Contribution of Arachidonic Acid Metabolites Derived Via Cytochrome P4504A to Angiotensin II–Induced Neointimal Growth

Fariborz A. Yaghini, Chunxiang Zhang, Jean-Hugues Parmentier, Anne M. Estes, Nauzanene Jafari, Susan A. Schaefer, Kafait U. Malik

Abstract—Angiotensin II and the arachidonic acid metabolite derived via cytochrome P450 20-hydroxyeicostetraenoic acid promote vasoconstriction and vascular smooth muscle cell (VSMC) proliferation. This study was conducted to determine if 20-hydroxyeicosatetraenoic acid contributes to angiotensin II-induced neointimal formation in balloon-injured rat carotid artery. In anesthetized rats, the drugs were infused into the clamped segment of the injured right common carotid artery for 60 minutes. The drug solution and catheter were withdrawn, the common carotid artery was ligated, and blood flow was restored. Exposure of the injured artery to angiotensin II (200 nmol/L) or arachidonic acid (10 µmol/L) increased neointimal thickening at day 14 (intima/media ratio 0.71 ± 0.14 with vehicle versus 1.65 ± 0.10 with angiotensin II or 1.31 ± 0.13 with arachidonic acid; P < 0.05). Cytochrome P450 4A1 antisense, but not scrambled, oligodeoxynucleotide (100 nmol/ml) reduced angiotensin II-induced or arachidonic acid-induced neointimal thickening (intima/media ratio 0.90 ± 0.07 for angiotensin II and 0.95 ± 0.06 for arachidonic acid). 20-hydroxyeicosatetraenoic acid (0.5 µmol/L) also increased neointimal thickening of injured artery (intima/media ratio 1.15 ± 0.03); this was not altered by cytochrome P450 4A1 antisense oligodeoxynucleotide. Angiotensin II, arachidonic acid, and 20-hydroxyeicosatetraenoic acid also induced the expression of cytochrome P450 4A and increased the number of CD45-positive cells; the latter effect of angiotensin II and arachidonic acid, but not 20-hydroxyeicosatetraenoic acid, was diminished by cytochrome P450 4A1 antisense oligodeoxynucleotide. These data suggest that arachidonic acid metabolites derived via cytochrome P450 4A, most likely 20-hydroxyeicosatetraenoic acid, mediate angiotensin II–induced neointimal thickening in injured rat carotid artery. (Hypertension. 2005;45:1182-1187.)

Key Words: angiotensin II • arachidonic acids • balloon injury • cytochrome p450 • vascular smooth muscle

Angiotensin II (Ang II), a biologically active octapeptide generated by the renin-angiotensin system, contributes to the regulation of blood pressure by maintaining vascular tone and salt and water balance. High levels of Ang II promote vascular smooth muscle cell (VSMC) hypertrophy, migration, and delayed hyperplasia, and cause inflammation, neointimal formation, and hypertension.1,2 Growth factors such as Ang II promote activation of transcription factors (nuclear factor κB and activating protein-1) and proinflammatory genes (cytokines, interleukins), upregulation of adhesion molecules (intercellular adhesion molecule, vascular cell adhesion molecule), stimulation of chemokine production (monocyte chemoattractant protein-1), and their receptors, and recruitment of inflammatory cells (monocytes, macrophages), all of which are critical processes involved in vascular inflammation and injury.2,3

Ang II also activates cytosolic phospholipase A2, which catalyzes the hydrolysis of phospholipids, leading to release of arachidonic acid (AA). AA is subsequently metabolized by cyclooxygenase (COX) into prostanoids, by lipoxygenase (LO) into 5(S)-, 12(S)-, and 15(S)-hydroxyeicosatetraenoic acid (HETE), and by cytochrome P450 (CYP) into 18-HETE, 19-HETE, 20-HETE and epoxyeicosatrienonic acids.4–6 AA metabolites generated via LO [12(S)-HETE] and CYP4A (20-HETE) have been implicated in the vascular actions of Ang II. For example, 12(S)-HETE contributes to Ang II-induced VSMC hypertrophy and stimulates monocyte adhesion to endothelial cells.7–9 Moreover, there is increased expression of 12-LO in rat carotid artery after balloon injury, and 12-LO hammerhead ribozyme decreases VSMC fibronectin expression and migration as well as neointimal growth.8,10,11

20-HETE has multiple effects as well. It is a potent vasoconstrictor in small arteries and increases reactivity of the mesenteric arteries in spontaneously hypertensive rats.12,13 It also mediates the vasoconstrictor effect of Ang II and the mitogenic effect of norepinephrine and contributes to the development of hypertension.14–18 Overexpression of

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From the Department of Pharmacology and Center for Vascular Biology, University of Tennessee Health Science Center, Memphis, Tenn.
Correspondence to Kafait U. Malik, DSc, PhD, Department of Pharmacology, College of Medicine, 874 Union Avenue, University of Tennessee Health Science Center, Memphis, TN 38163. E-mail kmalik@utmem.edu
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CYP4A1 in smooth muscle in renal arterial microvessels stimulates endothelial sprouting. The demonstration that: (1) Ang II promotes neointimal formation in balloon-injured rat carotid arteries; (2) CYP4A and its activity is expressed in various tissues including the vasculature: and (3) expression of CYP4A1 is increased in neointimal smooth muscle cells raises the possibility that CYP4A-derived metabolite(s) of AA contribute to the Ang II-induced increase in neointimal growth during vascular injury. To test this hypothesis, we examined the effects of CYP4A antisense and scrambled oligodeoxynucleotides (ODNs) on Ang II-induced neointimal growth as well as the effects of exogenous AA and 20-HETE in the presence or absence of these ODNs on neointimal growth in balloon-injured rat carotid arteries.

Materials and Methods
Rabbit polyclonal CYP450 4A, purified mouse anti-rat CD45, and polyclonal actin antibodies were purchased from Novus Biologicals (Littleton, Colo.), BD Biosciences (Palo Alto, Calif.), and Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif), respectively. Antigen Unmasking Solution, Vectastain ABC kit, DAB kit, and Vectamount were purchased from Vector Laboratories, Inc (Burlingame, Calif).

Oligonucleotides
Phosphorothioate antisense ODN was designed from nucleotide 10 to 30 of rat CYP4A1 cDNA (GenBank, M14972). The scrambled ODN had the same nucleotide content as antisense, but in random order. CYP4A1 antisense ODN (5'-CAG-TGC-AGA-GAC-GCT-CATGGT-3') and scrambled ODN (5'-CTG-ACC-GCA-GCA-CTT-AGA-TGG-3') were synthesized (Integrated DNA Technologies, Inc, Coralville, Iowa) following the recommendations of Stein to minimize nonspecific effects in vivo. The CYP4A antisense ODN used did not share homology with any other known cDNA sequence. The effects of CYP4A1 antisense and scrambled ODN on CYP4A protein level were examined in rat aortic VSMC in vitro as described in the data supplement. Ang II, AA, or 20-HETE in saline alone or in combination with antisense or scrambled ODN mixed with oligofectamine reagent (Invitrogen, Carlsbad, Calif) was applied to balloon-injured rat carotid arteries as described.

Cell Culture and Transfection
VSMCs from male Sprague-Dawley rats were isolated, cultured, and maintained as described. VSMCs were transfected with CYP4A1 antisense or scrambled ODN with transfection reagent, oligofectamine (Invitrogen) according to manufacturer’s instruction. VSMCs were then lysed and used for Western blot analysis (available in the data supplement at http://www.hypertension.ahajournals.org).

Vascular Injury Model, Vessel Preparation, Morphometric and Immunohistochemical Analyses
Treatment of the laboratory animals and the experimental protocols followed the guidelines of the University of Tennessee Health Science Center. Carotid artery balloon injury was performed as described. Briefly, 5 to 7 male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) (200 to 250 grams) were intraperitoneally injected and anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg) for each group of treatments, then the right common carotid artery was exposed, clamped, and injured with a 2-French Fogarty catheter. Drugs with or without CYP4A1 antisense or scrambled ODN were infused into the injured, clamped segment and incubated for 60 minutes. Exposure of rat injured carotid arteries for this or even shorter time period to phosphorothioate antisense ODN against growth factors has been reported to be effective in decreasing neointimal growth. After 60 minutes, the drugs and catheter were withdrawn, the external carotid artery was ligated, and blood flow was restored to the common carotid artery. The isolation and preparation of nonthrombotic arteries for morphometric and immunohistochemical analyses are described in the data supplement.

Western Blot Analysis
Three to 4 carotid arteries after 2 days from each treatment group were ground to a fine powder in liquid nitrogen (LN2). The lysates of these arteries or cultured VSMCs were prepared and subjected to Western blot analysis as described (data supplement).

Statistical Analysis
The results are presented as mean ± SEM. The unpaired Student t test was used to determine the difference between 2 groups. P<0.05 was considered statistically significant.

Results

Contribution of CYP4A to Ang II–Induced Neointimal Formation
CYP4A1 antisense but not scrambled ODN reduced CYP4A protein level in both cultured VSMCs and in vivo injured carotid arteries (Figure I, available at http://hyper.ahajournals.org). Balloon injury in rat carotid arteries caused neointimal growth as indicated by an increase in the ratio of intima to media (0.71±0.14 in vehicle-treated versus 0.12±0.02 in uninjured vessels; P<0.05, n=5). Exposure of injured carotid arteries to Ang II (200 nmol/L) for 60 minutes increased neointimal growth (Figure 1A and 1B); this short-term exposure to Ang II did not promote neointimal growth in the absence of balloon injury (data not shown). The effect of Ang II on neointimal growth was diminished in carotid arteries exposed to CYP4A1 antisense, but not scrambled, ODN (100 nmol/L; Figure 1A and 1B). The neointimal growth caused by injury alone was not altered by exposure to CYP4A1 antisense or scrambled ODN.

Contribution of CYP4A to AA-Induced Intimal Growth
To determine if exogenous AA mimics the action of Ang II and its effect is mediated through CYP4A1, balloon-injured carotid arteries were incubated with AA (10 μmol/L) for 60 minutes. AA enhanced the neointimal growth in injured carotid artery (Figure 2A and 2B). Treatment with CYP4A1 antisense, but not scrambled, ODN attenuated the effect of AA on neointimal growth (Figure 2A and 2B).

Effect of 20-HETE on Neointimal Formation
To evaluate the effect of AA-derived CYP4A metabolite on intimal growth, balloon-injured carotid arteries were incubated with 20-HETE (0.5 μmol/L) for 60 minutes. 20-HETE increased the growth of neointima (Figure 3A and 3B). The increase in neointimal growth caused by 20-HETE was not altered by exposure of injured carotid arteries to CYP4A1 antisense or scrambled ODN (Figure 3A).

Expression of Immunoreactive CYP4A in Injured Rat Carotid Artery
To determine expression of CYP4A, 0.5-μm sections of carotid arteries were immunostained with rabbit polyclonal CYP4A antibody as described in Materials and Methods. Exposure of balloon-injured carotid arteries to CYP4A1 antisense, but not scrambled, ODN attenuated the expression of CYP4A in arteries 14 days after treatment with Ang II,
AA, or 20-HETE (Figure 4). Higher magnification of this Figure is shown in online Figure II (20x/H11003).

Expression of Immunoreactive CD45-Positive Cells in Neointima After Injury

Ang II plays an important role in vascular inflammation and atherosclerosis.1 Also, Ang II activates immune cells and production of inflammatory mediators.2 To determine the effect of CYP4A antisense ODN on the density of inflammatory cells in neointima, we immunostained arterial sections with antibody to CD45, a leukocyte-common antigen. Ang II-induced, AA-induced, and 20-HETE–induced neointimal growth was associated with an increase in CD45-positive cells in the neointima of injured artery segments from 5 to 6 animals in each group. *Value different from the corresponding value obtained in the presence of vehicle and **value different from the corresponding value obtained in the presence of CYP4A1 scrambled ODN (P<0.05).

Discussion

This study, for the first time to our knowledge, demonstrates that a CYP4A-derived AA metabolite, most likely 20-HETE, contributes to the effect of Ang II on neointimal growth in vivo in balloon-injured rat carotid arteries. In the present study, exposure of carotid arteries to Ang II (200 nmol/L) for 60 minutes after balloon injury caused a marked increase in neointimal growth as indicated by increased intima-to-media ratio at day 14. This increase in neointimal growth was associated with enhanced expression of CYP4A in the neointima, which was reduced by treatment with CYP4A1 antisense, but not scrambled, ODN. These observations and our demonstration that treatment of injured arteries with CYP4A1 antisense, but not scrambled, ODN inhibited Ang II-induced increase in neointimal growth suggest involvement of CYP4A. Our finding that CYP4A1 antisense ODN did not alter the neointimal growth caused by injury alone indicates that the effect of Ang II to promote neointimal formation is caused by release of AA. Ang II promotes release of AA via activation of cytosolic phospholipase A2.30,31 Our novel observation that direct exposure of injured arteries to AA increased neointimal growth, which was inhibited by treatment with CYP4A1 antisense ODN, supports this view.

CYP4A1 is the most efficient ω-hydroxylation that catalyzes the oxygenation of AA to 20-HETE.32 20-HETE constricts small blood vessels, promotes endothelial sprouting in renal...

Figure 1. Effect of CYP4A1 antisense ODN on Ang II-induced neointimal formation. Sections of balloon-injured carotid artery were stained with H&E. A, Ang II (200 nmol/L) induced neointimal formation in carotid artery after injury compared with its vehicle (Veh). Treatment with CYP4A1 antisense (As), but not scrambled (Scr), ODN (100 nmol/L) diminished Ang II-induced intimal hyperplasia after injury. B, The bar graph shows the intima to media ratio (I/M) of carotid artery segments from 5 to 6 animals in each group. *Value different from the corresponding value obtained in the presence of vehicle and **value different from the corresponding value obtained in the presence of CYP4A1 scrambled ODN (P<0.05).

Figure 2. Effect of CYP4A1 antisense ODN on AA-induced neointimal growth. Sections of carotid artery were stained with H&E. A, AA (10 μmol/L) enhanced intimal proliferation in injured rat carotid artery compared with its vehicle (Veh). CYP4A1 antisense but not scrambled ODN (100 nmol/L) decreased AA-induced neointimal formation in injured arteries. B, The bar graph denotes the intima to media ratio (I/M) in carotid artery segments from 5 to 6 animals in each group. *Value different from the corresponding value obtained in the presence of vehicle and **value different from the corresponding value obtained in the presence of CYP4A1 scrambled ODN (P<0.05).
arterial microvessels, and exacerbates myocardial reperfusion injury. 12,32,33 20-HETE also mediates the vasoconstrictor effect of Ang II in the kidney. 14 Increased urinary excretion of 20-HETE has been reported to be associated with endothelial dysfunction. 33,34 Our finding that exposure of balloon-injured carotid arteries to 20-HETE increased neointimal growth that was not altered by simultaneous exposure to CYP4A1 antisense ODN, suggests that 20-HETE contributes to the effect of Ang II and AA on neointimal growth. However, the contribution of other CYP4A-derived AA metabolites to the neointimal growth caused by Ang II and exogenous AA cannot be excluded. The effect of CYP4A1 antisense ODN to reduce AA-induced but not 20-HETE–induced increase in neointimal growth confirms its selectivity in reducing the effect of AA on neointimal growth.

Balloon injury of arteries has been shown to promote infiltration of leukocytes to the adventitia around the site of injury and increase production of cytokines and chemokines that contribute to neointimal growth. 35,36 Ang II causes activation of immune cells and stimulates production of pro-inflammatory mediators that contribute to an increase in vascular permeability and infiltration of inflammatory cells, including leukocytes. 2 Our finding that Ang II and AA increased the number of leukocytes as detected by CD45-positive cells in arteries exposed to CYP4A1 scrambled ODN suggests that the inflammatory mediators released from these cells also contribute to neointimal growth. Because treatment of injured arteries with CYP4A1 antisense ODN decreased the number of CD45-positive cells and reduced neointimal growth, CYP4A-derived metabolites, most likely 20-HETE, stimulate the inflammatory process initiated by mediators released from leukocytes and smooth muscle cells during injury and exposure to Ang II. Polymorphonuclear leukocytes also synthesize 20-HETE, 37,38 which could also play an important role in the inflammatory process including increased adhesion of leukocytes to injured arteries and neointimal growth. The effect of CYP4A1 antisense ODN to reduce adventitial CYP4A levels and CD45-positive cells as indicated by immunostaining was not as marked as in neointima and media. This could be because of insignificant
lipase A2 and/or are generated during AA metabolism by oxygen species released by AA via activation of phospho-

growth factors stimulate neointimal formation. Finally, the degree of transfection of adventitia with CYP4A1 antisense ODN because it was applied intraluminally for only 60 minutes. Moreover, we cannot exclude the possibility of involvement of other mediator(s) of leukocyte recruitment in this part of the vessel wall.

Ang II via generation of 20-HETE may promote neointimal growth by activating one or more mitogen-activated protein kinase, such as extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase, JNK, or Akt.\textsuperscript{1,39} Both Ang II and 20-HETE increase the activity of one or more of these kinases in VSMCs, and inhibition of the activity of these kinases reduces neointimal growth caused by vascular injury.\textsuperscript{1,40–42} These kinases are also activated by reactive oxygen species generated by Ang II.\textsuperscript{3} A recent study has shown that reactive oxygen species produced by Ang II in adventitial fibroblasts and macrophages promote medial smooth muscle hypertrophy by paracrine action.\textsuperscript{43} Because AA has also been shown to stimulate production of reactive oxygen species in VSMCs,\textsuperscript{44} it is possible that the reactive oxygen species released by AA via activation of phospholipase A\textsubscript{2} and/or are generated during AA metabolism by CYP4A in one or more cell types of the vessel wall, as well as other inflammatory cells, contribute to the action of Ang II on neointimal growth.

Perspectives

The present study demonstrates that a CYP4A-derived AA metabolite(s), most likely 20-HETE, contribute(s) to the effect of Ang II in stimulating neointimal growth in balloon-injured rat carotid arteries. Because the LO and COX pathways have also been implicated in neointimal formation during injury, the relative contribution of various AA metabolites to Ang II-induced neointimal formation need to be determined. Moreover, identifying the cell types that generate AA metabolites and elucidating their mechanisms of action should enhance our understanding of how Ang II and other growth factors stimulate neointimal formation. Finally, the information generated from these studies could illuminate novel targets for the development of therapeutic agents for the treatment of restenosis, atherosclerosis, and other vascular diseases.

Acknowledgments

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Western Blot Analysis

Carotid arteries from 2-day treatment group were ground to a fine powder in liquid nitrogen and added into ice-cold 0.1% Triton lysis solution [10 mmol/L HEPES (pH 7.4), 50 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 5 mmol/L EDTA, 5 mmol/L EGTA, 100 µmol/L Na3VO4, 50 mmol/L NaCl, 0.1% Triton X-100, 500 µmol/L phenylmethylsulfonyl fluoride, and 10 µg/mL leupeptin] for 1 hr. Insoluble cell particles were separated from lysates by centrifuging and the protein content determined by Bradford method. The lysates of these arteries and cultured VSMC were resolved in 8% SDS-polyacrylamide gels and then transferred to a nitrocellulose membrane (1). After blocking with 2% milk and 2% BSA in TTBS buffer (20 mM Tris, pH 7.6, 137 mM NaCl and 0.05% Tween) for 1 hours, the membrane was incubated overnight with anti-CYP4A polyclonal antibody (Novus biologicals, Littleton, CO) at 1:1000 dilution in 5% BSA in TTBS buffer, followed by incubation with anti-mouse-IgG-horseradish-peroxidase antibody (1:1000 dilution in 5% BSA in TTBS) for 1 hour at 25°C. The immunoreactive protein was detected using supersignal chemiluminescent substrate (Pierce, Rockford, IL).

Morphometric and Immunohistochemical Analyses

The animals were sacrificed after 2 days for western blot analysis and 14 days for immunohistochemistry. The injured carotid artery was perfused with saline (3 min), followed by 10% buffered formalin (5 min) at a pressure of 100 mmHg. Animals in which thrombosis was observed in the injured vessel were not included in the study. The
isolated and cleaned arterial segments were incubated in 10% formalin, dehydrated with graded ethanol followed by xylene (1 hr), and embedded in paraffin. The embedded arteries were cut into sections (5 µm) and stained with hematoxylin-eosin for morphometric and immunohistochemical analysis as described (2). For morphometric analysis, the circumferences of the external and internal elastic laminae and the carotid artery lumen were measured using a computer image analysis system (Image J 1.32 program). From these values the intimal and medial areas and intima/media ratio were calculated.

CYP4A or CD45 expression in arterial sections was detected by immunohistochemical analysis performed according to manufacturer’s instructions (Vector Laboratories, Inc., Burlingame, CA). The slides were placed in warm antigen unmasking solution (10 min), incubated in 0.3% H₂O₂ (10 min) and rinsed. Then they were serially incubated in blocking solution (20 min), primary antibody (CYP4A or CD 45, 1:200 in PBS) (2 hrs), and secondary antibody (provided in the Vectastain ABC kit) (1 hr), and finally incubated with Vectastain solution (30 min) and washed. Following application of diaminobenzidine (5 min) and counterstaining with hematoxylin (Sigma, St Louis, MO), the slides were dehydrated with graded concentrations of ethanol. The stained cells were viewed with an Olympus inverted system microscope.

References

Figure legends.

**Figure I. Effect of CYP4A1 antisense and scrambled ODN on CYP4A protein level in rat cultured aortic VSMC and balloon-injured carotid artery.** A. The cultured aortic VSMC were transfected with CYP4A1 antisense or scrambled ODN (100 nmol/L) and the lysates were subjected to 10% SDS-PAGE followed by immunoblotting, as described in methods. CYP4A1 antisense, but not scrambled ODN suppressed CYP4A protein expression in VSMC (n=5). * value different from the corresponding value obtained in vehicle treated group (P < 0.05). B. The lysates of balloon-injured vessels alone, and those treated with CYP4A1 antisense or scrambled ODN (100 nmol/L) were prepared 2 days after surgery and subjected to 10% SDS-PAGE followed by immunoblotting as described in methods. CYP4A1 antisense, but not scrambled ODN reduced the expression of CYP4A protein in balloon-injured carotid artery (n=4). * value different from the corresponding value obtained in vehicle treated group (P < 0.05).

**Figure II. Effect of CYP4A1 antisense ODN treatment on CYP4A immunoreactivity in injured artery.** Injured carotid artery at 14 days after balloon injury was immunohistochemically analyzed using CYP4A polyclonal antibody (n=5). Immunoreactivity of CYP4A in CYP4A1 antisense (As) treated artery was reduced compared to that in vehicle. (Bar = 50 µm).

**Figure II. Effect of CYP4A1 antisense ODN treatment on CD45-positive cells in injured artery.** Injured carotid artery at 14 days after balloon injury was immunohistochemically analyzed using CD45 antibody (n=6). Exposure of the carotid artery to CYP4A1 antisense (As) ODN markedly reduced the density of CD45-positive cells in neointima compared to that in vehicle. (Bar = 50 µm).
Figure I.

A.

IB: CYP 4A

IB: Actin

kDa

CYP 4A Expression (arbitrary numbers)

Cont. CYP 4A1 Scr ODN CYP 4A1 As ODN

*
B.

**IB: CYP 4A**

![Image of IB: CYP 4A](image)

**IB: Actin**

![Image of IB: Actin](image)

Figure I.
Figure II.
Figure III.
Data supplement

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A.

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IB: Actin

kDa

- 50

CYP 4A Expression
(arbitrary numbers)

Cont.  CYP 4A1 Scr ODN  CYP 4A1 As ODN

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B.

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IB: Actin

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Figure III.