AT₃ Receptor Antagonism Reduces Endothelial Dysfunction and Intimal Thickening in Atherosclerotic Rabbits

Natalia de las Heras, Paloma Aragoncillo, Rosaura Maeso, Sandra Vazquez-Pérez, Josefa Navarro-Cid, Marc DeGasparo, Jessica Mann, Luis M. Ruilope, Victoria Cachofeiro, Vicente Lahera

Abstract—The effects of angiotensin (AT)₁ receptor antagonists on functional and morphological alterations associated with atherosclerosis are not well known. The current study was performed to examine the long-term effects of valsartan (3 or 10 mg/kg per day for 10 weeks) on endothelial function and structural changes in aorta from rabbits fed with either a control diet or a cholesterol-enriched diet. Rabbits fed with the cholesterol-rich diet showed higher (P<0.05) plasma levels of cholesterol than did controls. Treatment with valsartan (3 or 10 mg/kg per day) did not alter plasma cholesterol levels or systolic arterial pressure in any group. Contractions induced by angiotensin II were comparable in both control and hypercholesterolemic rabbits and were markedly reduced by treatment with valsartan. Relaxations induced by acetylcholine were lower in hypercholesterolemic rabbits than in controls. Treatment with valsartan (3 or 10 mg/kg per day) enhanced (P<0.05) this response in hypercholesterolemic rabbits but not in controls. Lumen and media cross-sectional areas were comparable in control and hypercholesterolemic rabbits. Vessel area was higher (P<0.05) in hypercholesterolemic rabbits than in controls. Intimal lesion was 29.5±6% in cholesterol-fed rabbits and nonexistent in control rabbits. Treatments with 3 and 10 mg/kg per day valsartan reduced (P<0.05) intimal lesion to 2.4±0.7% and 2.7±0.9%, respectively, and increased lumen area in hypercholesterolemic rabbits. No changes in either vessel or media cross-sectional areas were observed in these animals. In summary, angiotensin II, through AT₁ receptors, appears to play a key role in the development of the vascular functional and structural changes associated with hypercholesterolemia. AT₁ receptor antagonists, besides their antihypertensive effects, could be an important therapeutic tool to reduce the development of atherosclerosis. (Hypertension. 1999;34[part 2]:969-975.)

Key Words: angiotensin II receptors, angiotensin II atherosclerosis hypercholesterolemia endothelium

Vascular endothelium regulates vascular function and structure through the release of numerous factors such as nitric oxide (NO), endothelin-1 (ET-1), arachidonic acid derivatives, reactive oxygen species, monocyte adhesion molecules, growth factors, coagulation and fibrinolytic agents, and so on. In the presence of hypertension, diabetes, or hypercholesterolemia, dysfunctional endothelial cells lack their homeostatic role and mediate the functional and structural alterations associated with these cardiovascular risk factors. Endothelial dysfunction produced by hypercholesterolemia has been characterized by reduced endothelium-dependent relaxations in both humans and experimental animals, suggesting a reduced availability of NO. The major determinant of this phenomenon appears to be related to elevated concentrations of oxidized LDL (ox-LDL). This has been shown to decrease the expression of NO synthase in endothelial cells and to stimulate the production of superoxide anions, which inactivates NO, leading to the formation of peroxynitrate. Oxidized LDL are likewise able to increase ET-1 and thromboxane (TX) A₂, which could also account for functional alterations. All these factors could also be involved in the morphological modifications of the vessel wall associated with the development of atherosclerosis.

In addition to endothelium-derived vasoactive agents, angiotensin II (Ang II) can be considered as a proatherogenic agent because it is able to stimulate most of the processes involved in the development of atherosclerosis. Furthermore, hypercholesterolemia, and especially oxidized LDL, has been reported to augment Ang II production through the enhancement of angiotensin-converting enzyme (ACE) activity. All these facts justify the beneficial effects of ACE inhibitors in atherosclerotic patients and animals. In addition, an upregulation of AT₁ receptor gene expression by LDL in vascular smooth muscle cells...
has been shown recently.\textsuperscript{17} However, the effects of AT\(_1\) receptor antagonists on functional and morphological changes associated with atherosclerosis are not well known. Consequently, the current study was performed to examine the long-term effects of the AT\(_1\) receptor antagonist valsartan on vascular reactivity and structural changes in aorta from rabbits fed with a cholesterol-enriched diet.

Methods

General Procedure

Forty-eight male New Zealand rabbits (Granja Cunicular San Bernardo, Navarra, Spain) initially weighing 1957±40 g were used for the study. The animals were maintained under controlled light and temperature conditions and fed with either a normal rabbit chow or a diet containing 1% cholesterol (UAR) for 10 weeks with free access to tap water. Rabbits of each diet group were treated with valsartan (3 or 10 mg/kg per day) given in the food for the same period. All experimental procedures were approved by the Animal Care and Use Committee of Universidad Complutense, according to the guidelines for ethical care of experimental animals of the European Community.

Arterial Pressure Measurement

On the first day of the experiment, arterial pressure was directly measured in the medial ear artery in awake rabbits through a catheter connected to a pressure transducer (model P23XL; Spectromed) and recorded on a polygraph (7E; Grass Instruments).

Biochemical Measurements

After arterial pressure measurement, blood samples were collected in prechilled glass tubes containing EDTA at a final concentration of 10\(^{-7}\) mol/L through the catheter inserted in the ear artery of the rabbits. Plasma cholesterol levels were measured by use of a colorimetric reaction with a commercial kit (Boehringer-Mannheim).

Vascular Reactivity

After blood samples were taken, the animals were anesthetized with sodium pentobarbital (25 mg/kg IV); the descending thoracic aorta was exposed through a midline incision and excised. Aortic rings were prepared as previously described.\textsuperscript{18} Contractile responses to KCl (80 mmol/L) were used as references to express contractions to other vasoconstrictor agents. Contractile responses induced by Ang II (10\(^{-7}\) mol/L), phenylephrine (PE, 10\(^{-7}\) to 10\(^{-5}\) mol/L), ET-1 (10\(^{-9}\) to 10\(^{-7}\) mol/L), and the TXA\(_2\) receptor agonist U46619 (10\(^{-8}\) to 10\(^{-6}\) mol/L) were also evaluated. The vasorelaxing response to the endothelium-dependent vasodilator acetylcholine (ACh, 10\(^{-8}\) to 10\(^{-5}\) mol/L) and the endothelium-independent vasodilator sodium nitroprusside (SNP, 10\(^{-10}\) to 10\(^{-7}\) mol/L) were also studied. The vasorelaxing response to the endothelium-dependent vasodilator acetylcholine (ACh, 10\(^{-8}\) to 10\(^{-5}\) mol/L) and the endothelium-independent vasodilator sodium nitroprusside (SNP, 10\(^{-10}\) to 10\(^{-7}\) mol/L) were studied in aortic rings from normocholesterolemic and hypercholesterolemic rabbits precontracted with a submaximal dose of PE (10\(^{-6}\) mol/L).

To evaluate the possible mechanisms underlying the effects of hypercholesterolemia and/or valsartan treatment on relaxing responses to ACh, aortic rings were incubated with (1) N\(^\text{-}\)nitro-L-arginine methyl ester (L-NAME) (10\(^{-5}\) mol/L) to evaluate the role of NO; (2) either superoxide dismutase (SOD) (10\(^{-4}\) mol/L), catalase (10\(^{-4}\) mol/L), or deferoxamine (10\(^{-4}\) mol/L) to study the possible involvement of superoxide anions, H\(_2\)O\(_2\), and hydroxyl radicals; (3) either the TXA\(_2\) receptor antagonist iletroban (10\(^{-4}\) mol/L) or the ET\(_A/ET\_B\) receptor antagonist PD 154 (10\(^{-5}\) mol/L) to study the involvement of TXA\(_2\) and ET-1.

Histology and Morphometric Analysis

Aortic segments were fixed in 35% sodium phosphate-buffered formaldehyde, processed, impregnated, and embedded in paraffin and cut into 3- to 4-μm sections by microtome. The sections were stained with hematoxylin and eosin, Mason’s trichrome, and orcein. Morphometric (quantitative) determination of the area of the intima, media, and the vessel was performed with a MICROM image analyzer (Hardware IMCO 10, Kontron Bildanalyse, Software Mi- crom IP) as previously described.\textsuperscript{19} Briefly, all microscopic images of the sections were recorded on videotape with a videocamera, and the histological sections were digitalized, segmented-colored, and traced for calculation of the areas. To determine the luminal area, the cross-sectional area enclosed by the internal elastic lamina was corrected to a circle by applying the form factor (I/A\(_{\text{int}}\)) to the measurement of the internal elastic lamina, where I is the length of the lamina. Vessel area was determined by the cross-sectional area enclosed by the external elastic lamina corrected to a circle, applying the same form factor (I/A\(_{\text{ext}}\)) to the measurement of the external elastic lamina. This method avoids miscalculations of areas caused by eventual collapse of aortic segments.\textsuperscript{20} It should be mentioned that both functional and morphological studies were performed by 2 different blinded operators who were not aware of the group to which the animals belonged.

Drugs

Valsartan was kindly supplied by Novartis Pharma. All other drugs used for vascular reactivity experiments were purchased from Sigma Chemical Co. Stock solutions of these drugs were prepared in distilled water and diluted to desired concentrations with Krebs solution immediately before the experiment. Concentrations are expressed as final molar concentration in the organ chamber. Drugs and chemicals for morphological studies were purchased from Merck AG.

Calculations and Statistical Analysis

For vascular reactivity studies, the contractile response was expressed as percentage of the reference constrictor response to 80 mmol/L KCl. For agents that elicit relaxation of PE-preconstricted aortic rings, response is expressed as percent reduction of tension in the preconstricted state. Results are expressed as mean±SEM of rings from 8 rabbits unless otherwise specified. Vascular reactivity dose-response curves were compared by multivariate ANOVA for repeated measures with the use of the Complete Statistical System (CSS) program (Stutoft Inc). All other data were analyzed by use of 1-way ANOVA followed by a Newman-Keuls test if differences were noted. The null hypothesis was rejected when the probability value was <0.05.

Results

Blood Pressure and Cholesterol Levels

Rabbits fed with the cholesterol-enriched diet for 10 weeks showed higher (P<0.05) plasma levels of cholesterol than did animals fed with a control diet (Table 1). The concomitant treatment with valsartan, either 3 or 10 mg/kg per day for 10 weeks, did not alter plasma cholesterol levels in any group (Table 1). Systolic arterial pressures were similar in both diet groups and were not affected by the treatment with any dose of valsartan used (Table 1).

Vascular Reactivity

Maximal contractions induced by Ang II were comparable in both control and experimental rabbits and were markedly reduced by treatments with both doses of valsartan to a similar extent (Figure 1). Dose-related contractions induced by PE, ET-1, or U46619 were comparable in both control and experimental rabbits (Table 1). In hypercholesterolemic rabbits and were not affected by treatment with valsartan (data not shown). Dose-related relaxations induced by ACh were lower in hypercholesterolemic rabbits than in controls (Figure 2). Treatment with valsartan, either 3 or 10 mg/kg per day, did not modify this response in
control rabbits. However, both doses of valsartan enhanced the response to ACh in hypercholesterolemic rabbits to a similar extent (Figure 2). Dose-related relaxations induced by SNP were comparable in both control and hypercholesterolemic rabbits (maximal response 87.6±2.7 vs 92.1±2.8, % of PE contraction; control and hypercholesterolemic rabbits, respectively) and were not affected by valsartan treatment.

Incubation of aortic rings with L-NAME (10⁻⁵ mol/L) blocked relaxations to ACh in all groups (data not shown), indicating that NO is the main factor accounting for endothelium-dependent relaxations in aortic rings from control and hypercholesterolemic rabbits. Incubation of aortic rings with SOD, catalase, or deferoxamine did not show any effect on ACh-induced relaxations (data not shown), either in control or hypercholesterolemic rabbits, untreated or treated with valsartan. This rules out the involvement of these reactive oxygen species in any of the observed effects of hypercholesterolemia or valsartan treatments. Incubation of aortic rings with ifetroban did not modify dose-related relaxations to ACh in control rabbits but increased this response in hypercholesterolemic rabbits, indicating an enhancement of TXA₂ in this latter group (Figure 3). Incubation of aortic rings with ifetroban did not enhance this response to ACh in control rabbits, either 3 or 10 mg/kg per day (Figure 3). In contrast, incubation of aortic rings with PD154 did not affect ACh-induced relaxations (data not shown), either in control or hypercholesterolemic rabbits, untreated or treated with valsartan, ruling out the involvement of ET-1 (data not shown).

**Histological Studies**

Lumen and media cross-sectional areas were comparable in control and atherosclerotic rabbits (Table 2). However, vessel area was higher (P<0.05) in atherosclerotic rabbits than in control rabbits (Table 2). Intimal lesion was calculated as the area encompassed by the internal elastic lamina minus lumen area. In cholesterol-fed rabbits, the lesion occupied 29.5±6% of the intimal surface and was nonexistent in control rabbits. Treatments with 3 and 10 mg/kg per day valsartan reduced (P<0.05) the intimal lesion (2.4±0.7% and 2.7±0.9%, respectively) and increased lumen area in hypercholesterolemic rabbits (Table 2). No changes in either vessel or media cross-sectional areas were observed in these animals. Representative photographs of cross sections of aortic segments are included in Figure 4.

**Discussion**

The current study shows that in the absence of changes in both arterial pressure and plasma cholesterol levels, the AT₁ receptor antagonist valsartan was able to enhance the diminished relaxing response to ACh induced by hypercholesterolemia in rabbit aorta. Moreover, the intimal thickening observed in hypercholesterolemic animals was also prevented by valsartan treatment. These results suggest an important role of Ang II, through AT₁ receptors, in the functional and morphological alterations produced by hypercholesterolemia.

In the current study, aortic rings from hypercholesterolemic rabbits presented a reduced response to ACh but not to an endothelium-independent agent such as SNP. In contrast, endothelium-dependent or endothelium-independent contrac tile responses to Ang II, PE, ET-1, and the TXA₂ agonist U46619 were not affected by hypercholesterolemia. Comparable results have been previously reported in experimental animals and humans, indicating that during hypercholesterolemia and during the early stages of atherosclerosis, the most common vascular functional alteration is a reduction of endothelium-dependent relaxation.⁶⁻⁸ This effect could rely on a diminished availability of NO as the result of an enhanced production of ox-LDL, which has been demonstrated to reduce the expression of NO synthase by endothelial cells.¹⁰ In contrast, reactive oxygen species do not appear

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**Table 1. Plasma Cholesterol Levels and Systolic Arterial Pressure in Rabbits Fed Either a Diet Containing 1% Cholesterol or a Control Diet, Untreated or Treated With Valsartan (3 or 10 mg/kg per day, 10 Weeks)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cholesterol, mmol/L</th>
<th>Systolic Arterial Pressure, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.95±0.17</td>
<td>108±1</td>
</tr>
<tr>
<td>Control + valsartan, 3 mg/kg per day</td>
<td>1.01±0.40</td>
<td>104±3</td>
</tr>
<tr>
<td>Control + valsartan, 10 mg/kg per day</td>
<td>0.81±0.20</td>
<td>104±2</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>48.10±3.90</td>
<td>107±1</td>
</tr>
<tr>
<td>Cholesterol + valsartan, 3 mg/kg per day</td>
<td>42.80±2.32*</td>
<td>105±2</td>
</tr>
<tr>
<td>Cholesterol + valsartan, 10 mg/kg per day</td>
<td>43.62±1.42*</td>
<td>107±1</td>
</tr>
</tbody>
</table>

*P<0.05 compared with control rabbits.

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**Figure 1.** Contractile responses to Ang II (10⁻⁷ mol/L) in aortic rings from rabbits fed either a diet containing 1% cholesterol (CHO) or a control (C) diet, untreated or treated with valsartan (VAL, 3 or 10 mg/kg per day; 10 weeks). Values are mean±SEM of rings from 8 rabbits. *P<0.05 vs control.
to be involved in the diminished response to ACh observed in hypercholesterolemic rabbits because incubation of aortic rings with either SOD, catalase, or deferoxamine did not affect this relaxing response in any group. However, endogenous TXA₂ could have contributed to the reduced ACh relaxations because incubation of aortic rings with ifetroban enhanced this response in hypercholesterolemic rabbits. This is supported by a previous report showing that ox-LDL were able to stimulate TXA₂ by endothelial cells. In contrast, ET-1 does not appear to be involved in the diminished response to ACh because incubation of aortic rings with the ETA/ETB receptor antagonist PD 154 did not affect this response.

Another alteration that could contribute to the observed reduced relaxing response to ACh in hypercholesterolemic rabbits is the intimal thickening, which constitutes a physical barrier, preventing NO from reaching smooth muscle cells. Proliferation of cellular components, especially foam cells and smooth muscle cells migrating from media to subintimal space, could have contributed to intimal thickening. In agreement with previous reports, the study shows that despite the intimal thickening, a reduction in aortic lumen was not observed. This probably was due to an outward displacement of the vessel wall, which preserved the lumen from narrowing and was demonstrated by the increase in the vessel cross-sectional area, together with the maintenance of lumen area.

The results also show that the diminished relaxing response to ACh observed in hypercholesterolemic rabbits was en-

Figure 2. Line graph illustrating vasorelaxations induced by ACh (10⁻⁹ to 10⁻⁵ mol/L) in aortic rings precontracted with a submaximal dose of PE (10⁻⁶ mol/L) from rabbits fed with either a diet containing 1% cholesterol (CHO) or a control (C) diet. Values are mean±SEM of rings from 8 rabbits. *P<0.05 vs control; #P<0.05 vs CHO. Left panel, effect of treatment with 3 mg/kg per day valsartan (VAL) for 10 weeks; right panel, effect of treatment with 10 mg/kg per day valsartan for 10 weeks.

Figure 3. Line graph illustrating vasorelaxations induced by ACh (10⁻⁹ to 10⁻⁵ mol/L) in PE-precontracted aortic rings incubated or not incubated beforehand with the TXA₂ receptor antagonist ifetroban (10⁻⁶ mol/L) from rabbits fed with either a diet containing 1% cholesterol (CHO) or a control (C) diet. Values are mean±SEM of rings from 8 rabbits. *P<0.05 vs control; #P<0.05 vs CHO. Left panel, groups without valsartan treatment; right panel, groups treated with 10 mg/kg per day valsartan for 10 weeks. Results in rabbits treated with 3 mg/kg per day valsartan were comparable to those with the higher dose.
hanced by valsartan treatments. As expected, this effect was accompanied by a marked reduction of Ang II–induced contractions and suggests an important role of Ang II through AT₁ receptors in the endothelial dysfunction produced by hypercholesterolemia. In addition, it should be mentioned that either dose of valsartan produced a similar effect on ACh-induced relaxation, probably because the dose of 3 mg/kg per day produced the maximal effect possible on this parameter. Inhibition of several mechanisms activated by Ang II through AT₁ receptors, such as direct vasoconstriction or facilitation of sympathetic activity, could account for the observed amelioration of ACh relaxation in hypercholesterolemic rabbits. Inhibition of TXA₂ also could have contributed to the observed enhancement of ACh relaxations in hypercholesterolemic rabbits treated with valsartan. This notion is supported by the previous report showing that Ang II stimulates TXA₂ through the activation of AT₁ receptors. ¹² In the current study, the enhancement of ACh relaxation produced by the incubation with ifetroban in aortic rings from hypercholesterolemic rabbits was not observed in the animals treated with valsartan. This might indicate that AT₁ antagonism with valsartan could have reduced TXA₂ availability, and consequently incubation of aortic rings with ifetroban did not show any further effect. In contrast, neither inhibition of ET-1 or reactive oxygen species appear to be involved in the amelioration of ACh relaxations produced by valsartan in hypercholesterolemic rabbits. Increased NO availability also could have contributed to the observed amelioration of ACh relaxations in hypercholesterolemic rabbits treated with valsartan, although we do not have direct evidence of this notion. In previous studies in spontaneously hypertensive rats, we found that prolonged treatment with different AT₁ receptor antagonists enhanced ACh-induced relaxations in aortic, mesenteric, and renal vasculature from adult and senescent spontaneously hypertensive rats. ²⁷⁻³⁰ This improvement was mainly attributed to an enhancement of NO availability, and it was postulated that this could also involve the participation of AT₂ receptors. ³¹⁻³³

The reduction of the intimal lesion could have accounted for the observed improvement of endothelium-dependent relaxation in the hypercholesterolemic rabbits treated with valsartan. As was mentioned above for ACh relaxation, both doses of valsartan reduced intimal thickening to a similar extent, suggesting that the low dose of valsartan was enough to prevent the structural changes produced by a cholesterol-rich diet. Ang II can be considered as an important proatherogenic agent. ³⁴⁻³⁵ Although we do not have direct evidence from the current study, inhibition of several mechanisms activated by Ang II through AT₁

<table>
<thead>
<tr>
<th>Group</th>
<th>Vessel Area, mm²</th>
<th>Media Area, mm²</th>
<th>Lumen Area, mm²</th>
<th>Lesion Area, mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.44±0.69</td>
<td>2.17±0.17</td>
<td>4.64±0.37</td>
<td>…</td>
</tr>
<tr>
<td>Control+valsartan, 3 mg/kg per day</td>
<td>6.98±0.11</td>
<td>1.81±0.08</td>
<td>5.36±0.17</td>
<td>…</td>
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<tr>
<td>Control+valsartan, 10 mg/kg per day</td>
<td>7.60±0.30</td>
<td>2.25±0.14</td>
<td>5.64±0.31</td>
<td>…</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>8.26±0.20*</td>
<td>2.02±0.39</td>
<td>4.52±0.46</td>
<td>1.33±0.14</td>
</tr>
<tr>
<td>Cholesterol+valsartan, 3 mg/kg per day</td>
<td>8.29±0.77*</td>
<td>1.96±0.15</td>
<td>6.15±0.65*</td>
<td>0.16±0.07†</td>
</tr>
<tr>
<td>Cholesterol+valsartan, 10 mg/kg per day</td>
<td>8.23±0.47*</td>
<td>2.19±0.18</td>
<td>5.9±0.29*</td>
<td>0.14±0.09†</td>
</tr>
</tbody>
</table>

Values are mean±SEM of 8 rabbits.  
*P<0.05 compared with control rabbits; †P<0.05 compared with cholesterol rabbits.

Figure 4. Representative microphotographs (original magnification ×12.5) of cross sections of aorta from hypercholesterolemic untreated (A) or valsartan-treated (B, 3 mg/kg per day; C, 10 mg/kg per day) rabbits.
receptors could theoretically account for the observed reduction of the atherosclerotic lesion. Ang II through AT1 receptors stimulates modification of LDL.10,36 Ang II activates the transcription nuclear factor-xR, which promotes the expression of monocyte adhesion and chemotractant molecules (vascular cell adhesion molecule-1, monocyte chemotactic protein-1, and monocyte colony stimulating factor), enhancing monocyte adhesiveness to endothelium and their penetration to the subintimal space.37 Once monocytes are activated to macrophages, Ang II stimulates the expression of scavenger receptors in the cell membrane, facilitating the formation of foam cells. Furthermore, Ang II stimulates smooth muscle cell proliferation and migration from the media layer to the subintima.38,39 Consequently, the antagonism of these AT1-mediated effects of Ang II could have contributed to the observed reduction of atherosclerotic lesion in hypercholesterolemic rabbits. Finally, all these results further support the usefulness of drugs blocking the renin-angiotensin system (ACE inhibitors and AT1 receptor antagonists) to ameliorate the functional and structural vascular alterations associated not only with hypertension but with atherosclerosis.15,16,40

In summary, the current study suggests that Ang II, through AT1 receptors, plays a critical role in the development of the vascular functional and structural alterations associated with hypercholesterolemia. Furthermore, AT1 receptor antagonists, besides their antihypertensive effects, could be important therapeutic tools to reduce the development of the atherosclerotic process.

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