

Hypercholesterolemia Stimulates Angiotensin Peptide Synthesis and Contributes to Atherosclerosis Through the AT_{1A} 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 AT_{1A} 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 AT_{1A} 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. AT_{1A} 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. AT_{1A} 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 AT_{1A} 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 AT_{1A} receptor-deficient mice.

Conclusions—AT_{1A} 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 AT_{1A} receptor-deficient mice. These results demonstrate that hypercholesterolemia-induced stimulation of angiotensin peptide production provides a basis for the marked effect of AT_{1A} 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.¹ For example, evidence demonstrates that hypercholesterolemia increases AT₁ receptor density^{2,3} and functional responsiveness.² Moreover, ACE has been localized to atherosclerotic lesions, suggesting the capacity for local generation of angiotensin II (Ang II) in a hyperlipidemic environment.^{4,5}

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,^{6,7} whereas others found a decreased size of hypercholesterolemia-induced atherosclerotic lesions.^{8,9} 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.¹⁰ 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 AT_{1A} receptor exhibit reduced atherosclerosis compared with wild-type mice.¹¹

There is substantial evidence that Ang II promotes the development of atherosclerosis. There are many suggestions

Received September 26, 2004; revision received September 26, 2004; accepted October 21, 2004.

From the Division of Cardiovascular Medicine (A.D., D.L.R., H.L.) and the Graduate Center for Nutritional Sciences (A.D., L.A.C.), University of Kentucky, Lexington; and the Department of Biochemistry, Vanderbilt University, Nashville, Tenn (T.I.).

The online-only Data Supplement, which contains Figures I through III, can be found with this article at <http://www.circulationaha.org>.

Correspondence to Alan Daugherty, Wethington Building, Room 521, University of Kentucky, Lexington, KY 40536-0200. E-mail alan.daugherty@uky.edu

© 2004 American Heart Association, Inc.

Circulation is available at <http://www.circulationaha.org>

DOI: 10.1161/01.CIR.0000150540.54220.C4

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.¹² Potential direct mechanisms include effects on oxidative stress and enhanced inflammation mediated via elaboration of chemoattractants and adhesion molecules.¹³

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 AT_{1A} and AT_{1B}.^{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 AT₂ receptors oppose those of AT₁ receptors.²⁰ Therefore, there is the potential for enhanced AT₂ receptor stimulation in AT_{1A} 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 AT_{1A} 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 AT_{1A} 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 EEEQPTTSVQPGSPE, 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 carboxyl terminal and did not react with other angiotensin peptides. Conversely, the IgY against Ang II also reacted with angiotensinogen, Ang I, Ang III, Ang IV, Ang 4–8, and Ang 5–8.²⁴

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}

Mice and Diet

C57BL/6 mice were purchased from the National Cancer Institute. LDL receptor^{-/-} mice of either sex (B6.129S7-Ldlr^{tm1Her}; stock no. 002207) and AT_{1A}^{-/-} mice (B6.129P2-Agtr1a^{tm1Unc}; stock no. 002682) were obtained from the Jackson Laboratory. AT₂-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. TD88137) 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.

Genotyping by Polymerase Chain Reaction

AT_{1A} receptor genotyping used the following primers: antisense, 5'-AAATGGCCCTTAACTCTTCTACTG-3' and sense, 5'-ATTAGG-AAAGGGAACAGGAAGC. Resultant wild-type and deficient allele bands were 650 bp and 1.1 kb, respectively (Data Supplement Figure I). AT₂ receptor genotyping used the following primers: antisense, 5'-GGGATTCCTTCTTTGAGAC and sense, 5'-GTAAGAATTTGGAG-TTGCTG. Resultant wild-type and deficient allele bands were 500 bp and 1.1 kb, respectively (Data Supplement Figure I). LDL receptor genotyping used the following primers: 5'-AGGTGAGATGACAGGAGATC, 5'-AGGATGACTTCCGATGCCAG, and 5'-GCA-GTGCTCCTCATCTGACTTG. 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.^{26,27}

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 artifactual 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 Titers

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

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: AT_{1A}: sense, 5'-GACCAACTCAACCCAGAAAAAGC and antisense, 5'-CGAAGCGATCTTACATAGGTG; AT_{1B}: sense, 5'-GCAGCATTTAGCTAGACAGTTC and antisense, 5'-GCCTACGAAATCTTAACACAC; and AT₂: sense, 5'-CCTTTTGATAATCTCAACGCAACT and antisense, 5'-GACAACAAAACAGTGAGACCACAA. The Access RT-PCR system (Promega) was used to amplify mRNA expression. The annealing temperatures used were 58°C for AT_{1A} and AT_{1B} and 52°C for AT₂ receptors. Expected amplicon lengths were 340, 488, and 160 bp for AT_{1A}, AT_{1B}, 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,

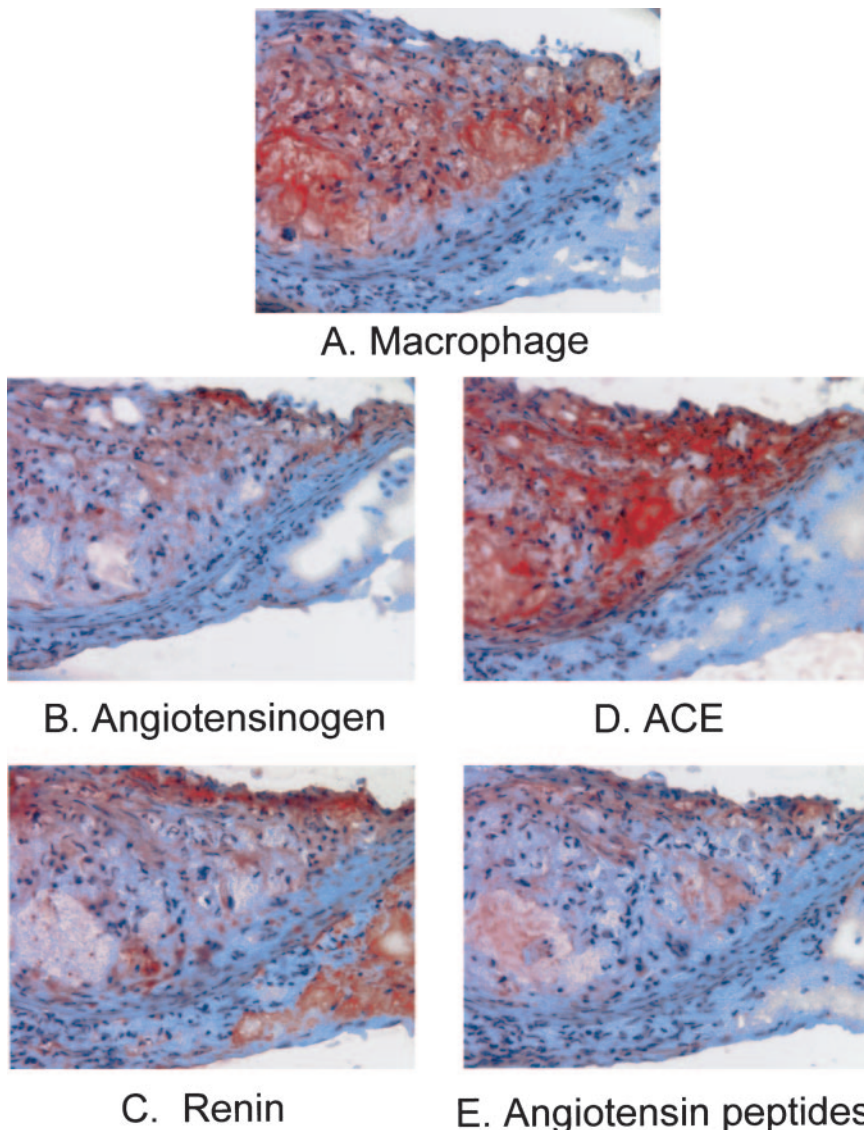


Figure 1. All components for angiotensin peptide synthesis are present in atherosclerotic lesions and are associated predominantly with macrophages. Immunocytochemistry was performed using a rabbit antiserum against mouse macrophages (A), chicken IgY against mouse angiotensinogen (B), chicken IgY against mouse renin (C), goat anti-mouse ACE IgG (D), and chicken IgY against angiotensin peptides (E). At dilutions used, controls (preimmune IgY, nonimmune IgG, and nonimmune serum) did not produce any discernible chromogen development.

frozen serial sections of aortic root were immunostained by use of commercially available antibodies against macrophages and ACE. In addition, sections were immunostained with newly developed chicken antibodies against angiotensinogen, renin, and angiotensin peptides (Figure 1). As noted previously in humans and monkeys, ACE immunostaining was detected in macrophage-rich regions of the core and shoulder of lesions.⁴ ACE was also detected on regions of the media. Interestingly, angiotensinogen, renin, and angiotensin peptides were present in macrophage-rich areas in abluminal regions. Negative controls included lack of chromogen development during incubation with the same concentrations of preimmune IgY, nonimmune IgG, or dilution of nonimmune serum (data not shown).

Deficiency of AT_{1A} Receptors Strikingly Reduces Atherosclerotic Lesion Size Independently of AT₂ Receptors

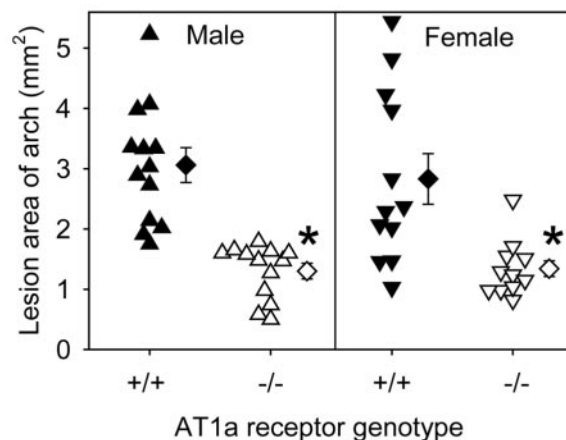
We determined the role of AT_{1A} and AT₂ receptors in hypercholesterolemia-induced atherosclerosis of LDL receptor^{-/-} mice fed a saturated-fat diet. Atherosclerosis was quantified on both the aortic intimal surface in the arch and in sections from the aortic root. The effect in the aortic intima was particularly striking, with a 2-fold decrease of atherosclerosis in both sexes of AT_{1A} receptor-deficient mice ($P < 0.001$ and $P < 0.004$ for males and females, respectively; Figure 2A). AT_{1A} 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 AT_{1A} 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 AT_{1A} receptor genotype (Table 1). Serum titers of MDA-LDL autoantibodies, a marker of oxidative stress, were not affected by AT_{1A} receptor genotype (Table 1). In addition, serum concentrations of the chemokine MCP-1 were not affected by AT_{1A} receptor genotype (Table 1). VCAM-1 has been implicated in the mechanisms by which Ang II induces atherosclerosis. In mature lesions from AT_{1A} 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 AT_{1A} receptor^{-/-} mice (Data Supplement Figure III).

In agreement with previous studies,³⁰ there was an increased concentration of renin in plasma from AT_{1A} receptor-deficient mice of both sexes. However, plasma aldosterone concentration was not significantly altered in AT_{1A} 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 AT_{1A} receptors has the potential to lead to a compensatory change in AT₂ receptor abundance. However, AT₂ receptor mRNA expression in the aorta of AT_{1A} receptor-deficient mice was not altered. In contrast, AT_{1B} receptor mRNA expression was increased in the aorta of AT_{1A} receptor-deficient mice (Figure 3).

A. Aortic intimal surface



B. Aortic root

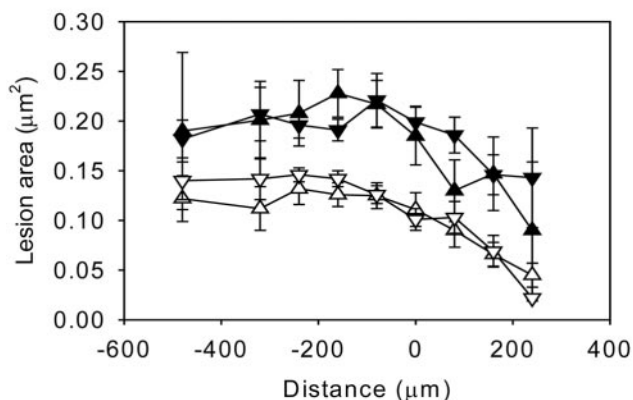


Figure 2. Atherosclerotic lesion development in LDL receptor deficiency is attenuated in both male and female AT_{1A} receptor-deficient mice. **A**, Atherosclerosis measured on aortic intimal surface. Triangles represent values for individual mice, diamonds represent means, and bars are SEM. * $P < 0.001$ and $P < 0.004$ between genotypes within male and female mice, respectively. **B**, Atherosclerosis in aortic root. Aortic roots were sectioned from appearance of valves to ascending aorta. Data are represented throughout aorta, with transition between sinus and ascending arch depicted as 0. This region was defined by appearance of aortic valvular stubs. Points are means, with closed and open triangles representing AT_{1A}^{+/+} and ^{-/-} mice, respectively. Upright and inverted triangles represent male and female mice, respectively. Bars represent SEM.

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

TABLE 1. Effects of AT_{1A} Genotype Deficiency on LDL Receptor^{-/-} Mice Fed a Fat-Supplemented Diet for 12 Weeks

| | Male | | Female | |
|---|---------------|---------------|---------------|---------------|
| | +/+ (n=13) | -/- (n=13) | +/+ (n=12) | -/- (n=11) |
| Body weight, g | 38.8±0.9 | 34.1±1.5 | 26.0±1.2† | 24.1±1.0† |
| Serum cholesterol, mg/dL | 1282±63 | 1351±66 | 1044±66† | 917±68† |
| Serum MDA-LDL autoantibody titers (ratio of MDA-LDL/LDL at 1:100 dilution) | 3.84±0.21 | 3.78±0.24 | ND | ND |
| Serum MCP-1, pg/mL | 390±44 | 296±43 | 337±47 | 313±47 |
| Plasma renin concentration, ng/mL | 0.057±0.02 | 0.131±0.02* | 0.069±0.02 | 0.127±0.02* |
| Plasma aldosterone, pg/mL | 246±22 | 205±22 | 242±23 | 198±24 |
| Systolic blood pressure, mm Hg | 116±5 | 105±4 | 119±5 | 111±5 |

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.

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.^{24,31} 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±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 AT_{1A} 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 AT_{1A} 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 AT_{1A} receptor-deficient mice.

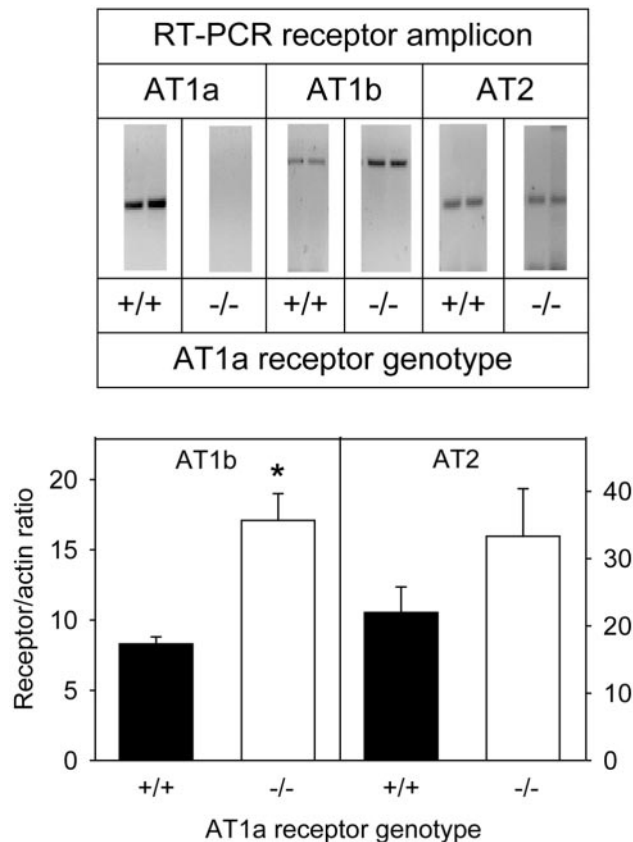


Figure 3. Abundance of AT_{1A}, AT_{1B}, and AT₂ receptor mRNA in aortic tissue from AT_{1A}^{+/+} and ^{-/-} mice. Top, Examples of gel that show examples of amplicons for AT_{1A} (340 bp), AT_{1B} (488 bp), and AT₂ (160 bp). Bottom, Quantification of mRNA abundance of AT_{1A} and AT₂ receptor mRNA in AT_{1A} receptor^{+/+} and ^{-/-} mice. Histograms are means of at least 6 mice, and bars are SEM. *P=0.005 for ^{+/+} compared with ^{-/-}. For AT₂, P=0.212.

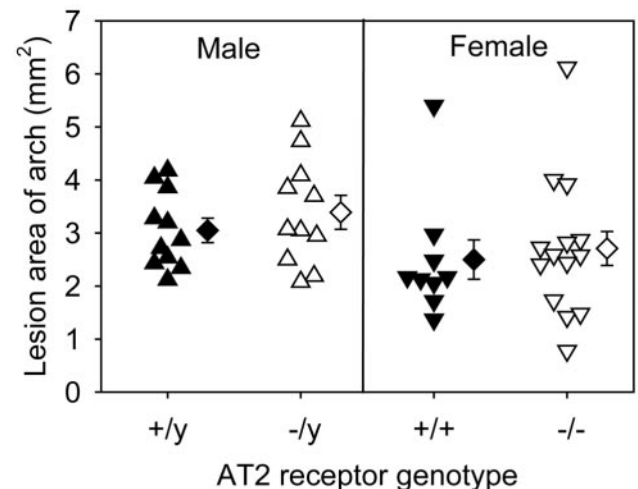


Figure 4. AT₂ receptor deficiency has no effect on atherosclerotic lesion development in aortic arch. Triangles represent values for individual mice, diamonds represent means, and bars are SEM. There was no statistical significance between genotypes or sexes.

TABLE 2. Effects of AT2 Genotype Deficiency on LDL Receptor^{-/-} Mice Fed a Fat-Supplemented Diet for 12 Weeks

| | Male | | Female | |
|--------------------------------|---------------|---------------|--------------|---------------|
| | +/y (n=11) | -/y (n=11) | +/+ (n=9) | -/- (n=14) |
| Body weight, g | 35.5±1.0 | 36.3±1.6 | 25.6±0.9* | 25.1±1.2* |
| Serum cholesterol, mg/dL | 1347±75 | 1433±97 | 964±56* | 955±46* |
| Serum MCP-1, pg/mL | 220±55 | 222±30 | 265±68 | 176±22 |
| Plasma aldosterone, ng/mL | 177±24 | 208±16 | 282±26 | 211±10 |
| Systolic blood pressure, mm Hg | 122±13 | 113±15 | 125±18 | 123±22 |

Values are represented as mean±SEM.

**P*=0.001 for male vs female groups. No significant differences were observed between genotypes.

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 precur-

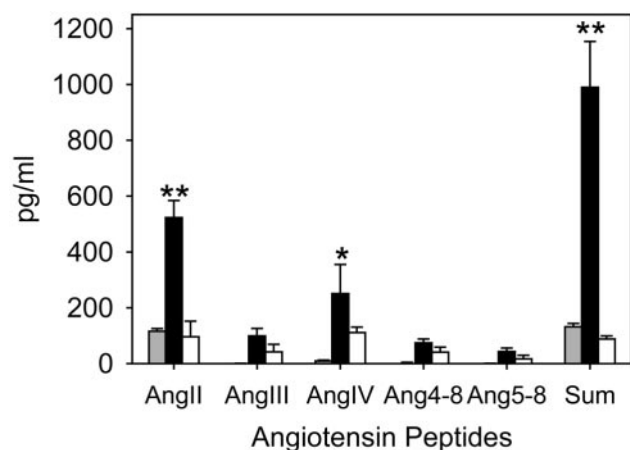


Figure 5. Hypercholesterolemia increases circulating angiotensin peptides, and AT_{1A} receptor deficiency results in reductions. Concentrations of angiotensin peptides were determined in age-matched male mice of the following genotypes of LDL receptors and AT_{1A} receptors, respectively: +/+ × +/+ (gray column), -/- × +/+ (closed column), -/- × -/- (open column). Blood was drawn and immediately placed in an antiprotease cocktail. Plasma was processed as indicated in text, and angiotensin peptides were resolved by reverse-phase HPLC. 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^{-/-} compared with +/+ mice. In LDL receptor^{-/-} mice with AT_{1A} receptor deficiency, angiotensin peptides were decreased to concentrations of wild-type mice. Histograms represent means of plasma from 4 to 10 individual mice, and bars are SEM. **P*<0.01, ***P*<0.001 for LDL receptor^{-/-} × AT_{1A} receptor^{+/+} group compared with other genotypes.

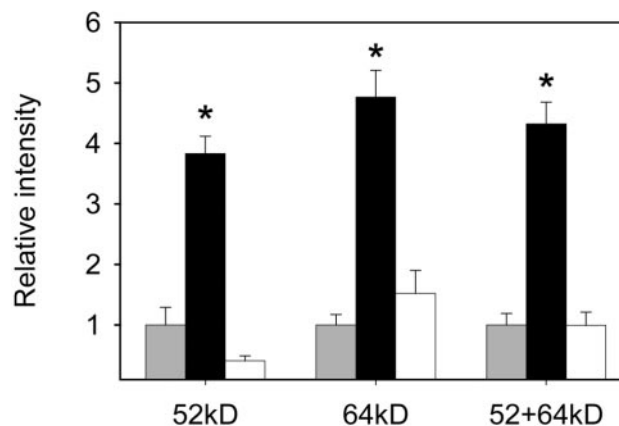
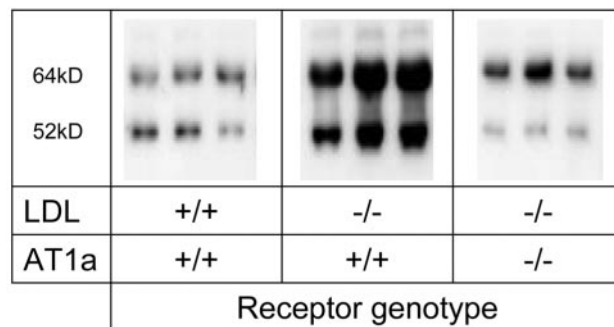


Figure 6. Hypercholesterolemia increases plasma concentrations of angiotensinogen that are normalized in mice with AT_{1A} receptor deficiency. Western blots were performed using a newly developed chicken IgY developed to carboxyl end of angiotensinogen. Top, Three examples per group. Bottom, Quantitative 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 AT_{1A} receptors, respectively: +/+ × +/+ (gray column), -/- × +/+ (closed column), -/- × -/- (open column). Histograms represent means of plasma from at least 6 individual mice and bars are SEM. **P*<0.001 for LDL receptor^{-/-} × AT_{1A} receptor^{+/+} group compared with other genotypes.

sors 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 AT₂ 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 AT₁ 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 AT₁ 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 AT₁ receptor^{+/+} versus ^{-/-} mice. Another proposed atherogenic mechanism for Ang II is the promotion of leukocyte chemoattraction and adhesion through activation of nuclear factor- κ B.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 mice⁴¹ but discordant with those obtained in AT_{1A} receptor- and apoE compound-deficient mice.¹¹ Recent findings¹¹ 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 AT₂ receptors can act as antagonists of AT₁ receptors in responses such as blood pressure, vascular reactivity, and apoptosis.^{42,43} AT₂ receptors have an increased role in mice with compound deficiencies of both subtypes of AT₁ receptors.¹⁸ Therefore, we speculated that one mechanism of decreased atherosclerosis in AT_{1A} receptor^{-/-} mice may be an augmented role of AT₂ receptors, which exert protective effects in Ang II-induced atherosclerosis.²⁸ We did not observe compensatory upregulation of AT₂ receptor mRNA expression in the aortas of AT_{1A} receptor^{-/-} mice. In addition, we were unable to demonstrate any effects of AT₂ receptor deficiency on the size of atherosclerotic lesions in LDL receptor^{-/-} mice. The lack of effect of AT₂ 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 AT₂ receptors in hypercholesterolemia-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 artifactual 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.⁴⁶ 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 AT_{1A} receptor-deficient mice. Although short-term administration of AT₁ receptor antagonists increases Ang II plasma concentrations,^{9,47} chronic administration decreases Ang II concentrations.⁴⁸ Our results demonstrate a reduction in plasma concentrations of both angiotensinogen and angiotensin peptides in AT_{1A} receptor-deficient mice. Considerable evidence demonstrates that Ang II exerts positive modulation of liver angiotensinogen mRNA expression, presumably through effects at the AT₁ receptor.^{49,50} Taken together, these findings suggest that the primary impact of AT_{1A} receptor deficiency is to lower circulating angiotensinogen as the substrate for production of angiotensin peptides. Moreover, reductions in circulating angiotensin peptides in AT_{1A} receptor-deficient mice further eliminates the possibility that AT₂ or AT_{1B} 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 AT_{1A} receptors on specific cell types are responsible for the development of atherosclerosis.

Acknowledgments

These studies were supported by National Institutes of Health grant HL-62846. We acknowledge the skilled technical assistance of Victoria English (angiotensin peptide quantification), Marc Helton (renin and aldosterone assays), and Deborah Howatt (atherosclerosis quantification).

References

- Ferrario CM, Smith R, Levy P, Strawn W. The hypertension-lipid connection: insights into the relation between angiotensin II and cholesterol in atherogenesis. *Am J Med Sci*. 2002;323:17–24.
- Nickenig G, Baumer AT, Temur Y, Kebben D, Jockenhovel F, Bohm M. Statin-sensitive dysregulated AT₁ receptor function and density in hypercholesterolemic men. *Circulation*. 1999;100:2131–2134.
- Nickenig G, Sachinidis A, Michaelsen F, Bohm M, Seewald S, Vetter H. Upregulation of vascular angiotensin II receptor gene expression by low-density lipoprotein in vascular smooth muscle cells. *Circulation*. 1997;95:473–478.
- Diet F, Pratt RE, Berry GJ, Momose N, Gibbons GH, Dzau VJ. Increased accumulation of tissue ACE in human atherosclerotic coronary artery disease. *Circulation*. 1996;94:2756–2767.
- Fukuhara M, Geary RL, Diz DI, Gallagher PE, Wilson JA, Glazier SS, Dean RH, Ferrario CM. Angiotensin-converting enzyme expression in human carotid artery atherosclerosis. *Hypertension*. 2000;35:353–359.
- Makaritsis KP, Gavras H, Du Y, Chobanian AV, Brecher P. α_1 -Adrenergic plus angiotensin receptor blockade reduces atherosclerosis in apolipoprotein E-deficient mice. *Hypertension*. 1998;32:1044–1048.
- Schuh JR, Blehm DJ, Friedrich GE, McMahon EG, Blaine EH. Differential effects of renin-angiotensin system blockade on atherogenesis in cholesterol-fed rabbits. *J Clin Invest*. 1993;91:1453–1458.
- Keidar S, Attias J, Smith J, Breslow JL, Hayek T. The angiotensin-II receptor antagonist, losartan, inhibits LDL lipid peroxidation and atherosclerosis in apolipoprotein E-deficient mice. *Biochem Biophys Res Commun*. 1997;236:622–625.
- Strawn WB, Chappell MC, Dean RH, Kivlighn S, Ferrario CM. Inhibition of early atherogenesis by losartan in monkeys with diet-induced hypercholesterolemia. *Circulation*. 2000;101:1586–1593.
- Sadoshima J. Novel AT₁ receptor-independent functions of losartan. *Circ Res*. 2002;90:754–756.
- Wassmann S, Czech T, Van Eickels M, Fleming I, Bohm M, Nickenig G. Inhibition of diet-induced atherosclerosis and endothelial dysfunction in apolipoprotein E/angiotensin II type 1A receptor double-knockout mice. *Circulation*. 2004;110:3062–3067.
- Weiss D, Kools JJ, Taylor WR. Angiotensin II-induced hypertension accelerates the development of atherosclerosis in apoE-deficient mice. *Circulation*. 2001;103:448–454.
- Weiss D, Sorescu D, Taylor WR. Angiotensin II and atherosclerosis. *Am J Cardiol*. 2001;87:25C–32C.
- Iwai N, Inagami T. Identification of two subtypes in the rat type I angiotensin II receptor. *FEBS Lett*. 1992;298:257–260.
- Sasamura H, Hein L, Krieger JE, Pratt RE, Kobilka BK, Dzau VJ. Cloning, characterization, and expression of two angiotensin receptor (AT-1) isoforms from the mouse genome. *Biochem Biophys Res Commun*. 1992;185:253–259.
- Itoh T, Ikeda T, Gomi H, Nakao S, Suzuki T, Itohara S. Unaltered secretion of beta-amyloid precursor protein in gelatinase A (matrix metalloproteinase 2)-deficient mice. *J Biol Chem*. 1997;272:22389–22392.
- Chen X, Li W, Yoshida H, Tsuchida S, Nishimura H, Takemoto F, Okubo S, Fogo A, Matsusaka T, Ichikawa I. Targeting deletion of angiotensin type 1B receptor gene in the mouse. *Am J Physiol*. 1997;272:F299–F304.
- Oliverio MI, Kim HS, Ito M, Le T, Audoly L, Best CF, Hiller S, Kluckman K, Maeda N, Smithies O, Coffman TM. Reduced growth, abnormal kidney structure, and type 2 (AT₂) angiotensin receptor-mediated blood pressure regulation in mice lacking both AT_{1A} and AT_{1B} receptors for angiotensin II. *Proc Natl Acad Sci U S A*. 1998;95:15496–15511.
- Kabayashi Y, Bardhan S, Takahashi K, Tsuzuki S, Inui H, Hamakubo T, Inagami T. Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition. *J Biol Chem*. 1993;268:24543–24546.
- Unger T. The angiotensin type 2 receptor: variations on an enigmatic theme. *J Hypertens*. 1999;17:1775–1786.
- Carey RM, Jin XH, Siragy HM. Role of the angiotensin AT₂ receptor in blood pressure regulation and therapeutic implications. *Am J Hypertens*. 2001;14:98S–102S.
- Ishibashi S, Goldstein JL, Brown MS, Herz J, Burns DK. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J Clin Invest*. 1994;93:1885–1893.
- Schreyer SA, Vick C, Lystig TC, Mystkowski P, LeBoeuf RC. LDL receptor but not apolipoprotein E deficiency increases diet-induced obesity and diabetes in mice. *Am J Physiol*. 2002;282:E207–E214.
- Cassis LA, Huang J, Gong MC, Daugherty A. Role of metabolism and receptor responsiveness in the attenuated responses to angiotensin II in mice compared to rats. *Regul Pept*. 2004;117:107–116.
- Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest*. 2000;105:1605–1612.
- Daugherty A, Pure E, Delfel-Butteiger D, Chen S, Leferovich J, Roselaar SE, Rader DJ. The effects of total lymphocyte deficiency on the extent of atherosclerosis in apolipoprotein E^{-/-} mice. *J Clin Invest*. 1997;100:1575–1580.
- Daugherty A, Whitman SC. Quantification of atherosclerosis in mice. *Methods Mol Biol*. 2003;209:293–309.
- Daugherty A, Manning MW, Cassis LA. Antagonism of AT₂ receptors augments angiotensin II-induced abdominal aortic aneurysms and atherosclerosis. *Br J Pharmacol*. 2001;134:865–870.
- Daugherty A, Whitman SC, Block AE, Rateri DL. Polymorphism of class A scavenger receptors in C57BL/6 mice. *J Lipid Res*. 2000;41:1568–1577.

30. Sugaya T, Nishimatsu S, Tanimoto K, Takimoto E, Yamagishi T, Imamura K, Goto S, Imaizumi K, Hisada Y, Otsuka A. Angiotensin II type 1a receptor-deficient mice with hypotension and hyperreninemia. *J Biol Chem*. 1995;270:18719–18722.
31. Cholewa BC, Mattson DL. Role of the renin-angiotensin system during alterations of sodium intake in conscious mice. *Am J Physiol*. 2001;281:R987–R993.
32. Schieffer B, Schieffer E, Hilfiker-Kleiner D, Hilfiker A, Kovanen PT, Kaartinen M, Nussberger J, Harringer W, Drexler H. Expression of angiotensin II and interleukin 6 in human coronary atherosclerotic plaques: potential implications for inflammation and plaque instability. *Circulation*. 2000;101:1372–1378.
33. Potter DD, Sobey CG, Tompkins PK, Rossen JD, Heistad DD. Evidence that macrophages in atherosclerotic lesions contain angiotensin II. *Circulation*. 1998;98:800–807.
34. Merat S, Casanada F, Sutphin M, Palinski W, Reaven PD. Western-type diets induce insulin resistance and hyperinsulinemia in LDL receptor-deficient mice but do not increase aortic atherosclerosis compared with normoinsulinemic mice in which similar plasma cholesterol levels are achieved by a fructose-rich diet. *Arterioscler Thromb Vasc Biol*. 1999;19:1223–1230.
35. Henriques TA, Huang J, D'Souza SS, Daugherty A, Cassis LA. Orchiectomy, but not ovariectomy, regulates angiotensin II-induced vascular diseases in apolipoprotein E-deficient mice. *Endocrinology*. 2004;145:3866–3872.
36. Zhou Y, Dirksen WP, Babu GJ, Periasamy M. Differential vasoconstrictions induced by angiotensin II: the role of AT₁ and AT₂ receptors in isolated C57BL/6J mouse blood vessels. *Am J Physiol*. 2003;285:H2797–H2803.
37. Warnholtz A, Nickenig G, Schulz E, Macharzina R, Brasen JH, Skatchkov M, Heitzer T, Stasch JP, Griendling KK, Harrison DG, Bohm M, Meinertz T, Munzel T. Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. *Circulation*. 1999;99:2027–2033.
38. Cyrus T, Pratico D, Zhao L, Witztum JL, Rader DJ, Rokach J, FitzGerald GA, Funk CD. Absence of 12/15-lipoxygenase expression decreases lipid peroxidation and atherogenesis in apolipoprotein E-deficient mice. *Circulation*. 2001;103:2277–2282.
39. Hernandez-Presa M, Bustos C, Ortego M, Tunon J, Renedo G, Ruiz-Ortega M, Egido J. Angiotensin-converting enzyme inhibition prevents arterial nuclear factor- κ B activation, monocyte chemoattractant protein-1 expression, and macrophage infiltration in a rabbit model of early accelerated atherosclerosis. *Circulation*. 1997;95:1532–1541.
40. Ito M, Oliverio MI, Mannon PJ, Best CF, Maeda N, Smithies O, Coffman TM. Regulation of blood pressure by the type 1A angiotensin II receptor gene. *Proc Natl Acad Sci U S A*. 1995;92:3521–3525.
41. Mangrum AJ, Gomez RA, Norwood VF. Effects of AT_{1A} receptor deletion on blood pressure and sodium excretion during altered dietary salt intake. *Am J Physiol*. 2002;283:F447–F453.
42. Siragy HM, Carey RM. Angiotensin type 2 receptors: potential importance in the regulation of blood pressure. *Curr Opin Nephrol Hypertens*. 2001;10:99–103.
43. Yamada H, Akishita M, Ito M, Tamura K, Daviet L, Lehtonen JY, Dzau VJ, Horiuchi M. AT₂ receptor and vascular smooth muscle cell differentiation in vascular development. *Hypertension*. 1999;33:1414–1419.
44. van Kats JP, Methot D, Paradis P, Silversides DW, Reudelhuber TL. Use of a biological peptide pump to study chronic peptide hormone action in transgenic mice: direct and indirect effects of angiotensin II on the heart. *J Biol Chem*. 2001;276:44012–44017.
45. Cole JM, Khokhlova N, Sutliff RL, Adams JW, Disher KM, Zhao H, Capecchi MR, Corvol P, Bernstein KE. Mice lacking endothelial ACE: normal blood pressure with elevated angiotensin II. *Hypertension*. 2003;41:313–321.
46. 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.
47. Muller P, Flesch G, de Gasparo M, Gasparini M, Howald H. Pharmacokinetics and pharmacodynamic effects of the angiotensin II antagonist valsartan at steady state in healthy, normotensive subjects. *Eur J Clin Pharmacol*. 1997;52:441–449.
48. Ichikawa S, Takayama Y. Long-term effects of olmesartan, an Ang II receptor antagonist, on blood pressure and the renin-angiotensin-aldosterone system in hypertensive patients. *Hypertens Res*. 2001;24:641–646.
49. Nakamura A, Iwao H, Fukui K, Kimura S, Tamaki T, Nakanishi S, Abe Y. Regulation of liver angiotensinogen and kidney renin mRNA levels by angiotensin II. *Am J Physiol*. 1990;258:E1–E6.
50. Brasier AR, Jamaluddin M, Han Y, Patterson C, Runge MS. Angiotensin II induces gene transcription through cell-type-dependent effects on the nuclear factor- κ B (NF- κ B) transcription factor. *Mol Cell Biochem*. 2000;212:155–169.