Impaired Endothelial Function in Transgenic Mice Expressing Both Human Renin and Human Angiotensinogen

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Background and Purpose—Chronic hypertension is a risk factor for carotid vascular disease and stroke. Mechanisms that account for alterations in carotid and cerebral vascular function during hypertension are poorly defined and based almost exclusively on studies in the spontaneously hypertensive rat, a model in which hypertension has an unknown etiology and in which the genetic background is dissimilar to the most commonly used normotensive control, the Wistar-Kyoto rat.

Methods—In this study we examined vascular function in a defined model of hypertension, double transgenic mice that overexpress both human renin (R+) and human angiotensinogen (A+). We studied vessels in vitro from R+/A+ mice as well as nontransgenic (R−/A−) and single transgenic (R−/A+ or R+/A−) littermate controls.

Results—After submaximal precontraction with U46619 or prostaglandin F2α, acetylcholine, which produces relaxation mediated by endothelial nitric oxide synthase, produced marked relaxation of carotid arteries in control mice but was impaired in R+/A+ mice. For example, 1 μmol/L acetylcholine relaxed the carotid artery by 79±4% versus 44±7% (P<0.01) in control and R+/A+ mice, respectively. Impaired responses to acetylcholine in R+/A+ mice could be restored toward normal with indomethacin (10 μmol/L). In contrast, relaxation of the carotid artery in response to nitroprusside and papaverine was similar in R+/A+ mice and control mice.

Conclusions—These findings indicate that acetylcholine-induced relaxation of carotid artery is impaired selectively in mice made hypertensive by expression of human renin and human angiotensinogen. The mechanism of this impairment may involve production of a cyclooxygenase-derived contracting factor. (Stroke. 2000;31:760-765.)

Key Words: acetylcholine ■ endothelium ■ hypertension ■ nitric oxide ■ nitroprusside

Chronic hypertension is a major risk factor for carotid vascular disease and stroke.1,2 For reasons that are not clear, hypertension is more strongly associated with stroke than with myocardial infarction.3 Recent findings suggest that carotid artery disease (carotid wall thickening) is associated with impairment of endothelium-dependent relaxation in patients with hypertension.4 In addition, a genetic analysis demonstrated cosegregation of impaired endothelium-dependent relaxation and a stroke-prone phenotype in chronically hypertensive rats.5 These findings reaffirm the importance of studying mechanisms of endothelial dysfunction in carotid artery and cerebral blood vessels under pathophysiological conditions, including hypertension.

At present, our understanding of changes in carotid and cerebral vascular function during hypertension is based almost exclusively on studies in spontaneously hypertensive rats (SHR) and stroke-prone spontaneously hypertensive rats (SHRSP), models in which hypertension has an unknown etiology and in which the genetic background is dissimilar to the most commonly used normotensive control, the Wistar-Kyoto rat (WKY).6 The first goal of the present study was to examine vascular function in a novel, defined model of hypertension, a transgenic mouse that overexpresses both human renin and human angiotensinogen genes (R+/A+).7–9 Using these mice, we examined the hypothesis that endothelium-dependent relaxation is impaired during hypertension. Some previous studies suggest that endothelial dysfunction during hypertension may involve production of an endothelium-derived contracting factor produced through the cyclooxygenase pathway.10 Thus, the second goal of the present study was to examine the hypothesis that impaired endothelial function in R+/A+ mice can be improved with indomethacin, an inhibitor of this enzyme system.

Materials and Methods

Animals

The experimental protocol was approved by our institution’s animal care and use committee. All breeding and genotyping was performed in the transgenic animal facility (directed by C.D.S.), located in a virus- and pathogen-free animal care facility. Double transgenic mice (R+/A+) were generated by crossing human renin (R+)...
mice with human angiotensinogen (A+) mice, as we have reported.\(^8,9\) The presence of the transgenes was assessed by gene- and species-specific polymerase chain reaction of DNA isolated from tail biopsy samples, as described previously.\(^8,9\)

We and others have found that the most accurate measurements of arterial pressure in mice are obtained with chronic indwelling catheters in conscious animals. We have shown previously\(^*\) that mean arterial pressure in conscious R+/A+ mice (measured with a chronic indwelling carotid catheter) is approximately 155 to 160 mm Hg compared with approximately 115 to 120 mm Hg in control animals (R−/−A−, nontransgenic controls).\(^8,9\) Because our goal was to study function of the carotid artery in these experiments, we did not implant carotid catheters in these mice. There are no differences in blood pressure between R−/−A− and single transgenic mice (R+/+A− or R−/−A+) owing to the strict species specificity in the enzymatic reaction between renin and angiotensinogen.\(^5\) Because of this specificity, mouse renin does not cleave human angiotensinogen, and human renin does not cleave mouse angiotensinogen.\(^8\) Because blood pressure is the same in all 3 mice, R−/−A−, R+/+A−, and R−/−A+ mice were all used as controls in the present study. Control and R+/+A+ mice averaged 7.5 months of age. Body weight of control and R+/+A+ mice was 26±4 and 25±3 g, respectively.

**Vascular Ring Preparation**

Mice (male and female) were anesthetized with pentobarbital (75 to 100 mg/kg IP), and both carotid arteries were quickly removed and placed in Krebs' buffer with the following ionic composition (mmol/L): NaCl 118.3, KCl 4.7, CaCl\(_2\) 2.5, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, K, HPO\(_4\) 1.2, NaHCO\(_3\) 25, glucose 11. Loose connective tissue in the adventitia was removed, and each carotid artery was cut into 2 rings (3 to 4 mm in length). Vascular rings were suspended in an organ bath containing 25 mL Krebs' solution maintained at 37°C. The rings were connected to a force transducer to measure isometric tension (contraction and relaxation). Resting tension was increased stepwise to reach the final tension of 0.2 to 0.25 g, and the rings were allowed to equilibrate for at least 60 minutes. This amount of resting tension is optimal for contraction in these arteries.

**Protocols**

Vessels were contracted submaximally (40% to 50% of maximum) with the thromboxane A\(_2\) mimetic U46619 (0.11-dideoxy-11α,9α-epoxy-methanoprostaglandin F\(_{2α}\)) (0.2 to 0.4 µg/mL) or prostaglandin F\(_{2α}\) (PGF\(_{2α}\)) (10 to 50 µmol/L). After a stable contraction plateau was reached, dose-response curves were obtained for acetylcholine, sodium nitroprusside, and papaverine. Acetylcholine was used to assess endothelial function, and nitroprusside and papaverine were used to examine direct effects on smooth muscle. We have shown previously that responses of the carotid artery to acetylcholine are mediated by release of nitric oxide by endothelial nitric oxide synthase and activation of soluble guanylate cyclase.\(^11\) In some experiments we examined effects of indomethacin (10 µmol/L) on vasomotor responses. We have used these techniques for studies of mouse vessels in vitro previously.\(^11\)–\(^14\)

At the end of each experiment, we obtained a dose-response curve for the carotid artery for U46619 to determine maximal contractile responses. U46619 produces greater maximal contraction of mouse carotid arteries than high concentrations of KCl.

**Statistical Analysis**

All data are expressed as mean±SE. Vasorelaxation responses were expressed as the percent relaxation from the amount of precontraction produced by U46619 or PGF\(_{2α}\). Comparisons were made with ANOVA or a t test, as appropriate. Statistical significance was accepted at P<0.05.

**Results**

**Vascular Responses in Control Mice and R+/A+ Mice**

Responses of the carotid artery to acetylcholine were similar in R−/−A−, R+/+A−, and R−/−A+ mice. Thus, these data were pooled into a single control group (n=15). In vessels from control mice, acetylcholine produced concentration-dependent relaxation (Figure 1). Relaxation in response to acetylcholine was impaired in carotid arteries from R+/+A+ mice (n=13), particularly at higher concentrations of acetylcholine (Figure 1). In contrast to arteries from control mice, higher concentrations of acetylcholine frequently caused transient contractures of the carotid artery from R+/+A+ mice (see Figure 2, top tracing, for an example).

Nitroprusside (Figure 3) and papaverine (Figure 4) produced similar relaxation of carotid arteries from control and R+/+A+ mice. Contraction of the carotid artery in response to U46619 was also similar in control and R+/+A+ mice (Figure 5). These findings indicate that impaired responses to acetylcholine in R+/+A+ mice were selective for the endothelium-dependent agonist acetylcholine and were not due to a generalized, nonspecific alteration in vascular function.

**Effect of Indomethacin on Vascular Responses**

In control mice, relaxation in response to acetylcholine was similar in the absence and presence of indomethacin (Figure 6, left panel). Contraction of the carotid artery in response to Acetylcholine in a R+/A+ Mouse

![Figure 2. Relaxation of the carotid artery in response to acetylcholine in a R+/A+ mouse in the absence (top tracing) and in the presence (10 µmol/L; bottom tracing) of indomethacin. Carotid rings were precontracted submaximally with U46619 before application of acetylcholine. Concentrations of acetylcholine are given above each tracing.](http://stroke.ahajournals.org/)

![Figure 1. Relaxation of the carotid artery in response to acetylcholine in a control (●; n=13) and R+/A+ (●; n=15) mice. Carotid rings were precontracted submaximally with U46619 or PGF\(_{2α}\) before administration of acetylcholine (see Methods for details). Values are mean±SE. *P<0.05 vs control.](http://stroke.ahajournals.org/)
U46619 was also not affected by indomethacin in control mice (data not shown).

In R+/A+ mice, relaxation in response to acetylcholine was enhanced in the presence of indomethacin (Figure 6, right panel). Transient contractions of the carotid artery from R+/A+ mice in response to higher concentrations of acetylcholine were absent in the presence of indomethacin (see Figure 2, bottom tracing, for an example). In contrast to effects of indomethacin on responses to acetylcholine in R+/A+ mice, indomethacin did not alter contraction of the carotid artery in response to U46619 (data not shown).

Discussion
There are several new findings in the present study. First, the relaxation of the carotid artery in response to acetylcholine is impaired in R+/A+ mice. In contrast, vasorelaxation in response to 2 endothelium-independent agonists, nitroprusside and papaverine, were not altered in R+/A+ mice. These findings indicate that vascular changes in R+/A+ are selective for endothelium and do not reflect some nonspecific alteration in vascular muscle. Second, altered responses of the carotid artery to acetylcholine in R+/A+ mice can be restored toward normal with indomethacin. These findings suggest that activity of the cyclooxygenase pathway contributes to vascular dysfunction in R+/A+ mice.

Potential Advantages of Studies Using R+/A+ Mice
Although some studies have examined endothelial function in carotid artery and intracerebral blood vessels previously, we suggest that the present approach of using mice that overexpress human renin and human angiotensinogen has distinct advantages. For example, in relation to studies of hypertension, the R+/A+ mouse represents a defined model in comparison to the commonly used SHR and SHRSP. SHR and SHRSP are models in which hypertension has an unknown etiology and in which the genetic background is quite dissimilar to WKY.6 Increasing evidence suggests that genetic background is an important additional variable in studies of cardiovascular biology. For example, genetic background differences with respect to susceptibility to atherosclerosis and vascular responses to acetylcholine have been described.15–17 In the present study we generated double transgenic mice (R+/A+) (and littermates that were used as controls) by crossbreeding human renin (R+) mice with human angiotensinogen (A+) mice, as we have reported.8,9 The genetic background of R+/A+ transgenic mice is nearly identical to that in the control animals since the mice used in these studies were derived from 4 to 5 generations of back-crossbreeding to C57BL/6J. With additional back-crossbreeding, future studies will be able to use mice with even greater homogeneity in the genetic background.

Figure 3. Relaxation of the carotid artery in response to nitroprusside in control (●; n=13) and R+/A+ (●; n=15) mice. Carotid rings were precontracted submaximally with U46619 or PGF$_{2\alpha}$ before administration of nitroprusside (see Methods for details). Values are mean±SE.

Figure 4. Relaxation of the carotid artery in response to papaverine in control (●; n=13) and R+/A+ (●; n=15) mice. Carotid rings were precontracted submaximally with U46619 or PGF$_{2\alpha}$ before administration of papaverine (see Methods for details). Values are mean±SE.

Figure 5. Contraction of the carotid artery in response to the thromboxane mimetic U46619 in control (●; n=13) and R+/A+ (●; n=15) mice. Values are mean±SE.

Figure 6. Relaxation of the carotid artery in response to acetylcholine in the absence (●) and presence (●; 10 μmol/L) of indomethacin (Indo). Left, Data for control mice (n=13); right, data for R+/A+ mice (n=15). Carotid rings were precontracted submaximally with U46619 or PGF$_{2\alpha}$ before administration of acetylcholine (see Methods for details). Values are mean±SE. *$P<0.05$ vs control.
previous studies examining effects of hypertension in SHR versus WKY have used strains that are genetically diverse, the results are clouded by the presence of genes in the genetic background that may themselves predispose or protect from hypertension or endothelial dysfunction.

Vascular Responses in Control and R+/A+ Mice
In control mice, acetylcholine, nitroprusside, and papaverine all produced marked relaxation of the carotid artery. Relaxation of the carotid artery in response to acetylcholine was not affected significantly by indomethacin. We have shown previously, using pharmacological approaches and endothelial nitric oxide synthase (eNOS)–deficient mice, that relaxation of the carotid artery in response to acetylcholine is mediated by endogenous production of nitric oxide (by eNOS) and activation of soluble guanylate cyclase. Vasorelaxation in response to nitroprusside (a donor of nitric oxide) is also mediated by activation of soluble guanylate cyclase. Because we have not repeated these experiments in R+/A+ mice, we assume that vasorelaxation in response to acetylcholine is mediated via the same mechanism in R+/A+ mice as in nontransgenic mice. In contrast to acetylcholine and nitroprusside, papaverine produces vasorelaxation that is endothelial independent and is not dependent on activity of soluble guanylate cyclase.

Endothelium-dependent relaxation is impaired in most studies of experimental animals and humans with chronic hypertension. For example, we and others have shown that dilatation of the basilar artery and cerebral arterioles in response to endothelium-dependent agonists is impaired in SHRSP. In addition, impaired relaxation of the carotid artery in response to acetylcholine has been observed in SHR and SHRSP and in rats with renovascular hypertension or deoxycorticosterone salt–induced hypertension. In contrast to endothelial-dependent stimuli, vasorelaxation in response to endothelial-independent agonists has been found to be relatively normal during chronic hypertension in most studies, which suggests that impairment of vascular function occurs primarily at the level of endothelium. In the present study we found that relaxation of the carotid artery in response to acetylcholine was impaired and responses to nitroprusside and papaverine were similar in control and R+/A+ mice. These new findings support the general concept that chronic hypertension is associated with endothelial dysfunction.

Recently, studies of endothelial function have been performed in a related model, transgenic rats that overexpress the mouse Ren2 gene [TGR(mRen2)27]. The results of these studies have been inconsistent in relation to findings on endothelial function. Endothelium-dependent relaxation in response to acetylcholine has been reported to be impaired in one study of aorta. In contrast, responses to endothelium-dependent agonists in TGR(mRen2)27 were not impaired in another study of aorta and studies of coronary arteries and the mesenteric circulation. Responses to endothelium-dependent agonists were enhanced in the renal circulation of TGR(mRen2)27 compared with nontransgenic controls. The explanation for why inconsistent results have been obtained in studies of endothelial function in TGR(mRen2)27 is not clear.

There have been very few studies of endothelial function in chronically hypertensive mice. In normal mice in which hypertension is produced by psychosocial stress, relaxation to acetylcholine in aorta and hind limb is paradoxically increased. Some data are available on vascular responses to endothelium-dependent agonists in other mice that are chronically hypertensive. For example, endothelial function has been studied in eNOS- and cGMP-dependent protein kinase I–deficient mice, which are moderately hypertensive compared with R+/A+ mice. Because responses to acetylcholine are completely abolished in the absence of these essential signaling mechanisms, these data are difficult to compare with the present findings. Thus, the effect of chronic hypertension on eNOS/cGMP signaling in blood vessels cannot be assessed in these models.

Mechanisms of Endothelial Dysfunction in R+/A+ Mice
Several studies have attempted to define mechanisms that account for impaired endothelium-dependent relaxation in blood vessels during chronic hypertension. These mechanisms include production of an endothelium-derived contracting factor produced through the cyclooxygenase pathway, degradation of nitric oxide by superoxide anion, and decreased expression of eNOS. In cerebral arterioles of SHR and SHRSP, impaired responses to endothelium-dependent agonists can be restored toward normal with indomethacin. To our knowledge, only 1 study examined mechanisms of carotid vascular dysfunction during hypertension and reported that impaired relaxation in response to acetylcholine in SHR was not altered by indomethacin. In contrast, we found that responses of the carotid artery to acetylcholine could be restored toward normal with indomethacin in R+/A+ mice. Thus, our findings differ in terms of mechanism when compared with the results obtained in carotid artery of SHR rats, thereby lending novelty to the present study. Our findings, using a model with better control of genetic background, support the overall concept that activity of cyclooxygenase contributes to vascular dysfunction during chronic hypertension.

In humans with essential hypertension, endothelium-dependent relaxation is impaired. Although several mechanisms have been proposed to account for this abnormality, indomethacin restores impaired endothelium-dependent responses toward normal in patients with hypertension. Thus, the findings in the present study in R+/A+ mice are consistent with data in at least some patients with hypertension and support that concept that impaired endothelial function in hypertension can be mediated, at least in part, by increased activity of cyclooxygenase.

In summary, endothelial function is impaired selectively in transgenic mice that express human renin and human angiotensinogen. The mechanism of this impairment may involve production of a cyclooxygenase-derived contracting factor.

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Editorial Comment

The first demonstration of the important role endothelial cells can play in augmentation of vasoconstriction was reported in 1982 by De Mey and Vanhoutte. Over the years, the concept that endothelial cells can produce and release vasoconstrictor substances in response to agonists and physical stimuli has been supported by numerous reports, including findings on human blood vessels. The mechanism underlying endothelium-dependent contractions is still not completely understood. Existing evidence suggests that activation of arachidonic acid metabolism via the cyclooxygenase pathway, with subsequent increased formation of vasoconstrictors prostaglandin H₂ and thromboxane A₂, leads to an increase in vascular tone.

In the present study Didion and colleagues studied vaso-motor reactivity of double transgenic mice overexpressing human renin (R¹) and human angiotensinogen (A¹). As a result of genetic manipulation, these animals are hypertensive with mean arterial pressure of 155 to 160 mm Hg (compared with approximately 115 to 120 mm Hg in control animals). The authors report impairment of endothelium-dependent relaxations to acetylcholine in isolated carotid arteries of genetically hypertensive mice. Endothelial function was normalized in arteries treated with the cyclooxygenase inhibitor indomethacin, which suggests that release of cyclooxygenase product(s) is responsible for impairment of endothelial function. This finding is agreement with previously reported effects of hypertension on endothelial function in spontaneously hypertensive rats and humans.

What are the possible clinical implications of these findings? The physiological and pathological role of endotheli-um-dependent contractions mediated by cyclooxygenase is unknown. Under physiological conditions the endothelial cells play a protective role in circulation by releasing substances (relaxing factors) that prevent vasoconstriction, platelet aggregation, smooth muscle cell proliferation, and white blood cell adhesion. However, under pathological conditions, the protective role of the endothelium diminishes and the activity of contracting factors becomes more prominent. Inhibition of cyclooxygenase may alter the balance between endothelium-derived relaxing and contracting factors in favor of the former, thus contributing to the beneficial effect of aspirin and aspirin-like drugs on vascular function.

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