Influence of the Status of the Renin-Angiotensin System on the Effect of Cilazapril on Neointima Formation After Vascular Injury in Rats

Jean-Paul Clozel, MD; Rita K.M. Müller, Dipl Nat; Sébastien Roux, MD; Walter Fischli, PhD; Hans-Rudolf Baumgartner, MD

Background. Angiotensin converting enzyme (ACE) inhibition has been shown to prevent neointima formation after vascular injury in rats. However, clinical results evaluating restenosis after angioplasty have been negative. The goal of the present study was to evaluate the influence of the renin-angiotensin system (RAS) status on the effect of ACE inhibition on neointima formation.

Methods and Results. Arterial injury was produced by balloononing the left carotid artery of rats, and neointima formation was evaluated by morphometry 2 weeks after balloononing. The effects of cilazapril were assessed in four experimental groups: normotensive rats, spontaneously hypertensive rats, hypertensive rats with a renal artery stenosis induced by clipping (two-kidney, one-clip rats), and hypertensive rats with uninephrectomy, high salt intake, and administration of deoxycorticosterone (DOCA). In parallel groups of rats, measurement of plasma renin activity was made in order to characterize (at least at the plasma level) the status of the RAS. As expected, renal artery stenosis markedly increased plasma renin activity, and DOCA decreased it to undetectable levels. Cilazapril had a marked preventive effect on neointima formation in normotensive rats, spontaneously hypertensive rats, and two-kidney, one-clip rats but was ineffective in DOCA rats.

Conclusions. We conclude that the status of the RAS has a major influence on the effect of cilazapril on neointima formation after vascular injury. (Circulation. 1993;88:1222-1227.)

KEY WORDS • angiotensin converting enzyme • hypertension • angioplasty • renin

Recently, it has been shown that one important risk factor for the occurrence of sudden death in hypertensive patients was the plasma renin level. One hypothesis to explain this finding was that an increase of plasma renin could lead to an increased production of angiotensin II. Angiotensin II would then induce proliferation of smooth muscle cells in the arterial wall and thus promote accelerated development of arteriosclerosis.1

The possible deleterious effect of angiotensin II was also suggested by the fact that angiotensin converting enzyme (ACE) inhibition2,3 or angiotensin II receptor blockade4 could prevent myointimal proliferation after vascular injury. Interestingly, the effect of ACE inhibition did not seem to be mediated by a decrease of arterial pressure, since a similar decrease of arterial pressure induced by verapamil did not prevent neointima formation.2 Recently, however, a major clinical trial (MERCATOR) evaluating the effects of ACE inhibition with cilazapril on restenosis showed negative results.4 One possible explanation for this negative result could be a different status of the renin-angiotensin system (RAS) between humans and rats. In addition, a recent experimental study suggests that the effect of ACE inhibition on neointima formation was not due to the decrease of formation of angiotensin II but to the accumulation of bradykinin, which is degraded by ACE. Therefore, it was important to evaluate the influence of the RAS status on the effect of cilazapril.

Several models of hypertension have been described, and their relation to the RAS has been well characterized.6 The two-kidney, one-clip (2KIC) model is a hypertension model with an initial high plasma renin level followed by a further normalization of the plasma renin level associated with a sustained overexpression of the tissular RAS.7,9 The deoxycorticosterone (DOCA) model is characterized by a very low plasma renin level.10 Spontaneously hypertensive rats have a normal or rather low plasma renin level.11,12 The goal of the present study was to evaluate whether the neointima formation induced by vascular injury was influenced by different hypertension models with different levels of stimulation of the RAS and whether ACE inhibition with cilazapril had similar effects on neointima formation in hypertension models with or without stimulation of the RAS.

Methods

Animals

Normotensive male Wistar-Kyoto (WKY) rats (Ibm: RORO, SPF) and spontaneously hypertensive rats (SHR) from the Okamoto strain (SHR/A3N) bred in Füllinsdorf, Switzerland, were used. All the rats were 12 to 16 weeks old.
Renal hypertensive rats (Goldblatt type 2K1C) were produced by the following procedure: 6-week-old male normotensive rats were anesthetized with ether, and the left renal artery was clamped with a silver clip (slit width, 0.22 mm). The right renal artery was left intact. In another group of normotensive rats (sham operated), the same surgical procedure was performed without implantation of a clip. Rats were used 6 weeks after surgery for the experiment.

DOCA-NaCl-hypertensive rats were prepared as follows: 7-week-old male normotensive rats, which were subjected to unilateral nephrectomy under ether anesthesia, received a subcutaneous implant of 25 mg of 11-DOCA acetate (Schering AG, Berlin-Bergkamen, Germany) as pellets during the same intervention. A second pellet was implanted 4 weeks after nephrectomy to keep the level of DOCA constant during the whole experiment. Six weeks after nephrectomy, the rats were used for the experiment. All rats were maintained under identical conditions of temperature (20 to 22°C), humidity (50% to 60%), and light/dark cycle and had free access to normal rat chow (NAFAG 850, Gossau, Switzerland). SHR-2K1C-hypertensive rats and normotensive rats received tap water, and DOCA-NaCl-hypertensive rats were given a 1% NaCl solution as drinking fluid. Hypertensive and normotensive rats with blood pressure values of 180 to 230 mm Hg and 110 to 150 mm Hg, respectively, were used for the experiment.

Arterial pressure and heart rate were measured indirectly by a tail-cuff plethysmographic technique.13

**Vascular Injury**

Endothelial denudation and vascular injury were achieved in the left common carotid artery of normotensive rats, as described.14 A balloon catheter (2F Fogarty, Edwards Laboratories, Santa Anna, Calif) was passed through the external carotid into the aorta; the balloon was inflated with sufficient water to distend the common carotid and was then pulled back to the external carotid. This procedure was repeated three times, and the catheter then was removed. Complete denudation of the endothelium was achieved throughout the common carotid, with some injury to medial smooth muscle cells, as assessed by morphological examination of random control rats 24 hours after the procedure. The rate of intimal thickening in the rat carotid in response to balloon injury slows considerably after 14 days.14 Therefore, at 14 days the animals were anesthetized and perfusion-fixed as described,14 with the modification that 2.5% glutaraldehyde and 90 mm Hg perfusion pressure were used. Carotid arteries were isolated from adherent tissue and embedded in Epon 812. Semithin sections (=1 μm) were stained with toluidine blue and basic fuchsin and processed for morphometric evaluation.15,16 Sections from the middle fifth of the carotid were analyzed, with the right carotid serving as a control.
Arterial Pressure and Quantitative Morphometry Results in the Experimental Groups

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>n</th>
<th>MAP (mm Hg)</th>
<th>Neointima area (10^-2 mm^2)</th>
<th>Neointima/media ratio</th>
<th>Media area (10^-2 mm^2) (ballooned)</th>
<th>Media area (10^-2 mm^2) (un ballooned)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>133±3‡</td>
<td>9.9±0.9</td>
<td>1.04±0.12</td>
<td>9.9±0.6</td>
<td>9.2±0.8</td>
</tr>
<tr>
<td>Cilazapril</td>
<td>7</td>
<td>106±3‡</td>
<td>3.4±1.1‡</td>
<td>0.41±0.14‡</td>
<td>8.2±0.3</td>
<td>8.7±0.3</td>
</tr>
<tr>
<td>SHR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>232±7¶</td>
<td>17.8±0.2[†</td>
<td>1.63±0.15[†</td>
<td>10.9±0.4[†</td>
<td>10.1±0.7[†</td>
</tr>
<tr>
<td>Cilazapril</td>
<td>10</td>
<td>128±3‡</td>
<td>2.7±0.6‡</td>
<td>0.32±0.08‡</td>
<td>8.4±0.5‡</td>
<td>7.6±0.3‡</td>
</tr>
<tr>
<td>DOCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11</td>
<td>188±11¶</td>
<td>6.5±1.3[†</td>
<td>0.57±0.11[†</td>
<td>11.4±0.5[†</td>
<td>10.9±0.5[†</td>
</tr>
<tr>
<td>Cilazapril</td>
<td>11</td>
<td>148±6†</td>
<td>4.8±1.2</td>
<td>0.45±0.10</td>
<td>10.3±0.5</td>
<td>9.4±0.4[†</td>
</tr>
<tr>
<td>2K1C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18</td>
<td>242±10¶</td>
<td>8.5±0.7</td>
<td>0.75±0.06[§</td>
<td>11.3±0.5[†</td>
<td>11.3±0.5[†</td>
</tr>
<tr>
<td>Cilazapril</td>
<td>18</td>
<td>120±4‡</td>
<td>1.0±0.5‡</td>
<td>0.10±0.05‡</td>
<td>9.8±0.4[†</td>
<td>9.3±0.5[†</td>
</tr>
</tbody>
</table>

Before drug intake, the variables of the hypertensive rats were compared with those of the normotensive rats only by ANOVA. MAP indicates mean arterial pressure; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; DOCA, deoxycorticosterone rats; 2K1C, two-kidney, one-clip rats.

Values are mean±SEM. *P<.05, †P<.01, ‡P<.001 vs control. §P<.05, ¶P<.01, ¶¶P<.001 vs WKY.

for the balloon-catheterized left carotid. Rats were selected randomly to be given either placebo or cilazapril (10 mg/kg per day mixed with normal food starting 2 days before balloonling and for 14 days). Animals were coded so that operation and analysis were performed without knowledge of which treatment individual animals received.

Biochemical Characterization of the RAS

To characterize the status of the RAS, we measured plasma renin activity in four other groups of rats of the same age that had the same interventions as described above (2K1C rats, DOCA rats, spontaneously hypertensive, and normotensive sham-operated rats). Plasma renin activity was measured once a week in each rat of each group for 6 weeks until a point corresponding to the balloon injury. Arterial pressure was also measured in these rats during these 6 weeks. Plasma renin activity was measured according to standard techniques, ie, (1) 225 μL of plasma, (2) 25 μL of 0.1 mol/L sodium phosphate, pH 7.4, and (3) 2.5 μL of 0.3 mol/L phenylmethylsulfonyl fluoride in ethanol were incubated for 3 hours at 37°C and 4°C, respectively. Fifty microliters of the samples were subsequently analyzed in triplicate with a standard radioimmunoassay kit (Clinical Assay, Cambridge, Mass) to quantitate the amount of angiotensin I generated. Net angiotensin I formation was estimated as the difference between samples maintained at 37°C and 4°C.

Study Design and Statistical Analysis

Vascular injury was induced in the normotensive and the hypertensive rats. In each group, the effects of cilazapril were assessed by unpaired Student’s two-tailed t test with a Bonferroni correction for multiple comparisons. The effects of vascular injury per se (without drug intake) on the different types of hypertensive rats were compared with the normotensive WKY rats by analysis of variance followed by Dunnnett’s t test. Changes of arterial pressure and plasma renin activity in the four additional groups used only for biochemical measurement were evaluated by descriptive analysis. All data are shown as mean±SEM. A value of P<.05 was considered significant.

Results

Arterial Pressure

Arterial pressure was significantly increased in the SHR, DOCA, and 2K1C rats compared with normotensive WKY rats (Fig 1). Sham operation had no effect on blood pressure (Fig 1). Under treatment with cilazapril, arterial pressure dramatically decreased in the SHR and 2K1C rats and much less in the DOCA rats (Fig 1 and Table). Cilazapril also slightly decreased arterial pressure in the normotensive WKY rats.

Effects of Vascular Injury in the Experimental Models

Neointima formation was increased only in SHR compared with normotensive WKY rats (see Table). Neointima formation was not increased in the 2K1C rats (compared with control WKY without or with sham operation). Neointima formation was decreased in DOCA rats compared with normotensive WKY rats.

Vascular Effects of Cilazapril in the Experimental Models

Cilazapril was effective in reducing neointima formation in every group of rats except the DOCA rats (see Table). This effect of cilazapril was observed when the neointima was expressed as absolute value or as a percentage of the media area. Despite the short treatment period, cilazapril significantly decreased the area of the media (both balloonled and un balloonled arteries) only in SHR and 2K1C rats.

Biochemical Characterization of the RAS

During the 6 weeks of renal stenosis and DOCA administration, arterial pressure increased (Fig 2). During the same period, arterial pressure stayed normal in the normotensive rats and increased in the spontaneously hypertensive rats (Fig 2). Plasma renin activity did
not change in normotensive rats and increased slightly in spontaneously hypertensive rats. Renal stenosis markedly increased plasma renin activity, which peaked at 1 week after renal artery stenosis and decreased progressively (Fig 3). Administration of DOCA decreased plasma renin activity to unmeasurable levels (Fig 3).

Discussion

The results of the study show that the effect of cilazapril on neointima formation after vascular injury strongly depends on the status of the RAS. In DOCA-hypertensive rats with a low plasma renin activity, cilazapril did not prevent neointima formation after vascular injury.

It is extremely difficult to evaluate the status of the RAS using plasma renin activity. It is well known that after a stimulus such as renal stenosis, the plasma renin activity increases and then tends to come back to baseline despite the fact that blood pressure stays increased due to a stimulation of components of the RAS present in tissues.7-9 In DOCA rats, plasma renin activity is decreased.10 In SHR, plasma renin activity is normal or low,11,12 but many studies have reported an increase of the component of the RAS in tissues.13-20 Our biochemical results confirm that we have used a high plasma renin model (2K1C rats) and a low plasma renin model (DOCA rats), but we have no information on the status of the RAS in tissues.

The present results do not support a role for the accumulation of bradykinin in the preventive effect of cilazapril. Cilazapril was ineffective in DOCA rats, in which, to our knowledge, only the RAS and not the bradykinin system is suppressed. Our results also show that arterial pressure alone does not substantially influence neointima formation after vascular injury. Despite the increased arterial pressure, 2K1C rats did not have a larger neointima after balloononing. DOCA-hypertensive rats had a smaller neointima compared with normotensive rats. This confirms our previous findings showing that verapamil at a dose decreasing arterial pressure to the same extent as cilazapril did not influence neointima formation.2 Moreover, DOCA is a mineralocorticoid. To our knowledge, in contrast with glucocorticoid, this type of drug has not been shown to influence neointima formation.

In the present study, one of the main determinants of the effect of cilazapril on neointima formation appeared to be the status of the RAS. As described before, cilazapril was very effective in preventing neointima formation in normotensive rats and in 2K1C rats in which the RAS is stimulated. However, cilazapril was ineffective in the DOCA rats, in which the RAS is inhibited. The extent of the effect of cilazapril in the normotensive WKY rats was similar compared with what has been described previously2 but was larger in 2K1C rats and SHR rats compared with WKY rats.
contrast, cilazapril was not effective in the DOCA rats, which might be easily explained by the fact that the RAS is already inhibited in these rats.\textsuperscript{10} Surprisingly, neointima formation was not increased in 2K1C rats, which have a stimulated RAS. One explanation is that the basal level of stimulation of the vascular RAS is already maximal in normotensive rats. Thus, no further stimulation of the system can be achieved. Another explanation is that the RAS (and most likely angiotensin II, its final product) acts as cofactor for the neointima formation. It seems necessary for the formation of neointima but not sufficient per se. Isolated stimulation of the RAS (such as in 2K1C rats) cannot increase neointima formation. In vitro, angiotensin II is, according to different authors, either very weak\textsuperscript{21} or not at all mitogenic\textsuperscript{22-25} for smooth muscle cells of normotensive rats. Infusion of angiotensin II has been shown to increase neointima formation.\textsuperscript{4,26} However, it is difficult to interpret these experiments in which exogenous angiotensin II has been administered since such infusions lead to plasma concentrations of angiotensin II that probably are never reached in physiological and pathological situations. It has been suggested that angiotensin II could act through stimulation of platelet-derived growth factor-A\textsuperscript{27} or epidermal growth factor expression.\textsuperscript{28,29} A role for transforming growth factor-\(\beta\) (TGF-\(\beta\)) has also been suggested.\textsuperscript{30,31}

The last finding of our study was the fact that for a similarly elevated arterial pressure, neointima formation was larger in SHR rats compared with DOCA and 2K1C rats. It is known that neointima formation is increased in SHR compared with normotensive rats.\textsuperscript{32,33} This was attributed to the high blood pressure,\textsuperscript{32,33} which does not seem to be the case in our study with DOCA or 2K1C rats. In vitro, it has been shown clearly that smooth muscle cells of SHR grow faster than smooth muscle cells of normotensive rats, pointing to differences of phenotype.\textsuperscript{34-37} Our study would suggest that in vivo, this difference is also present. However, it is not possible to exclude a role of the RAS in the increase of neointima formation after vascular injury compared with normotensive rats (also SHR). SHR rats have a normal or low plasma renin activity but an increased tissular expression of the RAS.\textsuperscript{16-19} It is also important to note that DOCA rats did not have an increased response to vascular injury despite the threefold increase of TGF-\(\beta\) mRNA\textsuperscript{37} or platelet-derived growth factor-\(\beta\) receptor mRNA level\textsuperscript{38} that has been shown in these rats. These results suggest that growth factors involved in medial thickening due to hypertension are different from those involved in vascular response to injury.

\textbf{Clinical Relevance}

The large multicenter trial MERCATOR, which had evaluated the effect of cilazapril on the prevention of restenosis, showed negative results. Several hypotheses can explain these negative results: The dose of cilazapril
could have been insufficient, cilazapril should have been given before angioplasty, or coronary spasm has masked the effect of cilazapril. However, the present study suggests that the effect of cilazapril is closely dependent on the RAS status. Thus, normotensive rats could have an RAS that is much more stimulated than that in humans. Differences of status of the RAS also could explain why cilazapril seems ineffective in other animal species such as pigs or monkeys, which points to the necessity of using several animal species for testing drugs that may prevent neointima formation after vascular injury.

References


Influence of the status of the renin-angiotensin system on the effect of cilazapril on neointima formation after vascular injury in rats.
J P Clozel, R K Müller, S Roux, W Fischli and H R Baumgartner