Effect of AT$_1$ receptor blockade on hepatic redox status in SHR: possible relevance for endothelial function?

Eva Cediel, David Sanz-Rosa, M. Pilar Oubiña, Natalia de las Heras, Francisco R. González Pacheco, Onofre Vegazo, Javier Jiménez, Victoria Cacho, and Vicente Lahera


Submitted 18 October 2002; accepted in final form 20 May 2003

Hypertension is associated with functional and structural alterations of the arterial wall, which seem to be responsible for most of the vascular complications of this disease (13, 40, 46). Moreover, endothelial dysfunction has been proposed as the most important vascular alteration in hypertension, which leads to the development of arteriosclerosis (13, 34). Hypertensive endothelial dysfunction has been mainly characterized by reduced endothelium-dependent relaxations and enhanced endothelium-dependent contractions (24, 26, 46). Reduced availability of nitric oxide (NO) seems to be a major cause for reduced endothelium-dependent relaxations (13, 24, 34). Diminished endothelial NO synthase (eNOS) expression or activity has been proposed as an important mechanism involved in reduced NO availability and subsequent endothelial dysfunction associated with hypertension. However, no general agreement on this matter exists because opposite results have been reported (2, 23, 26, 28, 47). Enhanced vascular production of reactive oxygen species, specifically superoxide anions, has been reported as another important mechanism contributing to hypertensive endothelial dysfunction due to inactivation of NO (29, 31). Liver has been described as the most important organ involved in the regulation of redox metabolism, due to the key enzymes responsible for reactive oxygen species clearance and the production of the most important systemic antioxidant agent, glutathione (GSH) (15, 18, 49). While providing GSH for their specific needs, the liver parenchymal cells export GSH to the outside, where it serves as a systemic source of thiol-reducing capacity (19). Thus it seems that hepatic antioxidant defense contributes to systemic redox status (6, 43), and consequently it might affect NO availability and endothelial function. However, this aspect has not been investigated yet.

Several studies in hypertensive animals and patients showed that antihypertensive treatment was able to enhance endothelium-dependent relaxations and reduced arterial wall thickness (37, 38, 41, 42, 44). However, the simple reduction of elevated arterial pressure does not seem to be the only mechanism responsible for the beneficial effects of antihypertensive drugs. In fact, angiotensin-converting enzyme in-
hbitors and ANG II receptor antagonists have been demonstrated as the most efficacious antihypertensive drugs in ameliorating endothelial dysfunction and vascular remodeling (37, 38, 41, 42, 44). The mechanisms responsible for the beneficial effects exerted by the mentioned antihypertensive drugs on endothelial dysfunction have been extensively investigated, yielding a variety of results, including enhancement of NO availability and reduction of vascular superoxide anion production (5, 13, 27, 29, 37, 38, 41, 42, 44). Consequently, because liver plays a key role in systemic antioxidant defense, the objective of the present study was to investigate whether the amelioration of endothelial dysfunction produced by candesartan in spontaneously hypertensive rats (SHR) was associated with modification of vascular oxidative stress and the hepatic redox system. To this end, endothelium-dependent relaxation, eNOS mRNA expression, and the mRNA expression of the subunit p22phox of NAD(P)H oxidase were evaluated in aorta from Wistar-Kyoto rats (WKY) and SHR untreated and treated with candesartan. In addition, the ratio between reduced and oxidized glutathione (GSH/GSSG), glutathione peroxidase (GSHPx), and glutathione reductase (GSHRed) activities, as well as malonyldialdehyde (MDA) levels, were measured in liver homogenates from the same rats.

**MATERIALS AND METHODS**

The study was conducted in 22-wk-old SHR and WKY following recommendations from the institutional animal care and use committee, according to the guidelines for ethical care of experimental animals of the European Union. Half of the animals were treated with candesartan cilexetil (2 mg·kg⁻¹·day⁻¹) for 10 wk. The dose of candesartan was chosen from previously published studies (48). Systolic arterial pressure (SAP) was measured by a tail-cuff plethysmograph (Narco Bio-Systems, Houston, TX) at the end of the treatment period as previously described (5, 38, 46).

**Aortic Endothelial Function**

Endothelial function was studied in aortic rings at the end of the treatment period in all animals. The day of the experiment, thoracic aorta was isolated, gently cleaned from surrounding tissue, and placed in oxygenated (95% O₂-5% CO₂) Krebs bicarbonate solution of the following composition (mmol/l): 118.5 NaCl, 4.7 KCl, 2.8 CaCl₂, 1.1 KH₂PO₄, 25.0 NaHCO₃, and 11.1 glucose, at 4°C. The thoracic aorta was then cut transversely in ring segments (2 mm long). Each ring was placed inside a 5-ml heated bath filled with Krebs buffer (37°C) bubbled with (95% O₂-5% CO₂) and suspended between two L-shaped stainless steel hooks. The upper one was attached to a force transducer (FT03, Grass) and coupled to a computerized system (Mc Lab 8E, AD Instruments) for measurement of isometric tension. Rings were allowed to equilibrate for 60–90 min with changes of buffer every 15 min and with several adjustments of length until baseline tension stabilized at 2 g. In previous studies, we found that 2 g of resting tension are optimal for the measurement of endothelial function when tension was stable, the experiments were initiated by obtaining a reference contractile response to 80 mmol/KCl. Endothelial function was studied by evaluating relaxations to ACh (10⁻¹⁰ to 10⁻⁸ mol/l) and contractions to ACh in the presence of the NOS inhibitor l-NAME (ACh + l-NAME; 10⁻⁸ to 10⁻⁶ mol/l). Endothelium-independent relaxations induced by sodium nitroprusside (SNP; 10⁻⁶ to 10⁻⁴ mol/l) were also carried out. Functional blockade of AT₁ receptors was evaluated through the response to ANG II (10⁻⁶ mol/l) in endothelium-denuded rings. This contracting response was reduced by 92% in rings from candesartan-treated SHR when compared with rings from untreated rats.

**Aortic eNOS mRNA Expression**

**RNA isolation.** One-hundred milligrams of pulverized frozen rat aortas were homogenized together with 1 ml of TriReagent (Molecular Research Center, Cincinnati, OH). RNA isolation was performed according to the Chomczynski method (7). RNA was quantified by measurement of optical density at 260 nm with a BioPhotometer (Eppendorf). RNAs were frozen at −80°C until their usage.

**Probe synthesis.** cDNA for eNOS was obtained by RT-PCR of 100 ng of total RNA. Primers were designed using rat GenBank and Basic Local Alignment Search Tool, BLAST (NCBI) and then synthesized (TIB MOLBIOL). Sequences were 5'-GGCATCACCAGGAGAAGAC-3' (sense) and 5'-CGAACCAGAGAACCAG-3' (antisense). A fragment of 485 base pairs (bp) was cloned in pGEM-T easy Vector (Promega, Madison, WI). We used DH5α as competent bacterial strain. Transformation of DH5α by plasmid was achieved by 42°C heat shock. After the transformed colony was grown and selected, plasmid DNA was sequenced. Once checked, transformed bacteria were grown in LB liquid medium. Plasmid DNA was digested by EcoRI (Amersham Pharmacia Biotech UK) and fractionated by agarose gel electrophoresis. cDNA was extracted from the gel (QIAquick gel extraction, QIAGEN) and quantified at 260 nm with a BioPhotometer (Eppendorf).

**Northern blot analysis.** Ten micrograms of total tissue RNA were used to perform a 1% formaldehyde agarose gel electrophoresis. After 3 h at 50 V, repeated washes of H₂O-DEPC, NaOH 50 mM, and SSC 10x were done. RNAs were transferred to a Hybond Nylon membrane (Amersham Pharmacia Biotech UK). Membrane was UV cross linked (BioRad) and prehybridized with Ultra-Hyb (Ambion, Austin, TX). A 485-bp fragment of rat eNOS was radiolabeled with [³²P]dCTP (Nuclear IbcIberica) by random primed DNA labeling kit (Boehringer Ingelheim). Membrane was hybridized together with radioactive probe for 24 h, and low-stringency washes, 2× SSC, 0.1% SDS and 0.1× SSC, 0.1% SDS, were done. Membrane was exposed to Kodak X-OMAT films with intensifying screens at −70°C. Films were scanned, and the amount of the radioactivity in the individual bands was quantified by a densitometer PC IMAGE (Foster Findlay). Data were normalized with 28S rRNA.

**Aortic p22phox mRNA Expression**

**RT.** Five micrograms of total RNA were heated with 2 μM random hexamer at 70°C for 5 min and quickly chilled in ice. Subsequently, a mixture of RNase inhibitor (0.7 U), 25 mM Tris·HCl (pH 8.3), 37 mM KCl, 1.5 mM MgCl₂, 10 mM DTT, dNTPs (0.4 mM each) and 2.5 U of mouse Moloney murine leukemia virus (MMLV) RT was added and incubated at 37°C for 60 min followed by heating at 95°C for 10 min and chilling on ice. Then the mixture was completed with DNase-free water to a final volume of 50 μl.

**Multiplex PCR.** Five microliters of above cDNA were taken for a multiplex PCR (MPCR) reaction (Maxim Biotech, San Francisco, CA). A mixture of MPCR buffer, Taq DNA polymerase (2.5 U), and specific MPCR primers for p22phox and GAPDH was added. The following time-temperature profile

---

AJP-Regul Integr Comp Physiol • VOL 285 • SEPTEMBER 2003 • www.ajpregu.org
was used to perform MPCR: two cycles of 96°C, 1 min and 58–60°C, 2 min; 28 cycles for amplification of p22phox and GAPDH genes of 94°C, 1 min and 58–60°C, 2 min; 1 cycle of 70°C, 10 min; and a final step of 25°C.

Sequences of the primers for p22phox were 5'-GCT-CATCTGTTCTCAGGAGTA-3' (sense) and 5'-ACGACCCTCATCTGTCTCAGGTA-3' (antisense); for GAPDH were 5'-TTGTATGACCATCAAGAAGGTTG-3' (sense) and 5'-CACCCTGTGGCTGTA-3' (antisense). Both were designed using the GenBank and Basic Local Alignment Search Tool (BLAST). Nucleotide sequences of GAPDH genes of 94°C, 1 min and 58–60°C, 2 min; 1 cycle of 70°C, 10 min; and a final step of 25°C.

Livers were isolated, cut in samples, immediately frozen in liquid nitrogen, and stored at −80°C until processing. Liver samples for GRed and GSH/GSSG determination were homogenized in potassium phosphate buffer, 50 mM, pH 7.5, containing 1 mM EDTA and 1 mg/ml BSA (1 g liver/2 ml buffer). Samples for GPx and MDA determination were homogenized in Tris-HCl buffer, 50 mM, pH 7.2, containing 5 mM EDTA and 1 mM 2-mercaptoethanol (1 g liver/6 ml buffer). After centrifugation (20 min, 3,000 rpm), supernatant was separated and redox parameters were measured. All procedures were run at 4°C. GSH/GSSG ratio, GPx, GRed, and MDA levels were measured by spectrophotometric (Hitachi 912 autoanalyzer) assays using commercial kits (Bioxytech GSH/GSSG-412, Bioxytech GPx-340, Bioxytech GR-340, Bioxytech LPO-586, Oxis Research). GRed assay is based on the oxidation of NADPH to NADP⁺ catalyzed by a limiting concentration of GRed. One GRed unit is defined as the amount of enzyme catalyzing the reduction of 1 micromole of GSSG per minute at ph 7.6 and 25°C. One molecule of NADPH is consumed for each molecule of GSSG reduced. Therefore, the reduction of GSSG is determined indirectly by measurement of the consumption of NADPH, as demonstrated by a decrease in absorbance at 340 nm as a function of time.

GPx assay is an indirect measure of the cellular GPx activity. GSSG produced on reduction of an organic peroxide by GSHPx is recycled to its reduced state by GSHRed. The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm, providing a spectrophotometric means for monitoring GPx activity. Measurement of GSSG in tissues requires the prevention of oxidation of GSH during sample preparation. The GSH/GSSG assay uses the thiol-scavenging reagent 1-methyl-2-vinylpyridinium trifluoromethanesulfonate at a level that rapidly scavenges GSH but does not interfere with GRed assay. The MDA assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenoxyindole, with MDA at 45°C. One molecule of MDA reacts with two molecules of the reagent to yield a stable chromophore with maximal absorbance at 586 nm. Results are expressed per milligram of protein (biuret method, Hitachi 912 autoanalyzer).

Aortic Morphometry

Aortic segments were fixed in 10% sodium phosphate-buffered formaldehyde, processed, and cut in sections (4 μm). To determine vessel or luminal area, the cross-sectional area enclosed by the external or internal elastic lamina, respectively, was corrected to a circle applying the form factor \( L^2/4\pi \) to the measurement of the lamina, where \( L \) is the length of the lamina. This method was used to avoid miscalculation of vessel and luminal areas, because aortic segments could be deformed during preparation. Media area was obtained by subtracting lumen area from the area encompassed by external elastic lamina. Measurements were made by tracing in digitized segmented-colored sections stained with hematoxylin-eosin using a QWIN Leica image analyzer (Leica Imaging Systems, Cambridge, UK) as previously described.

Hepatic Redox Parameters

Aortic eNOS mRNA Expression

Aortic eNOS mRNA expression was comparable in WKY and SHR. Treatment with candesartan increased (\( P < 0.05 \)) eNOS expression in both strains, although this effect was more marked in SHR.
Aortic p22phox mRNA Expression

Aortic mRNA expression of the subunit p22phox of NAD(P)H oxidase was higher ($P < 0.05$) in SHR than in WKY. Treatment with candesartan reduced ($P < 0.05$) p22phox expression only in SHR (Fig. 3).

Hepatic Redox Parameters

SHR presented lower ($P < 0.05$) GPx and higher ($P < 0.05$) GRed hepatic activity than WKY (Fig. 4). Furthermore, SHR presented lower ($P < 0.05$) hepatic GSH/GSSG ratio and elevated MDA levels when compared with WKY. Treatment with candesartan increased ($P < 0.05$) GSH/GSSG ratio, reduced ($P < 0.05$) MDA levels, but did not affect either GPx or GRed levels (Fig. 4). A positive correlation ($r = 0.564; P < 0.05$) between maximal relaxation induced by ACH and GSH/GSSG values was observed.

DISCUSSION

The present study shows that treatment with the AT$_1$ receptor antagonist candesartan was able to improve hepatic redox status in SHR, which was associated with a reduction of aortic p22phox mRNA expression and an enhancement of aortic eNOS mRNA expression. These effects could account for the observed amelioration of endothelial dysfunction and reduction of medial hypertrophy produced by candesartan in SHR.

As previously described, the present study showed diminished endothelium-dependent relaxations in re-
response to ACh in SHR, which suggest a reduced NO availability (13, 26, 34, 46). Two main causes could contribute to diminish NO availability: reduction of NO production due to diminished eNOS gene expression, protein expression, or activity, and enhanced inactivation of NO by superoxide anions (26, 31). A variety of results, including enhancement, reduction, or no modification of eNOS expression or activity, has been reported in hypertensive rats (2, 23, 26, 28, 47). The present results show that eNOS mRNA expression was comparable in WKY and SHR, indicating that, under the present experimental conditions, alterations of eNOS mRNA expression would not contribute in an important manner to the observed reduced endothelial dysfunction.

An enhanced aortic production of superoxide anions in SHR could be proposed because p22phox mRNA expression was higher in SHR than in WKY. This could contribute in an important manner to the observed endothelial dysfunction in SHR. This concept is supported by previous reports showing that enhancement of NAD(P)H oxidase as well as increased vascular production of superoxide anions importantly contributes to inactivation of NO and endothelial dysfunction in hypertension (30). In addition, the present study also showed diminished hepatic GPx activity in SHR, indicating that hypertension is associated not only with enhanced vascular superoxide anion production but with impaired hepatic redox defense, which could be reflected in increased systemic oxidant stress (6, 43). In fact, reduced hepatic GPx could be directly responsible for an enhanced availability of reactive oxygen species because this enzymatic activity catalyzes transformation of \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) (15, 49). It has been recently reported that deficiency of hepatic GPx exacerbates endothelial dysfunction in hyperhomocysteinemic mice and provides support for the contribution of a peroxide-dependent oxidative mechanism in endothelial dysfunction (8).

The results also show lower GSH/GSSG ratio in SHR than in WKY, which indicates diminished availability of GSH. This could be considered the most important systemic antioxidant agent, which is mainly produced by the liver and is systemically exported, serving as the main endogenous antioxidant agent (15, 18, 19, 49). Thus diminished hepatic GSH content in SHR might contribute to systemic and local oxidative stress and consequently to reduced NO availability and endothelial dysfunction. Furthermore, increased prevalence of reactive oxygen species is also supported by the increased MDA hepatic levels, which reflects an enhancement of enzymatic lipoperoxidation. In contrast, the meaning of elevated GRed levels observed in SHR is not clear, although it might be interpreted as an attempt to compensate excess of GSSG, which is the substrate for GRed activity. Thus, as suggested in different pathological experimental situations, it could be hypothesized that alterations of hepatic redox system and subsequent diminution of GSH could contribute to increase systemic oxidant stress, which also may affect endothelial NO availability.

### Table 1. Vessel, lumen, and media aortic areas in WKY and SHR untreated and treated with candesartan

<table>
<thead>
<tr>
<th></th>
<th>Vessel Area, mm²</th>
<th>Lumen Area, mm²</th>
<th>Media Area, mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>1.96 ± 0.12</td>
<td>1.46 ± 0.09</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>WKY + C</td>
<td>1.98 ± 0.08</td>
<td>1.50 ± 0.11</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>SHR</td>
<td>2.83 ± 0.25*</td>
<td>1.95 ± 0.10*</td>
<td>0.89 ± 0.08*</td>
</tr>
<tr>
<td>SHR + C</td>
<td>2.55 ± 0.13*</td>
<td>1.95 ± 0.19*</td>
<td>0.61 ± 0.03†</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. Wistar-Kyoto rats (WKY). †P < 0.05 vs. spontaneously hypertensive rats (SHR). C, candesartan.
availability associated with hypertension (10, 11, 19, 35, 45).

As in previous reports, the present study shows that treatment with the AT$_1$ receptor antagonist candesartan was able to enhance ACh-induced relaxation in SHR (25, 38). Several studies, using a variety of experimental approaches, suggested an increased availability of NO produced by AT$_1$ receptor antagonists in hypertensive rats as a key mechanism leading to the improvement of endothelial dysfunction (4, 12, 14, 25, 37, 38, 42). Diminution of vascular production of superoxide anions during AT$_1$ receptor blockade has been proposed to account in an important manner for the amelioration of endothelial dysfunction in hypertension (4, 51). The present results also support this concept because treatment with candesartan reduced the elevated p22phox mRNA expression in SHR. Because NAD(P)/H oxidase is considered the major source of superoxide anions in the vasculature (30, 50), it could be considered that candesartan would reduce aortic superoxide anion availability through the diminution of p22phox expression, thus contributing to increase NO availability.

In addition, the present results showed that treatment with candesartan increased hepatic GSH/GSSG ratio, which positively correlated with maximal relaxation of aortic rings to ACh. This result, together with the reduction of liver MDA levels, indicates an enhancement of hepatic antioxidant defense and reduction of systemic oxidative process. Thus increased hepatic GSH produced by candesartan could contribute to control systemic and local oxidative stress and consequently to enhance NO availability and to ameliorate endothelial dysfunction in SHR. Supporting the effects of systemic GSH on endothelial function are the results by Kugiyama et al. (22) showing that infusion of GSH improved vasomotor response to ACh in human coronary circulation. In addition, it should be mentioned that the results support the pathophysiological role of ANG II in the oxidative alterations associated with hypertension, not only at the vascular level but also on hepatic antioxidant defense. Thus it could be hypothesized that the effects of candesartan on oxidative stress are due to two mechanisms operating in the same direction: 1) the reduction of aortic p22phox and consequently aortic superoxide anion production, and 2) an effect on hepatic antioxidant defense, as demonstrated by the decrease of GSH/GSSG ratio.

In the present study, candesartan treatment enhanced eNOS mRNA expression in both strains although this effect was more marked in SHR. Previous studies showed that candesartan was able to stimulate eNOS mRNA and protein expression in the left ventricle from normotensive, ANG II-induced hypertensive rats, Dahl salt-sensitive and Goldblatt hypertensive rats, and stroke-prone SHR (3, 4, 17, 20, 21, 32). It should be considered that the similar effect of candesartan on eNOS mRNA expression in SHR and WKY, although it does not invalidate the physiological importance of eNOS, does suggest that the enhancement of eNOS mRNA expression would contribute in a secondary manner to increase NO availability and consequently to enhance ACh relaxations in SHR.

Another mechanism theoretically involved in the amelioration of endothelial function produced by candesartan could rely on the observed reduction of endothelium-dependent contraction. This notion is supported by previous studies showing that treatment with AT$_1$ receptor antagonists reduced the elevated endothelium-dependent contractions in aortic and coronary segments from SHR (24, 46). However, a secondary importance of the reduction of endothelium-depen-
dent contractions for the amelioration of endothelial function produced by candesartan could be proposed, because the treatment reduced Ach + L-NAME contractions in both strains.

Finally, candesartan treatment not only ameliorated endothelial dysfunction but also reduced medial hypertension as previously reported with ANG II receptor antagonists in different vascular territories (3, 42). Furthermore, smooth muscle cell function seemed to be also improved as indicated by the enhancement of relaxations to SNP. An explanation for this finding is the observed reduction of medial hypertrophy in these animals, which could improve contractile machinery of smooth muscle cells (33, 39). Hemodynamic stress produced in the arterial wall by elevated arterial pressure levels, as well as vasoactive agents such as ANG II and reactive oxygen species together with the reduced NO availability, have been proposed as important factors involved in vascular hypertrophy and remodeling (1, 16, 33, 39). Therefore, arterial pressure reduction, increased eNOS mRNA expression, and possibly stimulation of antioxidant defense produced by candesartan could have contributed to media area reduction and amelioration of smooth muscle contractile capacity.

In summary, the results also suggest that hypertension is not only associated with elevation of vascular oxidative stress but with alterations of hepatic redox system, where ANG II is clearly involved. Considering the importance of the liver in maintaining systemic redox balance and the importance of hepatic GSH, it could be hypothesized that amelioration of hepatic antioxidant status, together with the reduction of vascular p22phox expression produced by candesartan, could contribute to improve endothelial function in SHR. The results further support the key role of ANG II via AT1 receptors for the functional and structural vascular alterations produced by hypertension.

We thank Dr. G. Zalba for kindly providing p22phox primers for RT-PCR. We also thank Dr. C. Caramelo, B. Martínez, and A. Carmona for technical assistance.

DISCLOSURES

This work was supported by grants from Comisión Interministerial de Ciencia y Tecnología (SAF 2001–1864) and from Fondo de Investigaciones Sanitarias (FIS 01/0088–02).

REFERENCES


