Effect of AT₁ receptor antagonism on vascular and circulating inflammatory mediators in SHR: role of NF-κB/ΙκB system


Hypertension is a primary risk factor for atherosclerosis. The intrinsic mechanisms by which elevated arterial pressure levels lead to atherosclerosis are not totally established, although mechanical stress and subsequent endothelial dysfunction have been implicated (15, 25, 33). Endothelial dysfunction has been characterized mainly by impaired endothelium-dependent relaxation (27, 30, 35). Reduced availability of nitric oxide due to a diminution of its synthesis and/or enhanced degradation by superoxide anions has been implicated as a major cause of endothelial dysfunction in hypertension (5, 17, 30, 37). In addition, an increase in the production of cytokines and other mediators of inflammation has been associated with endothelial dysfunction (11, 20). Inflammation plays a key role in the development of atherosclerosis, which is considered a chronic inflammatory disease (3). However, the relationship between inflammation and hypertension is not well established because the effect of high blood pressure on inflammatory markers is not well determined. This is due to the variety of markers measured in different studies, the origin (local or circulating) of the markers, as well as the contradictory results reported in different studies (2, 16, 21, 23, 26, 32). In addition, the concomitant presence of other cardiovascular risk factors such as obesity, age, and diabetes could have increased inflammation and in turn been responsible for this variety of results (9, 21).

Angiotensin II, the main active component of the renin-angiotensin system, plays an important role in the functional and vascular alterations associated with hypertension. Furthermore, administration of either angiotensin-converting enzyme inhibitors or angiotensin II type 1 (AT₁) receptor antagonists is able to improve endothelial dysfunction and vascular remodeling in clinical and experimental hypertension (7, 14, 27, 28, 30). In addition, angiotensin II is considered a proinflammatory mediator that plays a pivotal role in the inflammatory process underlying development and complications of atherosclerosis (4, 24). This role involves activation of transcription factors such as NF-κB, which participates in the regulation of numerous inflammatory factors including cytokines, chemokines, and adhesion molecules (4, 19, 29). However, whether angiotensin II is involved in the inflammatory response associated with hypertension is not well established. Therefore, the aim of this study was to investigate 1) the role of angiotensin II in vascular and circulating inflammatory markers in spontaneously hypertensive rats (SHR) and 2) the possible involvement of the NF-κB/ΙκB system in the effect of angiotensin II on inflammatory mediators in SHR. To this end, we studied aortic mRNA expression and plasma levels of IL-1β, IL-6, and TNF-α in SHR untreated or treated with the AT₁ receptor antagonist candesartan. In addition, we evaluated the mRNA expression of the NF-κB p50 subunit precursor p105 and its inhibitor (ΙκB) in aortas from the same rats. To elucidate whether the changes produced by candesartan could be due to blood pressure reduction or AT₁ receptor blockade, we examined whether the changes produced by candesartan could be due to blood pressure reduction or AT₁ receptor blockade, we examined...
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Table 1. SAP levels and BW in WKY and SHR

<table>
<thead>
<tr>
<th>Group</th>
<th>SAP, mmHg</th>
<th>BW, g</th>
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<tbody>
<tr>
<td>WKY</td>
<td>126±2</td>
<td>403±6</td>
</tr>
<tr>
<td>SHR</td>
<td>199±5*</td>
<td>401±12</td>
</tr>
<tr>
<td>SHR + C</td>
<td>161±3*</td>
<td>407±8</td>
</tr>
<tr>
<td>SHR + TT</td>
<td>150±5*†</td>
<td>420±10</td>
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</table>

Values are means ± SE for 8 rats. SAP, systolic arterial pressure; BW, body weight; WKY, Wistar-Kyoto rats; SHR, spontaneous hypertensive rats; C, candesartan (2 mg·kg⁻¹·day⁻¹); TT, triple therapy (in mg·kg⁻¹·day⁻¹: 20 hydralazine + 7 hydrochlorothiazide + 0.15 reserpine). SHR were treated with vehicle (SHR), C, or TT for 10 wk. WKY served as controls. *P < 0.05 compared with WKY; †P < 0.05 compared with SHR treated with vehicle.

indicated the same parameters in SHR treated with the antihypertensive triple therapy (TT) of hydralazine + hydrochlorothiazide + reserpine. A group of Wistar-Kyoto rats (WKY) were used as normotensive reference.

METHODS

Studies were performed in male SHR (20–22 wk old; n = 24) from Harlam Interfauna Ibérica (Barcelona, Spain). Animals were fed a standard chow (A=04; Panlab, Barcelona, Spain) and had free access to drinking water. Animals were treated with vehicle, candesartan (2 mg·kg⁻¹·day⁻¹; AstraZeneca, Goeteborg, Sweden), or TT (in mg·kg⁻¹·day⁻¹: 20 hydralazine + 7 hydrochlorothiazide + 0.15 reserpine) given in the drinking water for 10 wk. The doses of candesartan and TT were chosen from previously published studies and adjusted to induce a comparable decrease in blood pressure (24, 36). WKY (n = 8) of the same age were used as a normotensive reference group. At the end of the treatment, systolic arterial pressure was measured as a tail-cuff plethysmograph (Narco Bio-Systems, Houston, TX) as previously described (28). On the day of the experiment, animals were killed by decapitation, and blood was collected in prechilled glass tubes containing EDTA. The aorta was isolated for molecular biology determinations. Isolation and manipulation of the aorta were always performed under sterile conditions. All experimental procedures were approved by the Animal Care and Use Committee of Universidad Complutense, according to the guidelines for ethical care of experimental animals of the European Union.

Plasma cytokine levels. Plasma IL-1β, IL-6, and TNF-α were measured with a quantitative sandwich enzyme immunoassay. A rat-specific monoclonal antibody for IL-1β, IL-6, or TNF-α was precoated onto microplates (R&D Systems, Minneapolis, MN). The minimum detectable dose was 5 pg/ml for IL-1β and TNF-α and 10 pg/ml for IL-6, with standard curve ranges of 3.9–2,000 pg/ml and 12.5–800 pg/ml for IL-1β and IL-6 and for TNF-α, respectively.

RNA isolation. Frozen rat aortas were pulverized in liquid nitrogen and homogenized together with 1 ml of Tri Reagent. RNA isolation was performed according to the Chomczynski method (10). RNA was quantified by optical density measurement at 260 nm with a BioPhotometer (Eppendorf). RNAs were frozen at −20°C until used.

Reverse transcription for cDNA synthesis. Five micrograms of total RNA were taken to perform reverse transcription. It was previously heated with 2 μM random hexamer at 70°C for 5 min and quickly chilled on ice. Subsequently, a mixture of 0.7 U RNase inhibitor, 25 mM Tris·HCl (pH 8.3), 37 mM KCl, 1.5 mM MgCl₂, 10 mM DTT, each dNTP at 0.4 mM, and 2.5 U of Moloney murine leukemia virus reverse transcriptase was added and incubated at 37°C for 60 min, followed by heating at 95°C for 10 min and chilling on ice. The mixture was then completed with DNase-free water for a final volume of 50 μl.

Multiplex polymerase chain reaction. Five microliters of the above-mentioned cDNA were taken for a multiplex polymerase chain reaction (MPCR) reaction (MPCR kit for Rat Inflammatory Genes Set-2; Maxim Biotech, San Francisco, CA). A mixture of MPCR buffer, Tag DNA polymerase (2.5 U), and specific MPCR primers for IL-6, IL-1β, TNF-α, NF-κB p50 subunit, IκB, and GAPDH was added. The following time-temperature profile was used to perform MPCR: 2 cycles of 1 min at 96°C and 2 min at 58–60°C; 27 cycles for amplification of IL-1β, p105, IκB, and GAPDH genes and 32 cycles for IL-6 and TNF-α of 1 min at 94°C and 2 min at 58–60°C; and 1 cycle of 10 min at 70°C and a final step of 25°C.

MPCR DNA product was fractioned electrophoretically on a 2% agarose gel containing 0.5 mg/ml ethidium bromide. The amplicon size of the genes was 532 bp for GAPDH, 453 bp for IL-6, 396 bp for p105, 351 bp for TNF-α, 294 bp for IL-1β, and 167 bp for IκB. Band intensity was measured with Gel Analysis Software (Syngene, Cambridge, UK). Data were normalized with GAPDH intensity data.

RESULTS

As shown in Table 1, SHR presented higher systolic arterial pressure levels than WKY (P < 0.05). Treatment with candesartan or TT markedly reduced systolic arterial pressure to levels that were not different between the treatments. All groups presented similar body weight at the end of the experiment (Table 1).

Plasma levels of IL-6 and IL-1β were higher (P < 0.05) in SHR compared with control animals (Fig. 1). Both candesartan and TT reduced plasma levels of IL-1β and IL-6 in SHR, this effect being more marked with candesartan than with TT. In fact, plasma levels of both cytokines were significantly lower in candesartan-treated rats compared with rats receiving TT. Neither hypertension nor treatments modified plasma levels of TNF-α.

mRNA expression of IL-6, IL-1β, and TNF-α in aorta homogenates was higher in SHR compared with WKY (Fig. 2).
Both candesartan treatment and TT reduced the increase in mRNA expression of the three cytokines in hypertensive animals (Fig. 2), this reduction being more marked in the case of candesartan. Indeed, mRNA expression of the three cytokines was significantly lower in candesartan-treated animals than in TT-treated animals.

Hypertension also increased the aortic mRNA expression of p105, the NF-κB p50 subunit precursor, because levels were higher in SHR than WKY (Fig. 3). Candesartan, but not TT, was able to reduce this (Fig. 3). In contrast, expression of the NF-κB inhibitor IκB was reduced in aortas from SHR compared with WKY. Candesartan increased IκB expression to the same extent as TT treatment.

DISCUSSION

The present data show that SHR presented an elevated aortic expression of cytokines (IL-6, IL-1β, and TNF-α) compared with WKY, suggesting an inflammatory process in the vascular wall associated with hypertension. This elevated vascular cytokine expression was accompanied by increased plasma levels of both IL-6 and IL-1β. The inflammatory process appears to be mediated by angiotensin II as well as an increase in hemodynamic forces associated with hypertension through the upregulation of NF-κB as well as a downregulation of its inhibitor, IκB.

The present study demonstrated that high arterial pressure is associated with an inflammatory process in the vascular wall because aorta from SHR showed an increase in the mRNA expression of IL-6, IL-1β, and TNF-α. Similarly, an increase in other markers of inflammation, including ICAM, VCAM, monocyte chemotactant protein (MCP-1), and IL-6 have been reported in vessels of hypertensive rats (16, 18, 21, 34).
This inflammatory process can play a key role in the progression of vascular damage associated with hypertension and could lead to the development of atherosclerosis, which is considered a chronic inflammatory disease (3). In addition, the elevated vascular expression of cytokines was accompanied by high circulating concentrations of IL-6 and IL-1β. Therefore, these data could suggest that hypertension is associated with a generalized inflammatory process because circulating levels may be generated from a variety of sources, including not only the vascular wall but also extravascular sources such as adipose tissue and blood cells (1, 6).

Mechanisms underlying the stimulation of both vascular and circulating inflammatory markers are not well established, although the participation of mechanical stress associated with hypertension can be suggested. This affirmation is based on the fact that reduction in either plasma concentrations or mRNA expression of inflammatory markers induced by both candesartan and TT was accompanied by a decrease in blood pressure. Therefore, these data support the notion that the stimulation of mechanoreceptors by elevated arterial pressure is involved in the inflammatory process observed in SHR. However, although the treatments produced a similar decrease in blood pressure, the effect on inflammatory markers was greater in candesartan-treated rats than in animals receiving an antihypertensive TT therapy that does not directly interact with AT1 receptors (36). Consequently, mechanical stress reduction seems not to be the only mechanism accounting for improvement in the inflammatory process induced by candesartan. The participation of angiotensin II through AT1 receptors in the inflammatory process associated with hypertension can, therefore, be proposed. Supporting this concept is the observation made by Tummala et al. (34) showing that the infusion of angiotensin II, but not of norepinephrine, for 6 days in rats made by Tummala et al. (34) showing that the infusion of angiotensin II, but not of norepinephrine, for 6 days in rats could lead to the development of atherosclerosis. Angiotensin II, in addition to hemodynamic changes induced by hypertension, could be involved in the stimulation of these inflammatory mediators through an upregulation of NF-κB as well as a downregulation of its inhibitor, IkB. The reduction in inflammatory mediators produced by AT1 receptor blockade seems to partially involve the prevention of these changes in the mRNA expression of the NF-κB/IkB system.

ACKNOWLEDGMENTS

We thank Blanca Martínez and Antonio Carmona for technical assistance.

REFERENCES
