Increased Angiotensin II Type 1 Receptor Expression in Hypercholesterolemic Atherosclerosis in Rabbits

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Abstract—Angiotensin II (Ang II) promotes vascular smooth muscle growth and may be involved in the initiation and progression of atherosclerosis. To examine whether Ang II receptor expression in vascular tissues is altered in atherosclerosis, male New Zealand White rabbits were fed a high-cholesterol diet (1% cholesterol + 4% coconut oil mixed with regular chow; hypercholesterolemic group, n = 12) or regular chow (control group, n = 8) for 10 weeks. At the end of this period, the serum cholesterol level in the rabbits fed the high-cholesterol diet was higher than that in the control group (3616 ± 144 versus 30 ± 1 mg/dL, P < 0.001). There was no atherosclerosis in the aortas of the control group, whereas 51 ± 6% of the aorta was covered with atherosclerosis in the hypercholesterolemic group. Total Ang II receptor expression in the atherosclerotic aortic tissues was increased 5-fold in the hypercholesterolemic rabbits (292 ± 28 versus 51 ± 32 fmol/mg tissue, mean ± SE, P < 0.001), and the increased Ang II receptor expression was entirely due to enhanced Ang II type 1 (AT1) receptor expression (289 ± 38 versus 38 ± 18 fmol/mg, P < 0.001), as Ang II type 2 receptor expression was unaltered (7 ± 5 versus 3 ± 2 fmol/mg, P = NS). AT1 receptors were localized primarily in the media and to some extent in the intima of the atherosclerotic aorta, as determined by immunohistochemistry with specific monoclonal and polyclonal AT1 receptor antibodies. Increased synthesis of AT1 receptor mRNA in atherosclerotic tissues was confirmed by reverse transcription–polymerase chain reaction. To evaluate the functional significance of increased AT1 receptor expression, the constrictor response of aortic rings to Ang II was examined and found to be markedly enhanced in atherosclerotic aortic rings (P < 0.01 versus control aortic rings). The endothelium-dependent relaxation of aortic rings from hypercholesterolemic rabbits was markedly attenuated (P < 0.001). This study shows that hypercholesterolemia in rabbits results in atherosclerosis, loss of endothelium-dependent relaxation, and increased Ang II receptor (entirely AT1 receptor) expression in aortic tissues, which may result in altered vasoreactivity.


Key Words: aorta ■ atherosclerosis ■ angiotensin II ■ endothelium ■ hypercholesterolemia

A ngiotensin II (Ang II) is a potent vasoactive peptide that produces systemic and local vasoconstriction.1 Relatively recent studies indicate that Ang II also has direct cellular effects on the pathobiology of vascular disease through growth-promoting activity. At least 2 distinct Ang II receptor subtypes, type 1 and type 2 (AT1 and AT2, respectively), have been identified.1 Binding of Ang II to its target AT1 in vascular tissues activates phospholipase C; raises intracellular calcium; activates nuclear elements that affect gene expression; and stimulates protein synthesis, mitogenesis, or hypertrophy.2,3 These phenomena are observed in quiescent vascular smooth muscle cells incubated with Ang II.4 In vascular smooth muscle cells, Ang II has been shown to activate fibroblast growth factor, platelet-derived growth factor, and transforming growth factor-β1.4,5 Ang II also mediates vascular smooth muscle cell growth and migration, monocyte/macrophage activation, and platelet activation and stimulates atherogenesis.6 Rakugi et al7 demonstrated induction of the angiotensin-converting enzyme (ACE) in the neointima of injured blood vessels and speculated on its possible role in restenosis after angioplasty. Viswanathan et al8 described increased Ang II receptor expression in the neointima after vascular injury, which would facilitate the action of Ang II. ACE has also been found in vascular smooth muscle cells in intimal lesions and in macrophages and vascular smooth muscle cells in the fibroproliferative lesion of human atherosclerotic plaques from atherectomy, surgical, and postmortem specimens.8 It is, therefore, possible that excessive Ang II synthesis, activity, or receptor expression contributes to the initiation or progression of atherosclerosis.

Abnormally high serum cholesterol levels are thought to be an important pathogenic factor in atherogenesis.9 An inherited form of hypercholesterolemia leads to early atherosclerosis in rabbits.10 Feeding of a high-cholesterol diet to rabbits often results in hypercholesterolemia and atherosclerosis.11 Al-
though the extent of atherosclerosis in different blood vessels of rabbits fed a high-cholesterol diet varies significantly, this model has been used extensively to study pathogenetic aspects of atherosclerosis and its modulation by various agents.\textsuperscript{1–13} The current study was designed to examine Ang II receptor expression in vascular tissues and its relationship with vasoreactivity and atherogenesis in a rabbit model of atherosclerosis induced by feeding of a high-cholesterol diet.

Methods

Hypercholesterolemic Rabbits
Male New Zealand White rabbits (\(~1\) kg weight) were housed individually under temperature-controlled conditions. During the 2-week period of adaptation, the rabbits were fed standard rabbit Chow and water ad libitum. Food intake was assessed on a daily basis. After stabilization, the rabbits were randomly assigned to 1 of the 2 regimens for 10 weeks: (1) standard rabbit Chow (control group, \(n=8\)) or (2) cholesterol-enriched rabbit Chow (1% cholesterol plus 4% coconut oil mixed with regular chow; high cholesterol–diet group, \(n=12\)). Only male rabbits were used to avoid the variability secondary to sex differences in this experimental model. A rabbit Chow containing 1% cholesterol and 4% coconut oil diet was chosen because other investigators have demonstrated that when given for a period of 10 to 12 weeks, it results in marked elevation in serum cholesterol and induction of diffuse, aortic atherosclerosis.\textsuperscript{11–13}

At the end of the 10-week dietary intervention, food was withdrawn for 12 hours, and the rabbits were weighed and then anesthetized with intravenous sodium pentobarbital (50 mg/kg). Venous blood samples were obtained for measurement of serum cholesterol, and aortas were excised for examination of atherosclerotic area (aorta), Ang II receptor binding, and determination of vasoreactivity. The total RNA was extracted from aortas from treated and untreated animals and analyzed by reverse transcription–polymerase chain reaction (RT-PCR). The study was approved by the appropriate animal research committees of the University of Florida and complied with the American Physiological Society guidelines.

Determination of Serum Cholesterol
Serum was analyzed for total cholesterol by an automatic analyzer at a commercial laboratory (Doctors’ Laboratory, Valdosta, Ga).

Determination of the Extent of Atherosclerosis

After dissection, the entire aorta (except the ascending part) was removed, opened longitudinally, and prepared for detection and quantification of areas of sudanophilia as directed by Holman et al.\textsuperscript{14} The aortic strips were immersed in 10% buffered formalin solution for 24 hours and then rinsed briefly in 70% alcohol. The tissues was immersed in Herxheimer’s solution that contained 5 g Sudan IV, 500 mL of 70% ethyl alcohol, and 500 mL acetone at room temperature for 15 minutes. The aortas were then transferred to 80% alcohol for 20 minutes, washed in running water for 1 hour, and then mounted and photographed. Aortic atherosclerosis was determined by planimetry of the distribution of sudanophilia in the photographs and expressed as percent of aortic area.

AT\(_1\) Receptor mRNA Expression by RT-PCR

Total RNA was isolated from aortas of control and high-cholesterol diet-fed rabbits by the guanidinium thiocyanate–phenol-chloroform method.\textsuperscript{22} The quality of isolated RNA was checked by gel electrophoresis. For this purpose, 2 \(\mu\)g of total RNA was denatured in a 50% formamide and 20% formaldehyde mixture for 10 minutes at 65°C. Denatured RNA was fractionated by gel electrophoresis on a 1% agarose gel containing 10% formaldehyde and examined under UV light after being stained with ethidium bromide. Five micrograms of the total RNA was digested with DNase I (RNase free) for 10 minutes at 37°C in the presence of 5 U of RNase inhibitor. After heat inactivation of DNase, RNA was reverse-transcribed for 50 minutes at 42°C with SuperScript II reverse transcriptase (Gibco-BRL) with oligo(dT) as the primer (Promega) in a 20-\(\mu\)L volume reaction mixture. The reaction was stopped by heating the samples for 15 minutes at 70°C. Ten percent of the single-stranded cDNA was used a template for amplification in the PCR with the use of Taq polymerase (Gibco-BRL). Primers were designed on the basis of the Ang II AT\(_1\) receptor sequence...
cloned from the rabbit kidney cortex. The sense primer sequence was 5' -TTTGGGAACAGCTTGGCGGT-3'; reverse primer was 5' -GCCAGCCAGCAGCCAAATAA-3'. After 5 minutes at 94°C, amplification was performed at 94°C for 45 seconds, 55°C for 45 seconds, and 68°C for 60 seconds for 40 cycles with a final incubation at 68°C for 7 minutes. Eight microliters of the PCR mixture was separated on a 1% agarose gel stained with ethidium bromide.

Statistical Analysis
All data are expressed as mean±SEM. Means of different groups were compared by ANOVA followed by Newman-Keuls test for paired and unpaired observations. A P value <0.05 was considered significant.

Results
High-Cholesterol Diet, Hypercholesterolemia, and Atherosclerosis
In the high cholesterol–diet group, 2 of 12 rabbits died of a lung infection at week 2. There were no deaths in the control group. At the end of the 10-week period, body weight was similar in the control (2.95±0.05 kg, n=8) and high cholesterol–diet (2.74±0.12 kg, n=10) groups. Serum cholesterol level in the high cholesterol–diet group was markedly higher than in the control group (3616±144 versus 30±1 mg/dL, P<0.001). The control group showed no atherosclerosis, whereas the high cholesterol–diet group showed 51±6% of the aorta covered with atherosclerosis (P<0.01 versus control).

Reactivity of Aortic Rings in Response to NE, Ang II, and ACh
The contractile response of aortic rings to Ang II and NE is shown in Figure 1. Aortic ring contraction in response to Ang II was markedly increased in all atherosclerotic rabbits compared with control rabbits (P<0.01). The greater contraction was seen in rings from all rabbits fed the high-cholesterol diet. The contractile response of aortic rings to NE was also greater in rings from hypercholesterolemic rabbits than in those from normal-cholesterol rabbits (P<0.01).

On exposure to the endothelium-dependent vasorelaxant ACh, aortic rings from control rabbits exhibited a typical concentration-dependent relaxation. In contrast, aortic rings from 4 hypercholesterolemic rabbits did not show any relaxation, and rings from the remaining hypercholesterolemic rabbits exhibited only modest relaxation in response to low concentration of ACh (10^{-7} mol/L) and a modest degree of contraction in response to a higher concentration of ACh (10^{-6} mol/L), a phenomenon not observed in any of the rabbits fed regular chow. The rightward shift in the ACh dose-response curve was highly significant (P<0.001). These data on the relaxant reactivity of aortic rings in response to ACh are also summarized in Figure 1.

Ang II Receptor Expression in Aortic Tissues by Autoradiography
Ang II receptor expression in aortic tissues was determined by autoradiography. Total Ang II receptor expression in the aortic tissues was several-fold higher in the hypercholesterolemic rabbits than in the control group (P<0.001). The increase in Ang II receptor expression was entirely due to an increase in AT_1 expression in the hypercholesterolemic rabbits (P<0.001), as AT_2 expression in the aortic rings was similar in the 2 groups. A representative autoradiogram is shown in Figure 2, and data from control and hypercholesterolemic rabbits are summarized in Figure 3. Although the precise localization of increased AT_1 receptor density could not be discerned from the autoradiographs, expression appeared to be predominantly in the media and intima.

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Contractile responses of rabbit aortic rings to NE and Ang II and relaxant responses of rabbit aortic rings to ACh. Contractile response of aortic rings was increased in hypercholesterolemic rabbits compared with control rabbits (P<0.01). Relaxant response of aortic rings to ACh was decreased in hypercholesterolemic rabbits (both P<0.01 versus control rabbits). Each data point is mean (±SEM) of 3 to 10 observations in different rabbits.

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Representative autoradiographs of aortic rings from control and hypercholesterolemic rabbits. Note marked increase in total Ang II receptor expression in hypercholesterolemic rabbit aortic segment. Increase in Ang II receptor expression was entirely due to an increase in AT_1 receptor expression, as AT_2 receptor expression was unaffected.
Immunohistochemical Localization of AT1 Receptors

AT1 receptor expression was localized in aortic tissues from hypercholesterolemic rabbits as well as from the control group by immunohistochemical staining. With a monoclonal antibody, AT1 receptor expression was predominantly in the smooth muscle layers, although there was immunopositivity in the intima as well. AT1 receptors were present in both the cytoplasm and cell membranes. Representative photomicrographs showing the distribution of immunopositivity are shown in Figure 4. With the use of a polyclonal antibody, expression of AT1 receptors was heavily concentrated in the media and to a much smaller extent in the endothelial lining (Figure 5). Although the immunocytochemical technique is not quantitative, the higher density of AT1-like receptors in the hypercholesterolemic aortas was striking.

Evidence for Increased AT1 mRNA by RT-PCR

Using AT1-specific primers, we amplified an expected size band of 341 bp (Figure 6). The aortic AT1 receptor mRNA level of the high-cholesterol group was markedly increased compared with the control group, wherein AT1 receptor mRNA was almost undetectable (Figure 6).

Discussion

Supplementation of the diet with cholesterol resulted in marked hypercholesterolemia, atherosclerosis, and increased Ang II receptor expression in the aortic tissues in this study. The enhanced Ang II receptor expression was almost entirely due to an increase in AT1 expression, which may have significant implications in atherogenesis and its treatment. The biological significance of enhanced AT1 receptor expression was evident from increased constrictor response of aortic rings to Ang II.

Abnormally high serum cholesterol levels are thought to be an important pathogenic factor in atherosclerosis.10 There is a positive correlation between total serum cholesterol and coronary mortality rates.24 In addition, there is strong clinical evidence that lowering the total serum cholesterol level...
The decrease in NO synthesis or an increase in its degradation decreases the incidence of cardiac events in patients with preexisting coronary artery disease and others at high risk of developing coronary artery disease and retards progression of symptomatic atherosclerosis. In the current study, feeding of a high-cholesterol diet to rabbits for 10 weeks resulted in marked hypercholesterolemia and development of atherosclerosis, which is consistent with several previous reports. These findings confirm an important role for hypercholesterolemia in atherogenesis.

Endothelium-derived relaxing factor (EDRF), most likely NO or a closely related compound, relaxes vascular smooth muscle and inhibits oxidative modification of LDL, cell adhesion, vascular smooth muscle proliferation, and platelet aggregation. Via release of EDRF/NO and prostacyclin, the vascular endothelium plays an important role in preventing the evolution of atherosclerosis. Several investigators have documented the involvement of endothelial dysfunction in atherosclerosis. The impaired EDRF-dependent vasodilatation in hypercholesterolemia and atherosclerosis has been documented in both rabbit and human arteries. The current study confirms that a high-cholesterol diet results in impaired relaxation of arterial tissues in response to the endothelium-dependent vasorelaxant ACh. The decreased relaxation in response to ACh implies a decrease in NO synthesis or an increase in its degradation.

An interesting and novel observation described in this report pertains to enhanced contraction of aortic tissues from hypercholesterolemic rabbits in response to Ang II. The contractile response to Ang II was enhanced in hypercholesterolemic rabbits compared with the controls. The increased contractile response of aortic rings paralleled the increase in Ang II receptor expression. We found that aortic ring contractile response to NE was also increased in hypercholesterolemic rabbits. It is possible that the greater contractile response to Ang II and NE is a manifestation of attenuated NO activity in these rings. It is also possible that hypercholesterolemia per se enhances contractile responses to multiple agonists, such as NE and thrombin. Hof et al have also shown an enhanced contractile response to Ang II as well as to NE in atherosclerosis. A recent study has shown that Ang II induces transcription and expression of α1-adrenergic receptors in vascular smooth muscle cells. This mechanism may also underlie the greater contractile response to NE in atherosclerosis. Dusting et al, however, could not find any difference in vasoconstrictor response to Ang II in control and hypercholesterolemic rabbits, but these investigators did not report on the extent of atherosclerosis and Ang II receptor expression. The variable response to vasoconstrictors may relate to differences in Ang II receptor expression and the extent of atherosclerosis in different blood vessels. It is possible that extensive plaque formation and distorted smooth muscle architecture in the atherosclerotic aorta leads to altered vasoconstriction. Notably, treatment with ACE inhibitors has been shown to alter vasoconstriction in an experimental setting and to decrease the facilitatory influence of Ang II on sympathetic stimulation. Because it is the AT1 receptor activation that results in vasoconstriction in response to Ang II, we believe that the upregulation of AT2 receptor number and affinity for the agonist accounts for the greater contractile response to Ang II in blood vessels from hypercholesterolemic animals observed in the current and other studies.

Direct evidence for increased AT1 receptor expression came from autoradiographic studies in rabbit aortas. We found that Ang II receptor expression was dramatically increased in aortic tissues from hypercholesterolemic rabbits. The increase in Ang II receptor expression was entirely due to an increase in AT1 expression, because AT2 expression was unaltered. Nickenig et al have recently shown that Ang II receptor gene expression is upregulated in rat aortic vascular smooth muscle cells by LDL.

Ang II receptor expression has been demonstrated in the neointima of experimentally injured vessels and vascular smooth muscle cells of human atherosclerotic plaques. The critical role of tissue Ang II receptor expression and activation in the pathobiology of intimal hyperplasia is supported by the observation of prevention of neointimal hyperplasia in response to vascular injury and atherosclerosis by ACE inhibitors and AT1 receptor antagonists. The protective effects of the AT1 receptor antagonists SR 47436 and losartan on Ang II–mediated proliferation of cultured human vascular smooth muscle cells after ischemic injury have been reported. It is noteworthy that the proliferation of vascular smooth muscle cells in these studies was not affected by the AT2 receptor antagonist PD 123,177. Weber et al showed that Ang II increased mitogenicity, intracellular calcium, phosphoinositol metabolism, and phosphorylation of a specific substrate for protein kinase C in cultured rat aortic smooth muscle cells and that the effect of Ang II was blocked by the AT1 receptor blocker losartan. Makita et al showed that AT1 receptor blockade with losartan blocked Ang II–stimulated as well as basal proliferation of human vascular smooth muscle cells. Collectively, these observations suggest that the AT1 receptor is expressed in large amounts in atherosclerotic tissues and probably participates in atherogenesis.

Ang II has cellular growth–promoting activity. In vascular smooth muscle cells, Ang II has been shown to activate fibroblast growth factor, platelet-derived growth factor, and transforming growth factor-β1. The endothelium is an important factor in modulating smooth muscle function and growth, and the presence of normal endothelium has been
shown to inhibit Ang II–induced growth-promoting effects.\textsuperscript{45,46} The endothelium-produced vasodilator substances, such as prostacyclin and NO, which have growth-inhibitory actions, help maintain vascular ultrastructure. In conditions associated with diminished synthesis of NO or excessive degradation of NO, the proliferative effect of Ang II is favored, and growth of vascular smooth muscle cells may result.\textsuperscript{47} Recent studies have shown that Ang II via AT\textsubscript{1} receptor activation enhances vascular superoxide formation via membrane NADH/NADPH oxidase activation and contributes to the increase in vascular tone.\textsuperscript{48} Other studies have documented an inhibitory effect of AT\textsubscript{1} receptor activation on NO synthetase mRNA and restoration of mRNA expression and NO-mediated vasorelaxation with blockade of AT\textsubscript{1} receptors.\textsuperscript{49,50} These studies provide a conceptual basis for the relationship between increased AT\textsubscript{1} expression and loss of NO-dependent relaxation in the presence of atherosclerosis.

The autoradiographic technique used in the current study does not permit precise localization of the Ang II receptors in the cytoplasm or the cell membrane. It is known that Ang II receptor sites are located mostly on the vascular smooth muscle cell membrane.\textsuperscript{51} Zambetis et al\textsuperscript{52} studied Ang II binding sites in rabbit arteries and found the binding sites to be dispersed throughout the media, with the highest levels of binding in the outer media as well as in the extra-adventitial inflammatory tissue of the atherosclerotic arteries. The immunohistochemical staining with a specific monoclonal antibody to AT\textsubscript{1} receptors in our study indicated that AT\textsubscript{1} receptor expression was predominantly in the media and to a small extent in the intima. Additional evidence from immunohistochemistry with a polyclonal antibody to AT\textsubscript{1} receptors also indicated that the enhanced AT\textsubscript{1} receptor expression was localized primarily in the medial layers of atherosclerotic aortas. It is possible that the immunostaining represented, at least in part, expression of AT\textsubscript{1} receptors in macrophages, monocyte-derived smooth muscle cells, or both.

The monoclonal antibody to the AT\textsubscript{1} receptor expression used in the current study was produced in Professor Vinson’s laboratory. This antibody was produced by immunization of BALB/c mice with synthetic peptides representing sequences from the extracellular domain (residues 8 to 17) or the intracellular domain (residues 229 to 237) of the AT\textsubscript{1} receptor. Initial characterization of the antibody showed specific immunofluorescence of vascular endothelium.\textsuperscript{19} This group subsequently demonstrated that the monoclonal antibody (6313/G2) to the AT\textsubscript{1} receptor is specifically directed against the N-terminal extracellular domain of the mammalian AT\textsubscript{1} receptor.\textsuperscript{53,54} This monoclonal antibody (6313/G2) to the AT\textsubscript{1} receptor has since been used by other investigators to identify AT\textsubscript{1} receptor expression in brain neurons.\textsuperscript{55,56} The polyclonal antibody was produced in our laboratory after immunization of rats with synthetic peptides, which represent amino acid sequence N-terminal 14–23 residues of the first extracellular domain of the AT\textsubscript{1} receptor and was anchored to the polylysine cores to form the multiple antigenic peptides. The specificity of the antibody was checked by Western blotting. A 65-kDa protein fraction was specifically selected by the antibody. This is the correct molecular weight of the mature, glycosylated AT\textsubscript{1} receptor.\textsuperscript{21}

We conducted RT-PCR to assess whether the increased AT\textsubscript{1} expression was due to increased AT\textsubscript{1} receptor mRNA. As shown in Figure 6, we found a marked increase in AT\textsubscript{1} receptor mRNA in aortic tissues of rabbits fed the high-cholesterol diet. In contrast, there was almost no detectable AT\textsubscript{1} receptor mRNA in aortic tissues of rabbits fed the control diet. There is preliminary evidence that ACE levels in atherosclerotic regions of hypercholesterolemic rabbits are significantly reduced,\textsuperscript{57} and it is conceivable that the reduction in tissue Ang II upregulates Ang II receptor expression. The finding of markedly increased AT\textsubscript{1} receptor expression in arteries from hypercholesterolemic animals is important for understanding the development and potential control of atherosclerotic plaque. A marked increase in AT\textsubscript{1} receptor expression may be the primary basis of an Ang II–mediated increase in smooth muscle cell migration, an effect enhanced by diminished NO release.\textsuperscript{58} Direct demonstration of a severalfold increase in AT\textsubscript{1} expression in the atherosclerotic aortas of rabbits in this study should provide impetus for the use of not only chemical blockade of AT\textsubscript{1} receptors but also of molecular biology–based techniques, such as the development of antisense oligonucleotides and antibodies to AT\textsubscript{1} receptors, to limit atherosclerosis and preserve vascular reactivity.

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