RhoA Activation Contributes to Sex Differences in Vascular Contractions


Objective—Studies have suggested that sex differences in endothelial function in part account for the lower incidence of cardiovascular disease in premenopausal women compared with men. Less is known about the role of smooth muscle. We hypothesized that signaling mechanisms that regulate calcium sensitivity in vascular muscle also play a role in determining sex differences in contractile function.

Methods and Results—In aorta, concentration-dependent contractions to serotonin were greater in male versus female mice whereas contractions to KCl and U46619 were similar. Nitric oxide or other endothelial-derived factors did not account for the difference in responses to serotonin because inhibition of nitric oxide synthase (NOS) with N\textsuperscript{G}-nitro-L-arginine, genetic deficiency of endothelial NOS, and removal of endothelium increased contractions but did not abolish the enhanced contractions in aorta from males. Contractions in aorta from both males and females were abolished by a serotoninergic 5HT\textsubscript{2A} receptor antagonist (ketanserin), however there was no sex difference in 5HT\textsubscript{2A} receptor expression. Activation of RhoA and Rho-kinase by serotonin was greater in aorta from males compared with females, but this was not related to greater expression of RhoA or Rho-kinase isoforms (ROCK1 and ROCK2). The sex difference in aortic contractions to serotonin was abolished by an inhibitor of Rho-kinase, Y27632.

Conclusion—We conclude that increased contractions to serotonin in aorta from male mice are attributable to differences in RhoA/Rho-kinase activation in smooth muscle independent of differences in the expression of RhoA or Rho-kinase. (Arterioscler Thromb Vasc Biol. 2007;27:1934-1940.)

Key Words: gender ■ Rho GTPase ■ serotonin ■ Rho-kinase

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Studies of sex differences in cardiovascular disease have generally focused on estrogen-mediated effects on endothelial function. Estrogen may improve endothelial function through several mechanisms including acting as an antioxidant, increasing the bioavailability of nitric oxide (NO), and increasing the expression of NO synthase (NOS).\textsuperscript{1} Although abnormal endothelial function is a marker of cardiovascular disease, studies in animal models suggest that alterations in endothelial function alone cannot account for the abnormal vascular responses in cardiovascular disease.\textsuperscript{2,3} For example, increased vasoconstrictor responses in models of vasospasm are attributable not only to abnormal bioavailability of NO from endothelium but also smooth muscle hyperreactivity.\textsuperscript{2} Thus, in addition to differences in endothelial function, sex differences in cardiovascular disease may be related to alterations in smooth muscle function.

We have previously demonstrated that contractile responses to serotonin are greater in carotid arteries from male compared with female mice.\textsuperscript{4} Serotonin, which may mediate platelet-induced vasospasm,\textsuperscript{5,6} produces changes in vascular tone through two opposing effects: vasodilation mediated by release of substances from endothelium and vasoconstriction mediated by direct activation of serotonin receptors in vascular muscle.\textsuperscript{7-10} In our previous work, genetic deficiency in eNOS augmented contractions to serotonin in arteries from both males and females but failed to abolish the sex difference.\textsuperscript{4} Although other sources of NO and endothelial vasodilators may upregulate to compensate for chronic deficiency of eNOS, we concluded that differences in NO from eNOS could not account for the sex difference in responses to serotonin.\textsuperscript{4} We did not rule out other endothelial-derived vasodilators or differences in smooth muscle function.

Contraction of vascular muscle occurs after increases in intracellular calcium or increased sensitivity of contractile proteins to calcium.\textsuperscript{11} The Rho/Rho-kinase pathway is a determinant of calcium sensitivity of contractile proteins and is activated by serotonin.\textsuperscript{12,13} Recent studies suggest that Rho-kinase activity is upregulated in cardiovascular diseases including vasospasm, hypertension, atherosclerosis, and stroke.\textsuperscript{14-17} The Rho/Rho-kinase pathway may also contrib-
ute to sex differences in function of vascular muscle. Inhibition of Rho-kinase had a greater effect on basal diameter of arteries from males in situ compared with females, suggesting a greater basal Rho-kinase activity. Our previous report of greater contractions to serotonin in carotid arteries from males compared with females may be attributable to differences in contractile mechanisms within vascular muscle mediated by Rho or Rho-kinase. The objective of this study was to determine whether increased contractions of arteries from males compared with females are attributable to sex differences in the RhoA/Rho-kinase pathway.

Materials and Methods

Experimental Model

The animal protocol was reviewed and approved by the VA Medical Center and the University of Iowa Animal Care and Use Committees. All animal procedures complied with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society. Male and female C57BL6 mice were used. eNOS deficient (eNOS−/−) mice were derived from breeding heterozygous eNOS+/- mice. This approach generated eNOS−/− mice as well as eNOS+/− mice used as littermate controls. Mice used for these experiments (both males and females) were derived from 7 to 8 generations of backcross breeding to C57BL6. Genotyping of mice was performed by polymerase chain reaction (PCR) of DNA from tail biopsies as described previously.

Measurements of Vascular Reactivity

Studies were performed in aorta because it is the most commonly used mouse model to date and allows us to compare vascular reactivity with expression and activity of Rho/Rho-kinase in the same blood vessel. Responses of aorta were measured using previously published methods. Mice (15 to 20 weeks of age) were heparinized (400 U/kg ip), anesthetized with pentobarbital (100 mg/kg ip), and the thoracic aorta rapidly removed and placed in ice-cold Krebs buffer (mmol/L: NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, and glucose 11). The aorta was cut into 4 rings (3 to 4 mm in length), mounted on wires connected to a force transducer in an organ bath filled with Krebs buffer at 37°C aerated with 20% O2, 5% CO2, and balance N2. Tension was increased to 0.75 g over 60 minutes. To measure relaxation, rings were precontracted with PGF2α (10 nmol/L), KCl (25 to 100 mmol/L), or U46619 (10 to 20 nmol/L) to maintain a stable contraction of 50% to 60% of maximal contractile response before addition of acetylcyanine (1 nmol/L to 10 µmol/L) or sodium nitroprusside (1 mmol/L to 10 µmol/L). Relaxation responses were expressed as percent decrease in tension from preconstriction values. Contractile responses to serotonin (10 nmol/L to 1 µmol/L), KCl (25 to 100 µmol/L), or the thromboxane mimetic U46619 (10 nmol/L to 1 µmol/L) were obtained by adding increasing concentrations to vessels without preconstriction. In general, 2 rings of aorta from each animal were used as controls and 2 were treated with inhibitors for 30 minutes before initiation of dose response curves. Responses were averaged and ‘n’ represents numbers of mice per group. The following inhibitors were used: for Rho-kinase (+)-trans-(4-(1-aminoethyl-N-4-pyridil) cyclohexanecarboxamide dihydrochloride (Y27632, 1 µmol/L), for NOS N^G-nitro-L-arginine (L-NNA, 10 µmol/L), for serotonergic 5HT2A receptors, ketanserin (1 µmol/L). To remove endothelium we gently rubbed the internal surface of the ring with suture.

Western Blot Analysis

Aorta were isolated and equilibrated in an organ bath for 30 minutes. Aorta (3 to 4 aorta pooled per sample) without and with serotonin (1 µmol/L) were flash frozen in liquid nitrogen (LN2), minced and sonicated in lysis buffer containing 25 mmol/L sucrose, 50 mmol/L MOPS, 2 mmol/L EDTA, 2 mmol/L EGTA pH 7.4. Complete Protease Inhibitor (Roche Molecular Biochemicals), NaF (50 mmol/L), Na pyrophosphate (20 mmol/L), p-Nitrophenol phosphate (1 mmol/L), and Microcystin LR (1 µmol/L). The homogenate was centrifuged at 14 000g for 15 minutes at 4°C and the resulting supernatant was centrifuged at 100 000g at 4°C for 1 hour. The membrane pellet was resuspended in lysis buffer with 1% Triton X-100. Protein concentrations were determined by the bicinchoninic acid method, and equal amounts of samples (10 to 30 µg) were separated by SDS-PAGE gel electrophoresis. After blocking in 8% milk, immunoblotting was performed on whole cell lysate using anti-RhoA (1:250, Santa Cruz), anti-ROCK1 and ROCK2 (1:500, BD Biosciences), ezrin-radixin-moesin (ERMs, 1:500, Chemicon) or phosphorylated-ERMs (PERMs, 1:500, Chemicon) and on membrane fractions using anti-5HT2A (1:1000, a generous gift from Bryan Roth at Case Western Reserve University Medical School) in 3% diluent followed by secondary antibodies conjugated with horseradish peroxidase. Immunoreactivity was visualized with enhanced chemiluminescence. Blots were digitized and normalized to actin (Sigma-Aldrich) for comparison (NIH Image).

RhoA Activation Assay

RhoA activation was measured by a modified ELISA (Cytoskeleton). Endothelium denuded aorta from male and female mice were flash frozen under basal conditions or after serotonin exposure (1 µmol/L). Samples were homogenized in lysis buffer (Cytoskeleton) with addition of phosphatase inhibitors NaF (50 mmol/L), Na pyrophosphate (20 mmol/L), p-Nitrophenol phosphate (1 mmol/L), and Microcystin LR (1 µmol/L). The homogenate was centrifuged at 14 000g for 5 minutes at 4°C to remove cellular debris. Active GTP-bound Rho bound to a Rho-GTP binding protein was measured with an anti-RhoA antibody and absorbance measured at 490 nm.

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Y27632 and ketanserin were purchased from Tocris Cookson Inc, PGF2α from Pfizer-Pharmacia, and U46619 from Biomol.

Statistical Analysis

All data are presented as mean±SEM. Dose response curves were compared by repeated measures analysis of variance. Data from immunoblots and RhoA activation were compared by Student t tests. Significance was defined at P<0.05.

Results

Differences in NO or Other Endothelium-Derived Substances Do Not Account for Greater Contractions of Arteries From Male Mice

Previous findings in carotid artery demonstrated enhanced contractions to serotonin in males compared with females. To determine whether sex-dependent differences are generalized, we compared responses in aorta. Serotonin produced concentration-dependent contractions of intact aorta from male and female mice (Figure 1A). Contractions of aorta to serotonin were approximately 2-fold greater in males compared with females (Figure 1A, P<0.05). Contractions to KCl and U46619 were similar between males and females (Figure 5) indicating that the sex difference in contractile response was specific for serotonin. Because serotonin can stimulate release of NO and other endothelium-derived substances which modulate contraction of smooth muscle, we determined whether NO accounted for differences in contractions between aorta from males and females. Responses to serotonin were measured after acute inhibition of NOS with L-NNA (10 µmol/L) in wild-type mice and in vessels from mice with genetic deletion of eNOS (eNOS−/−). Blockade or absence of
NOS was verified by confirming inhibition of relaxation to acetylcholine and intact relaxation to nitroprusside (data not shown). L-NNA increased contractions of arteries from both males and females to serotonin (Figure 1A, \( P<0.05 \) L-NNA versus without L-NNA). In eNOS-deficient mice, contractions to serotonin were increased to a level similar to L-NNA in both males and females (Figure 1B, \( P<0.05 \) eNOS\(^{-/-}\) versus eNOS\(^{+/+}\)). Although contractions increased in aorta from males and females after acute inhibition of NOS and with chronic eNOS deficiency, the magnitude of the difference in contractions between males and females was maintained. Relaxation to the NO-dependent vasodilator, acetylcholine (maximal response at 10 \( \mu \)mol/L: males 71\% ± 2%; females 78\% ± 7%) and the NO-independent vasodilator, nitroprusside (maximal response at 1 \( \mu \)mol/L: males 92\% ± 8%; females 96\% ± 2%) was similar between control males and females.

Because other endothelium-derived substances modulate vascular tone and could account for the sex-dependent differences,\(^1\) we tested whether removal of endothelium would abolish the difference in contractions to serotonin. Endothelial removal was verified by the absence of relaxation to acetylcholine but intact relaxation to nitroprusside (data not shown). After endothelial removal, contractions to serotonin were increased in aorta from both male and female mice (Figure 1C). But similar to acute (Figure 1A) and chronic NO deficiency (Figure 1B), contractions to serotonin were still greater in denuded aorta from males compared with females. These data demonstrate that although NO and endothelium modulate contractions to serotonin, they do not account for the sex-dependent difference in contractions to serotonin in mouse aorta.

Expression of Serotonergic 5HT\(_{2A}\) Receptors Is Similar in Males and Females

Sex differences in expression of serotonergic receptors which mediate aortic contractions may underlie the enhanced vasoconstriction in males. First, we determined whether activation of 5HT\(_{2A}\) receptors mediate contractions to serotonin in aorta\(^{21,22}\) by measuring responses to serotonin in the presence of the 5HT\(_{2A}\) receptor antagonist, ketanserin. Ketanserin abolished contractions to serotonin in both males and females (maximal contraction at 1 \( \mu \)mol/L: Male 0.029\% ± 0.010 g, \( n=6 \); Female 0.027\% ± 0.019 g, \( n=3 \)). Second, we compared expression levels of the 5HT\(_{2A}\) receptor in aortic membrane by immunoblotting. Expression was similar in aorta from males and females (Figure 2). Thus, contractions of mouse aorta to serotonin are mediated via the 5HT\(_{2A}\) receptor but differences in receptor expression levels do not account for the sex-dependent contractions to serotonin.

Activation of RhoA by Serotonin Is Greater in Aorta From Males Compared With Females

Serotonin activates RhoA and its effector Rho-kinase to regulate calcium sensitivity and muscle contraction.\(^{12,13}\) The small GTPase RhoA serves as a molecular switch to transduce extracellular stimuli to intracellular signaling pathways regulating muscle contractions, organization of the actin cytoskeleton, cell adhesion, and motility.\(^{16,23}\) To determine
whether this pathway mediates the sex difference in contractions, we measured RhoA activation in aorta from males and females under basal conditions and after contraction to serotonin (1 μmol/L) using a modified ELISA. Basal RhoA activity was approximately 2-fold greater in aorta from male mice compared with female (Figure 3A). In aorta from males, serotonin doubled RhoA activity (P<0.05 versus basal). Although serotonin tended to increase RhoA activation in aorta from females (P=0.065), the magnitude of RhoA activation was only a third of the level observed in males (Figure 3A). A possible explanation for the greater activation of RhoA in males compared with females is an increased expression of RhoA. Expression of RhoA in whole cell lysate did not differ in aorta from males and females (Figure 3B). These data suggest that serotonin induces a greater activation of RhoA in males that is not attributable to an increased expression of RhoA.

**Activation of Rho-Kinase by Serotonin Is Greater in Aorta From Males Compared With Females**

To increase vasoconstriction, a greater RhoA activation in males should result in a greater activation of downstream Rho-kinase. To assess Rho-kinase activity, we compared levels of phosphorylated ezrin, radixin, and moesin (PERMs), substrates for Rho-kinase. Western immunoblot was performed in whole cell lysate of aorta under basal conditions and after serotonin-induced contractions (1 μmol/L). Basal levels of PERMs as a percentage of total ERM expression were similar in aorta from males (1.02±0.38, n=5) and females (1.02±0.24, n=5). Serotonin produced an approximately 50% increase in the level of PERMs in males (Figure 4A and 4B). There was no significant change in the level of PERMs in females. One possible explanation for the greater increase in Rho-kinase activity in response to serotonin may be that Rho-kinase expression is greater in male mice. Two isoforms of Rho-kinase have been identified: ROCK1 and ROCK2. Western blot analysis was performed for both isoforms of Rho-kinase. Expression of ROCK1 and ROCK2 was similar in males and females (Figure 4C and 4D). These data suggest that the greater activation of RhoA and Rho-kinase are not attributable to a difference in the expression of either RhoA or Rho-kinase.

**Contractions of Aorta Mediated by Rho-Kinase Are Greater in Males Compared With Females**

We assessed a functional index of the increased RhoA and Rho-kinase activity by measuring contractions of aorta to serotonin in the absence and presence of a Rho-kinase inhibitor, Y27632 (1 μmol/L). Similar to the previous group (Figure 1), serotonin-induced contractions were greater in aorta from males compared with females (Figure 5A). Y27632 had no effect on basal tension. Inhibition of Rho-kinase had a marked effect on contractions to serotonin and a greater effect on responses of vessels from males (Figure 5A). Y27632 abolished the sex difference in serotonin-induced contractions. Y27632 had only a modest effect on contractions to KCl (Figure 5B). Although Y27632 tended to decrease contractions to the lowest concentration of U46619 used in this study, overall it had no significant effect on contractions to U46619 in aorta from either male or female mice (Figure 5C). Inhibition of Rho-kinase normalized contractions to serotonin in males and females demonstrating that the enhanced Rho-kinase activity in aorta from males mediates the sex difference.

**Discussion**

We reported several novel findings in this study. First, vasoconstriction of aorta to serotonin is dependent on sex.
Contractions to serotonin were greater in aorta from male compared with female mice. Second, although NO or other endothelium-derived factors modulate contractions to serotonin, the sex-dependent responses were not attributable to differences in NO or endothelial-mediated modulation of contractions to serotonin. Differences in vascular smooth muscle function must play a major role in sex difference in vasoconstriction. Third, although inhibition of 5HT$_{2A}$ receptors abolished contractions in both males and females, expression of the 5HT$_{2A}$ receptor was similar. Thus the sex difference in vasoconstriction to serotonin was not attributable to differences in expression of 5HT$_{2A}$ receptors. Fourth, both basal and agonist-induced activation of RhoA were greater in aorta from male mice compared with females. This was associated with greater activation of Rho-kinase. However, protein levels of RhoA and Rho-kinase were not different in aorta from male compared with female mice. Inhibition of Rho-kinase abolished the sex-dependent difference in vasoconstriction to serotonin. This suggests that activation of Rho and Rho-kinase are regulated differently in vessels from male compared with female mice.

**Sex Differences and NO-Mediated Vascular Reactivity**

In the present study we compared responses of mouse aorta to serotonin, a mediator of vasoconstriction in response to aggregating platelets which induces vasospasm under pathophysiological conditions.$^5,6$ Responses to serotonin are complex involving a balance of direct vasoconstriction through activation of 5HT$_{2A}$ receptors on smooth muscle and vasodilation through release of substances from endothelium.$^7-10$ If the sex difference in contractions to serotonin was solely attributable to greater agonist-induced release of NO from eNOS in females, pharmacological inhibition of NOS or responses in eNOS-deficient mice should have abolished this difference. In the present study, inhibition of NOS (L-NNA or eNOS$^{-/-}$ mice) or removal of endothelium augmented contractions to serotonin in aorta from both males and females. None of our approaches to remove NO normalized contractions to serotonin in males and females. The sex difference in serotonin-induced contractions was maintained. We also did not observe differences in relaxation to acetylcholine similar to other studies, suggesting that in mouse aorta NO-mediated responses are not modulated by sex.$^4,24$ The results of this study demonstrate that NO modulates contractions to serotonin, but it does not account for the sex difference in contractions to serotonin in mouse aorta. The results suggest that vascular muscle plays a major role in regulating the sex difference in contractile responses to serotonin.

**Sex Differences in Rho-Kinase**

Two recent studies suggest that sex differences in vascular reactivity may be related to the Rho/Rho-kinase pathway.$^{18,25}$ Inhibition of Rho-kinase decreased resting vascular diameter more in the cerebral circulation of male compared with female rats, suggesting that basal Rho-kinase activity is greater in males.$^{18}$ Despite these findings, protein levels of RhoA and Rho-kinase were not different.$^{18}$ Agonist-induced activation of RhoA and Rho-kinase was not compared in that study. In cultured vascular muscle, high levels of estrogen (0.1 to 100 μmol/L) decreased mRNA for ROCK2.$^{25}$ The results of the present study extend these findings by comparing effects of agonist-induced activation of Rho and Rho-kinase on sex differences in vascular function. Our results confirm that expression of RhoA and Rho-kinase was similar in aorta from males compared with females. Basal activity of RhoA but not Rho-kinase was greater in aorta from males. Serotonin-induced activation of both RhoA and Rho-kinase was greater in males. Thus differences in expression of RhoA
and Rho-kinase do not account for the greater Rho and Rho-kinase activity in males. The sex differences in activation of RhoA may be related to the family of Rho GTPase regulatory proteins, namely, guanine dissociation inhibitors, guanine exchange factors, and GTPase activating proteins which control the balance between active and inactive Rho.

**Sex-Dependent Contractions Are Agonist Specific**

In mouse aorta, a minor component of contractions to KCl was mediated by activation of Rho-kinase whereas contractions to U46619 were not. The role of Rho-kinase in mediating vasoconstriction to KCl and U46619 is not consistent and illustrates the dependence of the response on animal model and vascular bed. The contrasting role of Rho-kinase in mediating agonist-induced vasoconstriction may be related to the contribution of calcium-dependent and -independent mechanisms associated with different vascular beds. In addition, coupling of specific receptors subtypes to different G protein–mediated pathways may be an important determinant of the subsequent activation of Rho and Rho-kinase in different vascular beds. Determination of the specific G protein–coupled receptors coupled to Rho/Rho-kinase may provide clues for identifying mechanisms that regulate sex dependent responses in vascular muscle.

Calcium sensitivity of smooth muscle is under the control of at least 2 different mechanisms regulating activity of myosin light chain phosphatase (MLCP). The present study focused on the role of RhoA and Rho-kinase in the regulation of MLCP activity. Phosphorylation of MLCP by Rho-kinase inhibits its activity resulting in unopposed phosphorylation of MLC by a specific kinase. The end result is an increase in vascular contraction. Dephosphorylation of MLC by MLCP promotes relaxation of vascular smooth muscle. An alternative regulator of MLCP activity is protein kinase C activated CPI-17 (PKC-potentiated inhibitor protein of 17 kDa). CPI-17 is a potent inhibitor of MLCP leading to enhanced phosphorylation of MLC and contraction. We performed preliminary studies comparing the levels of CPI-17 in aorta from male and female mice and did not detect a significant difference in expression (data not shown). These data suggested that CPI-17 is not involved in the sex difference in contractile responses.

**Summary**

The results of the present study demonstrate that activation of RhoA and Rho-kinase in response to serotonin is greater in aorta from males compared with females resulting in greater contractions in males. The list of animal models of cardiovascular diseases in which Rho-kinase is augmented continues to increase. The Rho/Rho-kinase pathway plays a role not only in vasoconstriction but also cell migration, cardiac hypertrophy, and development of atherosclerosis. We propose that sex differences in the activation of Rho and Rho-kinase may contribute to the lower incidence of cardiovascular disease in premenopausal women compared with men.

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Disclosures

None.

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