

# Altered MAPK Signaling in Progressive Deterioration of Endothelial Function in Diabetic Mice

An Huang,<sup>1</sup> Yang-Ming Yang,<sup>1</sup> Changdong Yan,<sup>2</sup> Gabor Kaley,<sup>1†</sup> Thomas H. Hintze,<sup>1</sup> and Dong Sun<sup>1,2</sup>

We aimed to investigate specific roles of mitogen-activated protein kinases (MAPK) in the deterioration of endothelial function during the progression of diabetes and the potential therapeutic effects of MAPK inhibitors and agonists in the amelioration of endothelial function. Protein expression and phosphorylation of p38, c-Jun NH<sub>2</sub>-terminal kinase (JNK), and extracellular signal-regulated kinase (Erk) were assessed in mesenteric arteries of 3- (3M) and 9-month-old (9M) male diabetic and control mice. The expression of p38, JNK, and Erk was comparable in all groups of mice, but the phosphorylation of p38 and JNK was increased in 3M and further increased in 9M diabetic mice, whereas the phosphorylation of Erk was substantially reduced in 9M diabetic mice. NADPH oxidase-dependent superoxide production was significantly increased in vessels of two ages of diabetic mice. Inhibition of either p38 with SB203580 or JNK with SP600125 reduced superoxide production and improved shear stress-induced dilation (SSID) in 3M, but not in 9M, diabetic mice. Treating the vessels of 9M diabetic mice with resveratrol increased Erk phosphorylation and shear stress-induced endothelial nitric oxide synthase (eNOS) phosphorylation and activity, but resveratrol alone did not improve SSID. Administration of resveratrol and SB203580 or resveratrol and SP600125 together significantly improved SSID in vessels of 9M diabetic mice. The improved response was prevented by U0126, an Erk inhibitor. Thus, p38/JNK-dependent increase in oxidative stress diminished nitric oxide-mediated dilation in vessels of 3M diabetic mice. Oxidative stress and impaired Erk-dependent activation of eNOS exacerbates endothelial dysfunction in the advanced stage of diabetes. *Diabetes* 61:3181–3188, 2012

**D** diabetes is associated with various cardiovascular complications. In particular, the increased oxidative stress, which inactivates NO and hence impairs endothelium-dependent vasodilator responses and induces the dysfunctionality of endothelial progenitor cells (1–3), contributes significantly to the cardiovascular dysfunction in diabetes. We also demonstrated that inhibition of superoxide production improved endothelium-dependent shear stress-induced dilation (SSID) in arteries of young diabetic mice. In aged diabetic mice, however, impaired endothelial nitric oxide (NO) synthase (eNOS) activation prevented the anti-oxidative effect on ameliorating endothelial function (4). Thus, oxidative stress and impaired eNOS activation are two separate but mechanistically connected events,

especially during the cardiovascular complications in late stages of diabetes.

Among the family of mitogen-activated protein kinase (MAPK), p38 kinase (p38) and c-Jun NH<sub>2</sub>-terminal kinase (JNK) are activated in response to hyperglycemia, oxidative stress, and proinflammatory cytokines. Increased activation of p38 and JNK has become a fundamental mechanism responsible for cardiovascular dysfunction in diabetes (5,6). Indeed, inhibition of p38/JNK improved nitric oxide-mediated vasodilatation and reduced inflammation in hypercholesterolemic patients (7) and prevented tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )- and hypercholesterolemia-induced endothelial dysfunction (8,9). On the other hand, extracellular signal-regulated kinase (Erk), another member of the MAPK family, is mainly involved in regulating mitogen-induced cellular growth. Understanding of the specific role of Erk in endothelial dysfunction of diabetes remains incomplete, although some studies have suggested that the activation of Erk is increased in cultured endothelial cells isolated from subcutaneous tissues of type 2 diabetic subjects (10). However, in normal vascular endothelium, fluid shear stress quickly activates Erk-related signaling pathways (11,12), implying that Erk activation involves shear stress-induced regulation of endothelial function. Moreover, insulin and proinsulin C-peptide-induced eNOS activation are linked to the activation of Erk (13,14); and the cardiovascular protective effects of estrogen and estrogen receptor agonists are mediated through Erk-dependent mechanisms (15). Thus, the physiological activation of Erk is important for maintaining cardiovascular homeostasis. Despite the fact that the importance of MAPK in the regulation of vascular function has been described, changes in function of MAPK during the progression of diabetes have not yet been studied in resistance arteries. In particular, based on our previous findings that in addition to an increased oxidative stress, inactivation of eNOS plays a significant role in the endothelial dysfunction of 9-month-old (9M) diabetic mice (4), the question arises as to whether the specific modulation of MAPK activity can ameliorate endothelial function in advanced diabetes. Thus, in the current study, we aimed to assess the causative relationship between the MAPK activity and the endothelial dysfunction in blood vessels of diabetic mice. We hypothesized that an altered vascular MAPK is responsible for the exacerbation of endothelial dysfunction during the progression of diabetes, and therefore, normalizing MAPK activity improves endothelial function. To accomplish this goal, we used 3-month-old (3M) and 9M *Lepr*<sup>db<sup>-/-</sup></sup> mice as models for the early and advanced stages of type 2 diabetes. As observed, *Lepr*<sup>db<sup>-/-</sup></sup> mice develop obesity, hyperglycemia, and hyperinsulinemia after their first month and do not survive longer than 10 months. The heterozygous (*Lepr*<sup>db<sup>+/-</sup></sup>) littermates are lean and have normal plasma insulin and glucose and a normal life span. Therefore, age-matched male *Lepr*<sup>db<sup>+/-</sup></sup> mice were used as normal control mice.

From the <sup>1</sup>Department of Physiology, New York Medical College, Valhalla, New York; and the <sup>2</sup>Department of Physiology, Xuzhou Medical College, Xuzhou, China.

Corresponding authors: An Huang, an\_huang@nymc.edu, and Dong Sun, dong\_sun@nymc.edu.

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†Deceased.

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## RESEARCH DESIGN AND METHODS

**Animals and mesenteric artery isolation.** Six-week-old male homozygote type 2 diabetic mice (*Lepr<sup>db/db</sup>*; BKS.Cg-*Dock7<sup>m+/+</sup> Lepr<sup>db/db</sup>*/J) and their heterozygous littermates (*Lepr<sup>db/+</sup>*) were purchased from The Jackson Laboratory and maintained in our animal facilities with normal rodent chow diet until ages 3M and 9M when the experiments were performed. A total of 8 3M *Lepr<sup>db/+</sup>* and *Lepr<sup>db/db</sup>* mice and 14 9M *Lepr<sup>db/+</sup>* and *Lepr<sup>db/db</sup>* mice were used in experiments. On the day of experiments, mice were killed by inhalation of 100% CO<sub>2</sub>. The intestine and mesentery were excised and placed in a Petri dish containing cold (4°C) physiological salt solution (PSS) at pH 7.4. The PSS contained (in mmol/L) 142 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 5 dextrose, 2 pyruvate, 0.02 EDTA, and 3 MOPS. The tissue was pinned to the Silastic bottom of the dish. Superior mesenteric artery was cannulated and perfused with PSS to wash out the blood. With the use of microscissors, forceps, and an operating microscope, multiple first- and second-order mesenteric arteries were isolated for experiments of SSID and protein expression, superoxide production, and shear stress-induced nitrite production and eNOS phosphorylation. Experimental protocols were approved by the institutional animal care and use committee of New York Medical College, and conformed to the current guidelines of the National Institutes of Health and American Physiological Society for the care and use of laboratory animals.

**Immunoblotting.** Single first-order mesenteric arteries were pulverized in liquid nitrogen and incubated for 1 h in 20  $\mu$ L of 1 $\times$  laemmli sample buffer on ice. The buffer contained 5%  $\beta$ -mercaptoethanol and 1% protease and phosphatase inhibitor cocktails (Sigma-Aldrich). After the incubation, the samples were sonicated in ice water for two times (1-min duration and a 10-min interval). The samples were then heated at 95°C for 5 min. After centrifugation, the supernatant were separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and probed with antibodies to eNOS (BD Transduction Laboratories), p-eNOS (ser1177; Cell Signaling), p38, p-p38, JNK, p-JNK, Erk, p-Erk (Santa Cruz Biotechnology), and  $\beta$ -actin (Sigma-Aldrich), respectively. Prestained color protein markers (EZ-RUN, 170–10KD; Fisher Scientific) were used for monitoring protein separation and transfer efficiency. Immunoreactive bands were detected with an appropriate second antibody and visualized with a chemiluminescence kit (Pierce, Rockford, IL).

**SSID.** Second-order mesenteric arteries (~80  $\mu$ m in diameter and ~1 mm in length) were cannulated on glass micropipettes in vessel chambers and perfused with PSS at 37°C and pH 7.4. Intravascular pressure was maintained constant at 80 mmHg. Changes in internal diameter of vessels were measured using a microscope-image shearing device. After 1-h stabilization, vessels developed spontaneous tone that reduced the diameter to ~65% of their maximal diameter. Initial wall shear stresses ( $\tau$ ) of 20 and 40 dynes/cm<sup>2</sup> were applied to the vessels by increasing perfusate flow via a syringe pump. The flow rate ( $Q$ ) was determined by using the equation of  $\tau = 4Q\eta/\pi r^3$ , in which the radius ( $r$ ) was measured before the onset of flow. The viscosity ( $\eta$ ) of PSS at 37°C was 0.0069 poise. SSID was assessed in control and after administration of one of the following inhibitors: SB203580 (1  $\mu$ mol/L, inhibitor of p38), SP600125 (1  $\mu$ mol/L, inhibitor of JNK), VAS2870 (5  $\mu$ mol/L, NADPH oxidase inhibitor), and L-N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME; 300  $\mu$ mol/L, inhibitor of nitric oxide synthase). Multiple vessels isolated from the same animal were used in different protocols of SSID. In separate experiments, SSID was assessed in vessels of 9M mice in control and in the presence of resveratrol (100 nmol/L, Erk activator; 3,4',5-trihydroxystilbene; LKT Laboratories) and resveratrol plus one of following inhibitors: SB203580, SP600125, and VAS2870 without or with additional U0126 (1  $\mu$ mol/L, inhibitor of Erk). The agents used were administered directly into the perfusion chamber and incubated with vessels for 60 min before the assessment of SSID. None of these agents, at the concentration used, significantly affected the basal diameter of vessels. At the conclusion of experiments, suffusion solution was changed to a Ca<sup>2+</sup>-free PSS containing 1 mmol/L EGTA, in which vessels were incubated for 10 min at 80 mmHg. The diameter recorded at this condition was defined as the passive diameter.

**Shear stress-induced eNOS phosphorylation.** First-order mesenteric arteries (~250  $\mu$ m in diameter and ~10 mm in length) were isolated, cannulated, and perfused in vessel chambers with PSS at 37°C and pH 7.4. All side branches of the arteries were carefully ligated to prevent leakage. Intravascular pressure was maintained constant at 80 mmHg. The internal diameter of vessels was measured along the entire length of the vessel at 500  $\mu$ m interval, and the average diameter was calculated. The length of the arteries was also measured. After equilibration, a shear stress of 20 dynes/cm<sup>2</sup>, which was established by increasing perfusate flow calculated based on the average diameter of each vessel, was applied for 10 min in the absence or presence of resveratrol (100 nmol/L). Resveratrol was administered into both suffusion and perfusion solutions 1 h before the onset of the shear stress. After shear stress stimulation, vessels were snap-frozen in liquid nitrogen and kept in -80°C for assessing expressions of p-Erk and Erk, and p-eNOS and eNOS.

**Shear stress-induced nitrite formation.** Shear stress (20 dynes/cm<sup>2</sup>)-induced nitrite formation was assessed in first-order mesenteric arteries under control conditions and in the presence of resveratrol (100 nmol/L) and resveratrol plus L-NAME (300  $\mu$ mol/L). Shear stress was continuously applied to the vessels for 10 min, and perfusate was collected. Nitrite formation in the perfusate was assessed using 2,3-diaminonaphthalene (DAN) and a high-performance liquid chromatography (HPLC)/fluorescence detector-based assay to determine 1-(H)-naphthotriazole, a fluorescent product upon the reaction of nitrite and DAN (16,17). DAN was dissolved in N,N-dimethylformamide (5 mg/mL) and further diluted with 6 N HCl (0.05 mg/mL). DAN (20  $\mu$ L) was added to 200  $\mu$ L of perfusate and incubated for 10 min at room temperature. Then 20  $\mu$ L of 10 N NaOH was added. After a centrifugation, 20  $\mu$ L of supernatant was separated by an HPLC system (PU-2080 Plus; Jasco) with a C-18 reverse-phase column (Beckman Ultrasphere ODS, 5  $\mu$ m, 4.6  $\times$  250 mm). The mobile phase was composed of 35% acetonitrile and 50 mmol/L sodium phosphate buffer (pH 8.5) and run at a flow rate of 1 mL/min. The fluorescent signal of 1-(H)-naphthotriazole was detected at 375 nm (excitation) and 415 nm (emission) with a fluorescence detector (FP2020 Plus; Jasco). Standard curves of sodium nitrite (0–640  $\mu$ mol/L) were generated using PSS as a vehicle and used to calculate nitrite formation in the perfusate as picomoles per millimeter squared of the internal surface of vessels per minute.

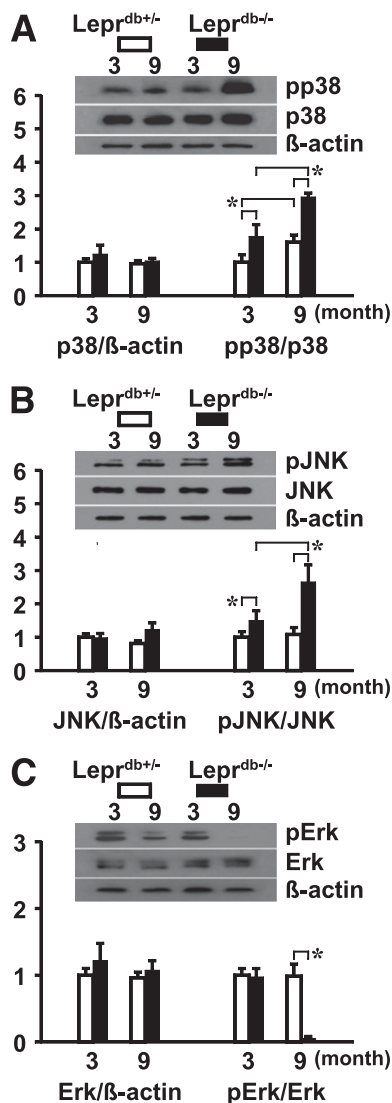
**Detection of superoxide.** Superoxide production in isolated first-order mesenteric arteries was measured by quantification of the chemiluminescence of 5  $\mu$ mol/L lucigenin in a liquid scintillation counter (LS6000IC; Beckman Instruments) (17). Vessels were incubated in Krebs-HEPES buffer (pH 7.4) at 37°C, in the control or after incubation with SB203580 (1  $\mu$ mol/L), SP600125 (1  $\mu$ mol/L), or VAS2870 (5  $\mu$ mol/L) for 60 min. The vessels were then transferred to vials containing 1 mL of Krebs-HEPES/lucigenin (5  $\mu$ mol/L) solution and counted in the scintillation counter over the next 10 min. Background signals (vials in the absence of the vessels) were subtracted from each sample to obtain the final readings. After that, vessels were completely digested with 50  $\mu$ L of 1 N NaOH and the amount of total protein was determined. The final superoxide production was expressed as counts per minute per microgram of protein.

Endothelial and smooth muscle superoxide formation in the cannulated first-order mesenteric arteries were determined by dihydroethidium (DHE) staining and confocal microscopy (18,19). DHE (10  $\mu$ mol/L) was administered intra- and extraluminally at 80 mmHg pressure for 30 min under dark conditions. After extra DHE was washed out with PSS, the vessels were cut longitudinally, removed from the cannulae, and fixed in 4% paraformaldehyde for 10 min. After a brief wash in PBS, the vessel segment adhered to glass slides with the endothelium facing up and coverslipped with antifading solution for confocal microscopy (Bio-Rad MRC 1024ES/Olympus 1  $\times$  70, UPlanFl  $\times$ 40 objective). Vessels without DHE staining were used as the background control. Two images were obtained per endothelial or smooth muscle layer of vessel segments. Fluorescent intensity of the image was measured by Photoshop histogram to determine the mean intensity and total pixels of DHE stains. The product of these two factors corresponded to the level of superoxide formation.

**Statistical analysis.** Data are expressed as means  $\pm$  SE. Changes in diameter of vessels in response to increases in shear stress were normalized to their passive diameters and expressed as percent passive diameter. Statistical significance was determined by ANOVA followed by a Tukey/Kramer multiple-comparison test. One-way and two-way ANOVA were used. Student's *t* test was also used as appropriate. Significance level was taken at *P* < 0.05.

## RESULTS

**Altered MAPK activation (phosphorylation) in mesenteric arteries of diabetic mice.** Protein expression of p38, JNK, and Erk are shown in Fig. 1. The total protein expression was comparable in all groups, but the activation, expressed as the ratio of phosphorylated versus total expression, was different between 3M and 9M, and between diabetic and control mice. As shown in Fig. 1A and B, the activation of p38 and JNK increased significantly in vessels of 3M diabetic mice and increased further in 9M diabetic mice compared with aged-matched control mice. The increased activation of p38 was also observed in vessels of 9M control mice, but it was significantly less than that of 9M diabetic mice. In contrast, the activation of Erk, which was unchanged in vessels of 3M diabetic mice, was dramatically reduced in 9M *Lepr<sup>db/db</sup>* mice (Fig. 1C).



**FIG. 1.** Protein expression of p38 (A), JNK (B), and Erk (C) in mesenteric arteries of 3M and 9M male Lepr<sup>db-/-</sup> and their heterozygous littermates (Lepr<sup>db+/+</sup>; a model of normal control mice). Summary data were obtained from 6 blots for each protein. The total expressions of p38, JNK, and Erk were normalized by  $\beta$ -actin. Densitometric ratios of phosphorylated and total protein expression of p38, JNK, and Erk were compared directly. Summary data were presented in comparison with the average expression in 3M Lepr<sup>db+/+</sup> group. \*Significant difference between groups.

**Inhibition of P38 or JNK increased shear stress-induced dilation in 3M diabetic mice.** To evaluate the specific role of the enhanced activation of p38 and JNK in the mediation of endothelial dysfunction in diabetic mice, SSID was assessed before and after inhibition of p38 and JNK. Figure 2 shows that shear stress (20 dynes/cm<sup>2</sup>)-induced dilation was significantly reduced in vessels of Lepr<sup>db-/-</sup> mice compared with that of Lepr<sup>db+/+</sup> mice. Inhibition of p38 with SB203580 (Fig. 2A) or JNK with SP600125 (Fig. 2B), enhanced SSID in vessels of 3M diabetic mice. The increased dilation in response to p38 and JNK inhibitor was prevented by L-NAME, suggesting a restored NO-mediated response. Similar to our previous findings (4), inhibition of NADPH oxidase with VAS2870 improved SSID. However, VAS2870-induced improvement of SSID was not further affected by the administration of SB203580, SP600125, or SB203580 plus SP600125 (Fig. 3A),

suggesting that the increased superoxide is caused by a p38/JNK-dependent activation of NADPH oxidase, which inactivates NO to impair SSID. Similar results were also obtained when 40 dynes/cm<sup>2</sup> shear stress was applied to these vessels (data not shown). In vessels of 9M Lepr<sup>db-/-</sup> mice, however, inhibition of p38 or JNK did not increase SSID (Fig. 2A and B). The combination of SB203580 and SP600125, or plus additional VAS2870, also failed to improve the dilation (Fig. 3B), implying that in addition to the enhanced oxidative stress, other mechanism(s) may also be involved in the endothelial dysfunction. Endothelium-independent dilator response to NO donor (acidified NaNO<sub>2</sub>) was not affected by the inhibition of p38 and/or JNK (Fig. 3A and B) at both ages of diabetic mice.

**p38- and JNK-dependent superoxide production in vessels of diabetic mice.** To further elucidate whether the beneficial effect of inhibiting p38 and JNK on SSID is of antioxidative stress in nature, vascular superoxide was measured. Superoxide production in vessels of Lepr<sup>db-/-</sup> mice was significantly increased (Fig. 4A). The increment was greater in 9M Lepr<sup>db-/-</sup> than in 3M Lepr<sup>db-/-</sup> mice. Inhibition of either p38 or JNK had no effect on superoxide level in vessels of Lepr<sup>db+/+</sup> mice, but eliminated the increased superoxide in vessels of diabetic mice. Furthermore, inhibition of NADPH oxidase with VAS2870 reduced superoxide, in both ages of diabetic mice, to a level similar to those caused by SB203580 and SP600125. Thus, these data support the notion that activation of p38 and JNK in vessels of diabetic mice increases oxidative stress via NADPH oxidase-dependent pathways.

Confocal microscopy of DHE staining was used to localize superoxide formation in endothelial and smooth muscle layers of vessels (18,19). Fluorescence intensity in the endothelial layer of 3M and 9M Lepr<sup>db+/+</sup> mice was comparable, but was increased significantly in Lepr<sup>db-/-</sup> mice, as shown in Fig. 4B that there were more than 18- and 36-fold increases in 3M and 9M diabetic mice compared with that in 3M control mice. Changes in fluorescent intensity in smooth muscle layers were relatively less than that in the endothelium. Figure 4C shows that there was an age-dependent increase in fluorescence intensity in both normal and diabetic mice, but the overall increase in smooth muscle cells of diabetic mice was ~2-2.5 fold higher than that of control mice. The absolute numbers of fluorescence intensity recorded from endothelial and smooth muscle layers of 3M Lepr<sup>db+/+</sup> mice were 350,743 and 8,146,681, respectively. The much greater fluorescence intensity of smooth muscle, compared with the endothelium, was mainly attributed to the large number of total pixels of DHE stains, rather than the mean fluorescence intensity. Thus, both a greater production of superoxide in smooth muscle cells and a greater increment in superoxide production in the endothelium contribute to the reduced NO bioavailability and exacerbation of endothelial dysfunction during the process of diabetes.

**Erk activation increases eNOS phosphorylation in vessels of 9M db/db mice.** Because the expression of p-Erk was obviously downregulated in vessels of 9M Lepr<sup>db-/-</sup> mice, we tested in the next series of experiments the effect of Erk activation on endothelial function of diabetic mice. Vessels of 9M Lepr<sup>db-/-</sup> mice were treated with resveratrol for 60 min before exposure to shear stress. Figure 5A and B shows that in response to 20 dynes/cm<sup>2</sup> shear stress, phosphorylation of Erk and eNOS, as well as the ratio of p-Erk/Erk and p-eNOS/eNOS, were significantly increased in resveratrol-treated compared

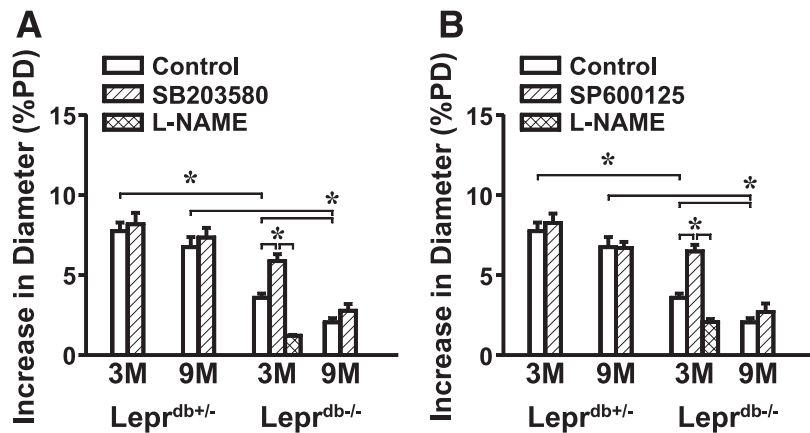


FIG. 2. Shear stress (20 dynes/cm<sup>2</sup>)-induced dilation in mesenteric arteries of 3M and 9M *Lepr<sup>db/+</sup>* and *Lepr<sup>db/-</sup>* mice, in control and in the presence of SB203580 (A), a p38 inhibitor, or SP600125 (B), a JNK inhibitor, respectively. L-NAME was used in the group of 3M *Lepr<sup>db/-</sup>* mice, in the presence of SB203580 or SP600125, to inhibit NO synthesis. *n* = 6–8 per group. \*Significant difference between groups. PD, passive diameter.

with untreated vessels. In parallel with the increased eNOS phosphorylation, eNOS activity, expressed as shear stress-induced nitrite formation, was also increased in resveratrol-treated vessels. Figure 5C depicts fluorescent signals of standard curves of sodium nitrite, measured by HPLC/fluorescence detector-based assay. The method is sensitive

enough to detect subpicomoles of nitrite in the perfusate. Figure 5D shows that resveratrol significantly increased perfusate nitrite in shear stress-stimulated vessels of 9M *Lepr<sup>db/-</sup>* mice; the response was sensitive to L-NAME. This suggests that resveratrol facilitates shear stress-induced eNOS activation. However, resveratrol alone failed to improve SSID in vessels of 9M diabetic mice (Fig. 6).

**Inhibition of oxidative stress and activation of Erk coordinately improve endothelial function in vessels of 9M diabetic mice.**

We assessed combined effects of inhibition of NADPH oxidase and activation of Erk/eNOS on SSID in vessels of 9M *Lepr<sup>db/-</sup>* mice. As shown in Fig. 7, coinubation of resveratrol and SB203580, resveratrol and SP600125, or resveratrol and VAS2870 significantly improved SSID. This improved dilation was prevented when the activation of Erk was inhibited by U0126.

**DISCUSSION**

We demonstrated in the current study that an altered MAPK signaling, characterized by potentiated p38/JNK activation and impaired Erk/eNOS activation, contributes significantly to the endothelial dysfunction in diabetic mice. Specifically, increased activation of vascular p38 and JNK were responsible for the enhanced formation of superoxide that scavenges NO, leading to an impaired SSID. This impaired SSID was reversible in the early stage of diabetes via inhibition of p38/JNK/reactive oxygen species (ROS) signaling. During the progression of diabetes, Erk signaling pathway was largely inactivated, accompanied by the inactivation of eNOS. As a consequence, endothelial dysfunction was reversed only in the presence of both the inhibition of p38/JNK/ROS and the activation of Erk/eNOS.

**P38/JNK-dependent oxidative stress impairs endothelial function in diabetes.** Our results, as shown in the present and previous studies (4), demonstrated that increased vascular superoxide production and reduced SSID accompany the progression of diabetes in *Lepr<sup>db/-</sup>* mice. It is known that oxidative stress activates p38 and JNK (5,6). Likewise, we found that the total expression of p38 and JNK was unchanged, but the phosphorylation of p38 and JNK was augmented in vessels of diabetic mice (Fig. 1), suggesting that the increased activation of p38 and JNK contribute significantly to the impaired vasodilator responses (Fig. 2). It is interesting that inhibition of p38 or JNK reduced superoxide production (Fig. 4), revealing a link between the

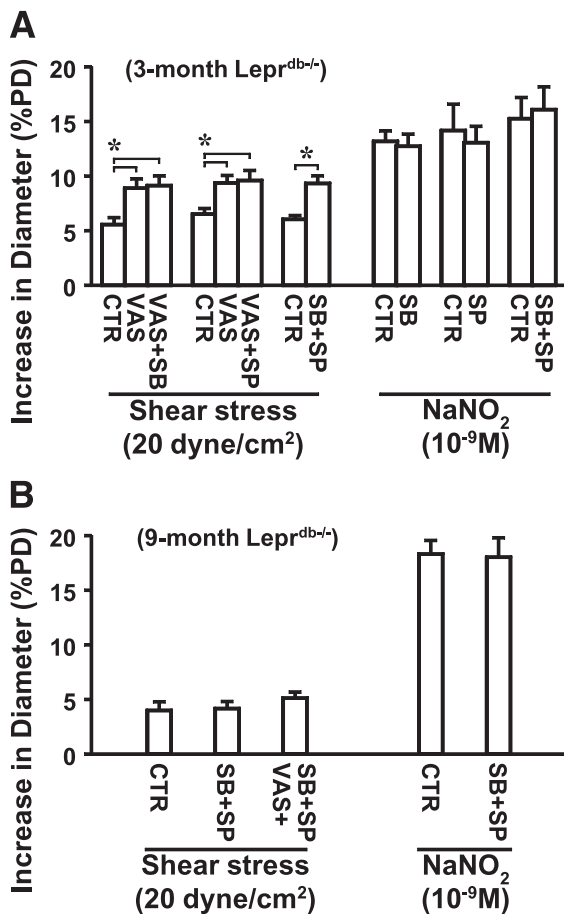
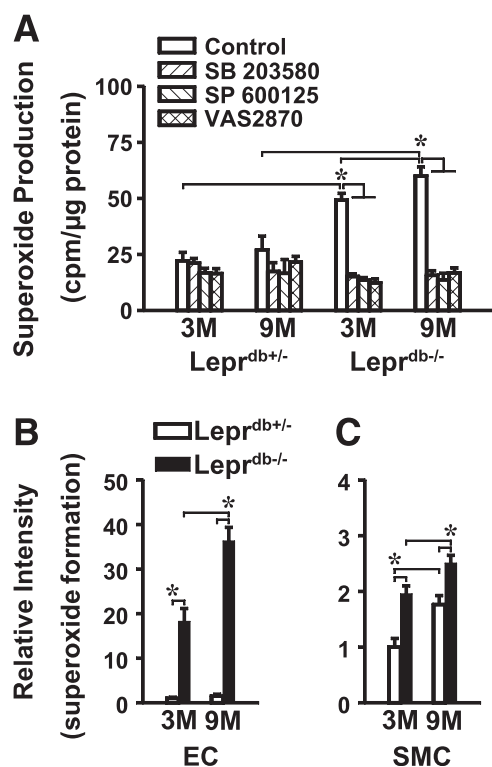


FIG. 3. Shear stress (20 dynes/cm<sup>2</sup>)- and NO (acidified NaNO<sub>2</sub>)-induced dilation in mesenteric arteries of 3M (A) and 9M (B) *Lepr<sup>db/-</sup>* mice in the control condition (CTR) and in the presence of VAS2870 (VAS; a NADPH oxidase inhibitor), SB203580 (SB), and SP600125 (SP), respectively. *n* = 8 per group. \*Significant difference between groups.



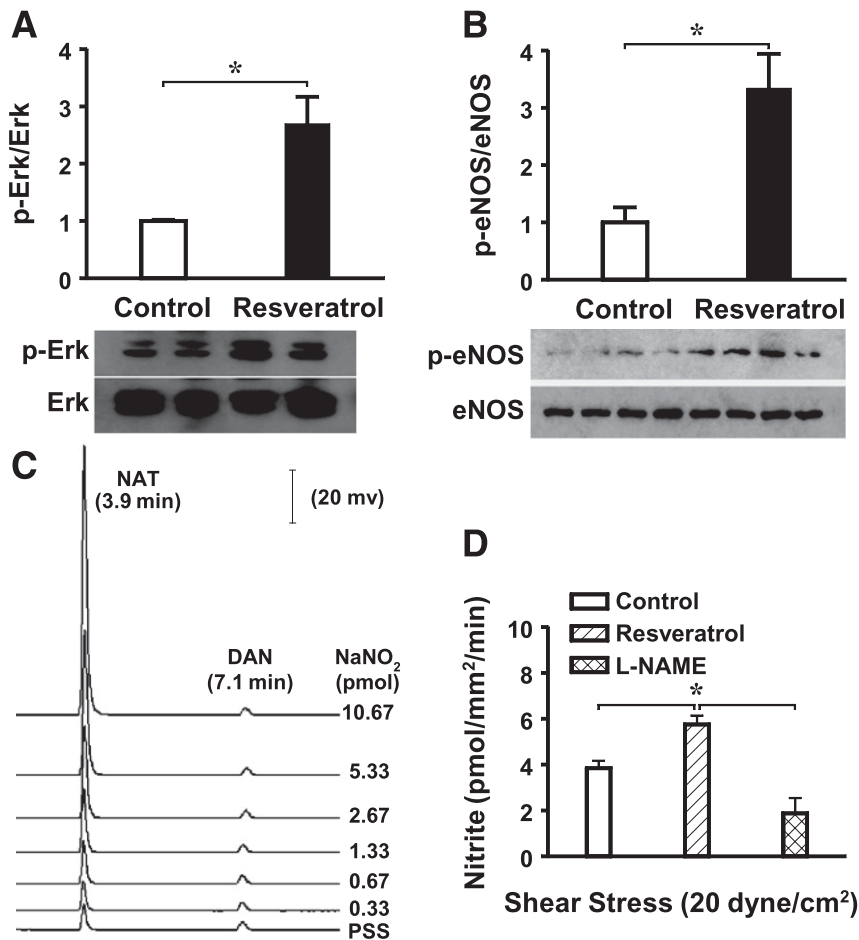


**FIG. 4.** Superoxide production in mesenteric arteries of 3M and 9M *Lepr<sup>db/+</sup>* and *Lepr<sup>db/-</sup>* mice. **A:** Lucigenin chemiluminescence detection of superoxide in control and in the presence of SB203580 (SB), SP600125 (SP), or VAS2870 (VAS), respectively.  $n = 6-8$  per group. **B and C:** Confocal image analyses of DHE staining in the endothelial cell (EC) and smooth muscle cell (SMC) layers of mesenteric arteries, respectively.  $n = 4$  per group. Data were normalized by the mean of 3M *Lepr<sup>db/+</sup>* group and expressed as relative fluorescent intensity of DHE staining. \*Significant difference between groups.

activated p38/JNK and NADPH oxidase-derived superoxide. This notion is supported by the fact that the effect of p38/JNK inhibition on superoxide production is identical to that of inhibition of NADPH oxidase (with VAS2870) (Fig. 4A) and moreover that inhibition of p38/JNK-initiated increases in SSID is not additive by additional presence of VAS2870 and vice versa (Fig. 3). Thus, the restoration of SSID via p38/JNK inhibitors is most likely mediated by the inhibition of NADPH oxidase-derived superoxide production. Owing to the limitation of obtaining sufficient samples from isolated single arteries of mice, we could not explore specific mechanisms in detail at this time. Other studies, however, provided evidence indicating that inhibition of p38 lowered blood pressure, improved renal hemodynamics, and enhanced acetylcholine-induced dilation in diabetic rats (20). Also, *in vivo* chronic inhibition of p38 has been reported to downregulate NADPH oxidase expression, attenuate superoxide production, and improve vascular function in a variety of animal models (21–23). Moreover, acute inhibition of p38 suppressed phorbol myristate acetate-induced activation of NADPH oxidase in neutrophils (24,25). Direct evidence for the p38-dependent activation of NADPH oxidase was provided by experiments conducted on cultured human lung endothelial cells (26), in which the activated p38 regulates the phosphorylation of NADPH oxidase subcomponent(s) to promote assembly and activation of the oxidase. Consistent with our findings, C-reactive protein-induced attenuation of NO-mediated dilation in isolated porcine coronary arterioles was reported to

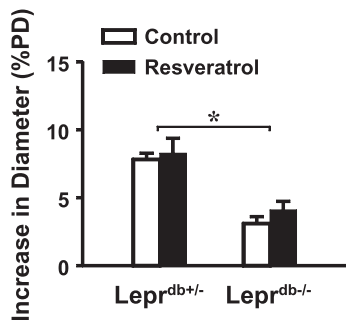
be mediated by p38-dependent activation of NADPH oxidase (27). Additionally, TNF- $\alpha$ -induced impairment of NO-mediated dilation was resulted from xanthine oxidase-derived superoxide, via JNK-dependent mechanisms (8). Thus, specific roles for p38- and/or JNK-dependent stimulation of oxidative stress as the mediator of endothelial dysfunction have been evaluated in a variety of disease models. On the other hand, alternative mechanisms of MAPK-dependent impairment of endothelial function have also been proposed. As reported, JNK directly phosphorylated eNOS-Ser(116), resulting in a reduced NO release (28). In the current study, however, inhibition of JNK did not further increase VAS2870-induced improvement of SSID (Fig. 3), excluding the possibility that JNK directly inhibits eNOS activity in the mesenteric arteries of diabetic mice.

**Hypoactivation of Erk impairs activation of eNOS in advanced diabetes.** Inhibition of p38 and JNK prevented the enhanced superoxide production in vessels of 3M and 9M diabetic mice, but improved SSID only in vessels of 3M diabetic mice (Fig. 3), suggesting that in addition of p38/JNK-dependent potentiation of oxidative stress, other independent mechanisms are involved in the exacerbation of endothelial dysfunction during the progression of diabetes. In this context, a specific role of Erk activation, which was greatly reduced in vessels of 9M diabetic mice (Fig. 1), attracted attention in terms of the possible crosstalk between Erk and eNOS signaling cascades in response to shear stress. Indeed, previous studies indicated that TNF- $\alpha$ -induced endothelial dysfunction was prevented by shear stress-stimulated activation of Erk (29). We therefore tested the hypothesis that recruiting Erk activity stimulates eNOS phosphorylation to improve NO-mediated SSID. Resveratrol, a polyphenolic phytoalexin found in grapes and red wine, has been shown to exert cardiovascular benefits. Resveratrol rapidly activates Erk and subsequently eNOS at nanomolar concentrations in vascular endothelial cells (30,31). Of note, resveratrol in a range of micromolar concentrations exerts a direct vasodilator effect, which was also involved in endothelial Erk activation (32). To this end, resveratrol at a concentration of 100 nmol/L was used in the current study, aimed to activate Erk without changing the basal tone of vessels. As expected, phosphorylation of Erk was significantly increased in the resveratrol-treated vessels of 9M diabetic mice, associated with increases in shear stress-stimulated eNOS phosphorylation and NO production (Fig. 5B and D). However, the functional significance of resveratrol in recruiting vascular Erk and eNOS activity seemed to be masked since it failed to improve SSID in these vessels (Fig. 6). But exposure of vessels to both resveratrol and inhibitors for p38/JNK or NADPH oxidase promoted normalization of SSID (Fig. 7). Thus, we interpret our data to mean that in the advanced stage in diabetic mice, increased superoxide production resulted from activated p38/JNK, and reduced NO release because of inactivation of Erk/eNOS, contributes independently but synergistically to the impaired SSID. On the other hand, we noted that the role of resveratrol in the improvement of endothelial function can also involve multiple mechanisms, such as the potentiation of endothelial SirT1 (33), and inhibition of inflammatory and oxidative signaling (34,35), all of which are beneficial to the activation of eNOS. Alternatively, high glucose-induced increases in adhesion molecule intracellular adhesion molecule-1 in cultured endothelial cells were prevented by resveratrol via a mechanism of inhibiting p38 activation (36). Furthermore, in the setting of



**FIG. 5.** Shear stress (20 dynes/cm<sup>2</sup>, 10 min)-stimulated Erk (A) and eNOS (B) phosphorylation (p) in mesenteric arteries of 9M *Lepr<sup>db/db</sup>* mice in control and after treatment with resveratrol (100 mol/L) for 60 min. Data were summarized from 3 blots. \*Significant difference between groups. C: HPLC/fluorescence detection of 1-(H)-naphthotriazole (NAT), a fluorescent product of nitrite and DAN. Traces show fluorescent signals and retention times of NAT and DAN, obtained from standard curves of sodium nitrite (0.33–10.67 pmol in 20 μL, equivalent to 20–640 μmol/L of sodium nitrite). D: Shear stress-induced release of NO (perfusate nitrite) in mesenteric arteries of 9M *Lepr<sup>db/db</sup>* mice in the control condition and in the presence of resveratrol and resveratrol plus L-NAME. *n* = 8 per group. \*Significant difference between groups.

insulin resistance, an altered activation of phosphoinositide 3-kinase/Akt pathway may also lead to the endothelial dysfunction (37,38). In the current study, however, we provide evidence that Erk inhibitor U0126 prevented resveratrol-induced improvement of SSID in 9M *Lepr<sup>db/db</sup>* mice (Fig. 7), confirming that resveratrol-specific

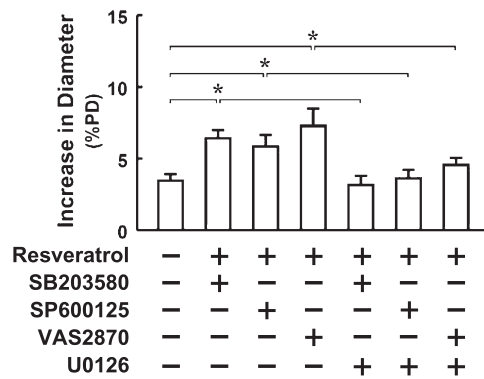


**FIG. 6.** Shear stress (20 dynes/cm<sup>2</sup>)-induced dilation in mesenteric arteries of 9M *Lepr<sup>db/db</sup>* and *Lepr<sup>db+/+</sup>* mice, in control and in the presence of resveratrol. *n* = 6–8 per group. \*Significant difference between groups. PD, passive diameter.

recruitment of Erk, followed by activation of eNOS, plays significant roles in the responses.

It is intriguing that *Lepr<sup>db/db</sup>* mice are hyperleptinaemia, which has been confirmed to play a pivotal role in obesity-related cardiovascular events, including insulin resistance (39). The effects of leptin on MAPK signaling may also contribute to the endothelial dysfunction in diabetes (40).

**Perspective and significance.** Treatment of isolated vessels with inhibitors for p38 and JNK reduced superoxide production and increased SSID in diabetic mice. Our data support the notion that p38 and JNK could serve as therapeutic targets for patients with cardiovascular disease. In this context, a novel therapy for diabetes with specific inhibition of MAPK signaling, such as cell-permeable JNK-inhibitory peptide, has been studied (7,41). As indicated, there are different mechanisms underlying endothelial dysfunction at different stages of diabetes. When the ROS-dependent mechanism contributes primarily to the endothelial dysfunction, an antioxidant therapy would be effective. When eNOS inactivation occurs, antioxidant therapy alone would not be sufficient; an optimal approach that activates eNOS such as resveratrol-mediated recruiting Erk and eNOS would then become necessary. We believe that the mechanistic insights provided by the current



**FIG. 7.** Shear stress (20 dynes/cm<sup>2</sup>)-induced dilation in mesenteric arteries of 9M *Lepr<sup>db-/-</sup>* mice in control and in the presence (+) or absence (-) of resveratrol, SB203580, SP600125, VAS2870, and U0126, respectively. *n* = 8 per group. \*Significant difference between groups. PD, passive diameter.

study may provide additional information for clinical treatment of diabetes.

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A.H. and D.S. designed the experiment, researched data, and wrote the manuscript. Y.-M.Y. and C.Y. researched data. G.K. and T.H.H. contributed to the discussion and reviewed and edited the manuscript. A.H. and D.S. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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