Role of the Renin-Angiotensin System in Neointima Formation After Injury in Rabbits

Philip Janiak, Olivier Libert, Jean-Paul Vilaine

Abstract We investigated the role of the renin-angiotensin system in neointima formation in a species in which converting enzyme inhibitors have been so far ineffective in suppressing abnormal vascular repair. The effects of converting enzyme inhibition by perindopril and selective blockade of angiotensin subtype 1 receptor by DuP 753 were assessed on neointima formation after balloon injury of rabbit carotid artery. Myointimal growth was measured by histomorphometric analysis. In rabbits treated 6 days before and for 14 days after injury, perindopril (2 mg/kg per day PO, n=7) significantly reduced neointima formation (~51%, P<.01). DuP 753 (1 mg/d, n=8) infused perivascularly for 14 days in the vicinity of injured carotid artery also markedly suppressed myointimal thickening (~60%, P<.01). To determine whether angiotensin subtype 2 receptor was implicated in this vascular response, we infused CGP 42112A, a specific subtype 2 receptor ligand, continuously for 14 days according to the same protocol of DuP 753 administration. CGP 42112A (1 mg/d) did not change the neointima-media ratio, indicating that angiotensin subtype 2 receptors were not involved in myointimal hyperplasia in rabbits. Thus in rabbits, the renin-angiotensin system plays a major role in neointima formation, and the protective effect of perindopril appears to be mediated mainly by inhibition of angiotensin II production, because blockade of the subtype 1 receptor reduced myointimal growth in a manner similar to that of converting enzyme inhibition, because intracarotid infusion of angiotensin II (500 ng/min) at the site of injury enhanced the vascular response (+39%, P<.05). Bradykinin (500 ng/min) administered in the same conditions as angiotensin II did not modify neointima formation. (Hypertension. 1994;24:671-678.)

Key Words • angiotensin-converting enzyme inhibitors • angiotensin II • hyperplasia • angioplasty

The renin-angiotensin system (RAS) plays a major role in the pathogenesis of neointima formation induced by vascular injury in rats. In this species, angiotensin-converting enzyme (ACE) inhibitors have been shown to suppress markedly the vascular response to balloon injury, suggesting that such therapy could be effective in humans to prevent restenosis after transluminal angioplasty. However, the recent results of the MERCATOR restenosis trials indicated that the ACE inhibitor cilazapril failed to reduce the rate of restenosis occurring in 30% to 40% of patients undergoing percutaneous angioplasty. Retrospectively, this lack of efficacy of cilazapril in humans correlated well with that observed in other species such as rabbits, swine, and nonhuman primates.

To understand the causes of therapeutic failures of cilazapril on restenosis in humans, we have investigated the role of the RAS in neointima formation in species in which ACE inhibitors have been so far ineffective in suppressing this excessive vascular repair. The first study conducted with cilazapril in rabbits did not allow us to conclude whether or not ACE inhibition limited myointimal thickening because the dose of ACE inhibitor used was only the maximally tolerated and not the most effective.

The aim of our study was first to determine whether long-term ACE inhibition by perindopril at a dose effective over a 24-hour period reduced neointima formation in rabbits. Since in rats the mechanism of action of ACE inhibitors on myointimal growth has been shown to involve both inhibition of angiotensin II (Ang II) formation and reduction of kinin catabolism, we examined the direct effect of Ang II and bradykinin on the vascular response. We also investigated the role of angiotensin subtype 1 (AT,) and 2 (AT,) receptors in the genesis of this lesion in rabbits because both receptor subtypes have been implicated in rats.

Methods

All studies were performed on male New Zealand White rabbits weighing 2 to 2.5 kg (Charles River Laboratories, Saint-Aubin-les-Elbeuf, France), and all procedures were carried out in accordance with the guidelines of the French Ministry of Agriculture for the use and care of laboratory animals.

Cardiovascular Studies

The first series of experiments was designed to determine the effective dose of perindopril over a 24-hour period after a single oral administration and after 6 days of chronic treatment. The duration of this treatment was identical to the pretreatment used for the subsequent histomorphometric studies. Rabbits were anesthetized by intramuscular injection of a mixture of xylazine (4 mg/kg) and ketamine (35 mg/kg), and the femoral artery and jugular vein were catheterized for arterial pressure measurement and drug administration, respectively. Both catheters were filled with heparinized saline and were tunnelled subcutaneously to exit between the shoulder blades. For postoperative analgesia, 2% lidocaine gel was applied to the wound edges. This procedure was followed after each surgical intervention. No analgesic was given to avoid possible effects on hemodynamic parameter measurements. Forty-eight hours after surgery, mean arterial pressure (MAP) and heart rate (HR) were monitored in conscious freely moving rabbits.

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Pulse pressure and MAP were displayed on a model ES1000 recorder (Gould Inc.) and HR was determined by a Gould Biotach cardiotachometer. After a 1-hour stabilization period, ACE activity was assessed by the pressor effects produced by intravenous injection of Ang II (500 ng/kg) before and after oral administration of perindopril (1, 2, and 3 mg/kg). The effectiveness of the inhibition was examined for 6 hours and at 24 hours after administration. For evaluation of ACE activity at the time of balloon injury, rabbits were treated orally with either perindopril (1 and 2 mg/kg) or its vehicle (distilled water, 1 mL/kg) for 4 days before the implantation of arterial and venous catheters as described above. Oral perindopril treatments were continued for the following 2 days. On day 7, MAP and HR were monitored, and the oral potency and duration of ACE inhibition were examined as reported above.

For the evaluation of whether the Ang II and bradykinin doses selected for the histomorphometric studies were pharmacologically active, the femoral and carotid arteries were catheterized with rabbits under xylazine-ketamine anesthesia as reported above for MAP measurement and drug infusion, respectively. In separate experiments, Ang II and bradykinin were infused in the carotid artery at 500 ng/min with a Harvard Apparatus pump (model 22-3, Ealing) (flow rate, 5 μL/h). Hemodynamic parameters were monitored before and for 4 hours after peptide infusion.

To determine the role of AT₁ and AT₂ receptor subtypes in the proliferative response to balloon injury, we infused chronically DuP 753 (1 mg/d) and CGP 42112A (1 mg/d), respectively, at the site of arterial injury. To achieve a blockade of the RAS similar to that obtained with perindopril, preliminary hemodynamic studies performed in rabbits showed that DuP 753 was orally effective on the pressor response to Ang II only at high doses (>30 mg/kg per day). Long-term oral treatment with such a high dose would have been associated with the loss of some of DuP 753, which were not available in our laboratory. It is known that balloon catheterization leads in more than 2 weeks to the development of a local proliferative lesion. So to study the role of AT₁ and AT₂ receptor subtypes in this pathological process, the essential point was to block chronically these receptors at the site of injury. Thus, for histomorphometric studies, DuP 753 and CGP 42112A were infused perivascularly at such a high dose. The choice of this period of administration came from a previous study performed in rats in which we showed that perivascular infusion of CGP 42112A markedly suppressed neointima formation, while in contrast, subcutaneous administration of this compound was ineffective in preventing this excessive vascular response. Such an administration route was not suitable for perindopril and was discarded from our protocol design because ACE is present in both vessel wall and plasma. Indeed, local delivery of perindopril would inhibit the production of Ang II only within the injured arterial wall and not in plasma. In separate experiments, we assessed the level of blockade of AT₁ receptors in the vessel wall by measuring the pressor effects of Ang II injected into the lumen of the carotid artery. For this purpose, rabbits were anesthetized by intravenous injection of pentobarbital (30 mg/kg) and ventilated with a respirator (Harvard Apparatus, model 683, Ealing). The left carotid artery was exposed, and DuP 753 (42 μg and 1 mg), CGP 42112A (1 mg), or vehicle was applied directly to the vessel. The doses of 42 μg and 1 mg corresponded to the amount of drug delivered in 1 and 24 hours, respectively, for histomorphometric studies. Forty-five minutes after drug application, the left carotid artery was isolated from the circulation by catheterization. A catheter was implanted in the common carotid artery via the external branch for arterial pressure measurement, and two catheters were introduced into the common and internal carotid arteries to perfuse in situ the isolated portion of the vessel and to collect the perfusates, respectively. The perfusion solution contained (mmol/L) NaCl 120.3, KCl 4.8, CaCl₂ 2.5, MgSO₄ 1.3, KH₂PO₄ 1.2, NaHCO₃ 24.2, glucose 5.8, pH 7.4, gassed with a mixture of 95% O₂ and 5% CO₂ and kept at 37°C. To maintain oncotic pressure and preserve the integrity of endothelium, 4% bovine serum albumin was added to the physiological solution. The flow rate was set for a basal pressure of 90 mm Hg. After a stabilization period of 15 minutes, AT₁ receptor activity was assessed by measuring the pressor effect of Ang II (500 ng) injected directly into the lumen of the carotid artery.

### Histomorphometric Studies

The first series of experiments was performed to determine the effects of ACE inhibition by perindopril on the neointima formation induced by balloon injury. Male New Zealand White rabbits (Charles River) weighing 2 to 2.5 kg were treated for 3 days before injury and for 14 days after injury with either perindopril (2 mg/kg per day PO) or its vehicle (distilled water, 1 mL/kg per day PO). The balloon injury procedure was performed as follows in anesthetized rabbits (4 mg/kg IM xylazine, 35 mg/kg IM ketamine). A 2F balloon embolectomy catheter (Baxter) was introduced through the facial artery into the left common artery. The vascular injury was performed by slowly pulling out the inflated balloon three consecutive times as described by Clowes et al. Postoperative analgesia was provided by 2% lidocaine applied to the wound edges. No further analgesia was given to avoid possible interaction with arterial repair. At the end of the 14-day treatment, ACE activity was assessed 24 hours after the last administration of perindopril. In conscious restrained animals, a catheter was introduced into the auricular artery with rabbits under local anesthesia (lidocaine 2%, subcutaneous) to measure MAP and HR. ACE inhibition was quantified by the pressor effects produced by intravenous injection of Ang I (500 ng/kg).

Since ACE inhibition suppresses Ang II formation and reduces the catabolism of kinins, in a second series of experiments, we investigated the direct effect of Ang II and bradykinin on myointimal growth. After the balloon injury procedure, a catheter connected to an osmotic minipump (model 2ML2, Alzet Corp.) was introduced into the carotid artery at the site of injury to deliver either Ang II (500 ng/min), bradykinin (500 ng/min), or vehicle (distilled water, 5 μL/h). To be able to carry out appropriate statistical comparison, both control groups of animals receiving Ang II or bradykinin also had an intraluminal catheter for infusion of vehicle solution. After 14-day chronic treatment with Ang II or bradykinin, a catheter was implanted into the auricular artery as reported above for measurement of MAP and HR.

A third series of experiments was designed to determine the role of AT₁ and AT₂ receptor subtypes in the proliferative response to arterial injury. Rabbits with no drug pretreatment underwent balloon injury of the left common artery as described above. At the end of the balloononing procedure, DuP 753 (1 mg/d), CGP 42112A (1 mg/d), or their respective vehicles (5 μL/h) were continuously infused perivascularly for 14 days in the vicinity of the injured carotid artery as reported elsewhere. In brief, a silicone elastomer cuff connected by a catheter to an osmotic minipump (Alzet model 2ML2) was placed and secured around the denuded artery to deliver a periadventitial drug infusion. Fourteen days after intimal injury, rabbits were anesthetized (4 mg/kg IM xylazine, 35 mg/kg IM ketamine) and received an intravenous injection of Evans blue (1.5 mL of 0.5% saline solution, pH 7.4) for evaluation of the level of endothelium regeneration. After perfusion and fixation (formaldehyde 4%) under 90 mm Hg of pressure, the carotid arteries were carefully removed and left in the same fixative until processed further. Only the portion of the carotid arteries that did not show any evidence of endoluminal regeneration (positive staining with Evans blue injection) was used for histomorphometric studies. The portion was divided into four segments (5 mm length) and embedded in paraffin. Nonserial cross sections (5 μm) were prepared and stained with orcein to measure medial and neointimal cross-sectional areas. The distance between two...
cross sections was 200 μm. All histomorphometric analysis was performed in a double-blind fashion with a computerized image-analysis system with 256 levels of gray and a 512x512 pixel grid (Biorad, Histo software). This system allows an overall analysis of the cross section. The internal elastic lamina was used as the border to distinguish the neointima from the media. For each carotid artery, neointimal and medial cross- sectional areas were averaged from the analysis of 12 cross sections (three cross sections per segment). The ratio of the cross-sectional area of the neointima to the cross-sectional area of the media was calculated and averaged over the 12 cross sections analyzed.

Drugs

The drugs used in the study were obtained from the following sources: Ang I, Ang II, bradykinin, and Evans blue dye were from Sigma Chemical Co; ketamine (Imalgene 1000) from Rhône Mérieux; xylazine from Bayer; and CGP 42112A [nicotinic acid-Tyr-(Nα-benzylxycarbonyl-Arg)-Lys-His-Pro-Ile-OH] from Neosystem. DuP 753 [potassium salt of 2-n-butyl-4-chloro-5-(hydroxymethyl)-1-[2'-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl]imidazole] was synthesized by the laboratory of Dr. J.L. Peglion at the Institut de Recherches Servier, Suresnes, France. Perindopril was also from the Institut de Recherches Servier.

Statistical Analysis

Data are expressed as mean±SEM. Cardiovascular studies were analyzed by either one-way ANOVA or two-way ANOVA with repeated measures. The post hoc test used was the Newman-Keuls test. Statistical analysis of histomorphometric studies was performed by Student's t test.

Results

Cardiovascular Studies

The initial series of experiments was conducted to define the most effective dose of perindopril active on ACE activity over a 24-hour period. The pressor response elicited by intravenous injection of Ang I (500 ng/kg) was used to determine the efficacy of perindopril on ACE activity. After a single oral administration, perindopril (1, 2, and 3 mg/kg) inhibited in a dose-dependent manner the pressor response to Ang I (Fig 1). At 24 hours after the dose, a significant inhibition was still observed and reached 58%, 72%, and 75% for 1, 2, and 3 mg/kg, respectively. Basal MAP and HR were not significantly changed by perindopril after a single administration (data not shown). Since the potency and duration of action of perindopril did not differ between 2 and 3 mg/kg PO, only 1 and 2 mg/kg was used for the long-term cardiovascular studies. Another set of experiments was performed to evaluate the extent of ACE inhibition by perindopril at the time equivalent to balloon injury after 6 days of pretreatment. In these conditions, 24 hours after the last administration, perindopril (1 and 2 mg/kg) significantly reduced in a dose-dependent manner basal MAP without altering basal HR (Table 1). The pressor response to Ang I was significantly decreased in the two perindopril-treated groups (Fig 1). This inhibitory effect was more pronounced in rabbits receiving 2 mg/kg per day of ACE inhibitor. Subsequent administration of perindopril at 2 mg/kg almost completely abolished the pressor action of Ang I for at least 6 hours. Therefore, this latter dose was used later to assess the effect of ACE inhibition on myointimal thickening. Rabbits treated 6 days before and 14 days after balloon catheterization by perindopril (2 mg/kg per day) showed a significantly lower MAP 24 hours after the last administration than those receiving vehicle (Table 1). The pressor effect of Ang I was significantly suppressed (~85%), and this inhibition was maintained after a new administration of perindopril (Fig 1).

Intracarotid infusion of Ang II (500 ng/min) significantly raised MAP but did not modify HR (Fig 2). The pressor response reached a maximum at 1 hour and then slightly decreased. At the same dose and under the same administration conditions, bradykinin significantly lowered MAP, but the maximal effect was achieved after 3 hours of infusion. These data indicated that the dose of 500 ng/min Ang II and bradykinin was hemodynamically active in rabbits and could be used to study the effect of these two peptides on neointima formation. In animals undergoing balloon injury, 14-day chronic infusion of Ang II (500 ng/min) significantly increased MAP (from 86±3 to 106±6 mm Hg, P<.05), whereas bradykinin (500 ng/min) infused in the same conditions markedly decreased MAP compared with control (from 90±5 to 73±4 mm Hg, P<.05).

The third set of experiments was designed to determine whether periadventitial administration of DuP 753 was efficient enough to block the AT1 receptor subtype. For this purpose, AT1 receptor activity was assessed after the topical application of DuP 753 (42 μg and 1 mg) or its vehicle on the isolated carotid segment by measuring the pressor response to intracarotid injection of Ang II (500 ng). Ang II injected directly into the lumen of the carotid artery produced a significant increase in MAP, which was blocked by the two doses of DuP 753 used (Table 2). To establish

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**Table 1. Effects of 6-Day and 20-Day Perindopril Treatment on Mean Arterial Pressure and Heart Rate in Conscious Rabbits**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>After 6-Day Treatment</th>
<th>After 20-Day Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle (n=5)</td>
<td>Perindopril (n=5)</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>1 mL/kg</td>
<td>1 mg/kg</td>
</tr>
<tr>
<td></td>
<td>104±6</td>
<td>83±4*</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>214±19</td>
<td>231±11</td>
</tr>
</tbody>
</table>

MAP indicates mean arterial pressure; HR, heart rate. Rabbits were treated for 6 and 20 days with either perindopril or vehicle (distilled water). Hemodynamic measurements were performed 24 hours after the last administration in conscious freely moving rabbits. Data were analyzed by one-way ANOVA. Post hoc test was the Newman-Keuls test. Values are mean±SEM.

*P<.01.
Fig 1. Line graphs show effects of oral administration of perindopril or its vehicle (distilled water, 1 mL/kg, n=5) on pressor response to intravenous injection of angiotensin I (500 ng/kg) in conscious rabbits. Top, Effects of perindopril (1 mg/kg, n=5; 2 mg/kg, n=5; 3 mg/kg, n=5) or its vehicle (n=5) after single administration. Middle, Effects of 6-day treatment with perindopril (1 mg/kg, n=5; 2 mg/kg, n=5) or its vehicle (n=5). Bottom, Effects of 20-day treatment with perindopril (2 mg/kg, n=7) or its vehicle (n=8). All perindopril doses acutely suppressed the pressor effect of angiotensin I significantly over 24 hours. Twenty-four hours after last drug administration of the 6- and 20-day treatments, pressor effect of angiotensin I was markedly reduced. This inhibitory effect was more pronounced with 2 mg/kg per day perindopril. MAP indicates mean arterial pressure. Data were analyzed by two-way ANOVA with repeated measures. Post hoc test was the Newman-Keuls test. Values are mean±SEM.

the effect of perivascular infusion of CGP 42112A on the AT1 receptor subtype, the same protocol was performed. CGP 42112A (1 mg per carotid artery) did not alter the pressor effect of intracarotid injection of Ang II (Table 2).

Histomorphometric Studies

Vascular injury of the carotid artery induced a neointima formation that was major 14 days after balloon catheterization. Perindopril (2 mg/kg per day) markedly suppressed neointima cross-sectional area (−52%, P<.01) and the neointima-media ratio (−51%, P<.01) (Figs 3 and 4). Media cross-sectional area was not changed by the ACE inhibitor.

In vehicle-treated groups, the presence of a delivery catheter at the site of injury compared with other routes of administration (ie, per os) significantly increased the cross-sectional area of the neointima without affecting the cross-sectional area of the media (Table 3). Consequently, the neointima-media ratio was greater in vehicle-treated groups with intraluminal catheters. Long-term intracarotid infusion of Ang II (500 ng/min) significantly enhanced neointima cross-sectional area (+35%, P<.05) and the neointima-media ratio (+39%, P<.05) compared with the control group with the intraluminal delivery catheter without affecting media cross-sectional area (Fig 5). Administered in the same conditions, bradykinin (500 ng/min, intracarotid infusion) had no effect on neointima and media cross-sectional areas or on the neointima-media ratio (Fig 5).

DuP 753 infused perivascularly at the site of denudation significantly decreased myointimal thickening (neointima cross-sectional area, −61%; neointima-media ratio, −60%). This treatment did not alter media cross-sectional area (Figs 3 and 4). In contrast, CGP 42112 administered in the same conditions (1 mg/d, perivascularly) had a marked effect on neointima but not on media cross-sectional areas (Fig 5).

Table 2. Effects of Perivascular Application of DuP 753 and CGP 42112A on Pressor Response to Angiotensin II Injection in Perfused Carotid Artery of Anesthetized Rabbits

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intracarotid Pressure Change, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>16±1</td>
</tr>
<tr>
<td>DuP 753 (42 µg)</td>
<td>3±2*</td>
</tr>
<tr>
<td>DuP 753 (1 mg)</td>
<td>1±1*</td>
</tr>
<tr>
<td>CGP 42112A (1 mg)</td>
<td>14±2</td>
</tr>
</tbody>
</table>

One hour after topical drug application, angiotensin II (500 ng) was injected into the lumen of perfused carotid arteries, and intracarotid pressure change was monitored (n=4 for each group). Data were analyzed by one-way ANOVA. Post hoc test was the Newman-Keuls test. Values are mean±SEM. *P<.01.
FIG 3. Photomicrographs show representative histological cross sections of rabbit carotid artery 14 days after balloon catheterization. Top left, injured carotid artery treated with vehicle; top right, treated with perindopril (2 mg/kg per day, PO); bottom left, treated with perivascular DuP 753 (1 mg per carotid artery per day); bottom right, treated with perivascular CGP 42112A (1 mg per carotid artery per day). Note strong reduction of neointimal area in carotid arteries from rabbit treated with perindopril and DuP 753. Bar, 100 μm.
vascular injury.

Data were analyzed by unpaired Student's t test. Values are mean±SEM. *P<.01. Cilazapril (30 mg/kg per day PO) was well tolerated by New Zealand White rabbits, which did not exhibit any signs of toxicity. At the time of balloon catheterization after 6 days of treatment, perindopril (2 mg/kg per day) significantly reduced MAP and suppressed ACE activity as assessed by the inhibition of the pressor action of Ang I. Thus, as in rats and guinea pigs, the RAS plays a major role in myointimal growth in rabbits. However, it is interesting to note that the magnitude of the proliferative response of the carotid artery is different between rats and rabbits, although the same procedure of balloon injury was applied. Indeed, the neointima-media ratio averaged 120% in rats,11,13,15,16 whereas it reached only 50% in rabbits. Moreover, the participation of the RAS in neointima formation might differ between these two species because despite the fact that perindopril inhibited ACE activity to the same extent and with the same time course in both species, the protective effect on myointimal thickening was not as marked in rabbits (−51%) as in rats (approximately 80%).

Neointima formation results mainly from three sequential events: proliferation, migration of vascular smooth muscle cells (VSMCs), and synthesis of extracellular matrix.

**Discussion**

The role of the RAS has been well characterized in the development of neointima induced by balloon injury in rats,1,13,15,16 but little was known in rabbits. Our findings indicate that in rabbits treated 6 days before and for 14 days after balloon catheterization, perindopril (2 mg/kg per day) significantly decreases neointima formation (−51%). Our results differ markedly from those reported by Clozel et al4 in which cilazapril used at the maximal tolerated dose did not prevent the vascular response to endothelial denudation, although this dose was high enough to reduce blood pressure and to inhibit plasma ACE activity almost completely. However, it is known from rat studies that the effective dose of cilazapril required for suppressing neointima formation (10 to 30 mg/kg per day PO) is higher than the antihypertensive dose,15 suggesting the implication of the local RAS in the genesis of this model. Taking this observation into account, Clozel and coworkers4 did not exclude the possibility that a more profound ACE inhibition could prevent this proliferative response in rabbits. In contrast with cilazapril, perindopril at 2 mg/kg per day PO was well tolerated by New Zealand White rabbits, which did not exhibit any signs of toxicity. At the time of balloon catheterization after 6 days of treatment, perindopril (2 mg/kg per day) significantly reduced MAP and suppressed ACE activity as assessed by the inhibition of the pressor action of Ang I. Thus, as in rats and guinea pigs, the RAS plays a major role in myointimal growth in rabbits. However, it is interesting to note that the magnitude of the proliferative response of the carotid artery is different between rats and rabbits, although the same procedure of balloon injury was applied. Indeed, the neointima-media ratio averaged 120% in rats,11,13,15,16 whereas it reached only 50% in rabbits. Moreover, the participation of the RAS in neointima formation might differ between these two species because despite the fact that perindopril inhibited ACE activity to the same extent and with the same time course in both species, the protective effect on myointimal thickening was not as marked in rabbits (−51%) as in rats (approximately 80%).

Neointima formation results mainly from three sequential events: proliferation, migration of vascular smooth muscle cells (VSMCs), and synthesis of extracellular matrix.

**Table 3. Comparison of Neointima Formation in Vehicle-Treated Groups With or Without Intraluminal Catheter in the Carotid Artery and Effect of Long-Term Intraluminal Infusion of Angiotensin II and Bradykinin on This Vascular Lesion**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Neointimal Cross-sectional Area, x1000 μm²</th>
<th>Medial Cross-sectional Area, x1000 μm²</th>
<th>Neointima-Media Ratio, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEH/IL−</td>
<td>12</td>
<td>138±15</td>
<td>343±15</td>
<td>41±5</td>
</tr>
<tr>
<td>VEH-Ang II/IL+</td>
<td>7</td>
<td>318±27*</td>
<td>341±32</td>
<td>100±14*</td>
</tr>
<tr>
<td>Ang II/IL+</td>
<td>8</td>
<td>431±44†</td>
<td>315±30</td>
<td>139±10†</td>
</tr>
<tr>
<td>VEH-BK/IL+</td>
<td>10</td>
<td>369±37</td>
<td>354±24</td>
<td>108±12</td>
</tr>
<tr>
<td>BK/IL+</td>
<td>7</td>
<td>427±37</td>
<td>366±23</td>
<td>119±13</td>
</tr>
</tbody>
</table>

*VEH/IL− indicates vehicle-treated group without intraluminal catheter; VEH-Ang II/IL+, group receiving vehicle of angiotensin II infused into injured carotid artery via intraluminal catheter; Ang II/IL+, angiotensin II-treated group via intraluminal catheter (500 ng/min); VEH-BK/IL+, group receiving vehicle of bradykinin infused into injured carotid artery via intraluminal catheter; and BK/IL+, bradykinin-treated group via intraluminal catheter (500 ng/min). Data were analyzed by unpaired Student's t test. Values are mean±SEM. *P<.01, VEH/IL− vs VEH-Ang II/IL+.
†P<.05, VEH-Ang II/IL+ vs Ang II/IL+.

**Fig 4.** Bar graphs show effects of perindopril (2 mg/kg per day PO, n=7), perivascular DuP 753 (1 mg/d, n=8), and perivascular CGP 42112A (1 mg/d, n=12) on neointima formation 14 days after balloon injury of rabbit carotid artery. Data were analyzed by unpaired Student's t test. Values are mean±SEM. *P<.01.

**Fig 5.** Bar graph shows effects of long-term intracarotid infusion of angiotensin II (500 ng/min, n=8), bradykinin (500 ng/min, n=7), and their respective vehicles on myointimal formation 14 days after balloon catheterization of rabbit carotid artery. Angiotensin II significantly enhanced neointima-media ratio, whereas bradykinin did not alter the extent of the vascular lesion. Data were analyzed by unpaired Student's t test. Values are mean±SEM. *P<.01.
ular matrix. Perindopril significantly increased cell density, indicating that the mechanism by which it inhibits the development of this vascular lesion involved the inhibition of proliferation and/or migration of VSMCs and also a reduction of matrix accumulation. Several mechanisms could be involved in the effect of perindopril on neointima because ACE does not cleave only Ang I but also other peptides, such as bradykinin. To elucidate whether these two pathways were necessary for perindopril to exert its beneficial effect, we studied the direct action of Ang II and bradykinin on myointimal growth. Fourteen-day infusion of Ang II at the site of injury enhanced neointima formation compared with control (+35%, P<.05), whereas bradykinin administered under the same conditions at a hypertensive dose (change in MAP: −20 mm Hg) did not alter this vascular response. Taken together, these results excluded a significant role of bradykinin in the protective effect of ACE inhibition on neointima formation in rabbits and differed from those obtained indirectly in rats with the use of a bradykinin antagonist.10,11 Indeed, in rats, Hoe 140, a B2 bradykinin antagonist, attenuated the effect of ACE inhibition on myointimal growth, suggesting that reduction in the breakdown of bradykinin is beneficial in limiting the extension of this lesion. However, this interpretation of this latter data has to be made with caution because long-term administration of Hoe 140 alone promoted neointima formation.12 This suggests either that Hoe 140 would behave as a partial agonist or that bradykinin would activate an antiproliferative pathway under basal conditions. Nonetheless, in rabbits, inhibition of Ang II formation appears to be the main mechanism by which ACE inhibitors act on this abnormal healing response. In rats, the mechanism of action of Ang II on myointimal thickening has been indicated to involve enhanced DNA synthesis in both neointima and media of denuded carotid arteries. However, differences between the hypertrophic and hyperplastic effects of Ang II have been reported in vitro in cultured VSMCs from different origins. For some investigators,12,13 Ang II possessed potent mitogenic properties or acted as a cofactor on proliferation, whereas for others,12,22,23 Ang II increased only protein synthesis. All these discrepancies might result from the ability of VSMCs to produce active transforming growth factor-β1 in response to Ang II. Indeed, Koibuchi et al24 indicated recently that cultured VSMCs responding with hypertrophy to Ang II expressed both transforming growth factor-β1 and basic fibroblast growth factor, whereas those responding with hyperplasia to Ang II synthesized only basic fibroblast growth factor. These data support the hypothesis of Dzau et al25 and Gibbons et al26 on a balance of proliferative and antiproliferative pathways controlling the growth of VSMCs.

In rat vasculature, two angiotensin receptor subtypes, AT1 and AT2, have been identified using the specific ligands DuP 753 and CGP 42112A, respectively.27,28 Although the prominent angiotensin receptor is the AT1 subtype in adult rats,29 we reported recently that both receptor subtypes were involved in the neointima formation induced by balloon injury.13 Our findings suggested that this excessive vascular repair could be linked to an increase in AT1 receptor activity. In the vasculature of adult rabbits, the proportion of AT2 receptors is very low (<10%) compared with AT1 receptors (>90%).30 However, the AT1-AT2 ratio might not be constant in rabbits and could be modulated with regard to physiological conditions, as has been already demonstrated in the myometrium31 and skin32 of other species. For these reasons we investigated the role of both AT1 and AT2 receptors in neointima formation. Periadventitial infusion of DuP 753 at the site of injury drastically inhibited myointimal thickening (−60%). Our data indicated that AT1 receptors played a major role in the pathogenesis of this lesion in rabbits, as previously reported in rats,12,13 but did not modify the neointimal cellularity, suggesting an inhibition of proliferation and/or migration of VSMCs (unpublished observations). The dose used (1 mg/d, perivascular route) was sufficient to block completely the vasoconstrictor effect of Ang II injected directly into the lumen of the carotid artery. In contrast, perivascular infusion of CGP 42112A (1 mg/d) did not alter the proliferative response to balloon catheterization or the pressor response to Ang II injected into isolated perfused carotid artery, even though in this latter case the daily dose of CGP 42112A (1 mg) was delivered topically at once instead of over 24 hours. The inefficacy of CGP 42112A suggested that AT1 receptors would not participate in the development of this lesion in rabbits, as opposed to rats, in which the same route of infusion of this selective AT2 ligand in the vicinity of the injured artery markedly suppressed neointima formation (approximately 75%).13 Finally, our findings showed that only AT1 receptors were more likely implicated in the vascular response to balloon injury, because long-term blockade of this Ang II receptor subtype produced the same reduction of neointima formation as ACE inhibition. Our results correlated well with other studies performed in rabbits regarding the role of the RAS in several pathophysiological models in which abnormal VSMC growth is the pathogenic factor. Thus, myointimal thickening resulting from autologous venous graft expressed only AT1 receptors in rabbits.33 Similar observations were made in the fibrous cap of atherosclerotic plaques of cholesterol-fed rabbits, in which the exclusive presence of the AT1 subtype has been reported,34 although long-term blockade of AT1 receptors by SC-51316 did not reduce plaque formation.35 The magnitude of the vascular response to balloon injury and the importance of the RAS in the development of this arterial lesion varied significantly within the same arteries of different species and also among arteries of the same species. It is important to emphasize that the difference of efficacy between perindopril and cilazapril on neointima formation might not result solely from a better tolerance of rabbits for perindopril but could also involve a greater biodisponibility and duration of action at the site of injury. So far, ACE inhibitors have failed to suppress myointimal proliferative lesions in response to injury in swine and baboon. Nonetheless, it would be interesting to establish whether the RAS is activated during neointima formation in these two species. If so, the identification of the mechanisms that counteract the potential benefit of ACE inhibition on myointimal thickening might be crucial to understanding the lack of efficacy of ACE inhibition on restenosis in humans.

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Role of the renin-angiotensin system in neointima formation after injury in rabbits.

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