Enhanced p22phox expression impairs vascular function through p38 and ERK1/2 MAP kinase-dependent mechanisms in type 2 diabetic mice

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1Department of Physiology, Hypertension and Renal Center of Excellence, Tulane University, New Orleans, Louisiana; 2Department of Physiology, College of Medicine, Yonsei University, Seoul, Korea; 3College of Nanoscale Science and Engineering, University at Albany, Albany, New York; and 4Department of Physiological Sciences, Eastern Virginia School of Medicine, Norfolk, Virginia

Submitted 4 November 2013; accepted in final form 6 December 2013

Kassan M, Choi S, Galán M, Lee Y, Trebak M, Matrougui K. Enhanced p22phox expression impairs vascular function through p38 and ERK1/2 MAP kinase-dependent mechanisms in type 2 diabetic mice. Am J Physiol Heart Circ Physiol 306: H972–H980, 2014. First published January 31, 2014; doi:10.1152/ajpheart.00872.2013.—Type 2 diabetes is associated with vascular complication. We hypothesized that increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit p22phox expression impairs vascular endothelial-dependent relaxation (EDR) in type 2 diabetes. Type 2 diabetic (db/db) and control (db/db+) mice were treated with reactive oxygen species (ROS) scavenger, polyethylene glycol superoxide dismutase (1,000 U/kg daily ip), or small interfering RNA p22phox (p22phox-lentivirus-small interfering RNA, 100 μg iv, 2 times/wk) for 1 mo. EDR was impaired in microvascular bed (coronary arteriole and femoral and mesenteric resistance arteries) from diabetic mice compared with control. Interestingly, ROS scavenger and p22phox downregulation did not affect blood glucose level or body weight but significantly improved EDR. Mitogen-activated protein kinases (ERK1/2 and p38) phosphorylation and NADPH oxidase activity were increased in arteries from diabetic mice and were reduced after ROS scavenger or p22phox downregulation in db/db− mice. The present study showed that enhanced p22phox expression causes vascular dysfunction through ERK1/2 and p38-mitogen-activated protein kinase-dependent mechanisms in male type 2 diabetic mice. Therefore, p22phox could be an important target to improve vascular function in diabetes.

type 2 diabetes; p22phox; endothelium-dependent relaxation; NADPH oxidase subunit p22phox

SUBSTANTIAL CLINICAL AND EXPERIMENTAL evidence suggest that vascular diseases are currently the principal causes of morbidity and mortality in patients with type 1 and type 2 diabetes mellitus (6). In patients with type 2 diabetes, insulin resistance as well as endothelial dysfunction appears to precede the development of hyperglycemia (38, 44, 45). Therefore, endothelial dysfunction may be a critical early target for preventing cardiovascular disease.

Endothelial dysfunction in diabetes is generally attributed to hyperglycemia through oxidative stress. Endothelial dysfunction and increased reactive oxygen species (ROS) levels are believed to be an underlying cause for vascular dysfunction and coronary artery disease in diabetes (34, 36). Several studies showed that nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, one of the most prominent sources of ROS in the endothelium, is increased in the diabetic vessels (35). NADPH oxidase is a multicomponent enzyme consisting of gp91phox, p22phox, p47phox, p67phox, and Rac-1 subunits. Recent studies have shown that subunit p22phox of NADPH oxidase is upregulated in the vasculature in type 2 diabetes (23). Additionally, there is evidence from both preclinical and clinical studies that p22phox is increased in patients with diabetes, indicating a role for NADPH-induced oxidative stress in the compromised cardiovascular system (1, 5, 7, 20, 40). Therefore, p22phox upregulation could be a potential target for cardiovascular diseases in diabetes.

Additionally, it has been reported that mitogen-activated protein kinases (MAP kinase; p38 kinase and ERK1/2) are increased in response to hyperglycemia and oxidative stress and are involved in cardiovascular complication in diabetes (43, 46, 37, 50), which highlight the need to delineate the link between NADPH oxidase and MAP kinases in the regulation of vascular function in diabetes.

In the present study, we tested whether p22phox plays a role in impaired microvascular endothelium-dependent relaxation (EDR) through MAP kinases (ERK1/2 and p38) in type 2 diabetic mice.

MATERIALS AND METHODS

General protocol in mice. All experiments were performed according to the “American Guidelines for the Ethical Care of Animals” and were approved by Tulane University Animal Care and Use Committee. Type 2 diabetic male mice (db/db, 8- to 10-wk-old males) and their homologous control (db/db+) were purchased from Jackson Laboratories (Bar Harbor, ME), housed in groups of five mice, maintained at a temperature of 23°C with 12-h:12-h light-dark cycle and fed a solid standard diet (Na+ content 0.4%) and water.

Mice were divided into 8 groups: 1) control mice infused with saline, (n = 10), 2) control mice treated with p22phox small interfering RNA (siRNA) (100 μg iv, 2 times/wk) for 1 mo (control + p22phox siRNA n = 10) as previously described (31), 3) control mice treated with scrambled p22phox (Scr p22phox) siRNA (100 μg iv, 2 times/wk) for 1 mo (control + Scr p22phox siRNA n = 10), 4) control mice treated with polyethylene glycol superoxide dismutase (PEG-SOD, 1,000 U/kg daily ip) for 1 mo (control + PEG-SOD, n = 10), 5) diabetic mice (db/db− mice infused with saline, n = 10), 6) diabetic mice treated with p22phox siRNA for 1 mo (db/db− + P22phox siRNA, n = 10), 7) diabetic mice treated with Scr p22phox siRNA for 1 mo (db/db− + Scr p22phox siRNA n = 10), and 8) diabetic mice treated with PEG-SOD for 1 mo (db/db− + PEG-SOD, n = 10).

Body weight and blood glucose levels were recorded weekly during the experimental period. Blood glucose measurements were obtained from tail blood samples using a blood glucose meter (Prestige Smart
System HDI; Home Diagnostic, Fort Lauderdale, FL) in all groups of mice after 6 h fast as previously described (13).

At the end of the treatment period, mice were anesthetized with isoflurane. Tissues (coronary arteries, MRAs, and femoral arteries) were harvested immediately, placed in physiological salt solution (PSS) consisting of (in mM) 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 7H₂O, 25 NaHCO₃, and 11 glucose 11 (pH 7.4) and processed appropriately for further studies.

Vascular reactivity: *ex vivo experiments*. The left anterior descending coronary arteries were isolated, cannulated with glass micropipettes, and perfused with PSS bubbled with a 95% O₂-5% CO₂ gas mixture. The vessels were pressurized to 50 mmHg using pressure-servo control perfusion system (Living Systems Instruments, St. Albans, VT) for a 40-min equilibration period. The vessel diameter was monitored by a video image analyzer as previously described (9, 12).

Intraluminal pressure was increased from 25 to 100 mmHg in a stepwise manner to assess myogenic tone. At the end of the experiments, vessels were incubated with a calcium-free PSS to determine passive diameter. Myogenic tone was calculated as the percentage between active and passive diameter. To determine the EDR, pressurized arteries were precontracted with thromboxane agonist (U-46619, 10⁻⁷ M) and cumulative concentrations (10⁻⁸–10⁻⁴ M) of acetylcholine were assessed. The nitric oxide synthase inhibitor N²-nitro-l-arginine methyl ester (L-NAME; 10⁻⁴ M) was added in the organ bath for 30 min before the assessment of the role of nitric oxide synthesis.

Isometric tension recording. Mesenteric resistance arteries (MRAs) and femoral arteries from control and *db/db* mice were carefully cleaned of fat and connective tissue and then cut into rings (2 mm in length). MRAs and femoral arteries were mounted in a small vessel, dual-chamber myograph for measurement of isometric tension. After a 30-min equilibration period in PSS solution bubbled with carbogen at 37°C and pH 7.4, arteries were stretched to their optimal lumen diameter for active tension development. After a second 30-min equilibration period, the vessels were exposed to phenylephrine (PE, 10⁻⁵ M), and the presence of functional endothelium was assessed by the ability of acetylcholine (10⁻⁶ M) to induce relaxation. After another wash step, rings of MRAs and femoral arteries were precontracted with PE (10⁻⁵ M), and at the steady maximal contraction, cumulative dose-response curves were obtained for acetylcholine (10⁻⁸–3·10⁻⁵ M).

To determine the role of endothelial nitric oxide synthase (eNOS) in the impaired EDR in *db/db* mice, femoral arteries and MRAs were incubated with L-NAME (100 μM) for 30 min, and then EDR was measured after precontraction with PE.

To further assess the role of ERK1/2 MAP kinase on the contraction response, rings were incubated with U0126 (ERK1/2 inhibitor 1 μM) for 30 min and were then contracted with PE for 20 min.

Western blot analysis. Freshly isolated hearts and MRAs from all groups were immediately frozen in liquid nitrogen and then homogenized in ice-cold lysis buffer as previously described (23). Western blot analysis was performed for phosphorylated and total eNOS (1:1,000 dilution; Cell Signaling, Boston, MA), phosphorylated and total ERK1/2 (1:1,000 dilution; Cell Signaling), phosphorylated and total p38 (1:1,000 dilution; Cell Signaling), and p22phox (1:500 dilution, Santa Cruz) using specific antibodies. Blots were stripped and then reprobed with β-actin (1:2,000 dilution, Santa Cruz). NADPH oxidase activity assay. Supernatant anion levels generated by NADPH oxidase were measured in lysates of heart, femoral arteries, and MRAs using lucigenin chemiluminescence. Briefly, lysates were prepared in a sucrose buffer containing (in mM) 50 KH₂PO₄, 1 EGTA, and 150 sucrose (pH 7.0) with protease inhibitor cocktail (Complete Mini, Roche Diagnostics) in a Tissue Dounce homogenizer on ice, and aliquots of the homogenates were used immediately. To start the assay, a volume of 100 μl of each lysate was used in a total volume of 1 ml PBS buffer preheated at 37°C, containing 5 μM lucigenin and 100 μM NADPH. Blank samples were prepared using 100 μl of sucrose buffer. Lucigenin activity was measured every 30 s for 10 min in a luminometer (Turner BioSystem 20/20, single tube luminometer) until enzymatic activity reached the plateau. Data are expressed as area under the curve of relative light units normalized to protein content (in μg protein).

Fluorescence in situ hybridization RNA. The fluorescence in situ hybridization (FISH) analysis target was a 476-bp DNA fragment containing bases from 2821 to 3299 of mouse eNOS included in National Center for Biotechnology Information reference sequence NM_008713.4, which was generated by amplification using as template the reverse-transcribed cDNA from total RNA extracted from heart tissue and the oligonucleotides: eNOS forward, 5'-gccgctgattcactgtc-3', and eNOS reverse, 5'-ctgctcattggactgtc-3'. The 476-bp amplicon was purified from agarose gel and cloned into pGEM-T vector (Promega, Madison, WI) and verified subsequently by sequencing using the T7 primer. Two clones of p-GEM-T-eNOS (476 bp) construct containing the insert in opposite directions were picked to synthesize the antisense probe for eNOS mRNA detection and the sense probe as a negative control for hybridization assay. 

Tab probes were synthesized and labeled with Alexa Fluor 594 dye using the FISH Tag RNA kit (Molecular Probes, Invitrogen). The RNA probes synthesis and labeling with fluorescent dye, hybridization, and detection of fluorescent signals were performed as described by the kit protocol and following the manufacturer’s instructions. eNOS mRNA was localized in frozen transverse sections from mesenteric and femoral arteries optimum cutting temperature (Tissue-Tek OCT, Sakura), embedded, cut into 10-mm-thick sections, and placed on a glass slide. The fluorescence intensity was quantified in captured images, taken with a fluorescent microscope (Nikon, Tokyo, Japan) with ×40 magnification, using the software NIS-Elements BR 3.0 (Nikon, Tokyo, Japan).

Drugs. PE hydrochloride, acetylcholine, and L-NAME were obtained from Sigma-Aldrich (St. Louis, MO). U0126 was obtained from LC Laboratories (Boston, MA). Thromboxane analog (U46619) was purchased from Calbiochem (USA and Canada).

Statistical analysis. Data are expressed as means ± SE. To compare concentration-response curves, statistical analysis was performed with the GraphPad Prism 4.03 software according to the extra sum of squares F-test principle. Other comparisons among groups were made using variance (2-way ANOVA) analysis followed by the post hoc Bonferroni’s test. Statistically significant values were considered when P < 0.05.

RESULTS

*General parameters*. Blood glucose levels and body weight were elevated in *db/db* mice (448 ± 9.75 mg/dl and 40.81 ±

### Table 1. Blood glucose and body weight measurements

<table>
<thead>
<tr>
<th>Mice</th>
<th>Body weight, g</th>
<th>Blood glucose, mg/dl</th>
<th>Systolic blood pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.59 ± 0.52*</td>
<td>155.81 ± 7.87*</td>
<td>101.14 ± 1.25</td>
</tr>
<tr>
<td>Control + p22phox siRNA</td>
<td>24.13 ± 0.23*</td>
<td>141.74 ± 4.42*</td>
<td>98.17 ± 2.16</td>
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<tr>
<td>Control + Scr p22phox siRNA</td>
<td>25.22 ± 0.13*</td>
<td>136.17 ± 5.05*</td>
<td>101.11 ± 3.81</td>
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<tr>
<td>Control + PEG-SOD</td>
<td>25.02 ± 0.11*</td>
<td>132.17 ± 3.17*</td>
<td>96.22 ± 0.81</td>
</tr>
<tr>
<td>db/db</td>
<td>40.81 ± 0.36</td>
<td>448.10 ± 9.75</td>
<td>107.12 ± 1.08</td>
</tr>
<tr>
<td>db/db + p22phox siRNA</td>
<td>41.22 ± 0.17</td>
<td>514.01 ± 19.72</td>
<td>105.12 ± 4.12</td>
</tr>
<tr>
<td>db/db + Scr p22phox siRNA</td>
<td>44.07 ± 0.13</td>
<td>498.72 ± 22.19</td>
<td>100.22 ± 7.31</td>
</tr>
<tr>
<td>db/db + PEG-SOD</td>
<td>42.66 ± 1.09</td>
<td>558.61 ± 14.44</td>
<td>98.76 ± 2.65</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 for control treated with polyethylene glycol superoxide dismutase (PEG-SOD) or p22phox small interfering RNA (siRNA) or scambled (Scr) p22phox siRNA vs. type 2 diabetic mice (db/db) treated with PEG-SOD or p22phox siRNA or Scr p22phox siRNA.
0.36 g, respectively) compared with control mice (155.8 ± 7.87 mg/dl and 25.59 ± 0.52 g, respectively) and were not affected by ROS scavenger or after p22phox downregulation (Table 1). Systolic blood pressure was similar in all groups.

p22phox and coronary artery reactivity. Myogenic tone was significantly increased in the coronary arteries from db−/db− mice compared with control mice and was normalized after treatment of ROS scavenger or p22phox downregulation (Fig. 1A). The EDR was also impaired in coronary arteries from db−/db− mice and was rescued after treatment of ROS scavenger or p22phox downregulation (Fig. 1A). To study the effect of p22phox downregulation on MAP kinases (ERK1/2 and p38) signaling, we performed Western blot analysis in coronary arteries from all groups. Our results indicate that phosphorylated and total ERK1/2 and p38-MAPK were increased in db−/db− mice compared with control and db−/+db− mice treated with p22phox siRNA or PEG-SOD (Fig. 1, F and G).

p22phox and EDR in MRA and resistance femoral artery. Our data demonstrated that EDR is impaired in MRAs and resistance femoral arteries in db−/db− mice, and the downregulation of p22phox by p22phox-siRNA or the use of ROS scavenger (PEG-SOD) significantly improved the EDR (Figs. 2A and 3A).

The inhibition of eNOS with L-NAME reduced EDR in control, db−/+db− and db−/db− mice treated with p22phox siRNA or PEG-SOD (Figs. 2B and 3B). Western blot analysis

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**Fig. 1.** Effect of polyethylene glycol superoxide dismutase (PEG-SOD) or p22phox small interfering RNA (siRNA) on myogenic tone and endothelium-dependent relaxation (EDR) in coronary arteries (n = 8). A: pressure-induced myogenic response in coronary arteries from control, type 2 diabetic mice (db−/+db−) treated with or without PEG-SOD or p22phox siRNA. B: EDR in response to cumulative doses of ACh (10⁻⁸ to 10⁻⁴ M) in coronary arteries from control, type 2 diabetic mice (db−/+db−) treated with or without PEG-SOD or p22phox siRNA. C: EDR in response to cumulative doses of ACh (10⁻⁸ to 10⁻⁴ M) in coronary arteries incubated with N⁵-nitro-L-arginine methyl ester [L-NAME; endothelial nitric oxide synthase (eNOS) inhibitor] from control, type 2 diabetic mice (db−/+db−) treated with or without PEG-SOD or p22phox siRNA. *P < 0.05 for db−/+db− vs. control, db−/+db− treated with PEG-SOD or p22phox siRNA. Western blot analysis for p22phox expression (D), phosphorylated (P)-eNOS and total (T)-eNOS (E), P-ERK1/2 and T-ERK1/2 (F), P-p38 and T-p38 (G) in coronary arteries from control, type 2 diabetic mice (db−/+db−) treated with or without PEG-SOD or p22phox siRNA (n = 4) is shown. *P < 0.05 for db−/+db− vs. control, db−/+db− treated with PEG-SOD or p22phox siRNA.
revealed that p22phox expression was elevated in db/db− mice compared with control and db−/db− mice treated with p22phox-siRNA and PEG-SOD (Fig. 2C). Additionally, in MRA from db/db− mice, eNOS activity was significantly reduced, whereas total and phosphorylated ERK1/2 and p38-MAPK were significantly augmented (Fig. 2, D–F). Interestingly, PEG-SOD or the downregulation of p22phox (p22phox-siRNA) increased eNOS activity and reduced p22phox expression, total and phosphorylated ERK1/2 and p38-MAPK (Fig. 2, C–F) in db/db− mice.

To further elucidate the mechanism of vascular endothelial dysfunction in diabetic mice linked to p22phox, we studied the role of the MAP kinase (ERK1/2) pathways. The inhibition of ERK1/2 by U0126 significantly decreased the contraction to PE in all groups. Interestingly, the decrease in the contraction was higher in db/db− mice than in control and db−/db− mice treated with p22phox-siRNA or PEG-SOD (Figs. 2G and 3C).

FISH RNA. eNOS mRNA was detected by FISH analysis in mesenteric and femoral arteries rings. Our data indicate that eNOS gene expression was reduced in db/db− mice compared with control mice. The downregulation of p22phox with p22phox-siRNA or the treatment of mice with PEG-SOD partially restored eNOS gene expression (Fig. 4).

NADPH oxidase activity in heart, MRAs, and femoral arteries. Our data demonstrated that NADPH oxidase activity was increased in diabetic group in all three vascular beds (Fig. 5, A–C). Interestingly, PEG-SOD or the downregulation of p22phox significantly reduced NADPH oxidase activity in coronary arteries, MRAs, and resistance femoral arteries (Fig. 5, A–C).

As control for all experiments using siRNA, the injection of scramble siRNA did not have any effect on the parameters we measured (Table 1 and Fig. 6, A–C).
DISCUSSION

In the present study, we determined that the upregulation of p22phox expression causes vascular endothelial dysfunction in type 2 diabetic mice. This observation came from our experiments where impaired EDR and augmented myogenic tone of resistance femoral artery, MRA, and coronary arteriole were rescued in type 2 diabetic mice treated with or without PEG-SOD or p22phox siRNA. Furthermore, we demonstrate that in type 2 diabetic mice (db/db) treated with or without PEG-SOD or p22phox siRNA. *P < 0.05 for db/db vs. db/db treated with PEG-SOD or p22phox siRNA; #P < 0.05 for db/db vs. control. C: key representative traces showing contraction curves to PE in control, type 2 diabetic mice (db/db) treated with or without PEG-SOD or p22phox siRNA before and after 20-min incubation with ERK inhibitor U0126.

Enhanced NADPH oxidase activity contribute to the impaired endothelium-dependent vasodilation in aorta from Otsuka Long Evans Tokushima Fatty rats as a model of type 2 diabetes (24). However, this study did not show the involvement of NADPH oxidase in endothelial dysfunction in resistance arteries. Taking into consideration that differential endothelial function in a conductance and resistance artery is well documented (32), we decided to study the effect of oxidative stress in resistance arteries (coronary arteries, MRAs, and femoral arteries) from type 2 diabetic mice that have been commonly and extensively used for the investigation of type 2 diabetes (43).

Among subtypes of NADPH enzymes, Nox1, Nox2, Nox4, and Nox5 are important sources of ROS in the vasculature, and their regulatory subunits, p22phox, p47phox, Noxa1 and p67phox, are expressed in blood vessels (30). Importantly, the p22phox subunit directly interacts with Nox1, Nox2, and Nox4 proteins in vasculature (4, 49). It also has been reported that p22phox plays a critical role in enhancing the expression of catalytic NOX components of NADPH oxidase. Thus p22phox appears to be the only unique requirement for activity of all Nox enzymes (31).

Myogenic response plays an important role in the local blood flow regulation (41). We and other investigators showed
an increase in myogenic tone in type 2 diabetes (12, 25). In this study we demonstrated that in type 2 diabetic mice, elevated myogenic response is a consequence of enhanced p22phox expression since the downregulation of p22phox by siRNA and ROS scavenger restored the myogenic tone in diabetic mice.

It is well known that vascular reactivity is also regulated by EDR mechanism. Our results indicate that EDR is impaired in three vascular beds (coronary arteriole, MRAs, and femoral resistance arteries) in type 2 diabetic mice. Additionally, we demonstrated that type 2 diabetes is associated with enhanced vascular NADPH oxidase subunit p22phox expression. Our data are in agreement with other studies reporting endothelial dysfunction and overexpression of p22phox protein in coronary arteries from diabetic mice (17). Thus the downregulation of p22phox and the use of ROS scavenger significantly improved EDR in femoral, mesenteric, and coronary artery. Our study is

Fig. 4. Fluorescence in situ hybridization RNA (n = 4). Representative figures for fluorescence in situ hybridization assay showing eNOS mRNA in MRAs and femoral arteries from control and type 2 diabetic mice (db/db) treated with or without PEG-SOD or p22phox siRNA. *P < 0.05 for db/db vs. control, db/db treated with PEG-SOD or p22phox siRNA.

Fig. 5. NADPH oxidase activity in coronary arteries (CA; A), MRAs (B), and femoral arteries (n = 8; C). NADPH oxidase activity quantified by lucigenin chemiluminescence in lysates of coronary arterioles, MRAs, and femoral arteries from control and type 2 diabetic mice (db/db) treated with or without PEG-SOD or p22phox siRNA is shown. RLU, relative light units. *P < 0.05 for db/db vs. control, db/db treated with PEG-SOD or p22phox siRNA.
in agreement with previous reports showing that treatment with a NADPH oxidase inhibitor restores EDR in conductance arteries (27, 29).

It is well known that protein kinases (MAPKs) such as ERK and p38-MAPK are in part implicated in the etiology of diabetes (42). In the present study, we showed augmented phosphorylated-p38-MAPK and phosphorylated-ERK1/2 in vessels from type 2 diabetic mice, suggesting that p38-MAPK and ERK1/2 are important factors in impaired vasodilator responses. Our data are in accordance with a recent study showing an increase in p-38-MAPK and ERK1/2 activities in MRAs from diabetic mice (22). ERK1/2 and p38-MAPK are known as stress-activated kinases and are stimulated by a variety of exogenous and endogenous stress-inducing factors, including hyperglycemia, ROS, and oxidative stress (16). Our results indicate that ERK1/2 activity was reduced after p22phox downregulation and the use of ROS scavenger, which is in accordance with other studies in which activation of ERK1/2 by NADPH-derived ROS has been reported in a variety of cells, including smooth muscle cells (10), endothelial cells (14), fibroblasts (18), kidney mesangial cells (19), and pulmonary mesangial cells (3). Additionally, Rygiel et al. (39) showed that deletion of the Rac GTPase activator impaired Nox function and decreased ERK1/2 phosphorylation.

On the other hand, our data showed that ROS scavenger and downregulation of p22phox resulted in a decrease in activated p38-MAPK. Several studies showed that in vivo chronic inhibition of p38-MAPK downregulates NADPH oxidase expression, attenuates superoxide production, and improves vascular function in a variety of animal models (8, 46, 49). Also, it has been reported that acute inhibition of p38-MAPK suppresses NADPH oxidase in neutrophils (26). Additionally, activated...
p22phox regulates smooth muscle contraction (21). In addition, it has been demonstrated in many studies that different agents that produce contractions of the smooth muscle activate ERK1/2 at the same time (11, 15). However, conflicting results were obtained regarding a role for ERK1/2 in smooth muscle cell contractile regulation. Our data indicate that the ERK1/2 pathway plays a key role in the regulation of femoral and MRA contraction. More importantly, the finding that inhibition of ERK1/2 greatly reduced PE-induced contraction in femoral and MRAs from diabetic versus nondiabetic mice suggests that type 2 diabetes enhances the implication of ERK1/2 in vascular contraction. However, how type 2 diabetes increases ERK1/2 phosphorylation and how it enhances vascular contraction are still unknown. Nevertheless, our results are in accordance with previous studies in which ERK inhibition reduced PE-induced contractions in MRAs from diabetic mice (28). However, these results are still not clear since a previous study showed that ERK1/2 inhibition did not affect PE-induced contraction in aorta from rats (47). These results suggest that the role of ERK1/2 in α1-adrenoceptor-mediated contraction shows considerable heterogeneity depending on vessel types. It is not clear at present whether and to what extent the enhanced ERK1/2 pathway in α1-adrenoceptor-mediated signaling affects the vascular tone of diabetic femoral and MRAs.

It has been shown that nitrosative/nitrative-induced stress is involved in the development of diabetic cardiac and vascular dysfunction (33). Our results demonstrate that oxidative stress regulates vascular dysfunction through the modulation of MAPKs activity. Thus we suggest that nitrosative/nitrative-induced stress could modulate MAPKs activity and therefore vascular dysfunction in type 2 diabetes, which need to be determined.

In summary, the results indicate that augmented p22phox expression causes microvascular dysfunction in type 2 diabetic mice likely through MAP kinases (ERK1/2 and p38). The role of the p22phox-ERK1/2 and p38-MAPK pathway and its mechanisms in the regulation of vascular reactivity in diabetes opens a new window for future studies.

Limitation. One limitation of the present study is that during diabetes, a portion of increased NADPH activity could originate from infiltrating immune cells. Additionally, ROS derived from inflammatory cells may also contribute to vascular inflammation/dysfunction in diabetes. Therefore, the chronic treatment with PEG-SOD or the downregulation of p22phox may indirectly attenuate the vascular dysfunction by decreasing ROS and inflammatory mediator generation in inflammatory cells. This option should firmly be determined.

Another limitation is that eNOS is regulated by multiple sites of phosphorylation (activator site at Ser1177 and inhibitory site at Thr495). The phosphorylation of eNOS at Ser1177 and dephosphorylation at Thr495 activates the enzyme, whereas inhibition results when Thr495 is phosphorylated and Ser1177 is dephosphorylated. However, Ser1177 can be phosphorylated along with other inhibitory residues that prevent the enzyme from being active, and as a consequence it reduces the amount of nitric oxide bioavailability. Recently, other phosphorylation sites of eNOS have been reported, including the stimulatory sites (Ser635 and Ser617) and the inhibitory sites (Thr495 and Ser116). Therefore, it is necessary to associate the eNOS phosphorylation with the EDR.

GRANTS

This work was supported by the National Heart, Lung, and Blood Institute Grants 1R01-HL-095566 (to K. Matrougui, principal investigator) and 5R01-HL-097111 (to M. Trebak, principal investigator).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.K., S.-K.C., and M.G. performed experiments; M.K., S.-K.C., M.G., Y.-H.L., M.T., and K.M. analyzed data; M.K., S.-K.C., M.G., Y.-H.L., M.T., and K.M. interpreted results of experiments; M.K., S.-K.C., and M.G. prepared figures; M.K. and S.-K.C. drafted manuscript; M.T. and K.M. edited and revised manuscript; K.M. conception and design of research; K.M. approved final version of manuscript.

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