Sex-dependent differences in Rho activation contribute to contractile dysfunction in type 2 diabetic mice

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1Department of Veterans Affairs Iowa City Health Care System and the Departments of 2Internal Medicine and 3Pharmacology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa

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Nuno DW, Harrod JS, Lamping KG. Sex-dependent differences in Rho activation contribute to contractile dysfunction in type 2 diabetic mice. Am J Physiol Heart Circ Physiol 297: H1469–H1477, 2009. First published August 7, 2009; doi:10.1152/ajpheart.00407.2009.—The objective of this study was to determine if mechanisms involved in vascular dysfunction in type 2 diabetes differ with sex. Vascular reactivity, expression, and activation of rhoA and rho kinase were measured in aorta from male and female nondiabetic C57BLKS/J and diabetic BKS.Cg-m+/+ Leprdb/J (db/db) mice, a model of type 2 diabetes. Relaxation to acetylcholine and nitroprusside was similar in aorta from nondiabetic male and female mice. Relaxation to acetylcholine was reduced ~50% in both male and female diabetic mice. Although inhibition of rho kinase with H-1152 increased relaxation to acetylcholine and nitroprusside in nondiabetic males, it had no effect on the response in either nondiabetic or diabetic females or diabetic males. Contraction to serotonin was increased similarly in male and female diabetic mice compared with nondiabetic mice and was reduced following inhibition of rho kinase with either fasudil or H-1152. Activation of rhoA and its downstream effector, rho kinase, was greater in aorta from diabetic males compared with nondiabetic males. In contrast, there were no differences in vascular activation of rhoA or rho kinase in diabetic females. The increased activity of rhoA and rho kinase in diabetic mice was not due to a change in protein expression of rhoA or rho kinase (ROCK1 and ROCK2) in vessels from either males or females. Although contractile dysfunction in vessels occurs in both male and female diabetic mice, the dysfunction in diabetic males is dependent upon activation of rhoA and rho kinase. Alternative mechanisms affecting rho kinase activation may be involved in females.

rhoprotein kinase; rhoA

DIABETES IS A major risk factor for the development of cardiovascular disease and is associated with accelerated atherosclerosis (46). In the United States, the age-adjusted prevalence of type 2 diabetes increased 98% in males but only 54% in females from 1980 through 2002, suggesting a sex difference in the incidence and/or diagnosis of diabetes. Although cardiovascular disease is reduced in premenopausal women compared with age-matched males, the protection from the development of cardiovascular disease afforded by sex is abolished following development of type 2 diabetes (23).

A consistent finding in blood vessels from animal models and patients with diabetes is impaired endothelium-dependent relaxation (20, 21, 46). Endothelial defects contributing to abnormal smooth muscle function in diabetes include a reduction in nitric oxide (NO) bioavailability and an increased release of contractile factors (33). While many studies have examined endothelial dysfunction in diabetes, fewer studies have examined the effect of diabetes on smooth muscle dysfunction. Study of vascular muscle in diabetic subjects is important since abnormal endothelial function alone cannot account for the increased vasoconstrictor responses observed in many cardiovascular diseases (12, 30, 33). Contractile function of smooth muscle regulated by protein kinase C (PKC) and rho/rho kinase is augmented in diabetes (10, 15, 18, 31, 40, 49) and a variety of other vascular diseases including hypertension, atherosclerosis, and cerebral and coronary vasospasm (3). In these diseases, activity of PKC and rho kinase is upregulated, leading to enhanced resting vascular tone and agonist-induced vasoconstriction. We have previously demonstrated sex-dependent differences in vasoconstriction in nondiabetic mice, in part, mediated by an increased activation of rho and rho kinase in arteries from males compared with females (32). The role of rho and rho kinase in vascular dysfunction in diabetic males compared with females has not been explored. The primary goal of the present study was to test the hypothesis that, in the presence of type 2 diabetes, vascular dysfunction is similar in arteries from males and females, but the underlying mechanisms contributing to dysfunction may differ.

Recent studies suggest that NO and rho/rho kinase signaling pathways interact in endothelium and vascular muscle to determine vascular tone and agonist-induced responses. RhoA and NO inversely affect the expression and activation of the opposing signaling pathways in some (6, 26, 41–44, 47) but not all studies (4, 29, 48). The magnitude and impact of this interaction may depend on cell type, tissue, animal, or disease state. Alterations in the cross talk between these two opposing pathways within endothelium and smooth muscle may determine the magnitude of vascular responses and contribute to development of contractile dysfunction in diabetes. The second objective of the present study was to determine the role of rhoA and rho kinase and the interaction with NO in the vascular dysfunction in male compared with female mice with type 2 diabetes.

MATERIALS AND METHODS

Experimental model. The animal protocol was approved by the Veterans Affairs Medical Center and the University of Iowa Animal Care and Use Committees and complied with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society. Male and female nondiabetic C57BLKS/J and homozygous leptin receptor-deficient diabetic mice (BKS.Cg-m+/+ Leprdb/J, db/db) were obtained from Jackson Laboratories. Weight and nonfasting blood glucose were measured following death (pentobarbital sodium, 150 mg/kg ip) on the day of study.

Measurement of vascular reactivity. Studies were performed in aorta, since it allowed us to compare vascular reactivity with biochemical end points in the same vessel type. Responses of aorta were
measured using previously published methods (25). Thoracic aorta were removed, placed in ice-cold Krebs buffer (in mM:mL: 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11 glucose), cut into rings (3–4 mm in length), and mounted on wires connected to a force transducer in an organ bath filled with Krebs (37°C aerated with 20% O2-5% CO2-balance N2). Tension was incrementally increased to 0.75 g over 45–60 min. A minimal contraction of 250 mg of each ring to KCI (75–100 mM) and >50% relaxation to nitroprusside (10 μM) was considered acceptable for inclusion in the study. To measure relaxation, rings were initially contracted with thrombinoxane A2 mimetic U-46619 (10–20 nM) before addition of acetylcholine (0.01–10 μM) or sodium nitroprusside (0.01–10 μM). Relaxation was expressed as percent change from the initial contraction. Contractions to serotonin (10 nM to 10 μM) and U-46619 (1–100 nM) were obtained under basal conditions. In response curves were performed in aorta in the presence of an inhibitor /H9262 before addition of acetylcholine (0.01–10 μM) or sodium nitroprusside (0.01–10 μM). Relaxation was expressed as percent change from the initial contraction. Contractions to serotonin (10 nM to 10 μM) and U-46619 (1–100 nM) were obtained under basal conditions. In general, two aortic rings from each animal were used as controls, and two were treated with a rho kinase inhibitor, fasudil (10 μM), or H-1152 (1 μM). In a separate group of animals, concentration-response curves were performed in aorta in the presence of an inhibitor of nitric oxide synthase (NOS), nitro-arginine (L-NA, 10 μM). Western blot analysis. In a separate group of animals, aorta (3–4/ sample) were isolated, flash-frozen in liquid nitrogen, minced, and sonicated in buffer [in mM: 25 sucrose, 50 3-(N-morpholino)propanesulfonic acid, 2 EDTA, 2 EGTA (Complete Protease Inhibitor; Roche Molecular Biochemicals), 50 NaF, 20 sodium pyrophosphate, 1 p-nitrophenyl phosphate, and 1 μM Microctin LR, pH 7.4]. The homogenate was centrifuged (14,000 g, 15 min, 4°C), and protein concentrations in the supernatant were determined by the bichinchoninic acid method. Equal amounts of protein were separated by SDSPAGE. After blocking, immunoblotting was performed using anti-rhoA (1:100; Santa Cruz), anti-ROCK1 and -ROCK2 (1:500; BD Biosciences), anti-ezrin-radixin-moesin (ERM)s and phosphorylated ERM s (PERMs; 1:500; Chemicon), and α-actin (1:500; Sigma-Aldrich) followed by secondary antibodies conjugated with horseradish peroxidase. The expression of PERMs to total ERMs was determined as an index of activated rho kinase (22). Immunoreactivity was visualized with enhanced chemiluminescence. Blots were digitized and normalized to actin for comparison (NIH Image).

RhoA activation assay. RhoA activation was measured using a modified enzyme-linked immunosorbent assay (G-LISA; Cytoskeleton). Aorta samples (2–3/sample) from male and female nondiabetic and diabetic mice were flash-frozen, homogenized in lysis buffer (with 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM P-nitrophe- nyl phosphate, and 1 μM Microctin LR; Cytoskeleton), and centrifuged at 14,000 g for 5 min at 4°C. Activated GTP-bound rho was measured as absorbance at 490 nm.

Materials. All chemicals were purchased from Sigma-Aldrich except fasudil (Tocris Bioscience), H-1152 (Alexis Chemical), and U-46619 (Bionol International).

Statistical analysis. Data are presented as means ± SE. Responses of multiple rings from a given animal treated similarly were averaged, and “n” represents numbers of mice per group. Concentration-response curves were compared by repeated-measures or a two-way analysis of variance followed by the Student-Newman-Kuel’s test. All n values for Western immunoblots and activation assays indicate the number of pooled samples. Data from immunoblots and rhoA activation were compared with ANOVA followed by Student-NewmanKuel’s test. Significance was defined as P < 0.05.

RESULTS

Development of type 2 diabetes in males vs. females. Table 1 lists the weight and blood glucose of nondiabetic and diabetic male and female mice used in all studies. Nondiabetic female mice weighed less, but blood glucose was similar to age-matched nondiabetic males (Table 1). Diabetic male and female mice were similar in weight and glucose levels.

Previous studies have reported similar levels of insulin in males and females of this model of type 2 diabetes (27). Thus, in this model, male and female mice developed similar levels of type 2 diabetes.

NO-mediated vasorelaxation in diabetes. Numerous studies have demonstrated impaired endothelial-dependent, NO-mediated vascular function in models of type 2 diabetes. We first compared NO-mediated responses to acetylcholine and nitroprusside in aortic rings from nondiabetic males and females. Acetylcholine (Fig. 1A) and nitroprusside (Fig. 1B) produced concentration-dependent relaxation of aorta that was similar in nondiabetic males (n = 8) and females (n = 6). Relaxation to acetylcholine was attenuated ~50% in diabetic males (n = 8; Fig. 1A) and females (n = 7; Fig. 1B), but relaxation to nitroprusside was only reduced in diabetic males (Fig. 1C) and not females (Fig. 1D). The impairment in the relaxation to nitroprusside was much less than the impairment in relaxation to acetylcholine in arteries from males. There were no differences in the EC50 values for acetylcholine or nitroprusside between any of the groups (data not included). Thus both endothelium-dependent and endothelium-independent NO-mediated relaxation was impaired in aorta from diabetic males, but only endothelium-dependent relaxation was impaired in aorta from diabetic females. These results suggest a modest impairment in vascular smooth muscle reactivity to NO-mediated vasodilators in males compared with females.

NO-mediated responses in aorta of nondiabetic mice were attenuated by rho kinase. To determine whether activation of rho kinase modulates NO-mediated vascular responses, we compared responses to acetylcholine and nitroprusside following inhibition of rho kinase with H-1152 in aorta. In nondiabetic mice, inhibition of rho kinase increased relaxation to both acetylcholine (Fig. 1A) and nitroprusside (Fig. 1B) in aorta from males but had no effect on the responses in aorta from females. There were no significant differences in EC50 values between any of the groups (data not included). In diabetic mice, H-1152 had no effect on relaxation to acetylcholine or nitroprusside in aorta from either males or females.

Because inhibition of rho kinase only affected relaxation of aorta from males, we compared responses to acetylcholine and nitroprusside before and after inhibition of rho kinase in the presence of L-NA in aorta from a separate group non-diabetic and diabetic male mice. Inhibition of NOS decreased relaxation to acetylcholine of aorta from nondiabetic mice (n = 8) to the levels seen in aorta from diabetic mice (n = 7). In the presence of L-NA, inhibition of rho kinase with H-1152 had no effect on relaxation to acetylcholine or nitroprusside in arteries from nondiabetic or diabetic mice (Fig. 2, A and B). There were no significant differences in EC50 values be-

Table 1. Weight and blood glucose of nondiabetic and diabetic male and female mice

<table>
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<th>Weight, g</th>
<th>Glucose, mg/dl</th>
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<tr>
<td>Nondiabetic male</td>
<td>31</td>
<td>28.6±0.5</td>
<td>163±6</td>
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<tr>
<td>Diabetic male</td>
<td>28</td>
<td>47.8±0.9*</td>
<td>480±10*</td>
</tr>
<tr>
<td>Nondiabetic female</td>
<td>17</td>
<td>21.5±0.6</td>
<td>177±9</td>
</tr>
<tr>
<td>Diabetic female</td>
<td>26</td>
<td>48.7±1.7*</td>
<td>465±19*</td>
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Values are means ± SE; n, no. of mice. *P < 0.05 vs. same sex nondiabetic mice and vs. nondiabetic male.
between any of the groups (data not included). Thus rho kinase has a modest effect to reduce the relaxation to both acetylcholine and nitroprusside in aorta from nondiabetic mice. However, when the endogenous or exogenous levels of NO are reduced, rho kinase activation does not play a role in the response to either agent.

Contractile dysfunction in aorta of diabetic mice. To determine whether impairment in vascular muscle function of type 2 diabetic subjects is sex dependent, we compared contractions in response to serotonin in aorta from nondiabetic and diabetic males and females. Serotonin produced similar concentration-dependent contractions of aorta from nondiabetic males (Fig. 3, A and C) and females (Fig. 3, B and D). Contractions in response to serotonin were augmented similarly in aorta from male and female diabetic compared with nondiabetic mice. Only the EC50 of serotonin in aorta from males was significantly shifted by diabetes (log EC50 nondiabetic males $\pm 6.32 \pm 0.07$; diabetic males $\pm 6.67 \pm 0.12$; $P < 0.05$). Thus contractions to serotonin were increased in aorta from male and female mice with type 2 diabetes.

Fig. 1. Relaxation in nondiabetic and diabetic males (A and B) and females (C and D) to acetylcholine and nitroprusside. Relaxation to acetylcholine and nitroprusside was similar in nondiabetic males and females. Inhibition of rho kinase with H-1152 increased relaxation to both acetylcholine and nitroprusside in nondiabetic males ($*P < 0.05$ vs. without H-1152). Relaxation in response to acetylcholine was impaired in both male and female mice with diabetes, but relaxation in response to nitroprusside was impaired only in males ($†P < 0.05$ nondiabetic vs. diabetic males).

Fig. 2. Relaxation in nondiabetic and diabetic males to acetylcholine (A) and nitroprusside (B) following inhibition of nitric oxide synthase with nitro-L-arginine (L-NA, 10 $\mu$M). L-NA inhibited relaxation to acetylcholine of arteries from nondiabetic mice but had no effect on the response of diabetic mice. In the presence of L-NA, inhibition of rho kinase had no effect on the response to either acetylcholine or nitroprusside.
NO-rho kinase interactions in contractile dysfunction in vasculature in diabetes. To assess the role of rho kinase in vasoconstriction to serotonin in nondiabetic and diabetic mice, responses were measured in the presence of two inhibitors of rho kinase, H-1152 and fasudil, in separate groups. Contractions of aorta in response to serotonin were inhibited significantly by both H-1152 (Fig. 3, A and B) and fasudil (Fig. 3, C and D) in nondiabetic male (left) and female mice (right). In the presence of H-1152 and fasudil, responses to serotonin were similar between nondiabetic male and female mice. Inhibition of rho kinase also significantly reduced contraction to serotonin in aorta from both male and female diabetic mice.

Because interactions between NO and rho kinase modulated relaxation of aorta from male mice, we tested whether this interaction affected contractile responses in nondiabetic and diabetic males by comparing responses to serotonin in the presence of L-NA. Although inhibition of NOS increased contractions to serotonin ($P < 0.05$), H-1152 produced a similar decrease in the serotonin-induced contractions (1 $\mu$M serotonin with L-NA nondiabetic mice $0.69 \pm 0.11$ g vs. nondiabetic + H-1152 $0.32 \pm 0.07$ g and diabetic $1.09 \pm 0.14$ g vs. diabetic + H-1152 $0.63 \pm 0.11$ g). These data suggest that, although NOS modulates contractions to serotonin, the rho kinase-mediated contribution to the contraction to serotonin is similar in the presence and absence of NO.

To determine if the difference in contractile function in type 2 diabetes is a generalized phenomenon or specific for serotonin, we compared responses of aorta from male and female nondiabetic and diabetic mice with the thromboxane A$_2$ mimic U-46619. Similar to serotonin, contractions to U-46619 are also mediated through activation of G protein-coupled receptors (17). U-46619 produced similar concentration-dependent contractions of aorta from nondiabetic males and females that were not different (Fig. 4, A and B). Contractions to U-46619 were reduced by H-1152 in aorta from nondiabetic males and females, but comparisons at individual concentrations did not reach statistical significance in females. In contrast to serotonin, contractions to U-46619 were not affected by diabetes in aorta from either males or females.

Activation of rho kinase is greater in aorta from diabetic males. To measure rho kinase activity, we compared levels of PERMs to total ERMs in aorta from nondiabetic and diabetic males and females. ERMs are selective substrates for rho kinase with minimal phosphorylation by PKC (22). The level of PERM/ERM was similar in aorta from nondiabetic males ($n = 7$) and females ($n = 6$; Fig. 5). In aorta from diabetic male mice, the level of PERM/ERM was increased significantly ($n = 7$; Fig. 5). In contrast, there was no change in the level of PERM/ERM in aorta from diabetic female mice ($n = 7$; Fig. 5).

To determine whether the increase in rho kinase activity was due to an increase in the expression of rho kinase, expression of ROCK1 and ROCK2 was compared in nondiabetic and diabetic males and females. There was no change in the expression of ROCK1 or ROCK2 in diabetic males or females compared with nondiabetic mice ($n = 7$ each; Fig. 6). Thus rho
kinase activation was increased in aorta from diabetic male mice compared with nondiabetic mice, in agreement with the H-1152-inhibited contraction in aortic rings. However, the increased rho kinase activation in aorta of male diabetic mice was not due to an increase in expression levels.

**Activation of rhoA by serotonin is greater in aorta from diabetic males.** Serotonin activates the small GTPase rhoA and its effector rho kinase through a G protein-coupled receptor to regulate calcium sensitivity of contractile proteins and muscle contraction (14, 28). 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 (36, 45). To determine whether a diabetic-induced increase in rhoA activation accounts for the increase in rho kinase activity, we measured rhoA activation in aorta from nondiabetic and diabetic males and females using a modified ELISA. Diabetes significantly increased rhoA activity in aorta from male mice (n = 6) but had no effect on rhoA activity in diabetic female mice (n = 4; Fig. 7A).

It is possible that the increase in rhoA activity in aorta from diabetic males is related to an increase in the expression levels of rhoA in males compared with females. In nondiabetic males, the expression of rhoA was not different from nondiabetic females (n = 7 each; Fig. 7, B and C). In addition, the expression of rhoA in diabetic males (n = 7) was not increased compared with nondiabetic males. Although expression of rhoA in diabetic females (n = 7) was not different from nondiabetic females, the expression levels of rhoA in diabetic females were significantly less than in diabetic males (Fig. 7, B and C). Thus both rhoA and rho kinase activation is greater in aorta from diabetic compared with nondiabetic male mice. In contrast, diabetes did not alter the activity of either rhoA or rho kinase in aorta from females.

**DISCUSSION**

There are several novel findings in this study. First, interactions between NO and rho kinase modulate relaxation of aorta from male but not female nondiabetic mice. This interaction is abolished when NO generation is inhibited or in the presence of diabetes when NO-mediated relaxation is reduced. Second, impairment in vascular relaxation was greater in diabetic males where there was a slight but significant decrease in the response to nitroprusside in addition to a decrease in the relaxation to acetylcholine. Third, contractile responses to serotonin were augmented to a similar degree in aorta from male and female mice with diabetes, and inhibition of rho kinase reduced these contractions. Fourth, activation of rhoA and rho kinase was greater in aorta from male mice with diabetes compared with those without diabetes. The increase in rhoA and rho kinase activation was not due to an increase in protein expression, since there were no differences in the expression of rhoA or either isoform of rho kinase (ROCK1 or ROCK2) in nondiabetic and diabetic males. Fifth, in contrast to males, neither rhoA nor rho kinase activation was increased in diabetic females compared with nondiabetic females. Thus, despite
similar increases in contractions in response to serotonin and reductions in contractile responses by two inhibitors of rho kinase, activity of rhoA and rho kinase was only increased in arteries of males. These data suggest that other mechanisms contribute to the vascular dysfunction in vessels from female mice with diabetes to account for the increased contractile responses.

Sex differences in NOS and rho kinase interactions modulate vascular reactivity. In the present study, there were no differences in relaxation to acetylcholine in aorta from nondiabetic males and females similar to previous findings (25, 32, 38). Although basal release of NO is greater in aorta from males compared with females, agonist-induced release was not different (38). These data suggest that, in normal mouse aorta, acetylcholine-induced NO-mediated responses are not sex dependent. Similar to previous studies (6, 34), responses to acetylcholine and nitroprusside were enhanced in arteries from nondiabetic males following inhibition of rho kinase. The effect of inhibitors of rho kinase on responses of aorta from males was absent when NO synthase was inhibited or when responses to NO-mediated vasodilators were reduced, as in diabetes. The NO-rho kinase interaction mediating the relaxation responses did not occur in arteries from either nondiabetic or diabetic females.

Several potential mechanisms may account for the increased NO-mediated dilation following inhibition of rho kinase of arteries from males. Rho kinase reduces NO bioavailability by decreasing activity of eNOS following phosphorylation (47), decreasing endothelial NOS (eNOS) expression (1) and decreasing levels of l-arginine, the substrate for eNOS (37). RhoA

Fig. 6. A: representative immunoblots of rho kinase (ROCK) 1, ROCK2, and actin in aorta from nondiabetic and diabetic males and females. The levels of rho kinase (normalized to actin) were not different in aorta from nondiabetic and diabetic males vs. females. B: ROCK1. C: ROCK2.

Fig. 7. A: RhoA activation (modified enzyme-linked immunosorbent assay) was greater in aorta from diabetic compared with nondiabetic males (n = 6 each, *P < 0.05, nondiabetic vs. diabetic), but there was no change in females (n = 4 each). OD, optical density. B: representative immunoblot of rhoA and actin expression in aorta from nondiabetic and diabetic males. C: RhoA expression (normalized to actin) was reduced in diabetic females compared with diabetic males (n = 7 each, †P < 0.05, diabetic males vs. diabetic females) but was not different in aorta from nondiabetic and diabetic males.
activation and eNOS expression and activation were inversely related in cultured human endothelium (26) and COS-7 cells (47), whereas in rat coronary microvascular endothelium there were no interactions between activation and expression of NO and rho kinase (48). Findings in rat aortic smooth muscle cells add to the complexity where increased levels of NO increased rhoA protein expression (43) but decreased its activation (42). Phosphorylation of rhoA by NO-induced cGMP-dependent protein kinase increased rhoA binding with guanine nucleotide dissociation inhibitor even in its GTP-bound state, leading to termination of rhoA activation (42). Contrast results have also been reported in studies of vascular reactivity. Although several studies suggest that NO-induced relaxation is, in part, attenuated by a rho kinase-mediated mechanism (2, 6), rho kinase-mediated contractions to phenylephrine were not affected by removal of endothelium, deficiency of eNOS, or inhibition of NOS and guanylyl cyclase (4, 29). It is unlikely that the acute administration of a rho kinase inhibitor as in the present study affected eNOS expression or the levels of L-arginine but rather activation of eNOS. The results with the endothelium-independent NO-mediated response to nitroprusside suggest that the interaction between NO and rho kinase occurred within vascular muscle and not endothelium. Marked increases in NO release and activation of guanylyl cyclase following upregulation of inducible NOS as in septic shock enhance agonist-induced NO-mediated relaxation and inhibit rho kinase-mediated contractile function of rat mesenteric arteries (9). The increased relaxation and impairment in contractile function in this model of septic shock occurred despite an increase in both rhoA and rho kinase expression. Inhibition of guanylyl cyclase completely restored the vascular responses and rho kinase activity, suggesting that guanylyl cyclase can suppress rho kinase-dependent function. We cannot rule out the possibility that the interaction between NO-guanylyl cyclase and rho kinase represents a physiological antagonism rather than a biochemical one. Alterations in the cross talk between these two opposing signaling pathways within endothelium or smooth muscle may determine the magnitude of vascular responses and contribute to the development of vascular dysfunction in diabetes.

In a genetic model of type 2 diabetes, we observed similar impairment in relaxation to acetylcholine in vessels from males and females. Numerous studies suggest that increases in reactive oxygen species reduce the bioavailability of NO in diabetics, contributing to reductions in NO-mediated relaxation (8, 10, 35). There was a modest, but significant, impairment in relaxation to nitroprusside in arteries from diabetic males. Impaired relaxation in response to exogenous NO or NO donors is not universally observed in diabetes but has been reported (8, 11, 24, 40). The impairment in smooth muscle function in arteries from diabetic males occurred at a time when smooth muscle responses to nitroprusside were normal in females. Although an upregulation of rho kinase within endothelium of mice with diabetes could result in a decrease in endothelium-dependent NO-mediated responses, such an effect cannot explain the diminished response of vascular muscle to exogenously administered NO. The reduction in relaxation to nitroprusside was modest compared with acetylcholine, but the change suggests an alteration in vascular muscle function in type 2 diabetic males. We conclude that there is no sex-dependent difference in the release or response to NO in aorta from nondiabetic mice, but, in the presence of type 2 diabetes, there is a modest impairment in vascular muscle response to NO that is dependent on sex.

Sex differences in rho kinase-mediated contractions in nondiabetic mice. We compared the role of rhoA and rho kinase in males and females since sex differences in vascular reactivity involving the rho/rho kinase pathway have been described (7, 16, 32). In cultured vascular muscle, high levels of estrogen (up to 100 μM) decreased mRNA for ROCK2 (16). Inhibition of rho kinase in vivo had a greater effect on resting diameter in the cerebral circulation of male compared with female rats, suggesting a greater rho kinase activity under basal conditions in males (7). This difference in rho kinase activity could not be explained by a sex-dependent difference in expression of rhoA or rho kinase (7). Agonist-induced activation of rhoA and rho kinase was not compared in that study. In our own study, expression of rhoA and rho kinase was also similar in aorta from males and females, but serotonin-induced activation of both rhoA and rho kinase assessed both biochemically and functionally was greater in males (32). Because differences in protein expression could not account for the greater rho and rho kinase activity in nondiabetic males, mechanisms regulating rhoA activation may differ. Factors regulating rhoA activation, including rho GTPase regulatory proteins (guanine dissociation inhibitors, guanine exchange factors, and GTPase-activating proteins) which control the balance between active and inactive rhoA and translocation of rhoA to the plasma membrane, may be involved. Preliminary data from our laboratory suggest that expression of guanine dissociation inhibitor is not different in males compared with females, but the role of other rho GTPase regulatory proteins and translocation of rhoA to the plasma membrane have not been ruled out.

In contrast to our previous studies in carotid artery and aorta, we did not observe a sex difference in contractions to serotonin in nondiabetic mice in this study (25, 32). Several factors may account for this difference. First, mice used in the present study have a different genetic background than the mice used in our previous study. In our previous work, we used C57BL/6 mice. Although the mice used in the present study were originally derived from the C57BL/6 background, they are not genetically identical. Several reports have demonstrated variable cardiovascular phenotypes in mice dependent upon genetic background (5, 19, 39), which highlight the importance of choosing appropriate genetic controls for comparisons. It is important to note that the appropriate genetic controls for the leptin receptor-deficient mice were used in these studies. Our results imply that genetic background plays an important role in determining sex differences in vascular responses.

Sex differences in rho kinase-mediated contractions in diabetic mice. In the present study, we determined whether the augmented contractile responses in diabetic mice were related to an increase in the expression and/or activation of rhoA and rho kinase in diabetic males and females. A previous study demonstrated an increase in rhoA but not rho kinase expression in diabetic db/db males compared with nondiabetic mice (49). In contrast to previous studies, we did not observe a difference in the expression of rhoA or rho kinase in aorta from diabetic mice compared with nondiabetic males despite increased activation of both. The contrasting results for rhoA expression may be related to a difference in age, since the study of Xie and coworkers (49) used mice 12–14 wk of age and in the present
study the age ranged from 12 to 40 wk. Surprisingly, there was no change in the activity or expression of either rhoA or rho kinase in aorta from diabetic females despite an increase in contractile function that was reduced by rho kinase inhibitors. Although fasudil and H-1152 are widely used as specific inhibitors of rho kinase, it is possible that unidentified nonspecific effects of these agents reduced contractile activity. Numerous studies have demonstrated an increase in PKC activation in males with diabetes (13, 15, 18). Conversely, acute hyperglycemia impaired relaxation to acetylcholine and increased expression of PKC-β2 in aorta from female and not male rats (13). It is possible that an upregulation of PKC makes a greater contribution to contractile dysfunction in diabetic females compared with males.

Clinical implications. Although numerous studies have reported an increased risk of cardiovascular disease in type 2 diabetic women compared with men, the difference is abolished when the rates are adjusted for other risk factors for cardiovascular disease such as age, hypertension, hypercholesterolemia, and smoking. Although the incidence of cardiovascular disease may vary with sex in the absence of diabetes, in the setting of type 2 diabetes, female sex no longer provides protection. However, sex may impact the mechanisms involved in the contractile dysfunction and may be an important variable in establishing therapeutic approaches for treatment to prevent or reverse the vascular dysfunction in patients with type 2 diabetes.

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