Angiotensin II increases vascular proteoglycan content preceding and contributing to atherosclerosis development[1]

Fei Huang,* Joel C. Thompson,* Patricia G. Wilson,* Hnin H. Aung,† John C. Rutledge,† and Lisa R. Tannock[2,*,§]

Division of Endocrinology and Metabolism,* University of Kentucky, Lexington, KY 40536-0200; Division of Endocrinology, Clinical Nutrition, and Vascular Medicine,† University of California, Davis, Davis, CA 95616; and Department of Veterans Affairs Medical Center,§ Lexington, KY 40511

Abstract Angiotensin II (angII) is known to promote atherosclerosis; however, the mechanisms involved are not fully understood. To determine whether angII stimulates proteoglycan production and LDL retention, LDL receptor-deficient mice were infused with angII (1,000 ng/kg/min) or saline via osmotic minipumps. To control for the hypertensive effect of angII, a parallel group received norepinephrine (NE; 5.6 mg/kg/day). Arterial lipid accumulation was evaluated by measuring the retention rate of LDL in isolated carotid arteries perfused ex vivo. Mice infused with angII had increased vascular content of biglycan and perlecan and retained twice as much LDL as saline- or NE-infused mice, although no group developed atherosclerosis at this time. To determine whether this increase in biglycan and perlecan content predisposed to atherosclerosis development, mice were infused with angII, saline, or NE for 4 weeks, then pumps were removed and mice received an atherogenic Western diet for another 6 weeks. Mice that had received angII infusions had 3-fold increased atherosclerosis compared with mice that had received saline or NE, and apolipoprotein B colocalized with both proteoglycans. Thus, one mechanism by which angII promotes atherosclerosis is increased proteoglycan synthesis and increased arterial LDL retention, which precedes and contributes to atherosclerosis development.—Huang, F., J. C. Thompson, P. G. Wilson, H. H. Aung, J. C. Rutledge, and L. R. Tannock. **Angiotensin II increases vascular proteoglycan content preceding and contributing to atherosclerosis development.** ***J. Lipid Res.*** **2008.** 49: **521–530.**

Supplementary key words low density lipoprotein retention • biglycan • extracellular matrix • norepinephrine

Angiotensin II (angII) is the major bioactive peptide of the renin-angiotensin system. There are abundant data from animal studies showing that angII administration potentiates the development of atherosclerosis, and antagonism of either angII formation or its interaction with its major receptor AT1a attenuates lesion progression (1–5). However, the mechanisms by which angII contributes to atherosclerosis development are not yet fully understood.

Numerous studies support the notion that angII has multiple actions at various levels in the development of vascular lesions. The atherogenicity of angII has been ascribed to several effects of angII, including its proinflammatory properties and its effect in stimulating the proliferation of vascular smooth muscle cells (6, 7). AngII is known to stimulate the secretion of extracellular matrix (ECM) components by vascular smooth muscle cells, such as laminin, fibronectin, collagen, elastin, and proteoglycans (8–12). However, it is not known whether this stimulation of ECM components leads to increased LDL retention in the artery wall.

As outlined in the "response-to-retention" hypothesis, the retention of atherogenic lipoprotein particles within the subendothelial space of the vascular wall is thought to be a critical step in the initiation of atherosclerosis (13). The interactions of LDL particles with ECM components facilitate changes of their structure, rendering them vulnerable to modification and uptake by macrophages (14). Apolipoprotein B (apoB), the major lipoprotein component of LDL, has been identified in close association with the vascular proteoglycans biglycan and decorin in human lesions (15–17), whereas in mouse atherosclerosis, apoB appears to colocalize with biglycan and perlecan (18). Proteoglycans bind lipoproteins via ionic interactions between negatively charged sulfate and carboxylic acid groups on the glycosaminoglycan chains with positively charged residues of apoB in the LDL particle

Manuscript received 19 July 2007 and in revised form 2 November 2007 and in re-revised form 21 November 2007.

Published, JLR Papers in Press, November 21, 2007.
DOI 10.1194/jlr.M700329-JLR200

[1] This work was presented in part at the Arteriosclerosis, Thrombosis, and Vascular Biology annual conference, Denver, CO, 2006.

[2] To whom correspondence should be addressed.
e-mail: lisa.tannock@uky.edu

(19, 20). Studies have demonstrated that angII stimulates the synthesis of proteoglycans by cultured vascular smooth muscle cells, with one study demonstrating increased LDL binding affinity of proteoglycans synthesized by vascular smooth muscle cells stimulated with angII (8, 12). The purpose of this study was to test the hypothesis that one mechanism by which angII induces atherosclerosis is via increased vascular proteoglycan synthesis with increased LDL retention in the artery wall.

MATERIALS AND METHODS

Chemicals and reagents

Cell culture media and additives were obtained from Invitrogen (Carlsbad, CA). All other reagents were from Sigma (St. Louis, MO) unless specified otherwise.

Mouse model

Female low density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice (C57BL6 background) and $AT1a^{-/-}$ $LDLR^{-/-}$ mice (21) were kindly provided by Dr. Alan Daugherty at the University of Kentucky. $LDLR^{-/-}$ mice were selected as the model because they do not develop atherosclerosis over the short term unless fed a high-fat, high-cholesterol diet. Mice were housed in a temperature-controlled facility with 12 h light/dark cycles. Mice consumed normal rodent chow ad libitum and had free access to water. In some experiments, mice received 6 weeks of Western diet (0.15% cholesterol and 21% fat; Harlan Teklad, catalog No. 88137) to induce atherosclerosis development. Animal care and experimental procedures were approved by and performed in accordance with University of Kentucky and University of California, Davis, Animal Care Committee guidelines, in conformity with Public Health Service policy on the humane care and use of laboratory animals.

Transient infusion of angII and norepinephrine

Mice reaching 20 g (generally at 10–12 weeks old) received angII (1,000 ng/kg/min) or saline for up to 28 days via Alzet osmotic minipumps (model 2004; ALZA Scientific Products, Mountain View, CA) implanted subcutaneously in the scapular region, as described previously (22). In some experiments, mice were infused with lower doses of angII (250 or 500 ng/kg/min). To control for the effects of angII to induce hypertension, parallel groups received either norepinephrine (NE; 5.6 mg/kg/day), selected to mimic the blood pressure increase induced by angII (3), or its vehicle, 0.2% ascorbate. In some experiments, the pumps were removed after 28 days to ensure complete cessation of infusions, then the mice were fed an atherogenic Western diet for the next 6 weeks. Systolic blood pressure was measured four to five times per week in conscious mice using an automatic tail cuff apparatus coupled to a personal computer-based data-acquisition system (RTBP1007; Kent Scientific, Litchfield, CT), with each measurement performed at the same time of day by the same operator. For each measurement, 10 chronological readings were obtained from each mouse to yield a mean blood pressure value for the day. Before pump implantation, mice were acclimatized to the system through 1 week of baseline blood pressure measurements.

Metabolic characterization

Serum total cholesterol concentrations were determined using an enzymatic cholesterol assay kit (Wako Chemical Co., Richmond, VA). Lipoprotein cholesterol distributions were evaluated in individual serum samples (50 μl) from three mice in each group after fractionation by fast-protein liquid chromatography gel filtration (Pharmacia LKB Biotechnology, Uppsala, Sweden) on a single Superose 6 column (23). Fractions were collected and cholesterol concentrations were determined with the cholesterol assay kit mentioned above. Transforming growth factor (TGF)-β was quantified in plasma collected after 3 days of infusions, with the TGF-β1 E_{max}® ImmunoAssay System (Promega, Madison, WI) according to the manufacturer's directions. Samples were acid-activated before quantification.

LDL isolation

LDL isolation was performed according to the method of Sattler, Mohr, and Stocker (24), and LDL protein was labeled with Alexa Fluor 546 (Molecular Probes, Eugene, OR) as described by the manufacturer's protocol (25). Briefly, postprandial blood samples were obtained from healthy volunteers at 3.5 h after consumption of a high-fat meal. These studies were approved by the Institutional Review Board of the University of California, Davis. LDL (d = 1.01–1.06 g/ml) was isolated from human plasma and obtained by sequential density gradient ultracentrifugation for 18 h at 14°C at 40,000 rpm in a 50.4Ti rotor. The labeled LDL (~2 mg protein/ml) was diluted in Krebs-Henseleit solution (final concentration, ~50 μg/ml) for use in these experiments.

Measurement of arterial lipid accumulation

Mice were anesthetized with intraperitoneal injection of pentobarbital (50 mg/kg body weight). The carotid arteries from the mice were dissected, cannulated, removed, and placed in a microscope viewing chamber, as described previously (26). Briefly, the vessels were alternately perfused with fluorescently labeled LDL and nonfluorescent buffer solution at a rate of 1.5 ml/min in physiological flow direction, pH, temperature, and pressure; thus, each artery serves as its own control. The nonfluorescent buffer was 1% BSA-Krebs-Henseleit buffer, and the perfusate contained 50 μg/ml fluorescently labeled LDL molecules. LDL accumulation was quantified during the washout phase as described previously (26).

Immunohistochemistry

Immunohistochemical staining was performed as described previously (22). Briefly, serial frozen sections (8 μm thick) were stained. The primary antibodies used were as follows: LF-159 for biglycan and LF-113 for decorin (both polyclonal, 1:200 dilution; generously provided by Dr. Larry Fisher, National Institutes of Health); K23300R for apoB (polyclonal, 1:50 dilution; which recognizes both mouse apoB-48 and human apoB-100; BioDesign, Saco, ME); and AB1033 for versican (polyclonal, 1:200 dilution; Chemicon, Temecula, CA). Perlecan was detected using RT-794-B1 (monoclonal, biotinylated, 1:100 dilution; Lab Vision-NeoMarkers, Fremont, CA). No biotinylated secondary antibody was needed for perlecan detection during the process. Negative controls were obtained with isotype-matched irrelevant antibodies, no primary antibody, or no secondary antibody.

Western blot analysis of proteoglycan expression

Thoracic aortas were stripped of adventitia, total protein was extracted and dissolved in 1% SDS, and concentration was determined by the method of Lowry et al. (27). Equal amounts of protein were separated by SDS-PAGE using 10% gels and then transferred to polyvinylidene fluoride membranes in 20% methanol. Membranes were blocked in 5% nonfat milk and 0.1%

Tween-20, then probed with antibodies against mouse biglycan (1:1,000 dilution; LF-159; National Institutes of Health). HRP-labeled secondary antibody was used at 1:25,000 dilution and detected using SuperSignal West Pico (34080; Pierce, Rockford, IL). Blotting for β-actin (ab8227; Abcam, Inc., Cambridge, MA) served as a loading control.

Cell culture experiments

Vascular smooth muscle cells isolated from rat (generously provided by Dennis Bruemmer, University of Kentucky) were cultured as described previously (28). After reaching confluence, the cells were serum-deprived (0.1%) for 48 h, then stimulated with angII (1 μmol/l) or saline, in the presence or absence of losartan (1 μg/ml), TGF-β neutralizing antibody 1D11 (10 μg/ml) (29), or irrelevant antibody 13C4 (10 μg/ml; both R&D Systems, Minneapolis, MN) for 24 h. Cells were metabolically labeled with $^{35}SO_4$ (100 μCi/ml) for the 24 h period. Sulfate incorporation into secreted proteoglycans was quantified using cetyl pyridinium chloride precipitation as described previously (30). The cell layer was washed with saline, then cell protein was quantified using a bicinchoninic acid protein assay kit (Bio-Rad, Hercules, CA). Sulfate incorporation was adjusted for cell protein content. The medium was collected, and secreted proteoglycans were concentrated and purified using separate DEAE-Sephacel minicolumns as described previously (30, 31). To estimate molecular weight, equal counts were applied to SDS-PAGE gels (3.5% stacking gel and 4–12% gradient resolving gel) (30, 31). Radiolabeled molecular weight markers were run in a parallel lane. Parallel wells were treated identically except without the metabolic labeling, and Western blot analysis was performed on the conditioned medium, as described above.

Quantification of atherosclerotic lesions

Atherosclerosis was quantified in three vascular beds: the aortic root, the aortic intimal surface, and the brachiocephalic artery (32). Aortic root sections (8 μm thick) collected every 72 μm and brachiocephalic artery sections (5 μm thick) collected every 125 μm were stained with Oil Red O and quantified using computer-assisted morphometry, as described previously (33). Aortic intimal surface lesions were evaluated by en face quantification of lesions as detailed previously (33). All quantifications were performed in duplicate by observers blinded to the group assignments of the mice.

Statistical analyses

Statistical differences of the experimental data were first assessed by one-way nonparametric ANOVA, followed by Dunn's multiple comparison test if a significant group difference was detected. All analyses were performed with SigmaStat (Jandel Scientific, San Rafael, CA). All data are normally distributed and presented as means ± SEM, except for that in Fig. 4, in which individual data points are shown. $P < 0.05$ was considered statistically significant.

RESULTS

AngII stimulates arterial LDL retention before the development of atherosclerosis

To determine whether angII stimulates arterial LDL retention, $LDLR^{-/-}$ mice were infused with angII (1,000 ng/kg/min) or saline for 4 weeks. Because angII infusions induce hypertension, parallel groups of littermate mice were infused with NE (5.6 mg/kg/min), which induces a similar degree of hypertension as does angII (3). During the infusion period, mice were fed normal laboratory chow, which does not stimulate atherosclerosis development. At the end of the infusion period, carotid arteries were isolated and perfused with LDL ex vivo. Carotid arteries from mice that received angII had a significantly higher rate of LDL retention ($P = 0.0006$) compared with carotid arteries from saline- or NE- infused littermate controls (**Fig. 1**).

AngII stimulates vascular biglycan content, which colocalizes with apoB

To determine whether this increase in arterial LDL retention was mediated by proteoglycans, adjacent sections of the perfused carotids were stained for apoB and the major vascular proteoglycans biglycan, perlecan, and decorin. Both biglycan and decorin exhibited strong staining in the adventitia in all groups, but carotids from angII-infused mice consistently demonstrated increased medial content of biglycan (**Fig. 2A**). Perlecan stained strongly in all mouse carotids, but total vascular content appeared to be increased in angII-infused mouse carotids compared with saline- or NE-infused mouse carotids (Fig. 2A). ApoB was detected in carotids from angII- and NE-infused mice, but not from saline-infused mice, and colocalized with both biglycan and perlecan, but not with decorin (Fig. 2). To confirm the immunohistochemical data, aortic protein was collected after 28 days of infusions and Western blot analysis demonstrated increased content of biglycan (Fig. 2B). Perlecan could not be evaluated by Western blot because of poor transfer. No difference was seen in decorin content between angII-, saline-, and NE-infused mice (data not shown).

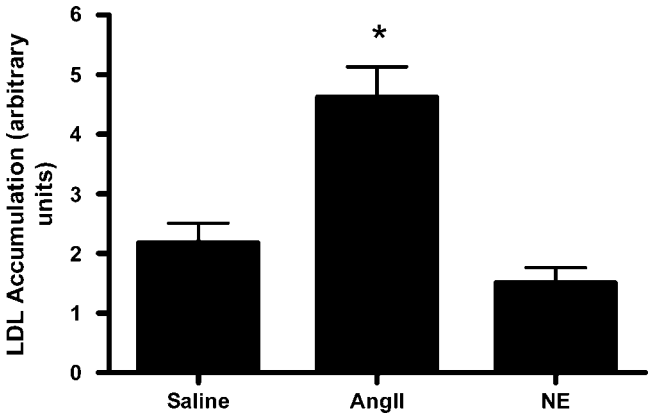

Fig. 1. Angiotensin II (angII) increases arterial LDL retention ex vivo in the absence of atherosclerosis. Chow-fed low density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice were infused with saline, angII, or norepinephrine (NE) for 28 days, then carotids were removed and LDL retention was quantified as described in Materials and Methods. Data shown are means ± SEM of n = 8 mice (saline group), n = 9 mice (angII group), and n = 4 mice (NE group). * $P < 0.01$ compared with the saline or NE group.

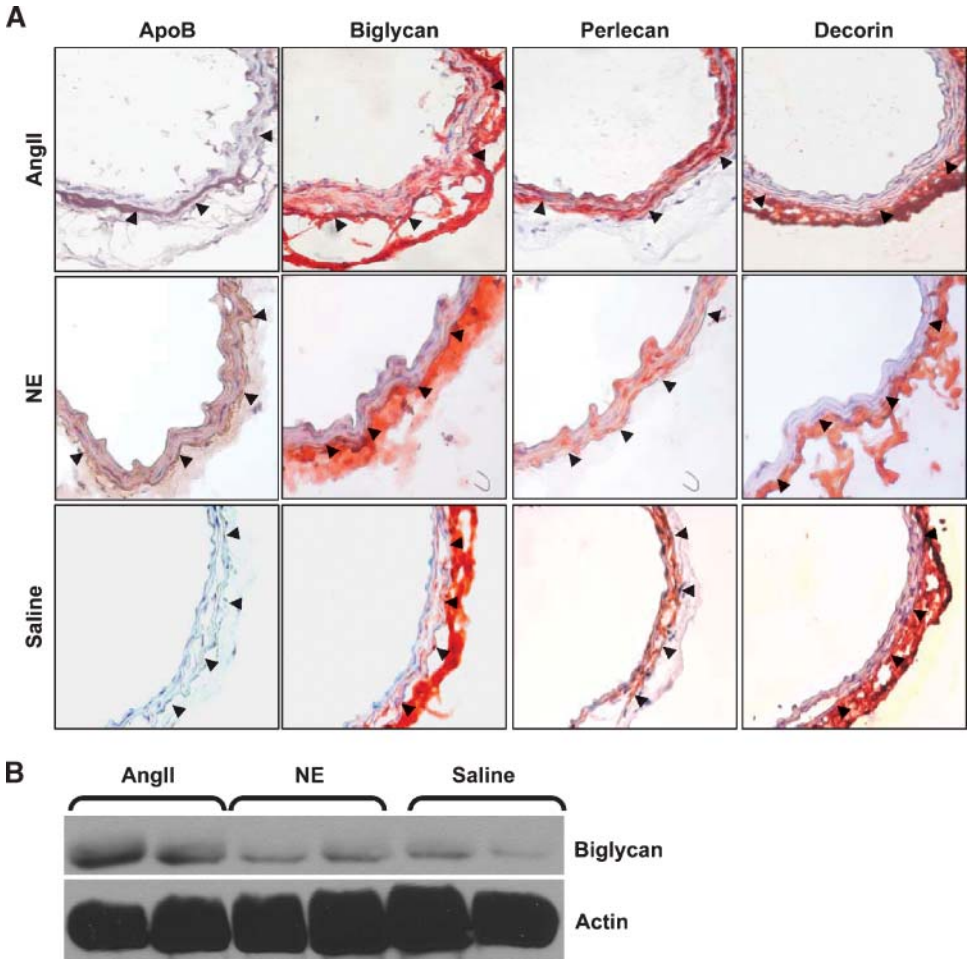

Fig. 2. AngII increases vascular biglycan. A: AngII-infused mice have increased carotid biglycan and perlecan compared with saline-infused mice, and apolipoprotein B (apoB) colocalizes with these proteoglycans in the absence of atherosclerosis. $LDLR^{-/-}$ mice were infused with angII, saline, or NE for 28 days, then carotids were removed and perfused ex vivo with LDL. Serial sections were stained for apoB (brown color product), biglycan, decorin, and perlecan (each red color product), demonstrating colocalization between apoB, biglycan, and perlecan. In each case, the lumen is on the upper left side of the image. In the case of biglycan and decorin, the adventitia stains strongly. The outer limit of the media is depicted by arrowheads in each image. Photos shown are representative of four mice per group, magnified 400×. B: AngII increases aortic biglycan in the absence of atherosclerosis. Thoracic aortas were obtained from mice after 28 days of angII, saline, or NE infusions, and Western blot analyses were performed. n = 5 per group. Western blots show two mice per group.

AngII stimulation of biglycan is dose-dependent and requires the AT1a receptor

To elicit mechanisms by which angII stimulates biglycan expression, mice were infused with saline or angII at 250, 500, or 1,000 ng/kg/min for 28 days. AngII increased blood pressure in a dose-dependent manner (average systolic blood pressure by group was 114, 129, 155, and 165 mm Hg for saline and angII at 250, 500, and 1,000 ng/kg/min, respectively; $P < 0.001$). AngII stimulated aortic biglycan content in a dose-dependent manner (**Fig. 3A**). AngII has been proposed to stimulate biglycan synthesis via increased TGF-β concentrations (34), and we and others have reported previously that TGF-β increases the synthesis of vascular proteoglycans, especially biglycan, and increases proteoglycan LDL binding affinity (30, 31, 35). Plasma TGF-β concentrations quantified after 3 days of infusions were increased by angII in a dose-dependent manner (Fig. 3B). To determine whether TGF-β was required for angII stimulation of biglycan, vascular smooth muscle cells in culture were stimulated with angII (1 μmol/l) in the presence or absence of an irrelevant antibody or a TGF-β-neutralizing antibody (13C4 and 1D11, respectively) (29). AngII exposure to cells caused increased total proteoglycan synthesis, as indicated by relative sulfate incorporation, and increased proteoglycan size; 13C4 had no effect, but the angII stimulation of sulfate incorporation and size was inhibited by 1D11 (Fig. 3C, D). Western blot analysis revealed stimulation of biglycan by angII, which was partially inhibited by 13C4 but completely inhibited by 1D11 and losartan (Fig. 3E). Losartan also inhibited the effect of angII to stimulate increased proteoglycan synthesis and size (Fig. 3C, D). AngII infusion

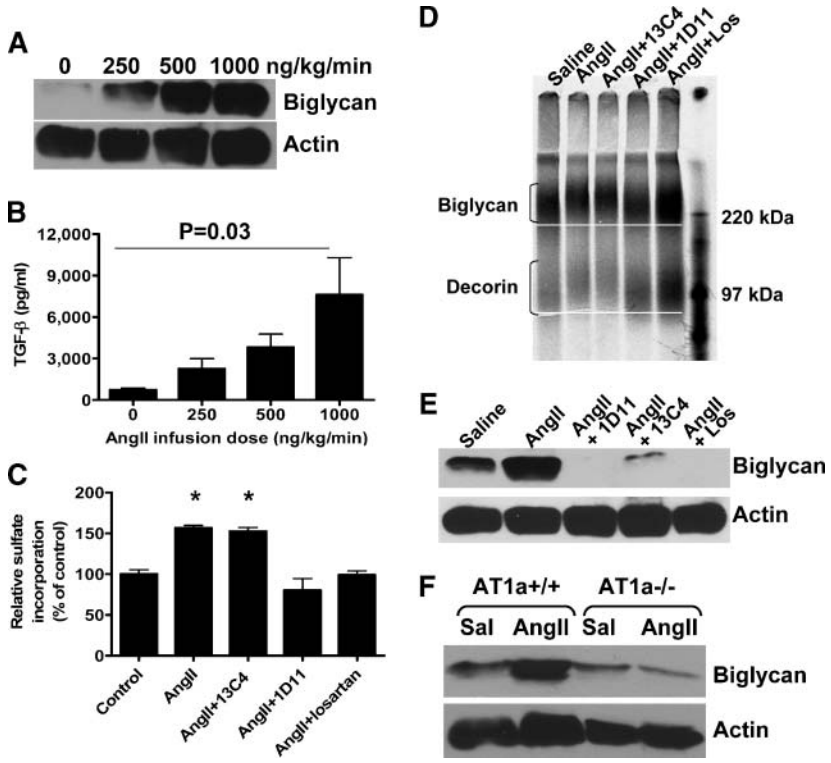

Fig. 3. AngII induction of biglycan requires transforming growth factor (TGF)-β and the AT1a receptor. A: AngII stimulates biglycan in a dose-dependent manner. Mice were infused with saline or angII at 250, 500, or 1,000 ng/kg/min for 28 days. Thoracic aortas were collected, and biglycan and actin were evaluated by Western blot. Shown are data for one mouse per group, representative of four mice per group. B: AngII stimulates TGF-β in a dose-dependent manner. TGF-β was determined in plasma samples collected after 3 days of infusions with angII at the indicated doses. Shown are means ± SEM from n = 4 mice per group. C: AngII stimulation of proteoglycan synthesis is inhibited by TGF-β-neutralizing antibody and by losartan. Confluent rat vascular smooth muscle cells were stimulated with angII (1 μmol/l) with or without losartan (1 μg/ml), TGF-β-neutralizing antibody 1D11 (10 μg/ml), or control antibody 13C4 (10 μg/ml) in the presence of 100 μCi/ml $^{35}SO_4$ for 24 h. Sulfate incorporation was determined as described in Materials and Methods. Data shown are means ± SEM relative to cells exposed to medium alone (control), which is expressed as 100%. Data are representative of two independent experiments performed in triplicate. * $P < 0.01$ versus control. D: AngII stimulation of proteoglycan size is inhibited by TGF-β-neutralizing antibody and by losartan. Proteoglycan size was estimated using SDS-PAGE (3.5% stacking gel with 4–12% gradient resolving gel). Cells were stimulated with the agents indicated, and secreted proteoglycans were isolated and purified. Lanes were loaded with equal counts. Horizontal white lines are superimposed on the image at the lower end of the biglycan and decorin bands from saline-exposed cells; the bands from angII and angII+13C4-exposed cells are shifted away from the lines, indicating their larger size. The gel shown is representative of two independent experiments. E: AngII stimulation of biglycan is inhibited by TGF-β-neutralizing antibody and by losartan. Lanes were loaded with equal amounts of protein, and biglycan and actin were evaluated by Western blot. F: AngII stimulation of biglycan requires the AT1a receptor. LDLR$^{-/-}$ mice wild type or deficient in the AT1a receptor were infused with saline or angII (1,000 ng/kg/min) for 28 days. Biglycan was determined on thoracic aortic protein by Western blot. The blot shows data for one mouse per group, representative of four to six mice per group.

of AT1a$^{-/-}$ LDLR$^{-/-}$ mice did not stimulate biglycan content (Fig. 3E), demonstrating that the presence of the AT1a receptor is required for angII to stimulate biglycan.

AngII predisposes to increased diet-induced atherosclerosis

To determine whether the induction of vascular biglycan content and the stimulation of LDL retention predisposed to accelerated atherosclerosis, mice received infusions (angII at 1,000 ng/kg/min, saline, NE, or ascorbic acid) for 4 weeks while fed normal chow, which does not induce atherosclerosis. After 4 weeks, the pumps were removed to ensure the cessation of the delivery of agents, and the mice were fed high-fat, high-cholesterol Western diets for another 6 weeks. Blood pressure was monitored throughout the 10 weeks. Both angII and NE infusion increased systolic blood pressure to a similar extent (systolic pressure of 171.5 ± 2.1 and 161.1 ± 2.5 mm Hg for the angII group and the NE group, respectively) compared with the vehicle infusion controls (systolic pressure of 123.7 ± 2.8 and 121.2 ± 1.1 mm Hg for the saline group and the ascorbate group, respectively) during the 4 weeks of infusions. Systolic blood pressure returned to baseline

levels after the removal of the minipumps (systolic pressure of 121.8 ± 2.0 mm Hg in the angII group, 120.6 ± 1.9 mm Hg in the NE group, 118.2 ± 3.6 mm Hg in the saline group, and 119.2 ± 2.8 mm Hg in the ascorbate group). Initiation of the Western diet substantially increased hyperlipidemia in all four groups after 6 weeks (total plasma cholesterol during infusions while fed normal rodent chow was 141 ± 8 mg/dl; total plasma cholesterol after 6 weeks of the Western diet was 985 ± 11 mg/dl), but at no time were any differences found in body weight, plasma cholesterol levels, or lipoprotein cholesterol distribution between groups (data not shown). None of the groups had detectable atherosclerosis after 4 weeks of infusions (data not shown); however, all four groups developed atherosclerosis after 6 weeks of diet-exacerbated hyperlipidemia. The group that had received 4 weeks of angII infusion before diet feeding had significantly increased atherosclerotic lesion areas compared with the other three groups, as confirmed by the quantification in aortic root, en face thoracic aorta, and brachiocephalic arteries (**Fig. 4A–C**; $P < 0.001$ for each). Immunohistochemical analyses demonstrated that mice previously infused with angII had increased staining for biglycan and perlecan, but not for decorin or versican, in the subendothelial region compared with the other groups (**Fig. 5A**). ApoB was colocalized with both biglycan and perlecan in atherosclerotic lesions in all groups, but it had strongest staining in the mice previously infused with angII (Fig. 5B).

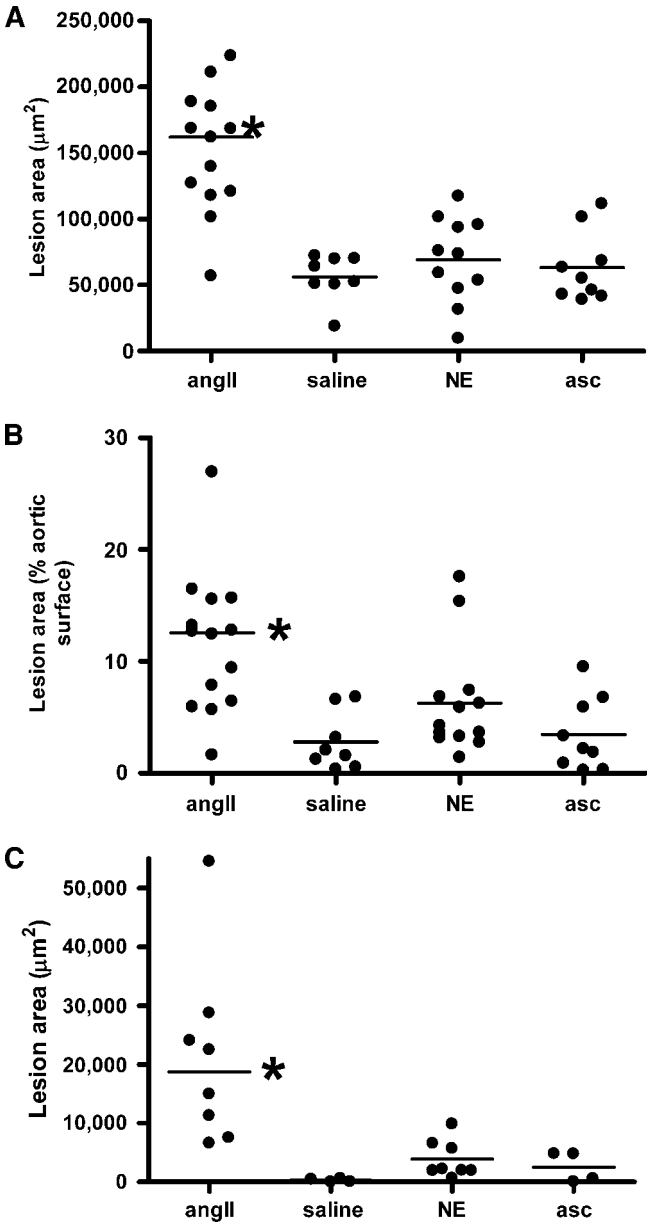

Fig. 4. Previous angII infusion predisposes to increased atherosclerosis. LDLR$^{-/-}$ mice were infused with angII, saline, NE, or ascorbate (asc; vehicle for NE) for 28 days while fed normal rodent chow. After 28 days, the pumps were removed and mice were fed a Western diet for 6 weeks. Atherosclerotic area was determined in the aortic sinus (A), the en face aortic surface (B), and the brachiocephalic artery (C) as described in Materials and Methods. Each symbol represents the lesion area of an individual mouse, and each horizontal line represents the median for the group. In each case, the angII group is significantly different from all other groups (* $P < 0.01$), and the other groups do not differ significantly from each other.

DISCUSSION

The purpose of this study was to determine whether angII promoted proteoglycan-mediated vascular lipid retention, thought to be one of the key steps in the initiation of atherosclerosis, as outlined in the response-to-retention hypothesis (13). Our data demonstrate that angII stimulates increased vascular content of proteoglycans, especially biglycan, in vivo, and increased LDL retention before the development of atherosclerosis. Upon subsequent challenge with a high-fat, high-cholesterol diet, mice that had previously been infused with angII had increased atherosclerosis development compared with mice previously infused with NE or saline. Immunohistochemistry demonstrated that apoB strikingly colocalized with biglycan and perlecan in carotids perfused with human LDL ex vivo and in the atherosclerotic lesions in aortic roots from mice after 6 weeks of diet-induced hyperlipidemia. Thus, we now demonstrate that increased vascular proteoglycan content precedes and contributes to the development of atherosclerosis, rather than increasing in context with the developing atherosclerotic lesion.

Proteoglycans bind lipoproteins through ionic interactions between the negatively charged sulfate and carboxylic groups on glycosaminoglycan chains and the positively charged amino acid residues on apoB or apoE. It has been shown that lipoproteins can migrate in and out of the artery wall, and it has been reported that lipoprotein entry does not differ between atherosclerosis-susceptible and atherosclerosis-resistant areas of the vasculature (36). Thus, the retention of lipoproteins appears to be critical in atherosclerosis. Several studies have shown that the proteoglycan content differs between atherosclerotic and normal regions of the artery wall (37, 38), but to date,

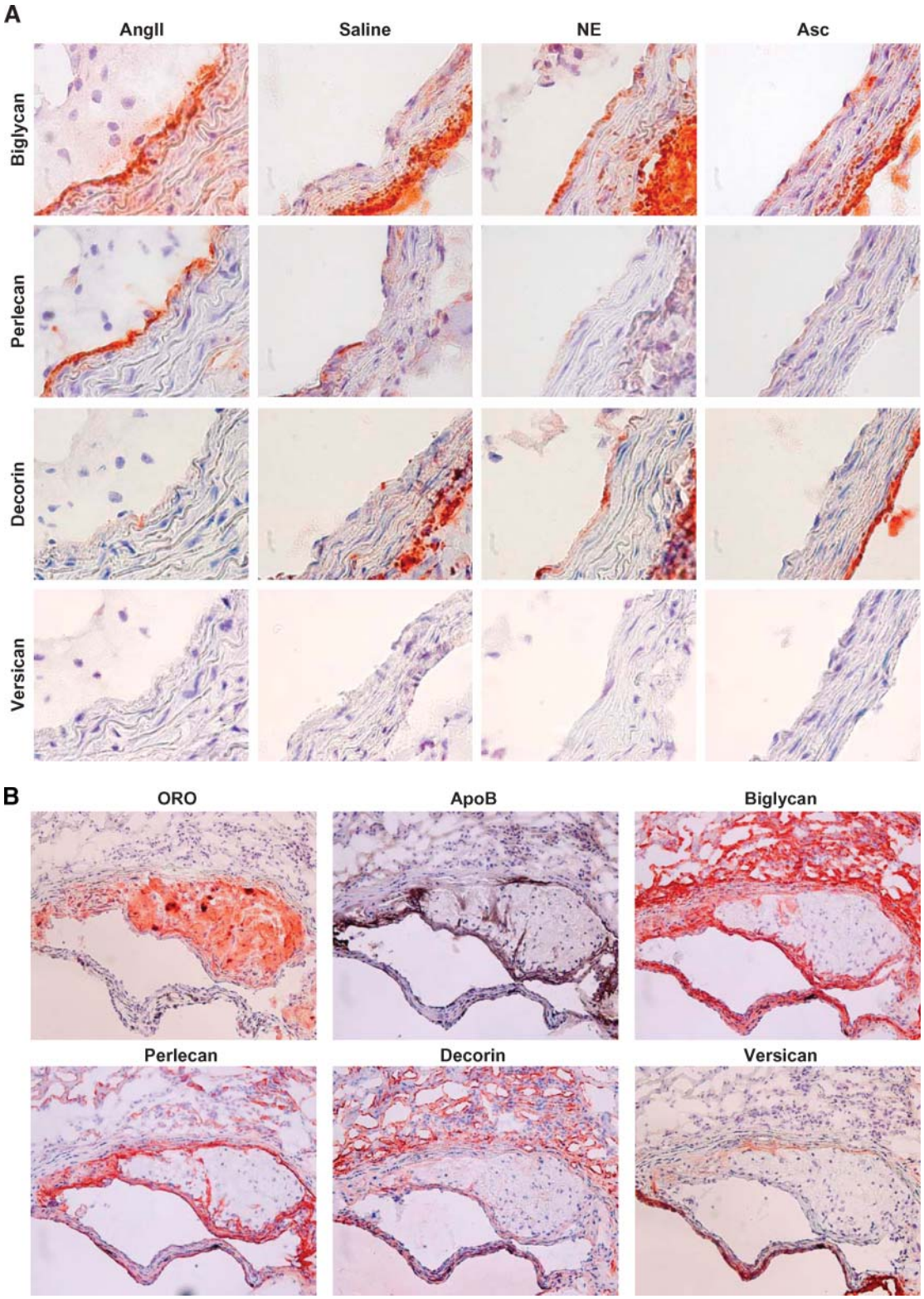

Fig. 5. Previous angII infusion predisposes to increased aortic root biglycan and perlecan, which colocalize with apoB in atherosclerotic lesions. LDLR$^{-/-}$ mice were infused with angII, saline, NE, or ascorbate (Asc) for 28 days while fed normal rodent chow. After 28 days, the pumps were removed and mice were fed a Western diet for 6 weeks. A: Adjacent sections from atherosclerotic lesions were immunostained for biglycan, perlecan, decorin, or versican (each red color product). Photos are representative of n = 6 mice for the angII and NE groups and n = 4 mice for the saline and ascorbate groups, magnified 1,000×. In each photo, the lumen is on the upper left side of the image. Strong adventitial staining of biglycan and decorin can be seen in some images. B: Serial sections from an atherosclerotic lesion in the aortic sinus were stained with Oil Red O (ORO), apoB (brown color product), biglycan, perlecan, decorin, or versican (each red color product). Photos are representative of a mouse from the angII-pretreated group, magnified 200×. In each photo, the lumen is at the bottom of the image.

data demonstrating the role of proteoglycans in atherosclerosis development are limited. Biglycan has been reported to be the proteoglycan most colocalized with apoB in both human (15, 17) and mouse (18) atherosclerosis. However, apoB has also been seen colocalized with decorin in humans (17) and with perlecan in mice (18), suggesting that these other proteoglycans may also play a role in mediating lipoprotein retention. A previous study reported that male apoE$^{-/-}$ mice heterozygous for perlecan had less atherosclerosis after 12 weeks of normal chow diet (39); however, after longer follow up, this protection from atherosclerosis was lost, and in female apoE$^{-/-}$ or male LDLR$^{-/-}$ mice, no protection from atherosclerosis was observed in perlecan heterozygotes (39). These authors proposed that decreased LDL retention may have contributed to their findings of reduced atherosclerosis in young male apoE$^{-/-}$ mice, and our data support this concept. However, as lesions progress and mature, other mechanisms promoting atherosclerosis come into play, and the role of proteoglycan-mediated LDL retention may become less prominent.

A role for lipoprotein lipase in mediating the retention of LDL at later stages of atherosclerosis has been demonstrated (40–42). Decorin overexpression in apoE$^{-/-}$ mice was shown to decrease atherosclerosis development, with decreased plaque macrophage and collagen content (43). LDL retention was not quantified; however, decorin overexpression was associated with decreased triglyceride and plasma TGF-β concentrations, suggesting that the effect of decorin overexpression to attenuate atherosclerosis development may be mediated via multiple mechanisms. Although there are no data available regarding altered biglycan content in vivo on atherosclerosis development, overexpression of biglycan in smooth muscle cells in vitro was found to cause increased lipoprotein retention on the extracellular matrix (44). Thus, this finding, taken in conjunction with our finding of increased vascular biglycan content and increased LDL retention in vivo, supports a proatherogenic role for biglycan.

The mechanism by which angII stimulates vascular proteoglycan synthesis and LDL retention is not fully understood. Several in vitro studies demonstrate that angII stimulates proteoglycan synthesis by vascular smooth muscle cells (8, 12) as well as by other cell types pertinent to atherosclerosis, including macrophages (45), cardiac myocytes (46), and fibroblasts (34). The mechanism by which angII stimulates proteoglycan synthesis is not fully understood, but it appears to be mediated through the AT1 receptor, although one report suggests that both AT1 and AT2 receptors are involved (12). AT1a$^{-/-}$ mice have a striking protection from atherosclerosis (21), and it is the expression of AT1a on resident vascular cells (rather than on bone marrow-derived cells such as macrophages) that is critical for atherosclerosis development (47). Our finding that AT1a$^{-/-}$ mice do not have increased proteoglycan synthesis with angII infusion suggests that the lack of proteoglycan stimulation by AT1a$^{-/-}$ resident vascular cells may be part of the mechanism by which AT1a$^{-/-}$ mice are protected from atherosclerosis.

Several studies have demonstrated a link between angII and TGF-β. We and others have shown that TGF-β potently upregulates vascular proteoglycan synthesis and stimulates the synthesis of longer glycosaminoglycan chains with increased LDL binding affinity (30, 35, 48). Our findings that angII infusions increased plasma TGF-β levels, and that TGF-β-neutralizing antibodies inhibited the effect of angII to increase proteoglycan synthesis and size in vitro, suggest that angII-induced increased TGF-β is a mechanism by which angII increases LDL retention and atherosclerosis development. In this context, TGF-β plays a proatherogenic role, via its effects to increase proteoglycan-LDL binding interactions. In vivo experimental data regarding the role of TGF-β are limited; however, two separate studies have demonstrated that the inhibition of TGF-β signaling decreased atherosclerotic plaque fibrosis but increased plaque inflammation (49, 50). Thus, the role of TGF-β in atherosclerosis development appears complicated.

Although angII infusions increase blood pressure, our data suggest that the effect of angII to increase vascular proteoglycan synthesis and LDL retention is direct, rather than subsequent to the development of hypertension. Infusion of mice with NE at a dose that led to a similar degree of hypertension as angII did not stimulate proteoglycan synthesis, LDL retention, or subsequent stimulation of atherosclerosis after diet-exacerbated hyperlipidemia. Furthermore, our data are consistent with the study by Ayabe et al. (51), who found that a 2 week angII preinfusion (1,000 ng/kg/min) did not induce atherosclerosis at the end of the infusion, although it did 14 weeks later in apoE-deficient mice.

One limitation of our data is that we used human LDL in the carotid perfusion studies, rather than mouse LDL. However, like human LDL, LDL from LDLR$^{-/-}$ mice contains predominantly apoB-100, rather than apoB-48 (52). Moreover, previous detailed studies using gene-targeted mice expressing only apoB-48 or only apoB-100 have demonstrated equal atherogenicity of these lipoproteins (53). Both apoB-100-containing LDL and apoB-48-containing LDL bind to purified biglycan (54), which could account for the fact that apoB-48 and apoB-100 are equally atherogenic (53). Furthermore, another study demonstrated no difference in arterial LDL accumulation between hamster and human LDL (55). Our data demonstrate that angII-infused mice have increased retention of human LDL in carotids perfused ex vivo and increased development of atherosclerosis, which supports our hypothesis that one mechanism by which angII is proatherogenic is by increasing proteoglycan-mediated LDL retention in the artery wall.

In summary, we conclude that angII can promote vascular proteoglycan synthesis and increase arterial lipid retention before the development of atherosclerosis. This study provides in vivo data in support of the response-to-retention hypothesis of atherosclerosis (13). Further research is required to determine whether biglycan is the key proteoglycan responsible for LDL retention and atherosclerosis development.

This work was supported in part by National Institutes of Health Grants HL-082772 (to L.R.T.) and HL-55667 (to J.C.R.) and by an Atorvastatin Research Award to L.R.T. sponsored by Pfizer, Inc. The authors gratefully acknowledge support by the University of Kentucky Hospital under the Physician Scientist Program.

REFERENCES

1. Candido, R., K. A. Jandeleit-Dahm, Z. Cao, S. P. Nesteroff, W. C. Burns, S. M. Twigg, R. J. Dilley, M. E. Cooper, and T. J. Allen. 2002. Prevention of accelerated atherosclerosis by angiotensin-converting enzyme inhibition in diabetic apolipoprotein E-deficient mice. *Circulation.* **106:** 246–253.
2. Daugherty, A., and L. Cassis. 1999. Chronic angiotensin II infusion promotes atherogenesis in low density lipoprotein receptor$^{-/-}$ mice. *Ann. N. Y. Acad. Sci.* **892:** 108–118.
3. Weiss, D., J. J. Kools, and W. R. Taylor. 2001. Angiotensin II-induced hypertension accelerates the development of atherosclerosis in apoE-deficient mice. *Circulation.* **103:** 448–454.
4. Keidar, S., R. Heinrich, M. Kaplan, T. Hayek, and M. Aviram. 2001. Angiotensin II administration to atherosclerotic mice increases macrophage uptake of oxidized LDL: a possible role for interleukin-6. *Arterioscler. Thromb. Vasc. Biol.* **21:** 1464–1469.
5. Sugiyama, F., S. Haraoka, T. Watanabe, N. Shiota, K. Taniguchi, Y. Ueno, K. Tanimoto, K. Murakami, A. Fukamizu, and K. Yagami. 1997. Acceleration of atherosclerotic lesions in transgenic mice with hypertension by the activated renin-angiotensin system. *Lab. Invest.* **76:** 835–842.
6. Dzau, V. J. 2001. Theodore Cooper Lecture. Tissue angiotensin and pathobiology of vascular disease: a unifying hypothesis. *Hypertension.* **37:** 1047–1052.
7. Prescott, M. F., R. L. Webb, and M. A. Reidy. 1991. Angiotensin-converting enzyme inhibitor versus angiotensin II, AT1 receptor antagonist. Effects on smooth muscle cell migration and proliferation after balloon catheter injury. *Am. J. Pathol.* **139:** 1291–1296.
8. Figueroa, J. E., and P. Vijayagopal. 2002. Angiotensin II stimulates synthesis of vascular smooth muscle cell proteoglycans with enhanced low density lipoprotein binding properties. *Atherosclerosis.* **162:** 261–268.
9. Hahn, A. W., S. Regenass, F. Kern, F. R. Buhler, and T. J. Resink. 1993. Expression of soluble and insoluble fibronectin in rat aorta: effects of angiotensin II and endothelin-1. *Biochem. Biophys. Res. Commun.* **192:** 189–197.
10. Kato, H., H. Suzuki, S. Tajima, Y. Ogata, T. Tominaga, A. Sato, and T. Saruta. 1991. Angiotensin II stimulates collagen synthesis in cultured vascular smooth muscle cells. *J. Hypertens.* **9:** 17–22.
11. Regenass, S., T. J. Resink, F. Kern, F. R. Buhler, and A. W. Hahn. 1994. Angiotensin-II-induced expression of laminin complex and laminin A-chain-related transcripts in vascular smooth muscle cells. *J. Vasc. Res.* **31:** 163–172.
12. Shimizu-Hirota, R., H. Sasamura, M. Mifune, H. Nakaya, M. Kuroda, M. Hayashi, and T. Saruta. 2001. Regulation of vascular proteoglycan synthesis by angiotensin II type 1 and type 2 receptors. *J. Am. Soc. Nephrol.* **12:** 2609–2615.
13. Williams, K. J., and I. Tabas. 1995. The response-to-retention hypothesis of early atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **15:** 551–561.
14. Sartipy, P., G. Bondjers, and E. Hurt-Camejo. 1998. Phospholipase A2 type II binds to extracellular matrix biglycan: modulation of its activity on LDL by colocalization in glycosaminoglycan matrixes. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1934–1941.
15. O'Brien, K. D., K. L. Olin, C. E. Alpers, W. Chiu, K. Hudkins, T. N. Wight, and A. Chait. 1998. Comparison of apolipoprotein and proteoglycan deposits in human coronary atherosclerotic plaques: co-localization of biglycan with apolipoproteins. *Circulation.* **98:** 519–527.
16. Riessen, R., J. M. Isner, E. Blessing, C. Loushin, S. Nikol, and T. N. Wight. 1994. Regional differences in the distribution of the proteoglycans biglycan and decorin in the extracellular matrix of atherosclerotic and restenotic human coronary arteries. *Am. J. Pathol.* **144:** 962–974.
17. Nakashima, Y., H. Fujii, S. Sumiyoshi, T. N. Wight, and K. Sueishi. 2007. Early human atherosclerosis: accumulation of lipid and proteoglycans in intimal thickenings followed by macrophage infiltration. *Arterioscler. Thromb. Vasc. Biol.* **27:** 1159–1165.
18. Kunjathoor, V. V., D. S. Chiu, K. D. O'Brien, and R. C. LeBoeuf. 2002. Accumulation of biglycan and perlecan, but not versican, in lesions of murine models of atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **22:** 462–468.
19. Boren, J., K. Olin, I. Lee, A. Chait, T. N. Wight, and T. L. Innerarity. 1998. Identification of the principal proteoglycan-binding site in LDL: a single point mutation in apolipoprotein B100 severely affects proteoglycan interaction without affecting LDL receptor binding. *J. Clin. Invest.* **101:** 2658–2664.
20. Kovanen, P. T., and M. O. Pentikainen. 1999. Decorin links low-density lipoproteins (LDL) to collagen: a novel mechanism for retention of LDL in the atherosclerotic plaque. *Trends Cardiovasc. Med.* **9:** 86–91.
21. Daugherty, A., D. L. Rateri, H. Lu, T. Inagami, and L. A. Cassis. 2004. Hypercholesterolemia stimulates angiotensin peptide synthesis and contributes to atherosclerosis through the AT1A receptor. *Circulation.* **110:** 3849–3857.
22. Daugherty, A., M. W. Manning, and L. A. Cassis. 2000. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J. Clin. Invest.* **105:** 1605–1612.
23. Cole, T. G., R. Kitchens, A. Daugherty, and G. Schonfeld. 1990. An improved method for separation of triglyceride-rich lipoproteins by FPLC. *Pharmacia Biocommunique.* **4:** 4–6.
24. Sattler, W., D. Mohr, and R. Stocker. 1994. Rapid isolation of lipoproteins and assessment of their peroxidation by high-performance liquid chromatography postcolumn chemiluminescence. *Methods Enzymol.* **233:** 469–489.
25. Pitas, R. E., T. L. Innerarity, J. N. Weinstein, and R. W. Mahley. 1981. Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. *Arteriosclerosis.* **1:** 177–185.
26. Walsh, B. A., A. E. Mullick, R. L. Walzem, and J. C. Rutledge. 1999. 17Beta-estradiol reduces tumor necrosis factor-alpha-mediated LDL accumulation in the artery wall. *J. Lipid Res.* **40:** 387–396.
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
28. Bruemmer, D., F. Yin, J. Liu, T. Kiyono, E. Fleck, A. J. Van Herle, and R. E. Law. 2003. Expression of minichromosome maintenance proteins in vascular smooth muscle cells is ERK/MAPK dependent. *Exp. Cell Res.* **290:** 28–37.
29. Dasch, J. R., D. R. Pace, W. Waegell, D. Inenaga, and L. Ellingsworth. 1989. Monoclonal antibodies recognizing transforming growth factor-beta. Bioactivity neutralization and transforming growth factor beta 2 affinity purification. *J. Immunol.* **142:** 1536–1541.
30. Schonherr, E., H. T. Jarvelainen, M. G. Kinsella, L. J. Sandell, and T. N. Wight. 1993. Platelet derived growth factor and transforming growth factor-beta1 differentially affect the synthesis of biglycan and decorin by monkey arterial smooth muscle cells. *Arterioscler. Thromb.* **13:** 1026–1036.
31. Schonherr, E., H. T. Jarvelainen, L. J. Sandell, and T. N. Wight. 1991. Effects of platelet-derived growth factor and transforming growth factor, beta1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. *J. Biol. Chem.* **266:** 17640–17647.
32. Williams, H., J. L. Johnson, K. G. Carson, and C. L. Jackson. 2002. Characteristics of intact and ruptured atherosclerotic plaques in brachiocephalic arteries of apolipoprotein E knockout mice. *Arterioscler. Thromb. Vasc. Biol.* **22:** 788–792.
33. Daugherty, A., and S. C. Whitman. 2003. Quantification of atherosclerosis in mice. *Methods Mol. Biol.* **209:** 293–309.
34. Tiede, K., K. Stoter, C. Petrik, W. B. Chen, H. Ungefroren, M. L. Kruse, M. Stoll, T. Unger, and J. W. Fischer. 2003. Angiotensin II AT(1)-receptor induces biglycan in neonatal cardiac fibroblasts via autocrine release of TGFbeta in vitro. *Cardiovasc. Res.* **60:** 538–546.
35. Little, P. J., L. Tannock, K. L. Olin, A. Chait, and T. N. Wight. 2002. Proteoglycans synthesized by arterial smooth muscle cells in the presence of transforming growth factor-beta1 exhibit increased binding to LDLs. *Arterioscler. Thromb. Vasc. Biol.* **22:** 55–60.
36. Schwenke, D. C., and T. E. Carew. 1989. Initiation of atherosclerotic lesions in cholesterol-fed rabbits. I. Focal increases in arterial LDL concentration precede development of fatty streak lesions. *Arteriosclerosis.* **9:** 895–907.

37. Hollmann, J. A., A. Schmidt, D-B. Von Bassewitz, and E. Buddecke. 1989. Relationship of sulfated glycosaminoglycans and cholesterol content in normal and atherosclerotic human aortas. *Arteriosclerosis.* **9:** 154–158.
38. Volker, W., A. Schmidt, W. Oortmann, T. Broszey, V. Faber, and E. Buddecke. 1990. Mapping of proteoglycans in atherosclerotic lesions. *Eur. Heart. J.* **11 (Suppl. E):** 29–40.
39. Vikramadithyan, R. K., Y. Kako, G. Chen, Y. Hu, E. Arikawa-Hirasawa, Y. Yamada, and I. J. Goldberg. 2004. Atherosclerosis in perlecan heterozygous mice. *J. Lipid Res.* **45:** 1806–1812.
40. Tabas, I., Y. Li, R. W. Brocia, S. W. Xu, T. L. Swenson, and K. J. Williams. 1993. Lipoprotein lipase and sphingomyelinase synergistically enhance the association of atherogenic lipoproteins with smooth muscle cells and extracellular matrix. A possible mechanism for low density lipoprotein and lipoprotein(a) retention and macrophage foam cell formation. *J. Biol. Chem.* **268:** 20419–20432.
41. Williams, K. J., G. M. Fless, K. A. Petrie, M. L. Snyder, R. W. Brocia, and T. L. Swenson. 1992. Mechanisms by which lipoprotein lipase alters cellular metabolism of lipoprotein(a), low density lipoprotein, and nascent lipoproteins. Roles for low density lipoprotein receptors and heparan sulfate proteoglycans. *J. Biol. Chem.* **267:** 13284–13292.
42. Gustafsson, M., M. Levin, K. Skalen, J. Perman, V. Friden, P. Jirholt, S. O. Olofsson, S. Fazio, M. F. Linton, C. F. Semenkovich, et al. 2007. Retention of low-density lipoprotein in atherosclerotic lesions of the mouse: evidence for a role of lipoprotein lipase. *Circ. Res.* **101:** 777–783.
43. Al Haj Zen, A., G. Caligiuri, J. Sainz, M. Lemitre, C. Demerens, and A. Lafont. 2006. Decorin overexpression reduces atherosclerosis development in apolipoprotein E-deficient mice. *Atherosclerosis.* **187:** 31–39.
44. O'Brien, K. D., K. Lewis, J. W. Fischer, P. Johnson, J. Y. Hwang, E. A. Knopp, M. G. Kinsella, P. H. Barrett, A. Chait, and T. N. Wight. 2004. Smooth muscle cell biglycan overexpression results in increased lipoprotein retention on extracellular matrix: implications for the retention of lipoproteins in atherosclerosis. *Atherosclerosis.* **177:** 29–35.
45. Keidar, S., M. Kaplan, A. Hoffman, and M. Aviram. 1995. Angiotensin II stimulates macrophage-mediated oxidation of low density lipoproteins. *Atherosclerosis.* **115:** 201–215.
46. Rosenkranz, S. 2004. TGF-beta1 and angiotensin networking in cardiac remodeling. *Cardiovasc. Res.* **63:** 423–432.
47. Cassis, L. A., D. L. Rateri, H. Lu, and A. Daugherty. 2007. Bone marrow transplantation reveals that recipient AT1a receptors are required to initiate angiotensin II-induced atherosclerosis and aneurysms. *Arterioscler. Thromb. Vasc. Biol.* **27:** 380–386.
48. Tannock, L. R. 2006. Proteoglycans can mediate renal lipoprotein retention. *Diabetologia.* **49:** 1115–1116.
49. Lutgens, E., M. Gijbels, M. Smook, P. Heeringa, P. Gotwals, V. E. Koteliansky, and M. J. Daemen. 2002. Transforming growth factor-beta mediates balance between inflammation and fibrosis during plaque progression. *Arterioscler. Thromb. Vasc. Biol.* **22:** 975–982.
50. Mallat, Z., A. Gojova, C. Marchiol-Fournigault, B. Esposito, C. Kamate, R. Merval, D. Fradelizi, and A. Tedgui. 2001. Inhibition of transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. *Circ. Res.* **89:** 930–934.
51. Ayabe, N., V. R. Babaev, Y. Tang, T. Tanizawa, A. B. Fogo, M. F. Linton, I. Ichikawa, S. Fazio, and V. Kon. 2006. Transiently heightened angiotensin II has distinct effects on atherosclerosis and aneurysm formation in hyperlipidemic mice. *Atherosclerosis.* **184:** 312–321.
52. Ishibashi, S., J. Herz, N. Maeda, J. L. Goldstein, and M. S. Brown. 1994. The two-receptor model of lipoprotein clearance: tests of the hypothesis in "knockout" mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc. Natl. Acad. Sci. USA.* **91:** 4431–4435.
53. Veniant, M. M., V. Pierotti, D. Newland, C. M. Cham, D. A. Sanan, R. L. Walzem, and S. G. Young. 1997. Susceptibility to atherosclerosis in mice expressing exclusively apolipoprotein B48 or apolipoprotein B100. *J. Clin. Invest.* **100:** 180–188.
54. Flood, C., M. Gustafsson, P. E. Richardson, S. C. Harvey, J. P. Segrest, and J. Boren. 2002. Identification of the proteoglycan binding site in apolipoprotein B48. *J. Biol. Chem.* **277:** 32228–32233.
55. Rutledge, J. C., M. M. Woo, A. A. Rezai, L. K. Curtiss, and I. J. Goldberg. 1997. Lipoprotein lipase increases lipoprotein binding to the artery wall and increases endothelial layer permeability by formation of lipolysis products. *Circ. Res.* **80:** 819–828.