Hypercholesterolemia Stimulates Angiotensin Peptide Synthesis and Contributes to Atherosclerosis Through the AT1A Receptor

Alan Daugherty, PhD, DSc; Debra L. Rateri, BS; Hong Lu, PhD; Tadashi Inagami, PhD; Lisa A. Cassis, PhD

Background—Hypercholesterolemia-induced atherosclerosis is attenuated by either pharmacological antagonism of AT1 receptors or AT1A receptor deficiency. However, the mechanism underlying the pronounced responses to angiotensin II (Ang II) antagonism has not been determined. We hypothesized that hypercholesterolemia stimulates the production of angiotensin peptides to provide a rationale for the profound effect of AT1A receptor deficiency on atherogenesis.

Methods and Results—Atherosclerotic lesions were analyzed in LDL receptor–deficient mice. Immunocytochemical analysis demonstrated that atherosclerotic lesions contained all the components of the conventional pathway for Ang II synthesis. AT1A receptor deficiency caused a marked decrease in atherosclerotic lesion size in both the aortic root and arch of male and female mice, without a discernible effect on composition. AT1A receptor deficiency–induced reductions in atherosclerosis were independent of systolic blood pressure and measurements of oxidation and chemoattractants. Aortic AT2 receptor mRNA expression was not altered in AT1A receptor–deficient mice, and AT2 receptor deficiency had no effect on lesion area or cellular composition. Hypercholesterolemia greatly augmented the systemic renin-angiotensin system, as demonstrated by large increases in plasma concentrations of angiotensinogen and angiotensin peptides (Ang II, III, IV, and 4–8). These increases were ablated in hypercholesterolemic AT1A receptor–deficient mice.

Conclusions—AT1A receptor deficiency had a striking effect in reducing hypercholesterolemia-induced atherosclerosis in LDL receptor–negative mice. Hypercholesterolemia was associated with increased systemic angiotensinogen and angiotensin peptides, which were reduced in AT1A receptor–deficient mice. These results demonstrate that hypercholesterolemia-induced stimulation of angiotensin peptide production provides a basis for the marked effect of AT1A receptor deficiency in reducing atherosclerosis. (Circulation. 2004;110:3849-3857.)

Key Words: atherosclerosis ■ blood pressure ■ angiotensin ■ hypercholesterolemia

Hypercholesterolemia accelerates the development of atherosclerosis. Interactions between hypercholesterolemia and the renin-angiotensin system (RAS) in lesion formation have been suggested. For example, evidence demonstrates that hypercholesterolemia increases AT1 receptor density and functional responsiveness. Moreover, ACE has been localized to atherosclerotic lesions, suggesting the capacity for local generation of angiotensin II (Ang II) in a hyperlipidemic environment.

Further evidence for a functional role of the RAS in atherosclerosis comes from a consistent literature demonstrating that inhibition of ACE reduces atherosclerotic lesions in a wide variety of experimental models. Studies using AT1 receptor antagonists in atherosclerosis have not been consistent. Several studies failed to demonstrate any effect on atherosclerosis, whereas others found a decreased size of hypercholesterolemia-induced atherosclerotic lesions. Losartan has been the predominant AT1 receptor blocker used in studies that demonstrate a reduction in atherosclerosis. Although an effective AT1 receptor antagonist, this drug has several ancillary properties that may confound mechanistic interpretation. Therefore, pharmacological approaches have provided intriguing, but not compelling, evidence for a role of AT1 receptors in hypercholesterolemia-induced atherosclerosis. More convincingly, a recent study demonstrated that male apolipoprotein E (apoE)–deficient mice that lack the AT1A receptor exhibit reduced atherosclerosis compared with wild-type mice.

There is substantial evidence that Ang II promotes the development of atherosclerosis. There are many suggestions...
for potential mechanisms by which Ang II promotes lesion formation. These mechanisms include indirect consequences of changes in blood pressure, although this does not appear to be a major factor. Potential direct mechanisms include effects on oxidative stress and enhanced inflammation mediated via elaboration of chemoattractants and adhesion molecules.

Although there is convincing evidence of a major role for Ang II in atherogenesis, no rationale has been presented for the profound effect of inhibition of RAS on hypercholesterolemia-induced atherosclerosis that forms in the absence of exogenous Ang II delivery. One area of speculation has focused on Ang II receptors. AT1 receptors have 2 subtypes of separate gene products that are called AT1A and AT1B.14,15 Deficiencies of either receptor subtype cause relatively modest phenotypes, although compound deficiency leads to retarded growth and renal defects similar to that seen in ACE- and angiotensinogen-deficient mice.16–18 A single gene encodes the AT2 receptor.19 Several studies have suggested that the effects of AT1 receptors oppose those of AT1 receptors.20 Therefore, there is the potential for enhanced AT1B receptor stimulation in AT1 receptor–negative (−/−) mice.21 However, the role of the AT2 receptor in hypercholesterolemia-induced atherosclerosis has not been defined. In addition, interactions between hypercholesterolemia and the RAS, which may have contributed to effects from AT1A receptor deficiency, have not been defined.

On the basis of suggestions of interactions between hypercholesterolemia and the RAS, we hypothesized that hypercholesterolemia would augment specific components of the RAS. These mechanisms were studied in LDL receptor−/− mice of both sexes.22 Feeding these mice a diet enriched in saturated fat leads to hypercholesterolemia associated with obesity and insulin resistance.23 These studies demonstrate that hypercholesterolemia greatly promotes angiotensin peptide concentrations and provide a basis for the profound effects of AT1A receptor deficiency on atherogenesis in hypercholesterolemic states.

Methods

Development of Chicken Antibodies
Antibodies were developed against mouse angiotensinogen, Ang II, and renin by use of the peptides EEEQPTSTVSQGPSE, DRVYIHPF, and RKFYTEFDRHNNR, respectively (Aves Laboratory). Peptides were conjugated to keyhole limpet hemocyanin and injected into chickens. Purified IgY was isolated from egg yolks by affinity chromatography. The sequence used for angiotensinogen was at the root as described previously.24 The anti–Ang II IgY exhibited 100% cross-reactivity to Ang II, Ang III, and Ang IV and attended via elaboration of chemoattractants and adhesion molecules.

Genotyping by Polymerase Chain Reaction
AT1A receptor genotyping used the following primers: antisense, 5′-AAATGGCCCTTACCTCTCTACTG-3′ and sense, 5′-ATTAGGGAAAGGGAACAGGAAGC. Resultant wild-type and deficient allele bands were 650 bp and 1.1 kb, respectively (Data Supplement Figure I). LDL receptor genotyping used the following primers: antisense, 5′-AGGATTCCTTCTTTGAGAC and sense, 5′-GTAAGAATTTGGAGTTGGCT. Resultant wild-type and deficient allele bands were 500 bp and 1.1 kb, respectively (Data Supplement Figure I). AT1B receptor genotyping used the following primers: 5′-AGGGATTCTCTCTTGTACAGGCAC-3′ and sense, 5′-GTAAGAATTTGGAGTTGGCT. Resultant wild-type and deficient allele bands were 383 and 800 bp, respectively.

Quantification of Atherosclerosis
Atherosclerosis was quantified both on the aortic intima and in the root as described previously.25,26 Blood Pressure Measurements
Systolic blood pressure was measured on conscious, restrained mice by use of the Visitech (Visitech Systems) tail-cuff system as described previously.28 Measurement of Serum Components
Lipids
Serum cholesterol concentrations and lipoprotein cholesterol distributions were determined as described previously.25 Renin-Angiotensin System
Plasma renin concentration was measured by generation of Ang I during incubation of plasma as described previously.24 Plasma aldosterone concentrations were quantified by radioimmunoassay by use of a commercially available kit (Diagnostic Systems Laboratories). To quantify angiotensin peptides, plasma was collected directly into an antiproteolytic cocktail to curtail artificial production or degradation of peptides. Plasma concentrations of angiotensin peptides were resolved by high-performance liquid chromatography (HPLC) and quantified by radioimmunoassay.24 The anti–Ang II IgY exhibited 100% cross-reactivity to Ang II, Ang III, and Ang IV and 80% with Ang 4–8.

Monocyte Chemotactic Protein-1
Serum monocyte chemotactic protein (MCP)-1 concentrations were quantified by ELISA (JE/MCP-1, R&D).

Oxidation Autoantibody Titters
Serum autoantibodies to malondialdehyde (MDA)-LDL were measured by ELISA as described previously.26 Briefly, human LDL was modified by MDA. Either LDL or MDA-LDL was coated on ELISA plates by overnight incubation at 4°C. BSA was used to block nonspecific binding sites. Dilutions of mouse sera (1:100) were secondary antibodies were detected by use of ABC kits (Vector Laboratories) with AEC as chromogen.25,26

Mice and Diet
C57BL/6 mice were purchased from the National Cancer Institute. LDL receptor−/− mice of either sex (B6.129S7-Ldltm1Her; stock no. 002207) and AT1A−/− mice (B6.129P2-Agrt1atm1Unc; stock no. 002682) were obtained from the Jackson Laboratory. AT1−/− deficient mice were obtained from Dr Inagami. All genotypes had been backcrossed 10 times into a C57BL/6 background. Littermates were selected for the studies reported in this article. All mice were maintained in a barrier facility and fed normal mouse laboratory diet.

To induce hypercholesterolemia, mice were fed a diet supplemented with fat (21% wt/wt) and cholesterol (0.15% wt/wt; Harlan Teklad; diet no. T688137) beginning at 8 weeks of age for a total of 12 weeks. All studies were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

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Immunocytochemistry
Immunocytochemistry was performed on frozen sections, with appropriate negative controls, as described previously.25 Macrophages were detected with rabbit anti-mouse macrophage serum (Accurate Chemicals; AI-AD31240). ACE was detected by use of a goat anti-mouse IgG (Santa Cruz Biotechnology; sc-12184). Angiotensinogen, Ang II, and renin were detected by use of the chicken IgYs discussed above. CD106 (vascular cell adhesion molecule 1, VCAM-1) was detected by use of a rat anti-mouse IgG (PharMingen; 01811D). Biotinylated labeled secondary antibodies were detected by use of ABC kits (Vector Laboratories) with AEC as chromogen.25,26

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added and incubated for 4 hours. A biotinylated anti-mouse antibody (1:200; BA-9200, Vector Laboratories), ABC (Vectastain, PK-6100), and ATBS (Sigma, A-9941) were used to detect reactivity of sera.

mRNA Abundance of Angiotensin Receptors
RNA was harvested from mouse aortas by use of the SV Total Isolation System (Promega). Polymerase chain reaction (PCR) was performed with 100 ng of total RNA. The following primers were used: AT1A: sense, 5′-GACCAACTCAACCCAGAAAAGC and antisense, 5′-CGAGCGATCTTTACATAGGTG; AT1B: sense, 5′-GCAGCATTTAGCTAATAGGTG and antisense, 5′-GGTACGAAAATCCTAAACACAC; and AT2: sense, 5′-CCTTTTGATAATCCTACCGCAACT and antisense, 5′-GACAACAAAAACAGTGGAGACCACAA. The Access RT-PCR system (Promega) was used to amplify mRNA expression. The annealing temperatures used were 58°C for AT1A and AT1B and 52°C for AT2 receptors. Expected amplicon lengths were 340, 488, and 160 bp for AT1A, AT1B, and AT2 receptors, respectively. Products were resolved by agarose gel electrophoresis and visualized with ethidium bromide. Quantitative assessment of band densities was performed with Kodak Image software (Image Station, 440CF, Kodak). mRNA abundance was determined by comparison with β-actin.

Western Blot Analysis of Plasma Angiotensinogen
Plasma proteins were resolved by SDS polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). Western blot analyses were performed as described previously.29 PVDF membranes were incubated with chicken anti-mouse angiotensinogen IgY (1:200). Blots were then incubated with peroxidase-conjugated rabbit anti-chicken IgG (1:5000 dilution, Jackson Immuno-Research). Immunoreactivity was visualized with an enhanced chemiluminescence Western blotting detection kit (Pierce). Angiotensinogen protein was evaluated as the densitometric value/mean control value (C57BL/6 mouse plasma) ratio.

Statistics
Data were analyzed with 2-way ANOVA by use of SigmaStat. Data were tested for use of parametric or nonparametric post hoc analysis, and multiple comparisons were performed by use of Tukey tests. Values of P<0.05 were considered to be statistically significant. All data are represented as mean±SEM.

Results
Hypercholesterolemia-Induced Atherosclerotic Lesions Contain All the Components for Local Synthesis of Angiotensin Peptides
To determine whether the components required for angiotensin peptide production were present in atherosclerotic lesions,
Deficiency of AT1A Receptors Strikingly Reduces Atherosclerotic Lesion Size Independently of AT2 Receptors

We determined the role of AT1A and AT2 receptors in hypercholesterolemia-induced atherosclerosis of LDL receptor−/− mice fed a saturated-fat diet. Atherosclerosis was quantified on both the aortic intimal surface in the arch and in sections from the aortic root. The effect in the aortic intima was particularly striking, with a 2-fold decrease of atherosclerosis in both sexes of AT1A receptor−deficient mice (P<0.001 and P<0.004 for males and females, respectively; Figure 2A). AT1A receptor deficiency also profoundly reduced atherosclerotic lesion size throughout the aortic root in both males and females (Figure 2B). The cellular contents of the lesions were predominantly macrophages in both AT1A receptor+/+ and −/− mice (Data Supplement Figure II).

Although there were sex-specific changes in body weight, plasma cholesterol concentrations, and lipoprotein-cholesterol distribution, these parameters were not affected by AT1A receptor genotype (Table 1). Serum titers of MDA-LDL autoantibodies, a marker of oxidative stress, were not affected by AT1A receptor genotype (Table 1). In addition, serum concentrations of the chemokine MCP-1 were not affected by AT1A receptor genotype (Table 1). VCAM-1 has been implicated in the mechanisms by which Ang II induces atherosclerosis. In mature lesions from AT1A receptor wild-type animals, VCAM-1 was present throughout the endothelium, macrophages, and smooth muscle cells underlying lesions. There was no discernible difference in the distribution of VCAM-1 protein in lesions from AT1A receptor−/− mice (Data Supplement Figure III).

In agreement with previous studies, there was an increased concentration of renin in plasma from AT1A receptor−deficient mice of both sexes. However, plasma aldosterone concentration was not significantly altered in AT1A receptor−deficient mice. Moreover, although there was a trend in males, there were no significant differences in systolic blood pressure among any groups (P=0.3 for genotype; Table 1).

Absence of AT1A receptors has the potential to lead to a compensatory change in AT1 receptor abundance. However, AT2 receptor mRNA expression in the aorta of AT1A receptor−deficient mice was not altered. In contrast, AT1A receptor mRNA expression was increased in the aorta of AT1A receptor−deficient mice (Figure 3).

To further evaluate whether AT2 receptors contributed functionally to hypercholesterolemia-induced atherosclerosis, we developed AT2 receptor−deficient animals in an LDL receptor−/− background. There was no effect of AT2 receptor deficiency on the size of atherosclerotic lesions in either male or female mice (Figure 4). The absence of AT2 receptors had no effect on body weight, serum cholesterol, MCP-1, plasma aldosterone, or systolic blood pressure (Table 2).

Hypercholesterolemia Increases Plasma Angiotensinogen and Angiotensin Peptide Concentrations

Under normolipidemic conditions, we determined plasma concentrations of total angiotensin peptides to be 116±9...
pg/mL. This was predominantly in the form of Ang II, although measurable concentrations of angiotensin III, IV, and 4–8 peptides were also present, as described previously.\(^2^4,3^1\) In LDL receptor–deficient mice fed a high-saturated-fat diet, there was a highly significant increase in plasma concentrations of the sum of the detectable angiotensin peptides to \(990 \pm 164\) pg/mL (\(P < 0.001\)). Under hyperlipidemic conditions, Ang II remained the predominant form in plasma. However, there were also significant increases in angiotensins III, IV, 4–8, and 5–8 peptides. Despite the observed elevations in plasma renin concentration in AT1A receptor–deficient mice (see Table 1), the plasma concentrations of Ang II, III, and IV were decreased (Figure 5). To define mechanisms for regulation of angiotensin peptide concentrations with hypercholesterolemia and with AT1A receptor deficiency, we determined relative plasma angiotensinogen concentrations by Western blotting (Figure 6). The pattern of changes in angiotensinogen concentration in plasma closely mirrored changes in plasma angiotensins, with marked elevations in plasma angiotensinogen with hypercholesterolemia that were normalized in AT1A receptor–deficient mice.

<table>
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<tr>
<th>Table 1. Effects of AT1a Genotype Deficiency on LDL Receptor (^{-/-}) Mice Fed a Fat-Supplemented Diet for 12 Weeks</th>
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<td><strong>Male</strong></td>
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<td>+/+ (n=13)</td>
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<tr>
<td>Body weight, g</td>
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<td>Serum cholesterol, mg/dL</td>
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<tr>
<td>Serum MDA-LDL autoantibody titers (ratio of MDA-LDL/LDL at 1:100 dilution)</td>
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<td>Serum MCP-1, pg/mL</td>
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<td>Plasma renin concentration, ng/mL</td>
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<td>Plasma aldosterone, pg/mL</td>
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<td>Systolic blood pressure, mm Hg</td>
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Values are represented as mean±SEM. ND indicates not determined.

*\(P=0.001\) for renin concentrations comparing genotypes within sexes.
†\(P<0.001\) for cholesterol concentrations and body weight comparing sexes within genotypes.

**Figure 3.** Abundance of AT1a, AT1b, and AT2 receptor mRNA in aortic tissue from AT1a\(^{+/+}\) and AT1a\(^{-/-}\) mice. Top. Examples of gel that show examples of amplicons for AT1a (340 bp), AT1b (488 bp), and AT2 (160 bp). Bottom. Quantification of mRNA abundance of AT1a and AT2 receptor mRNA in AT1a receptor \(^{+/+}\) and \(^{-/-}\) mice. Histobars are means of at least 6 mice, and bars are SEM. *\(P=0.005\) for \(^{+/+}\) compared with \(^{-/-}\). For AT1b, \(P=0.212\). **Figure 4.** AT2 receptor deficiency has no effect on atherosclerotic lesion development in aortic arch. Triangles represent values for individual mice, diamonds represent means, and bars are SEM. There was no statistical significance between genotypes or sexes.
Discussion

Hypercholesterolemia-Induced Atherosclerotic Lesions Contain All the Components for Local Synthesis of Angiotensin Peptides

The only protein in the classic synthetic pathway of Ang II that has been detected previously in atherosclerotic lesions is ACE.4,5,32 Using a commercially available antibody, we also demonstrated that ACE protein is widely distributed throughout mouse lesions, as described in human and monkey atherosclerosis.4,33 To detect mouse renin and angiotensinogen, we developed antibodies using peptide sequences specific to the mouse protein to enable the first description of these proteins in atherosclerotic lesions. Both renin and angiotensinogen were detected predominantly in macrophage-rich shoulder regions of atherosclerotic lesions. The epitope of the anti–Ang II IgY was common to both precursors of Ang II, as well as other angiotensins. The newly developed purified IgY exhibited 100% reactivity against Ang II, III, IV, and 4–8, with more limited reactivity against 5–8. Marked elevations in Ang II and other angiotensins were observed in LDL receptor /−/− mice compared with /+/+ mice. In LDL receptor /−/− mice with AT1A receptor deficiency, angiotensin peptides were decreased to concentrations of wild-type mice. Histobars represent means of plasma from at least 6 individual mice and bars are SEM. **P<0.001 for LDL receptor /−/− AT1A receptor /−/− group compared with other genotypes.

Figure 6. Hypercholesterolemia increases plasma concentrations of angiotensinogen that are normalized in mice with AT1A receptor deficiency. Western blots were performed using a newly developed chicken IgY developed to carboxyl end of angiotensinogen. Top, Three examples per group. Bottom, Quantitative analysis of relative intensities of different glycosylated forms of angiotensinogen. Angiotensinogen was determined in age-matched male mice of the following genotypes of LDL receptors and AT1A receptors, respectively; /+/+ (gray column), /−/− × /−/− (gray column), /−/− × /−/− (open column). Histobars represent means of plasma from at least 6 individual mice and bars are SEM. **P<0.001 for LDL receptor /−/− AT1A receptor /−/− group compared with other genotypes.
ors and products of Ang II. Therefore, we have referred to the immunoreactivity as “angiotensin peptides,” which were diffusely distributed throughout lesions.

Although we defined the presence of all components for the synthesis of angiotensin peptides within atherosclerotic lesions, it is not clear that they were synthesized at this locus. For angiotensinogen, renin, and ACE, there is the potential for each of these proteins to be derived from the systemic sources of liver, kidney, and lung, respectively. Future studies will determine the source of lesion RAS components in the development of atherosclerosis.

Effects of AT$_{1A}$ and AT$_{2}$ Receptor Deficiency on Atherogenesis

A recent study has demonstrated that AT$_{1A}$ receptor deficiency reduces atherosclerosis in apoE-deficient mice.$^{11}$ There are potential differences in the atherogenic mechanisms between apoE$^{-/-}$ and LDL receptor$^{-/-}$ mice, because the latter respond to high-fat diets with obesity and diabetes.$^{23,34}$ The ability of AT$_1$ receptor deficiency to decrease lesion size in these different models of atherosclerosis highlights the important role of the AT$_{1A}$ receptor subtype in the development of atherosclerosis. In addition, the profound effect of AT$_{1A}$ receptor deficiency to reduce lesion size was noted in 2 vascular beds and in both male and female mice, demonstrating that vascular location and sex do not influence AT$_{1A}$ receptor–mediated atherosclerosis.$^{35}$

The mouse aorta has been reported to express both the AT$_{1A}$ and AT$_{1B}$ receptors. A recent study demonstrated that AT$_{1A}$ receptor deficiency had no effect on Ang II–induced contractions of mouse aortic tissue.$^{26}$ These responses were presumed to be caused by AT$_{1B}$ receptors, because AT$_1$ receptor antagonists inhibited Ang II contractions in AT$_{1A}$ receptor–deficient mice.$^{36}$ Our results demonstrate that deficiency of AT$_{1A}$ receptor results in compensatory upregulation of AT$_{1B}$ receptor expression in the aorta. However, profound reductions in the development of atherosclerosis were observed even with increased AT$_{1B}$ receptor expression. Thus, the AT$_{1A}$ receptor appears to be the predominant receptor involved in the development of atherosclerotic lesions.

Many mechanisms have been proposed for the atherogenic effects of Ang II. One of the most prominent is increased oxidant stress.$^{37}$ Although there is no generally accepted measurement of oxidant stress, titers of autoantibodies against modified LDL have been used as an indication.$^{38}$ Using this criterion, we were unable to discern differences in oxidant stress in AT$_{1A}$ receptor$^{-/-}$ versus$^{-/-}$ mice. Another proposed atherogenic mechanism for Ang II is the promotion of leukocyte chemotraction and adhesion through activation of nuclear factor-κB.$^{39}$ However, we were also unable to discern any changes in plasma concentrations of MCP-1 or abundance and distribution pattern of VCAM-1 in lesions.

The initial reports on AT$_{1A}$ receptor–deficient mice noted a decrease in systolic and diastolic blood pressure that was gene-dosage–related.$^{30,40}$ The extent of the decrease was dependent on the technique used to measure blood pressure.$^{40}$ Profound changes in systolic blood pressure were noted in mice that lacked both AT$_{1A}$ and AT$_{1B}$ receptors.$^{18}$ In the present study, we observed that AT$_{1A}$ receptor deficiency in LDL receptor$^{-/-}$ mice on a C57BL/6 background had no significant effect on systolic blood pressure. This is in agreement with findings in C57BL6 mice$^{41}$ but discordant with those obtained in AT$_{1A}$ receptor– and apoE compound–deficient mice.$^{11}$ Recent findings$^{11}$ demonstrate that hydralazine-induced reductions in blood pressure in apoE$^{-/-}$ mice did not influence the development of atherosclerosis. Collectively, these results demonstrate that reductions in blood pressure with AT$_{1A}$ receptor deficiency are not the primary mediator of Ang II–induced atherosclerosis.

There is evolving evidence that AT$_2$ receptors can act as antagonists of AT$_1$ receptors in responses such as blood pressure, vascular reactivity, and apoptosis.$^{42,43}$ AT$_2$ receptors have an increased role in mice with compound deficiencies of both subtypes of AT$_1$ receptors.$^{18}$ Therefore, we speculated that one mechanism of decreased atherosclerosis in AT$_{1A}$ receptor$^{-/-}$ mice may be an augmented role of AT$_2$ receptors, which exert protective effects in Ang II–induced atherosclerosis.$^{38}$ We did not observe compensatory upregulation of AT$_2$ receptor mRNA expression in the aortas of AT$_{1A}$ receptor$^{-/-}$ mice. In addition, we were unable to demonstrate any effects of AT$_2$ receptor deficiency on the size of atherosclerotic lesions in LDL receptor$^{-/-}$ mice. The lack of effect of AT$_2$ receptor deficiency on lesion area, compared with the dramatic decrease in the size of atherosclerotic lesions in AT$_{1A}$ receptor–deficient LDL receptor$^{-/-}$ mice, is not consistent with a prominent role of AT$_2$ receptors in hypercholes terolemia–induced atherosclerosis.

Hypercholesterolemia Increases Plasma Angiotensinogen and Angiotensin Peptide Concentrations

The present study defined the effect of hypercholesterolemia on plasma concentrations of angiotensin peptides. Despite the intense interest in the physiological and pathological effects of Ang II, there is a relative paucity of data on angiotensin peptide concentrations in plasma of many species, most particularly in mice. The few publications in mice show widely differing concentrations, ranging from 14 to $\approx$3000 pg/mL.$^{31,44,45}$ The quantification of angiotensin peptides is potentially complicated by their artificial destruction and/or production after acquisition of plasma. In the present study, we used an antiproteolytic cocktail added to freshly removed blood to prevent postacquisition production or degradation of angiotensins.$^{24}$ The measurement of Ang II is also complicated by the antibody recognizing an epitope that is present in many angiotensin peptides. Therefore, we used HPLC to resolve angiotensin peptides before radioimmunoassay. Use of this technique demonstrated that Ang II and IV are the major peptides in the plasma of normolipidemic mice, as described previously.$^{24}$ Furthermore, it demonstrates that hypercholesterolemia promotes large increases in angiotensin peptides. This increase was predominantly in Ang II, but we also observed significant increases in III, IV, and 4–8 peptides.

To define mechanisms for increases in systemic angiotensin peptide concentrations, we focused on angiotensinogen as the only known precursor to Ang II. Recent studies demonstrate that, in contrast to humans and rats, in which circulating
renin is rate-limiting, in mice, angiotensinogen is the determining factor in Ang II synthesis. The magnitude of elevation in plasma angiotensinogen concentration with hyperlipidemia was similar to the robust effect of hyperlipidemia to elevate angiotensin peptides (4-fold elevations in both). These novel results demonstrate that hypercholesterolemia exerts stimulatory effects on angiotensinogen, contributing to a robust activation of the RAS.

An additional novel finding of this study is that the circulating concentrations of angiotensin peptides were reduced in AT1A receptor-deficient mice. Although short-term administration of AT1 receptor antagonists increases Ang II plasma concentrations, chronic administration decreases presumably through effects at the AT1 receptor. Taken in plasma concentrations of both angiotensinogen and angiotensin peptides in AT1A receptor-deficient mice. Moreover, reductions in circulating angiotensin peptides in AT1A receptor-deficient mice further eliminates the possibility that AT1 or AT1B receptor effects contribute to hypercholesterolemia-induced atherosclerosis.

Conclusions

The present study demonstrates that hypercholesterolemia stimulates the synthesis of angiotensin peptide production as a mechanism contributing to the ability of AT1 receptor deficiency to profoundly decrease lesion formation. Future studies will define the role of Ang II production by local sources in the vascular wall in the development of atherosclerosis. Also, it will be important to define whether AT1A receptors on specific cell types are responsible for the development of atherosclerosis.

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References


44. Lum C, Shesely EG, Potter DL, Beierwaltes WH. Cardiovascular and renal phenotype in mice with one or two renin genes. Hypertension. 2004;43:79–86.


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