Effects of Angiotensin Converting Enzyme Inhibitors and of Hydralazine on Endothelial Function in Hypertensive Rats

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The function of the endothelium is impaired in hypertension. In spontaneously hypertensive rats (SHR), acetylcholine-induced relaxation is decreased and serotonin-induced constriction is increased. The goal of our study was to evaluate the effect of a long-term treatment with cilazapril, a new angiotensin converting enzyme inhibitor, or hydralazine, a vasodilator, on the endothelium-dependent responses in aorta of SHR. Wistar-Kyoto rats were used as normotensive reference. Isolated aortic rings with or without endothelium were suspended in organ chambers. The rings with intact endothelium were contracted with norepinephrine. Acetylcholine-induced relaxation was markedly enhanced by cilazapril treatment. The tension achieved at maximal relaxation was 8±4% of norepinephrine contraction in the cilazapril-treated SHR versus 55±5% in the untreated SHR (p<0.001). Hydralazine had no significant effect. The effect of serotonin was also markedly modified by cilazapril. In untreated SHR, serotonin induced the release of a vasoconstrictor substance by the endothelium as assessed by the ratio of maximal tension induced by serotonin in rings with endothelium over maximal tension in rings without endothelium, which was greater than 1. This ratio was reversed in cilazapril-treated SHR but not in hydralazine-treated SHR. Captopril had effects similar to cilazapril. Finally, evaluation of carotid arteries showed that cilazapril also prevented morphological changes of the intima in SHR (i.e., infiltration by mononuclear cells). We conclude that angiotensin converting enzyme inhibitors prevent the functional and morphological alterations in endothelium that are found in hypertension and speculate that this action might participate in their antihypertensive effect. (Hypertension 1990;16:532-540)

It is now becoming clear that the endothelium plays a fundamental role in the regulation of vascular tone. It produces vasodilating (prostacyclin, endothelium-derived relaxing factor [EDRF]), and vasoconstricting (leukotrienes, thromboxane A2, endothelin) substances. It also contains enzymes that can activate (angiotensin converting enzyme [ACE]) or inactivate (monoamine oxidase) vasoactive substances. It finally has an ideal location to detect biochemical or dynamic and mechanical changes in the blood stream.

Chronic hypertension is associated with structural changes of the endothelium: cell replication is accelerated, cell size and volume are increased, and there is an increased permeability to high molecular weight substances with changes in gap junction morphology. Chronic hypertension is also associated with functional changes in the vascular endothelium: endothelium-dependent relaxation to acetylcholine, adenosine diphosphate, and serotonin is impaired; acetylcholine induces endothelium-dependent contractions; and serotonin-induced contractions are increased.

Cilazapril, a new ACE inhibitor, is a potent antihypertensive drug. Hydralazine is a vasodilator also used in essential hypertension. The goal of our study was to investigate whether administration of cilazapril or hydralazine might prevent the altered endothelium-dependent responses in aorta of spontaneously hypertensive rats (SHR). For this purpose, we measured endothelium-dependent relaxation to acetylcholine and evaluated the role of endothelium in the constriction induced by serotonin on aortic rings of SHR treated or not treated with cilazapril or hydralazine. Normotensive Wistar-Kyoto (WKY) rats were used as reference.

Methods

Animals

Male SHR and WKY rats, 10–13 weeks of age, were used. All rats were housed in similar conditions...
and had free access to water. The WKY rats and the untreated group of SHR were fed a standard rat chow for 20 weeks. During the whole 20-week period, one group of SHR received cilazapril (10 mg/kg/day mixed with the chow) and another group of SHR received hydralazine (2.5 mg/day in drinking water). To evaluate the effects of a short-term administration of cilazapril, a group of SHR fed a normal diet received a daily dose of cilazapril (10 mg/kg) for 4 days. Cilazapril in this group was not mixed with the chow but was administered by gastric gavage because this short time would not have allowed the rats to get used to the new taste. Finally, in a separate experiment, a last group of SHR received another ACE inhibitor, captorplin, given by gastric gavage at a dose of 100 mg/kg/day for 4–6 days. The doses of cilazapril, captorplin, and hydralazine were maximal effective doses, as shown in preliminary experiments made over a 2-week period. At 30 weeks, mean blood pressure and heart rate were measured by the tail-cuff method.

**Measurement of Contraction of Isolated Aortic Rings**

At the age of 30–34 weeks, the rats were anesthetized with ether and exsanguinated, and the thoracic aorta was excised, dissected, and cut in 5-mm rings. Two rings were used for each rat: in one ring the endothelium was left intact, in the other the endothelium was removed by gentle rubbing of the intimal surface. In each experiment, the rings of three rats, from three different groups, were studied in parallel. Each ring was suspended in a 10 ml isolated organ bath filled with a Krebs-Henseleit solution (in mM: NaCl 115, KCl 4.7, MgSO4 1.2, KH2PO4 1.5, NaHCO3 25, CaCl2 2.5, glucose 11.1) kept at 37°C and gassed with a 95% O2–5% CO2 mixture. The rings were connected to force transducers, and isometric tension was recorded (recorder Linearorder mark VII, Graphtec Corp., Tokyo).

The rings were stretched to a resting tension of 3 g. The same resting tension was chosen for all the rings because preliminary experiments in de-endothelialized rings showed no difference in the optimal point of the length–tension relation for norepinephrine and potassium chloride between SHR and WKY rats, as confirmed in other studies. After an equilibration period, the rings with intact endothelium were contracted with 10^{-5} M norepinephrine, and cumulative doses of acetylcholine (10^{-9} to 10^{-4} M) were added. We used equivalent doses of norepinephrine rather than equipotent doses, so that all the rings would be exposed to the same dose of norepinephrine. We found in separate experiments that in SHR the tension achieved after acetylcholine-induced relaxation, expressed in percentage of norepinephrine contraction, was not affected by the tension induced by norepinephrine within the range described in our study. Indeed, the maximal effect induced by acetylcholine in aortic rings precontracted with norepinephrine (10^{-7} M) to a tension of 0.96±0.04 g (similar to the contraction obtained in untreated SHR and WKY rats) was the same as in aortic rings precontracted with norepinephrine (3×10^{-9} M) to a tension of 0.60±0.04 g (similar to the contraction obtained in cilazapril-treated SHR) (37±4% and 40±4% of norepinephrine contraction, respectively, n=6). The concentration of acetylcholine inducing 50% maximal relaxation (IC50) and the maximal relaxation were measured. On rings where endothelium was rubbed, the absence of endothelium was confirmed by the absence of relaxation to 10^{-5} M acetylcholine. After the rings were washed and returned to a stable baseline, the constricting effect of serotonin was evaluated on all the rings by adding cumulative doses of serotonin (10^{-4} to 10^{-6} M). The concentration of serotonin exhibiting 50% maximal contraction (EC50) and the maximal contraction were measured. In addition, the ratio of the maximal contraction in a ring with endothelium over the maximal contraction in a ring without endothelium from the same rat was calculated.

In some experiments, indomethacin (Sigma Chemical Co., St. Louis, Mo.) freshly dissolved in 0.1 M sodium carbonate was added to the organ bath 10 minutes before either norepinephrine or serotonin. The vehicle of indomethacin was added as a control in another aortic ring from the same rat.

In other experiments, the relaxing effect of sodium nitroprusside (Roche, Basel, Switzerland) was evaluated in rings denuded of their endothelium. Cumulative doses of sodium nitroprusside (10^{-10} to 10^{-7} M) were added after contraction of the rings with 10^{-7} M norepinephrine.

**Morphological Evaluation**

Morphological evaluation of the endothelium of carotid arteries was performed in parallel in some WKY rats, untreated SHR, and SHR treated for 4 months with cilazapril. After pentobarbital sodium (Nembutal, Abbott Laboratories, North Chicago, Ill.) anesthesia, a cannula was implanted into the left ventricle. The rat was perfused at a pressure of 90 mm Hg, first with 5 ml Krebs-Henseleit solution containing 10^{-5} M adenosine, then with 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.4, room temperature) for 15 minutes. The carotid artery was removed and further fixed for 90 minutes in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4, 4°C). After washing with 0.1 M sodium cacodylate plus 7% sucrose (pH 7.4, 4°C), and osmium fixation, the artery was dehydrated with ethanol, cut in five segments, and embedded in Epon 812, Fluka Chemie AG, Buchs, Switzerland. Semithin sections (1 μm) stained with toluidine blue and basic fuchsin were investigated by light microscopy. Ultrathin sections were stained with uranylacetate and lead citrate and examined with a transmission electron microscope (CM12/STEM, Philips Electron Optics, Eindhoven, The Netherlands).
TABLE 1. Characteristics of Systolic Blood Pressure (Tail-Cuff Method) and Heart Rate Measured at 30 Weeks In Different Experimental Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY</th>
<th>SHR</th>
<th>Hydralazine</th>
<th>Cilazapril (4 months)</th>
<th>Cilazapril (4 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure (mm Hg)</td>
<td>147±3*</td>
<td>210±5</td>
<td>171±4*</td>
<td>124±4*</td>
<td>186±3*</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>377±16</td>
<td>495±13</td>
<td>508±4</td>
<td>418±22*</td>
<td>446±18†</td>
</tr>
</tbody>
</table>

WKY, Wistar-Kyoto rats.

* p<0.001 compared with untreated spontaneously hypertensive rats (SHR).
† p<0.05 compared with untreated SHR.

Statistical Analysis

Student’s unpaired t test was used to compare untreated SHR with WKY rats, and to compare each group of treated SHR (long-term or short-term treatment with cilazapril, long-term treatment with hydralazine) with untreated SHR. Because the use of WKY rats as normotensive controls has been questioned,21 we did not compare the treated SHR with WKY rats but simply used the WKY rats as a reference. The effect of indomethacin was assessed by a paired t test.

All the results are expressed as mean±SEM. The n represents the number of rats used. A level of p<0.05 was considered significant.

Results

Blood pressure was significantly higher in SHR than in WKY rats and significantly lower in SHR treated with cilazapril and hydralazine than in untreated SHR (Table 1). Heart rate was decreased by cilazapril but slightly increased by hydralazine. SHR had significantly higher heart rate than WKY rats (Table 1).

In the rings with intact endothelium, norepinephrine (10^-7 M) induced comparable contractions in WKY rats and SHR (1.17±0.12 and 1.16±0.06 g, respectively) but significantly reduced contractions in the hydralazine-treated SHR (0.72±0.06 g, p<0.001) and even more reduced in the cilazapril-treated SHR (0.54±0.08 g, p<0.001). However, in the rats treated with cilazapril for 4 days, the contraction to norepinephrine was not different from untreated SHR (1.04±0.12 g).

Acetylcholine induced endothelium-dependent relaxation that reached a maximum at 10^-5 M. With higher doses, acetylcholine induced smaller relaxation, probably because of the increasing constricting effect. This was pronounced in all the SHR groups but minimal in the group of WKY rats (Figure 1). The maximal relaxation to acetylcholine was much greater in cilazapril-treated SHR than in untreated SHR. The tension induced by 10^-6 M acetylcholine was 55±5% of norepinephrine contraction in SHR versus 8±4% after long-term treatment with cilazapril (p<0.001) and 22±7% after short-term treatment with cilazapril (p<0.001). In contrast, hydralazine had no significant effect on maximal relaxation (53±7%, NS) (Figure 1). The maximal relaxation induced by acetylcholine in WKY rats (46±5%) was slightly but not significantly greater than in untreated SHR.

The IC50 for acetylcholine-induced relaxation was not significantly different in the cilazapril-treated groups and in control SHR (2.9±0.5×10^-8 M and 2.1±0.5×10^-8 M, respectively, for long-term and short-term cilazapril versus 5.3±3.0×10^-8 M for untreated SHR). In hydralazine-treated SHR and WKY rats, IC50 was also not significantly different from untreated SHR (7.5±4.2×10^-8 M and 7.7±3.7×10^-8 M, respectively). The relaxation induced by sodium nitroprusside on rings without endothelium precontracted with norepinephrine was not significantly different in SHR and WKY rats. In both strains, sodium nitroprusside-induced 100% maximal relaxation and the IC50 were similar (6.5±1.4×10^-9 M and 1.1±0.2×10^-9 M, respectively, in SHR and WKY rats). A 4-day treatment with cilazapril did not modify the response to sodium nitroprusside (100% maximal relaxation, IC50 of 6.2±1.0×10^-9 M) (Figure 2).

Figure 1. Line graph showing concentration-response curves of acetylcholine administered to rings with intact endothelium contracted with norepinephrine (10^-7 M) in the different experimental groups. For the number of experiments, see Table 1. ***Maximal relaxation significantly different from maximal relaxation in untreated spontaneously hypertensive rats (SHR) (p<0.001). WKY, Wistar-Kyoto rats.
Like acetylcholine, the effects of serotonin were strikingly modified by cilazapril treatment. The ratio of the maximal tension induced by serotonin in rings with intact endothelium over the maximal tension induced in rings without endothelium was greater than 1 in SHR (Figure 3). In the cilazapril-treated groups (both long-term and short-term treatment), this ratio was significantly decreased ($p<0.001$ and $p<0.05$, respectively) and was below unity as in WKY rats ($p<0.001$ compared with untreated SHR). In contrast, this ratio remained greater than 1 in hydralazine-treated SHR (Figure 3). In rings with intact endothelium, the SHR treated with cilazapril for 4 months had significantly lower maximal response (0.99±0.10 versus 2.32±0.19 g, $p<0.001$) and higher $EC_{50}$ (6.3±0.9×10$^{-6}$ M versus 1.2±0.2×10$^{-6}$ M, $p<0.001$) to serotonin than untreated SHR. Hydralazine and short-term treatment with cilazapril increased $EC_{50}$ significantly (1.7±0.2×10$^{-6}$ M for hydralazine and 2.4±0.3×10$^{-6}$ M for short-term cilazapril treatment versus 1.2±0.2×10$^{-6}$ M for SHR controls, $p<0.05$ and 0.01, respectively) but did not affect maximal response to serotonin (Figure 4A). Cilazapril had not only an effect on the endothelium but also an effect on smooth muscle cell reactivity to serotonin, as shown in Figure 4B. In rings without endothelium, the maximal response to serotonin was significantly lower in SHR chronically treated with cilazapril than in untreated SHR (1.48±0.08 versus 2.07±0.14 g, $p<0.01$), and the sensitivity to serotonin was significantly lower in cilazapril-treated SHR than SHR controls ($EC_{50}=14.8±3.5×10^{-7}$ M versus 3.4±1.1×10$^{-7}$ M, $p<0.05$). In contrast, neither hydralazine nor short-term cilazapril treatment significantly affected maximal tension or $EC_{50}$.

In another series of experiments, the effects of indomethacin on endothelial function in untreated SHR and in SHR treated for 4 days with cilazapril were studied. In untreated SHR, incubation of aortic rings with indomethacin (10$^{-6}$ M) decreased significantly the ratio of maximal contraction to serotonin in rings with endothelium over maximal contraction to serotonin in rings without endothelium (from 1.40±0.08 to 1.15±0.10, $n=8$, $p<0.01$). In cilazapril-treated SHR, incubation of the rings with indomethacin also decreased this ratio significantly (from 1.04±0.03 to 0.83±0.02, $n=6$, $p<0.001$). The magnitude of the effect of indomethacin was the same in aortic rings of untreated SHR and of cilazapril-treated SHR (Figure 5).

Captopril administered for 4–6 days to SHR had effects similar to cilazapril. Captopril increased the maximal relaxation to acetylcholine (28±6% of norepinephrine contraction) to about the same extent as cilazapril (22±7% of norepinephrine contraction) and decreased the ratio of the maximal contraction to serotonin in rings with endothelium over rings without endothelium by 16% (versus 22% after 4 days of cilazapril). After captopril, this ratio decreased from 1.29±0.05 in control SHR to 1.09±0.03 ($n=9$, $p<0.01$). Finally, captopril did not modify the dose-
response curve to sodium nitroprusside in denuded aortic rings contracted with norepinephrine. In captopril-treated SHR, sodium nitroprusside relaxed the rings by 100%, with an IC₅₀ of 5.0±0.8×10⁻⁹ M as compared with 6.5±1.4×10⁻⁹ M in untreated SHR (Figure 2).

Morphological evaluation was performed on carotid arteries fixed in a relaxed state. Cilazapril completely normalized the morphological alterations seen in SHR. In SHR, the alterations included bulging of endothelial cells and thickening of the subendothelial layer (Figure 6A), which was infiltrated with mononuclear cells, collagen fibers, and amorphous material (Figure 7, A and B). In cilazapril-treated SHR, the endothelial layer was flat (Figure 6B). The subendothelial layer was thin and no mononuclear cells could be seen (Figure 7, C and D). In WKY rats, the morphological aspect of the intima by light microscopy (Figure 6C) and electron microscopy (Figure 7, E and F) was similar to that of cilazapril-treated SHR.

Discussion

Our results show that long-term treatment with cilazapril but not hydralazine can reverse or prevent
two main alterations in endothelial function observed in SHR: the depressed acetylcholine-induced relaxation and the increased contraction to serotonin.

Acetylcholine-induced relaxation, which was depressed in SHR, as described in several previous studies,\textsuperscript{11,13,22,23} was dramatically improved after cilazapril treatment. The reduction in endothelium-dependent relaxation to acetylcholine in SHR may be due to a decreased release or action of EDRF,\textsuperscript{10,13,14} or to the release of a prostanoid vasoconstrictor by the endothelium. Indeed, in isolated aortic rings\textsuperscript{17} and renal arteries\textsuperscript{11} of SHR but not of WKY rats, acetylcholine may induce endothelium-dependent contractions, which are prevented by indomethacin, and indomethacin normalizes acetylcholine-induced endothelium-dependent relaxation in SHR.\textsuperscript{17} The decrease in endothelium-dependent relaxation in SHR aorta is not due to a decreased smooth muscle "relaxability" because we found that the effect of sodium nitroprusside for relaxing aortic rings contracted with norepinephrine is similar in SHR and WKY rats, as already described in cerebral arterioles\textsuperscript{12} and mesenteric arteries\textsuperscript{13} of stroke-prone SHR. Thus, the decrease in acetylcholine-induced relaxation in SHR is due to an endothelial dysfunction. Because cilazapril does not increase the smooth muscle relaxability of SHR (the effect of sodium nitroprusside is not modified by the ACE inhibitors), our results suggest that the effect of cilazapril on acetylcholine-induced relaxation is due to a change in endothelial function.

In addition, long-term treatment with cilazapril markedly decreased the contractions induced by serotonin. Serotonin, like acetylcholine, has both a direct...
constricting effect on smooth muscle cells and a relaxant effect due to the release of EDRF. Moreover, in SHR, serotonin, like acetylcholine, induces the release of a vasoconstrictor substance by the endothelium. This was suggested in previous studies and confirmed by our results, which show that serotonin induces, in SHR but not in WKY rats, a greater contraction in rings with endothelium than in rings without endothelium, as shown by a “ratio” greater than 1. Our data, therefore, show that cilazapril, which decreases the maximal contraction to serotonin to a much greater extent in rings with endothelium than in rings without endothelium and therefore reverses the ratio of maximal response to serotonin in rings with endothelium over rings without endothelium, prevents the endothelium-dependent part of the constriction induced by serotonin in SHR.

In addition to its effect on endothelial function, cilazapril decreased the direct constricting effect of serotonin on smooth muscle cells, as shown by the decreased response to serotonin in endothelium-denuded rings. Cilazapril and other ACE inhibitors have been shown in SHR and in rats with renovascular hypertension to decrease the thickness of the media in the aorta and myocardial arterioles, leading to improved compliance. One can speculate that the decrease in smooth muscle volume may be associated with a decrease in contractility because in resistance vessels of SHR, increased wall thickness and increased contractility are associated. We conclude, therefore, that cilazapril decreases smooth muscle contractility but also has a direct effect on endothelial function, both leading to a lesser contractile response to serotonin.

The mechanism by which cilazapril alters endothelial function, leading to decreased contraction to serotonin and increased relaxation to acetylcholine, is not explained by the present study. Both serotonin and acetylcholine induce the release, or increase the action, of a vasoconstrictor prostanoid by the endothelium of SHR, as shown by the significant decrease of the serotonin ratio by indomethacin as well as by previous reports. Cilazapril might act by inhibiting the synthesis or release of this prostanoid. Alternatively, cilazapril might increase the synthesis or release of a vasodilator substance by the endothelium, which might be EDRF or a vasodilator prostaglandin. Indeed, ACE inhibitors may affect eicosanoid production. Captopril increases plasma levels of a prostaglandin E2 metabolite in humans, and the formation of the prostacyclin metabolite 6-ketoprostaglandin F1alpha by cultured endothelial cells (see Reference 27). ACE inhibitors may act by increasing the level of kinins that stimulate phospholipase activity, or by redirecting the metabolism of endoperoxides toward vasodilator prostaglandins. However, the results of the experiments on the effects of indomethacin in cilazapril-treated SHR may suggest that cilazapril does not work by the same mechanism as indomethacin (i.e., inhibition of the synthesis of the vasoconstrictor prostanoid) because indomethacin applied to aortic rings of SHR treated for 4 days with cilazapril had the same effect as in aortic rings of untreated SHR.

The effect of cilazapril on endothelial function was already pronounced after only 4 days of treatment despite a relatively small effect on blood pressure. Captopril increases plasma levels of a prostaglandin E2 metabolite in humans, and the formation of the prostacyclin metabolite 6-ketoprostaglandin F1alpha by cultured endothelial cells (see Reference 27). ACE inhibitors may act by increasing the level of kinins that stimulate phospholipase activity, or by redirecting the metabolism of endoperoxides toward vasodilator prostaglandins. However, the results of the experiments on the effects of indomethacin in cilazapril-treated SHR may suggest that cilazapril does not work by the same mechanism as indomethacin (i.e., inhibition of the synthesis of the vasoconstrictor prostanoid) because indomethacin applied to aortic rings of SHR treated for 4 days with cilazapril had the same effect as in aortic rings of untreated SHR.

Cilazapril not only had an effect on endothelial function but induced remarkable changes in morphology. Cilazapril completely reversed or prevented the intimal thickening in SHR. Intimal thickening has been described in carotid arteries and aorta in SHR and in other models of hypertension. This intimal thickening is due to increased migration of blood-borne mononuclear cells and increased deposition of extracellular material and is associated with alterations in the shape of endothelial cells. We found that all those alterations were present in carotid arteries of SHR but not WKY rats, and all were prevented by cilazapril. The infiltration of mononuclear cells in the intima of SHR was particularly striking, and one could question whether part of the alteration in endothelial function might not be attributed to the presence of mononuclear cells and the factors they might secrete. If those mononuclear cells are actually playing a role in this endothelial dysfunction in SHR, then the effect of cilazapril in preventing endothelial dysfunction may essentially be due to prevention of mononuclear infiltration. It has recently been shown that cilazapril prevents neointima formation after endothelial denudation induced by balloon in rats. It is tempting to believe that cilazapril acts on the mononuclear...
cells and on the smooth muscle cells proliferating in the intima by a similar mechanism.

In conclusion, chronic ACE inhibition may protect the "endothelium" from the functional and morphological changes seen in hypertension. ACE inhibitors are known to induce peripheral vasodilatation by inhibiting the production of angiotensin II and therefore its direct constricting effect. Our present study suggests an additional mechanism for the antihypertensive effect of ACE inhibitors.

Acknowledgments

We thank Ursula Wolfgang, Andréa Roeckel, Doris Stamm, and Jean-Claude Brun for technical help; Jemesitas Lobsiger for typing the manuscript; Laurence Hilfiger for graphical work; and Prof. Hans-Rudolf Baumgartner for critical reading of the manuscript.

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35. Key Words • cilazapril • hydralazine • endothelium • chronic hypertension • serotonin • angiotensin converting enzyme inhibitors • rat studies
Effects of angiotensin converting enzyme inhibitors and of hydralazine on endothelial function in hypertensive rats.
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Hypertension. 1990;16:532-540
doi: 10.1161/01.HYP.16.5.532

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