Losartan but Not Verapamil Inhibits Angiotensin II–Induced Tissue Endothelin-1 Increase
Role of Blood Pressure and Endothelial Function

Livius V. d’Uscio, Sidney Shaw, Matthias Barton, Thomas F. Lüscher

Abstract—Endothelin partially mediates angiotensin (Ang) II–induced vascular changes in vivo. This study investigated the effects of the angiotensin type 1 receptor antagonist losartan and the calcium channel blocker verapamil on vascular reactivity and tissue endothelin-1 levels in aortas of Wistar-Kyoto rats treated for 2 weeks with Ang II (200 ng · kg⁻¹ · min⁻¹). Ang II increased systolic blood pressure (39±4 mm Hg, P<0.05). Concomitant treatment with losartan abolished the Ang II–induced pressure increase (P<0.05), whereas verapamil reduced it only partially (P<0.05). In the aortas of rats with Ang II–induced hypertension, tissue endothelin-1 content was increased threefold and contractions to endothelin-1 were impaired (P<0.05). Interestingly, these alterations were normalized by losartan (P<0.05) but not by verapamil. Hence, there was a strong, negative correlation between contractions to endothelin-1 and tissue endothelin-1 content (r = -0.733, P<0.0001). In contrast, both antihypertensive drugs normalized impaired endothelium-dependent relaxations to acetylcholine and reduced the sensitivity of vascular smooth muscle to sodium nitroprusside compared with Ang II–treated rats (P<0.05). Ang II–induced hypertension enhanced endothelium-dependent contractions to acetylcholine, and these were normalized by either drug. In conclusion, these findings suggest that long-term treatment with Ang II modulates endothelin-1 protein expression in the rat aorta. Although both antihypertensive agents lowered blood pressure and normalized endothelial function, only losartan prevented the increase in tissue endothelin-1 content, suggesting that angiotensin type 1 receptor antagonists but not calcium antagonists modulate tissue endothelin-1 in vivo. (Hypertension. 1998;31:1305-1310.)

Key Words: angiotensin II • endothelin • endothelium • losartan • verapamil • aorta

Angiotensin II is an important mediator contributing to cardiovascular diseases such as hypertension, congestive heart failure, and renal failure.1–3 Ang II has multiple effects as a local modulator of vascular tone in an autocrine and paracrine manner via specific angiotensin receptors.2 Ang II–induced proliferation of rat VSMCs is mediated via the AT₁ receptor.4 Ang II also stimulates the release of relaxant factors such as NO5–7 and constricting factors such as prostanoids8 and ET-1 in endothelial cells.9–11 ET-1 is a potent vasoconstrictor peptide1,9 and acts as a mitogen and trophic factor in cultured VSMCs.12,13 The Ang II–induced expression of pre-pro-ET mRNA and subsequent ET-1 synthesis occur not only in cultured vascular endothelial cells9–11,14 but also in nonendothelial cells such as rat VSMCs through its interaction with the AT₁ but not the AT₂ receptor.15,16 Long-term treatment with Ang II increases tissue ET-1 content and induces vascular hypertrophy of small arteries, effects that are totally prevented by ETA receptor blockade,17 suggesting that this interaction between the vascular renin-angiotensin system and ET-1 is also operative in vivo. Furthermore, in mesenteric arteries of spontaneously hypertensive rats, Ang II induces vascular production of ET-1 and thereby augments the contractile responses to NE in an endothelium-dependent manner.18 Although calcium channel blockers lower arterial pressure and improve endothelium-dependent relaxations in experimental models of hypertension,19,20 the long-term effects of calcium channel blockade in Ang II–induced hypertension and vascular ET production in this model are not known.

The present study was designed to investigate the effects of long-term treatment with the AT₁ receptor antagonist losartan and the calcium channel blocker verapamil in Ang II–induced hypertension, with special emphasis on the ET pathway and its effects on endothelium-dependent and -independent vascular reactivity of isolated rat aortas.

Methods

Experimental Animals
Male normotensive Wistar-Kyoto rats 9 weeks old were obtained from IFFA Credo (L’Arbresle, France) and maintained on standard rat chow with free access to drinking water at the animal facilities of the University Hospital Bern. All experimental protocols for animal research were approved by the local authorities of Bern, Switzerland.
At the age of 10 weeks, rats were randomly assigned to 1 of the following 4 groups: (1) a control group (fed standard chow and water), (2) an Ang II group, (3) an Ang II plus losartan group, and (4) an Ang II plus verapamil group. Ang II was administered by subcutaneously implanted osmotic pumps (model 2002, Alzet Corp) delivering 200 ng·kg⁻¹·min⁻¹ for 14 days. The dosages of losartan and verapamil were based on previous studies, and the drugs were administered with the powdered chow at an average dose of 14.2 ± 63.1 ± mg·kg⁻¹·d⁻¹, respectively. Body weights of the rats were monitored before and at the end of treatment. SBP and heart rate were measured in conscious rats by a tail-cuff method with the use of a pulse transducer (model LE 5000, Letica) before treatment and at the time when the rats were used for the following experiments at the age of 12 weeks. Then the rats were anesthetized (thiopental, 50 mg/kg body weight IP) and decapitated. The aortas were isolated and placed immediately into cold (4°C) modified Krebs-Ringer bicarbonate solution (in mmol/L): NaCl 118.6, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.1, EDTA 0.026, and glucose 10.1.

**Experimental Setup**

In Krebs solution each thoracic aorta was dissected free from connective tissue while being viewed under a microscope (Leica Wild M3C) and cut into rings 4 mm long. Rings were mounted horizontally between fixed and movable stainless stirrups, connected to a force transducer for recording isometric tension (Statham Universal UTC2), and then placed in an organ bath filled with 25 mL Krebs solution. The solutions were maintained at 37°C and aerated continuously with 95% O₂ /5% CO₂ gas. After an equilibration period of 30 minutes, the rings were progressively stretched to their optimal passive tension (2.5 ± 0.2 g) as assessed by the response to 100 mmol/L KCl in modified Krebs solution, which was prepared by equimolar replacement of NaCl with KCl in Krebs-Ringer bicarbonate solution. Contractions to KCl did not differ between the rats in the control group (3.75 ± 0.06 mN/mm) and those treated with Ang II (3.81 ± 0.09 mN/mm), Ang II plus losartan (3.94 ± 0.08 mN/mm), or Ang II plus verapamil (3.81 ± 0.07 mN/mm). The stretched ring segments were equilibrated for 30 minutes before the experiment.

**Protocols**

For investigation of endothelium-dependent relaxations, aortic rings from the four experimental groups were incubated with or without SQ30741 (a prostaglandin H₂/thromboxane A₂ receptor antagonist) at 10⁻² mol/L for 30 minutes or SOD (a superoxide anion scavenger) at 150 U/mL for 5 minutes. Rings were precontracted with NE (1 to 3x10⁻² mol/L) and then relaxed with ACH (10⁻³ to 10⁻⁴ mol/L). For endothelium-independent relaxation, rings were precontracted with NE (1 to 3x10⁻² mol/L) and relaxed with 10⁻¹⁰ to 10⁻⁹ mol/L SNP. Precontractions to NE were comparable in all treatment groups.

Endothelium-dependent contractions were tested in quiescent rings incubated with the NO synthase inhibitor L-NAME at 10⁻⁴ mol/L, for 30 minutes alone or with SQ30741 (10⁻² mol/L). Cumulative concentrations of ACH (10⁻⁹ to 10⁻⁴ mol/L) were then added to the organ baths. Contractions to ET-1 (10⁻¹⁰ to 10⁻⁷ mol/L) were studied in aortic rings pretreated with or without SQ30741 at 10⁻⁷ mol/L. Contractile responsiveness of vascular smooth muscle to NE (10⁻¹⁰ to 10⁻³ mol/L) and U46619 (10⁻¹⁰ to 10⁻⁶ mol/L) was also obtained.

**Measurement of Tissue ET-1 Levels**

Dissected aortic rings were frozen in LN₂ and kept at −80.25°C until assayed. In addition, rat aortas from control and Ang II groups were denuded of endothelium by scraping with a scalpel blade. Measurements were performed in a blinded fashion (n=6 per group). Frozen vessels were crushed, weighed, and homogenized as described elsewhere. Eluates were dried in a vacuum centrifugal evaporator and reconstituted in working assay buffer for radioimmunoassay. The overall recovery of ET-1 added to chloroform/methanol vessel homogenates and taken through all extraction steps was 65% ± 3%, with interassay and intraassay coefficients of 5.6% and 10%, respectively (n=6).

**Drugs**

The following drugs were used for the protocols: ACH HCl, L-NE bitartrate, U46619 (9,11-dideoxy-11a,9α-epoxymethanoprostaglandin F₂). SNP dihydroxyl, CuZn SOD (from bovine erythrocytes; 4400 U/mg protein), L-NAME, Ang II (all from Sigma Chemical Co), and SQ30741 (Squibb Institute for Medical Research, Princeton, NJ). ET-1 was purchased from Novabiochem/Calbiochem AG. Losartan and verapamil were provided by Merck Sharp & Dohme-Chibret AG and Knoll AG, respectively. All drugs were dissolved in distilled water except ET-1 and SQ30741, which were prepared in 0.1% BSA solution and in 10% ethanol, respectively. Then all drugs were diluted in Krebs solution and expressed as final molar concentrations in the organ bath.

**Statistical Analysis**

The contractions were expressed as a percentage of 100 mmol/L KCl–induced contractions, which were obtained at the beginning of each experiment. Results are presented as mean ± SEM. In all experiments, n equals the number of rats per experiment. For statistical analysis, the sensitivity of the vessels to the drugs was expressed as the negative logarithm of the concentration that caused half-maximal relaxation or contraction. Maximal relaxation (expressed as a percentage of precontraction) or contraction was determined for each individual concentration-response curve by nonlinear regression analysis with the use of MatLab software. For simple comparison between two values, paired Student’s t test was used. For multiple comparisons, results were analyzed by ANOVA followed by Bonferroni’s correction. Pearson’s correlation coefficients were calculated by linear regression. A value of P<0.05 was considered significant.

**Results**

**Characteristics of Animals**

The Ang II–induced increase in SBP was abolished by concomitant oral treatment with losartan but was prevented only partially by verapamil (P<0.05 versus Ang II group; Figure 1A and Table 1). Heart rate was lowered only in rats that received verapamil (P<0.05 versus Ang II group; Table 1). Body weights are also shown in Table 1.

**Tissue ET-1 Levels**

The ET-1 tissue content in the aortas with an intact endothelium was increased threefold by Ang II treatment (P<0.05 versus control; Figure 1B). In aortas without an endothelium, basal ET-1 levels were 65±7% of those with endothelium in the control group and 64±5% of those in the Ang II group (P=NS). Losartan but not verapamil completely prevented the Ang II–induced increase in peptide content (P<0.05 versus Ang II group; Figure 1B).
Reduced vascular reactivity to ET-1 was almost normalized by long-term administration of losartan only (P<0.05 versus Ang II group for the maximal response). Maximal contractions to ET-1 and local tissue ET-1 concentrations were inversely correlated (r = 0.733, P<0.0001, n = 24; Figure 2B). Contractions to ET-1 were unaffected by SQ30741 in all groups (data not shown; n = 6 or 7 per group). In contrast to contractions to ET-1, long-term treatment with either losartan or verapamil normalized the impaired contractions to NE observed in the Ang II–treated rats (P<0.05 for the maximal response; Table 2).

Long-term treatment with Ang II increased endothelium-dependent contractions to ACH (P<0.05; Table 2) in quiescent aortic rings with an intact endothelium in the presence of L-NAME. Concomitant treatment with either losartan or verapamil normalized endothelium-dependent contractions (P<0.05; Table 2). SBP was highly correlated with endothelium-dependent contractions to ACH (r = 0.804, P<0.0001, Figure 1. Net increase in SBP (A) and tissue ET-1 levels (B) in rat aortas after 2 weeks of treatment with different regimens. Results are mean±SEM. *P<0.05 vs control; †P<0.05 vs Ang II group (ANOVA plus Bonferroni's correction).

**TABLE 1.** Characteristics of the Rats Measured After 2 Weeks of Treatment With Different Regimens

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Ang II</th>
<th>Ang II+Los</th>
<th>Ang II+Ver</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>137±2</td>
<td>175±5*</td>
<td>140±1†</td>
<td>153±4†</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>322±9</td>
<td>366±22</td>
<td>327±8</td>
<td>268±8†</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>319±10</td>
<td>282±10*</td>
<td>311±9</td>
<td>303±8</td>
</tr>
</tbody>
</table>

Los indicates losartan; AT, receptor antagonist; Ver, verapamil, L-type calcium channel blocker. Figure 1A presents the net increase in SBP during the 2-week treatment. Data are mean±SEM. *P<0.05 vs control; †P<0.05 vs Ang II–treated rats (ANOVA plus Bonferroni’s correction).

**Figure 2.** A, Contractions to ET-1 in rat aortic rings after 2 weeks of treatment with different regimens. Contractions are expressed as percentage of 100 mmol/L KCl, and results are mean±SEM. *P<0.05 vs control; †P<0.05 vs Ang II group (ANOVA plus Bonferroni). B, Linear inverse relationship between maximal contractions to ET-1 and tissue concentrations of ET-1 in rat aortas.

**TABLE 2.** Maximal and Half-Maximal Contraction and Sensitivity Values of Different Vasoactive Agents in Aortas of Wistar-Kyoto Rats After 2 Weeks of Treatment With Different Regimens

<table>
<thead>
<tr>
<th>Vasoactive Agent</th>
<th>Control</th>
<th>Ang II</th>
<th>Ang II+Los</th>
<th>Ang II+Ver</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACH</td>
<td>Max</td>
<td>5.6±0.9</td>
<td>12.4±1.4*</td>
<td>6.5±0.5†</td>
</tr>
<tr>
<td></td>
<td>1/2Max</td>
<td>5.6±0.1</td>
<td>5.2±0.1*</td>
<td>5.6±0.1†</td>
</tr>
<tr>
<td>U46619</td>
<td>Max</td>
<td>137±4</td>
<td>139±4</td>
<td>131±4</td>
</tr>
<tr>
<td></td>
<td>1/2Max</td>
<td>7.3±0.1</td>
<td>7.4±0.1</td>
<td>7.3±0.1</td>
</tr>
<tr>
<td>NE</td>
<td>Max</td>
<td>115±4</td>
<td>92±7*</td>
<td>114±6†</td>
</tr>
<tr>
<td></td>
<td>1/2Max</td>
<td>7.7±0.1</td>
<td>7.5±0.1</td>
<td>7.7±0.1</td>
</tr>
</tbody>
</table>

Los indicates losartan; Ver, verapamil. Maximal (Max) responses to the agonist are percent reaction to 100 mmol/L KCl, 1/2Max values are the log of the molar concentration of agonist causing half-maximal contraction. Data are mean±SEM (n=6 to 8). *P<0.05 vs control; †P<0.05 vs Ang II–treated rats (ANOVA plus Bonferroni’s correction).
hypertension are completely normalized by the AT1 receptor alterations of the vascular ET pathway in Ang II–induced hypertensive animals, endothelium-dependent relaxations to ACH in the aorta were markedly impaired compared with control (Figure 3A; \( P<0.05 \) vs control; \( P<0.05 \) vs Ang II group; Figure 3B).

**Vascular Relaxations**

In Ang II–induced hypertensive animals, endothelium-dependent relaxations to ACH in the aorta were markedly impaired compared with control (Figure 3A; \( P<0.05 \)). Both losartan and verapamil improved endothelium-dependent relaxations to a similar degree (Figure 3A; \( P<0.05 \)), which were unaffected by SQ30741 or SOD (data not shown). The sensitivity of the concentration-response curves to ACH did not differ among the groups.

Maximal endothelium-independent relaxations to the NO donor SNP were comparable in all groups, but the sensitivity was shifted to the right in the Ang II group (\( P<0.05 \) versus control group). Both losartan and verapamil normalized endothelium-independent relaxations (\( P<0.05 \) versus Ang II group; Figure 3B).

**Discussion**

In this study, we have demonstrated for the first time that alterations of the vascular ET pathway in Ang II–induced hypertension are completely normalized by the AT1 receptor antagonist losartan. In contrast, the calcium channel blocker verapamil did not affect Ang II–induced increases in tissue ET-1 content or contractions to this peptide in the aorta, despite verapamil’s beneficial effects on SBP, endothelial dysfunction, and impaired reactivity to other vasoconstrictors.

The renin-angiotensin system plays an important role as a modulator of vascular structure and function in arterial hypertension.\(^2\) However, it is still unclear whether and to what extent endothelial dysfunction contributes to vascular changes and whether these changes in the vascular ET system are due to changes in endothelial function or blood pressure. In this study, the calcium channel blocker verapamil only partially prevented the increase in SBP induced by long-term administration of Ang II at a dose that was comparable to those that have been shown to prevent elevation of arterial pressure in different animal models of hypertension.\(^19,27,28\)

In line with previous observations, losartan abolished the Ang II–induced increase in SBP.\(^33\) Whereas losartan prevented the Ang II–induced increases in local aortic ET-1 concentrations, verapamil had no effect on tissue levels of this peptide. Ang II has been previously reported to stimulate expression of pre-pro-ET mRNA\(^9,11,14\) and to increase the release of the mature peptide in cultured endothelial cells.\(^10\) VSMCs\(^15,16\) and mesangial cells\(^17\) in vitro. In this study, however, vascular ET-1 was found to be mainly located in nonendothelial cells, as shown by the levels measured in intact aortas without an endothelium in both control rats and those receiving Ang II.

Possible explanations are that (1) ET-1 is produced in endothelial cells\(^8\) and then most of the peptide is released abluminally toward the underlying SMCs\(^38\) or that (2) ET-1 is synthesized locally in VSMCs of the media.\(^35\) The latter explanation is more likely, because VSMCs do synthesize ET-1 and are quantitatively dominant in intact blood vessels. Furthermore, inhibition of Ang II–induced ET production by losartan suggests that AT1 receptors, which are expressed in VSMCs, are modulated by vascular ET-1 production in vivo, thus confirming results obtained in cell culture systems.\(^16,29\) In the present study, verapamil had no effect on tissue ET-1 levels despite its beneficial effects on blood pressure and restoration of endothelial function. The reason for this phenomenon is unclear. It can be speculated that calcium antagonists, which may interfere with endothelial ET-1 release,\(^10,31,32\) do not affect the ET system in VSMCs, which were shown to be the primary location of ET-1 induced by Ang II in this study. This hypothesis may also help explain the different antihypertensive effects of losartan and verapamil, which is also supported by their differential effects on tissue ET-1 content. Indeed, blockade of ET\(_A\) receptors, which lowered blood pressure to the same extend as verapamil, completely abolished the Ang II increase in aortic ET-1 content.\(^33\) Interestingly, in healthy mountaineers, nifedipine was ineffective in lowering increased plasma ET-1 levels induced by high-altitude hypoxia.\(^34\)

A relationship between increased aortic ET-1 content and functional vascular changes is further suggested by the observation that aortic contractions to exogenous ET were inversely related to tissue levels of this peptide. Thus, it can be argued that the contribution of ET to the elevation of blood pressure is negligible, since elevated tissue ET levels are compensated for by decreased vasoconstriction efficacy.
However, we and others have previously shown that an ET₃ receptor antagonist prevents part of the pressure rise in the same model of hypertension. The decreased contractions to ET-1 in Ang II-induced hypertension are likely due to receptor downregulation, because these contractions were inversely related to the local peptide content. Furthermore, alterations of the signal transduction pathways appear unlikely to explain the blunted constriction to ET-1 in Ang II-induced hypertension, because ET-1 shares common signaling pathways with other agents and reactivity to these agents was improved by verapamil. This statement is supported by experiments that show that calcium channel antagonists do not inhibit the contractions to ET-1 in rat aortas and in human mammary arteries in vitro.

Although verapamil did not modulate elevated ET-1 levels, endothelium-dependent and -independent relaxations were improved, an effect shared by losartan. The improvement in endothelium-dependent relaxations therefore appears to be related to an improved sensitivity of VSMCs to NO, as illustrated by the results obtained with the NO donor SNP. Long-term blockade of ET₃ receptors only partially improved endothelial function, whereas endothelium-independent relaxations to SNP were unaffected. This finding suggests that the improvement in endothelial function is mediated at least partially via the selective activation of ET₃ receptors, which, in turn release NO or prostacyclin. Indeed, a recent study has confirmed our previous observations.

Endothelium-dependent contractions to ACH were enhanced in Ang II-induced hypertension. Concomitant treatment with either drug normalized the contractions to ACH. Responses of VSMCs to an agonist acting at the thromboxane A₂/prostaglandin H₂ receptor level, such as the thromboxane analogue U46619, were, however, comparable, suggesting enhanced production of prostanoids (which may interfere with endothelium-derived NO) as a likely mechanism.

These data are in line with previous reports in renin-angiotensin II–induced hypertension, because ET-1 shares common signal transduction pathways with other agents and reactivity to these agents was improved by verapamil. This statement is supported by experiments that show that calcium channel antagonists do not inhibit the contractions to ET-1 in rat aortas and in human mammary arteries in vitro.

In conclusion, this study shows that in Ang II–induced hypertension, losartan but not verapamil prevents Ang II–induced increases in tissue ET-1 levels and impaired contractions to ET-1. In contrast, blockade of AT₁ receptors and L-type calcium channels lowered SBP, normalized aortic endothelium-dependent relaxations to ACH, and decreased the release of endothelium-derived contracting factors. Thus, local vascular ET protein expression in Ang II–induced hypertension appears to be at least partly independent of blood pressure and endothelial function.


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