering with the NF-κB and AP-1 activation pathways, although additional mechanisms cannot be discarded (Fig. 7).
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


