Angiotensinogen Modulates Renal Vasculature Growth

Marcus Brand, Noël Lamandé, Curt D. Sigmund, Etienne Larger, Pierre Corvol, Jean-Marie Gasc

Abstract—We have shown previously that angiotensinogen, like other members of the serine protease inhibitor family, has antiangiogenic properties in vitro on cultured endothelial cells and in ovo in the chick chorioallantoic membrane assay. The aim of this study was to show the effects of angiotensinogen on vascular wall remodeling in vivo. We measured the vessel wall thickness (tunica media) stained with an antibody to α-actin. In the kidney, arterioles were 21.5% thinner in male transgenic mice overexpressing human angiotensinogen than in male control animals. In other vascular beds, the arterial wall thickness was not affected. By using in situ hybridization and Northern blot analysis, human angiotensinogen expression was detected at a high level in the male kidney and at a much lower level in other organs. There is a relationship between the effect of angiotensinogen on arterial wall thickness and the local expression level of angiotensinogen in this model of transgenic mice. Because human angiotensinogen is not cleaved to a significant extent by mouse renin, the reduction in kidney arterial wall thickness is because of angiotensinogen itself and not angiotensin II, and we show that the reduction was not because of hypoplasia or hypotropia. In contrast, a marked difference in the expression of platelet-derived growth factor receptor-β was observed in the kidney arterioles at day 5 when compared with controls. Altogether, these observations provide the first quantitative evidence that a high level of angiotensinogen expression can inhibit the growth of kidney artery walls in vivo. (Hypertension. 2006;47:1067-1074.)

Key Words: angiotensinogen ■ muscle, smooth, vascular ■ remodeling ■ vessels ■ animals, transgenic

Angiotensinogen (AGT) is a 452-amino acid protein that is synthesized mainly in the liver.1 It is the only known precursor of angiotensin I (Ang I), an inactive decapeptide that is further converted by Ang I-converting enzyme into angiotensin II (Ang II), the main effector of the renin–angiotensin system.1 AGT belongs to the family of noninhibitory serpins, as revealed by its gene organization, amino acid sequence, and secondary structure.2 Several serpins (eg, maspin, pigment epithelium-derived factor, antithrombin III, and kallistatin) are known to have antiangiogenic effects.3–6 We showed recently that AGT also displays antiangiogenic properties in vitro and in ovo.7 AGT inhibits the proliferation of cultured endothelial cells, blocks the formation of capillaries on Matrigel, and impairs the formation of capillaries in the chick chorioallantoic membrane assay.7 In addition, an adenovirus encoding human AGT exhibits strong antiangiogenic and thereby antitumoral effects in different experimental tumor models in mice.8

Several indirect observations suggest the implication of AGT in vascular wall remodeling. As shown by Northern blot analysis and in situ hybridization studies, AGT is produced locally in vascular smooth muscle cells (VSMCs), that is, the tunica media of large blood vessels.9,10 Physiologically, AGT may also be involved in the stability of the blood brain barrier: Kakinuma et al11 demonstrated that AGT-deficient mice exposed to cold stress for several days exhibit a massive and irreversible edema of the brain because of impaired blood–brain barrier permeability, which could be corrected by Ang II or angiotensin IV administration. The same group showed that renin-deficient mice and wild-type (WT) mice did not exhibit this phenotype,12 suggesting that the observed vascular brain barrier abnormality in AGT-deficient mice was either because of a lack of local AGT, per se, or because of an alternative pathway for generating angiotensin peptides. The purpose of the present study was to determine whether human AGT is directly implicated in vascular wall remodeling. We measured the vessel wall thickness of a wide size range of kidney arterioles in 5-, 15-, and adult 60-day-old male and female transgenic mice overexpressing human AGT (Hu-AGT-TG mice).13 We used mice that overproduced human AGT to study the role of AGT, per se, because mouse renin does not cleave human AGT to a significant extent.

In 60-day-old male Hu-AGT-TG mice, the walls of kidney arterioles are thinner than those from male control animals. A reduction in arteriolar wall thickness of kidney was already observed in 15-day-old male Hu-AGT-TG mice, whereas no reduction in vessel wall thickness of kidney arteries was observed in 5-day-old Hu-AGT-TG mice in comparison with
their controls. A reduction in arterial wall thickness was not observed in other organs that do not express large amounts of the human AGT transgene (heart, white adipose tissue, and mesenteric artery system). This suggests a direct relationship between the level of AGT expression and arterial wall thickness. Human AGT, therefore, exerts a paracrine rather than an endocrine action on the arterial walls of male Hu-AGT-TG mice.

Methods

Animal Models

All of the animal procedures for the care and use of laboratory animals were conducted according to the guidelines of our institution and Federation of European Laboratory Animal Science Association.

Hu-AGT-TG Mice

Hu-AGT-TG mice were generated as described previously. Briefly, the transgene consisted of all 5 exons of the human AGT gene and the intervening introns plus ~1.2 kb upstream and 1.4 kb downstream of the human gene. This 14-kb genomic construct was injected into C57BL/6J × SJL/J 1-cell fertilized mouse embryos, thus generating transgenic mice. Germ line transmission was ~50%, with an equal distribution between males and females. Hu-AGT-TG mice were maintained in a heterozygous state by consecutive 10 backcross breeding to C57BL/6 mice. The Ang II plasma concentration, blood pressure, and phenotype of Hu-AGT-TG mice were monitored using the IPLab software (IPLab, Scanalytics) following parameters were directly measured on the computer monitor using the IPLab software (IPLab, Scanalytics, Figure 1C): (1) the surface (S) of the vessels, corresponding with the area between the inner and outer limits of the vessel wall, that is, the tunica media, as defined by α-actin immunostaining; and (2) the length of the major axis (L), that is, the longest external dimension (diameter) of the vessel section (Figure 1C, arrow). The S/L ratio was calculated to normalize wall thickness to the vessel diameter. Under the assumption that the vessel section is a circle or an ellipse, this parameter is directly proportional to the vessel wall thickness.

Vessel Wall Measurement

Histologic sections were immunostained with an antibody to smooth muscle α-actin, a marker of the tunica media of arteries (Figure 1A and 1B). The blood vessels examined were cut in a plane perpendicular to the axis of the vessels. Each vessel was scanned with a digital camera (Digital Microscope Camera, Polaroid), and the following parameters were directly measured on the computer monitor using the IPLab software (IPLab, Scanalytics, Figure 1C): (1) the surface (S) of the vessels, corresponding with the area between the inner and outer limits of the vessel wall, that is, the tunica media, as defined by α-actin immunostaining; and (2) the length of the major axis (L), that is, the longest external dimension (diameter) of the vessel section (Figure 1C, arrow). The S/L ratio was calculated to normalize wall thickness to the vessel diameter. Under the assumption that the vessel section is a circle or an ellipse, this parameter is directly proportional to the vessel wall thickness.

In Situ Hybridization

Histologic sections were immunostained with an antibody to smooth muscle α-actin, a marker of the tunica media of arteries (Figure 1A and 1B). The blood vessels examined were cut in a plane perpendicular to the axis of the vessels. Each vessel was scanned with a digital camera (Digital Microscope Camera, Polaroid), and the following parameters were directly measured on the computer monitor using the IPLab software (IPLab, Scanalytics, Figure 1C): (1) the surface (S) of the vessels, corresponding with the area between the inner and outer limits of the vessel wall, that is, the tunica media, as defined by α-actin immunostaining; and (2) the length of the major axis (L), that is, the longest external dimension (diameter) of the vessel section (Figure 1C, arrow). The S/L ratio was calculated to normalize wall thickness to the vessel diameter. Under the assumption that the vessel section is a circle or an ellipse, this parameter is directly proportional to the vessel wall thickness.

Figure 1. Parameters of vessel wall measurement: (A) histologic section of a kidney of a control animal. Kidney arteries were stained with a VSMC-specific α-actin antibody and scanned by a digital camera. (B) enlargement of a scanned kidney artery. (C) the following vessel wall parameters were measured: vessel wall surface (S) corresponding with the area between the inner and outer limits of the vessel wall; major axis (L, arrow), that is, the external diameter of the vessel. The ratio S/L was calculated and is directly proportional to the vessel wall thickness. Scale bar: (A) 15 μm.
Culture of VSMC

Human umbilical arterial smooth muscle cells (HUSMCs), human aortic smooth muscle cells (HAoSMCs), and rat aortic smooth muscle cells were obtained from Clonetics (Cambrex BioScience) and cultured in the medium provided by the supplier. For proliferation experiments, HUSMCs or HAoSMCs were seeded at a density of 10,000 cells/well in collagen-coated wells (24-well culture plates). After 24 hours, HUSMCs or HAoSMCs were starved in 0.2% fetal calf serum for 30 hours. Proliferation, as assessed by measuring the amount of 3H-thymidine (Amersham) incorporated in 24 hours, was stimulated by adding 2.5 ng/mL of human basic fibroblast growth factor (Cambrex BioScience). Then cells were treated with 10% trichloroacetic acid and solubilized (200 mmol/L NaOH). The radioactivity incorporated in the DNA was measured by liquid scintillation counting. AGT at concentrations between 1 and 5000 nmol/L was tested on cell proliferation.

Hormone Measurements

Blood was collected into chilled tubes containing EDTA. Plasma concentration of human AGT was measured indirectly, as described by Clauser et al., using recombinant human renin. Mouse plasma renin concentration (PRC) was measured as described previously. Briefly, aliquots of mouse plasma samples were incubated for 15 minutes at 37°C in the presence of binephrectomized rat plasma as a source of AGT. Produced Ang I concentrations were determined by radioimmunoassay.

Statistics

The Mann–Whitney U test was used to compare control and genetically engineered animals. Data are represented as SEM. Statistical tests were considered significant if P≤0.05.

Results

Expression of Human AGT mRNA in Various Tissues of Male and Female TG Mice

To investigate whether human AGT is directly implicated in vessel wall remodeling, we used a mouse line carrying human AGT as a transgene in addition to the endogenous mouse AGT. Plasma concentration of human AGT was higher in male (5393 nmol/L±1571 nmol/L) than in female mice (3716 nmol/L±682 nmol/L; Figure 2); these values correspond with concentrations that are 200- to 280-fold (for male) and 90- to 150-fold (for female) higher than in normal human plasma (20 to 30 nmol/L). The higher plasma human AGT value observed in male animals can be related to the marked AGT expression in male kidney when compared with female kidney (see below). To assess the absence of significant cleavage of human AGT by mouse renin, we measured mouse PRC, a highly sensitive indicator of angiotensin peptide levels, in Hu-AGT-TG mice and control mice. The comparison of mouse PRCs between male and female Hu-AGT-TG mice and their controls showed no difference between any of the groups (Figure 3).

The kidney of male Hu-AGT-TG mice is a major site of human AGT expression, because in situ hybridization revealed a high level of human AGT mRNA in the epithelial cells of the proximal convoluted tubules of male Hu-AGT-TG mice (Figure 4A and 4B). Only a low level of human AGT mRNA expression was detected in the proximal convoluted tubules of female Hu-AGT-TG mice (Figure 4C). As a control, no human AGT mRNA was detected in the kidneys of WT mice (Figure 4D). Human AGT mRNA was observed at low level in cardiomyocytes (Figure 4E and 4F) and was hardly detectable in the adipose tissue of both male and female transgenic mice (Figure 4G and 4H). Northern blot analysis showed a high level of human AGT mRNA in the male kidney and a very low level of transgene expression in the female kidney (data not shown). Human AGT mRNA was hardly detectable in the heart, brain, and adipose tissue of both sexes (data not shown).

Altogether, these results show that the human AGT transgene is expressed variably, according to tissues and gender. Among the tissues studied, human AGT is strikingly expressed in the male kidney and at much lower levels in all other tissues. For this reason, we compared renal artery wall thickness to other territories.

Arterial Wall Thickness in Various Tissues of Male and Female Transgenic Mice

The vascular wall thickness of arterioles in the kidney was measured in 20 to 25 vessels of every animals studied (six 60-day-old male Hu-AGT-TG mice and 11 age- and sex-matched control mice). Arteriole wall thickness was 21.5% lower in male Hu-AGT-TG mice than in male control mice (P=0.002; Figure 5A) in arteries of which the maximum external diameter was between 10 and 150 μm. Larger arteries (diameter between 250 and 600 μm) did not show this dystrophy. The measurement of vascular wall thickness
of kidney arterioles in 20 vessels of five 15-day-old male Hu-AGT-TG mice and 4 age-and-sex-matched control mice showed a significant reduction in arterial wall thickness of 24.7% ($P<0.001$; Figure 5A). Measurement of the vessel wall thickness of kidney arterioles of 20 vessels in three 5-day-old male Hu-AGT-TG mice and 3 age- and sex-matched control mice showed no difference in arterial wall thickness. The vessel wall thickness of kidney arterioles was also measured in four 60-day-old female Hu-AGT-TG mice and in 4 age- and sex-matched control mice: no difference in arterial wall thickness was observed between female Hu-AGT-TG mice and control mice (Figure 5B). In other organs and vascular beds of male and female mice, adipose tissue and heart (Figure 5B), and mesenteric artery tree (data not shown), no difference in vessel wall thickness was observed between Hu-AGT-TG mice and their controls.

Density of VSMC in the Vessel Wall
To evaluate whether the reduction in vascular wall thickness of renal arterioles in male Hu-AGT-TG mice is because of hypotrophy or hypoplasia of the smooth muscle cell layer, we measured the density of VSMCs in kidney vessels of three 60-day-old WT and 3 age- and sex-matched Hu-AGT-TG mice. In each mouse, the density of VSMCs was evaluated in 4 vessels. No difference in VSMC density was obtained between WT and Hu-AGT-TG (data not shown). This means that the reduction in vascular wall thickness of kidney vessels in male Hu-AGT-TG mice is because of a lower number of

Figure 4. Distribution of human AGT mRNA in male and female Hu-AGT-TG and control mice. (A) in situ hybridization analysis showing the distribution of human AGT mRNA in the proximal tubules of the kidney in a male Hu-AGT-TG mouse (arrows, glomeruli). (B) high-level expression of human AGT mRNA in the proximal tubules surrounding a renal artery (*) in a male Hu-AGT-TG mouse. (C) low-level expression of human AGT mRNA in the proximal tubules of the kidney in a female Hu-AGT-TG mouse (arrow, glomerulus). (D) absence of human AGT mRNA signal in the kidney of a control mouse (*kidney artery; arrow, glomerulus). (E and F) low expression level of human AGT mRNA in cardiomyocytes (arrows) of the left ventricle surrounding a coronary artery (*) in a male (E) and female (F) Hu-AGT-TG mouse. (G and H) very low expression level of human AGT mRNA in adipocytes of a male (G) and female (H) Hu-AGT-TG mouse. Scale bars: (A–D) 40 μm; (E–H) 15 μm.
cells and not because of cells of a smaller size in the smooth muscle cell layer.

Apoptosis and VSMC Proliferation

Our data indicate that a high expression of human AGT regulates negatively the growth of renal arterioles in male mice. This may be a consequence of increased apoptosis of VSMCs in the vascular wall or reduced proliferation of VSMCs. Alternatively, it might be because of a reduced recruitment of pericytes, which are the progenitor cells of the differentiated vascular wall cells. To distinguish between these possibilities, we measured apoptosis by using activated caspase-3 immunostaining and the TUNEL technique. No specific apoptosis signal was detected in kidney arteries or arterioles from 5-day-old male and female Hu-AGT-TG or 60-day-old male and female Hu-AGT-TG mice (results not shown). By using the proliferating cell nuclear antigen and Ki-67 immunostaining, a specific marker for cell proliferation, no difference in VSMC proliferation rate was observed between 5-day-old male and female Hu-AGT-TG mice (results not shown). A putative in vitro effect of human AGT on VSMC proliferation was evaluated by measuring 3H-thymidine incorporation in cultured HUSMCs and HAoSMCs. Concentrations of human AGT in the range of 1 to 200 nmol/L did not alter the proliferation rate of either cell line (Figure 6A and 6B). Even very high doses of human AGT (5000 nmol/L) did not alter the proliferation of HUSMCs, HAoSMCs or rat aortic smooth muscle cells (data not shown).

If the reduced growth of kidney small arterioles is not because of smaller cells (hypotrophy), a lower proliferation rate, or increased apoptosis, the cause for the differential growth between control and Hu-AGT-TG must not be intrinsic to the vascular wall of the small arterioles but rather a consequence of cell recruitment from the surrounding stroma, that is, pericyte recruitment. PDGF-B and its receptor (PDGF-Rβ) play a major role in pericytes recruitment. We evaluated the level of PDGF-B mRNA and PDGF-Rβ mRNA in male mice at day 5 and day 15 by in situ hybridization. The level of PDGF-B mRNA expression was low and did not vary between day 5 and 15 in Hu-AGT-TG and was similar to that of control mice (data not shown). In contrast, the level of PDGF-Rβ mRNA is age- and genotype-specific (Figure 7). Using a scale of gradation from 1+ to 4+, the level of PDGF-Rβ expression in small arterioles and also glomeruli was the highest at day 5 in Hu-AGT-TG males (mean value of 5 mice, 3.6), and then decreased from day 5 to day 15 (mean value of 6 mice, 2.7). A similar decrease occurred in control mice but starting at a lower level at day 5 (mean value of 3, 3.0) and reaching almost background noise level at day 15 (mean value of 5, 1.2). Therefore, during the first days of life, PDGF-Rβ expression was more marked in renal arterioles in Hu-AGT-TG than in WT mice.

Discussion

The present study shows that a high level of expression of human AGT is associated with a clear decrease of renal arteriole thickness, suggesting that AGT, per se, can influence vascular remodeling in rodents. Previous studies have shown that the lack of AGT in transgenic gene–deficient mice is accompanied by a marked increase in renal artery thickness.18–20 However, this observation could not be unambig-

![Figure 5. Arterial wall thickness in controls and in male and female Hu-AGT-TG mice. (A) arterial wall thickness (S/L) in the kidneys of 5-, 15-, and 60-day-old control and male Hu-AGT-TG mice. (B) arterial wall thickness in the kidneys of control and female Hu-AGT-TG mice and in adipose tissue and heart of control and male Hu-AGT-TG mice. S/L indicates ratio between the surface area (μm²) and major axis (μm) of each vessel; n, number of mice studied (20 to 25 vessels were studied in each animal). Comparison with control mice, ns indicates not significant. Values are mean±SEM.](http://hyper.ahajournals.org/).
and female Hu-AGT-TG mice was comparable with their controls; PRC is a highly sensitive marker of intrarenal angiopeptidase levels as a slight increase in Ang II concentration downregulates renin production and circulating renin concentration (PRC) by negative feedback.\(^\text{26}\) Third, moreover, if high levels of Ang II were to be produced in Hu-AGT-TG mice, they would have been expected to have a reverse effect of what was observed, because Ang II exerts a well-documented growth-stimulatory effect on vessels.\(^\text{27}\) Finally, however, it may be that AGT, at this supraphysiological concentration, is cleaved by another and still unknown pathway leading to the generation of peptide, such as Ang (1-7), or/and angiotensin IV. Ang (1-7)\(^\text{7}\) has been reported to attenuate neointimal formation after stent implantation in the rat.\(^\text{28}\)

There is a relationship between the local level of human AGT expression and the vascular effect. There is a 21% reduction in renal arteriole thickness only in male Hu-AGT-TG mice where the transgene is markedly expressed, compared with females. The onset of difference in arteriole wall thickness between WT and Hu-AGT-TG mice occurs between day 5 and day 15. This period corresponds to the last wave of glomeruli and nephron differentiation. In female TG mice, where human AGT is expressed at a much lower level in the kidney, there is no appreciable AGT effect on renal artery thickness. In other organs where there is a low level of AGT expression in male and female Hu-AGT-TG mice (heart, adipose tissue, and mesenteric artery tree), there is no dystrophy in the vascular wall thickness. This is in favor of a relationship between AGT level expression and the degree of vascular reduction. However, a final conclusion cannot be drawn, because in other organs, in which a high level of human AGT transgene expression was detected, in the liver and in the adrenals, almost all of the vessels were \(\alpha\)-actin negative and their wall thickness could not be evaluated.

In the proximal convoluted tubules, human AGT mRNA was detected, and it could be hypothesized that AGT is released into the lumen of the tubules. However, our data are rather in favor of a local effect in the interstitium where blood vessels are located. The absence of the AGT effect in other vascular territories despite high circulating levels further suggests that AGT exerts a paracrine rather than an endocrine action on the kidney arterial walls of male Hu-AGT-TG mice.

Our data raise the question of the mechanism of action by which AGT modulates vascular wall remodeling. Remodeling involves VSMC growth and migration, as well as production of extracellular matrix.\(^\text{29}\) AGT affects endothelial cell proliferation,\(^\text{7}\) but an effect on VSMC proliferation or migration has not been reported. In our study, there was no evidence for proapoptotic effects of AGT in renal arterioles or in other vascular beds of newborn and adult Hu-AGT-TG mice. There was also no evidence for an in vitro antiproliferative effect of AGT on VSMCs.

Another possible mechanism to explain the difference in arteriole wall thickness between control and Hu-AGT-TG mice involves the fate of pericytes during embryogenesis. Pericytes are precursor cells of the differentiated vascular wall cells.\(^\text{30}\) During embryonic vascular wall remodeling, an excess of AGT may affect recruitment, proliferation, migra-
tion, or differentiation of pericytes into VSMCs. In the present study, we find that AGT does not operate through hyperplasia, thus supporting the hypothesis of a role for pericytes recruitment in the regulation of arteriole growth. Several factors are involved in vascular wall remodeling, among them the PDGF system for small vessels and the angiopoietin-1/tie-2 ligand receptor pair for large blood vessels. The observation of PDGF-Rβ expression evaluated by in situ hybridization shows that, at the time of terminal kidney differentiation, PDGF-Rβ progressively decreases to reach a minimal level at day 15 in control animals, whereas it remains high in Hu-AGT-TG mice at the same age. The persistence of a high level of expression of PDGF-Rβ is somewhat paradoxical, because it occurs in animals with a high AGT circulating concentration, a condition responsible for the thinning of vascular wall thickness. Relatively high PDGF-Rβ expression may be part of a counterregulation mechanism for the AGT effect on VSMCs. In any case, it may be proposed that the small arterioles in the kidney have a regulation process different from other arteries.

Perspectives

The data reported raise several questions. First, the mechanism of action of AGT on vascular remodeling; because of its effect on endothelial cells, AGT may interfere with the PDGF system and alter vessel maturation. Alternatively, AGT may interact with extracellular matrix proteins or cell surface proteins. Maspin, another member of the serpin family with antiangiogenic and antitumoral effects, interacts with extracellular matrix collagen I and III. Second, the physiological role, if any, of AGT in vessel wall remodeling because AGT effects were observed only in supraphysiological expression of human AGT in the kidney. High local levels of AGT may contribute to abnormal vascular remodeling, together with its action on neoangiogenesis. Such an effect accounts for the marked antangiogenic and antitumoral effects of AGT in 2 experimental tumor models in mice, the human MDA mammary carcinoma and the murine B16-F10 melanoma.

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