

Vascular NADH Oxidase Is Involved in Impaired Endothelium-Dependent Vasodilation in OLETF Rats, a Model of Type 2 Diabetes

Yong K. Kim, Mi-S. Lee, Seok M. Son, In J. Kim, Won S. Lee, Byung Y. Rhim, Ki W. Hong, and Chi D. Kim

Superoxide anion can modulate vascular smooth muscle tone and is potentially involved in diabetic vascular complications. The present study was undertaken to characterize both vascular production and the enzymatic source of superoxide anion in type 2 diabetic rats. In the thoracic aorta of OLETF rats, endothelium-dependent relaxation was markedly attenuated compared with that of control (LETO) rats in association with a significant increase in superoxide production ($2,421.39 \pm 407.01 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$). The increased production of superoxide anion was significantly attenuated by diphenyleneiodonium (DPI; $10 \mu\text{mol/l}$), an inhibitor of NAD(P)H oxidase. The production of superoxide anion in response to NADH as a substrate was markedly increased in the vascular homogenates, but NADPH, arachidonic acid, xanthine, and succinate produced only small increases in chemiluminescence. In line with these results, studies using various enzyme inhibitors, such as DPI, allopurinol, rotenone, N^G -monomethyl-L-arginine, and indomethacin, suggest that the predominant source of superoxide anion in vascular particulate fraction is NADH-dependent membrane-bound oxidase. Furthermore, the expression of p22phox, a major component of vascular NAD(P)H oxidase, was markedly increased in the aorta from OLETF rats compared with that of LETO rats. These findings suggest that upregulated expression of p22phox mRNA and enhanced NADH oxidase activity contribute to the impaired endothelium-dependent vasodilation in OLETF rats. *Diabetes* 51:522–527, 2002

Vascular complications are responsible for the excess mortality associated with diabetes (1), and impaired endothelium-dependent vasodilation has been described in humans and in animal models of the disease (2–6). A variety of mechanisms have been proposed to explain these phenomena; in particular, oxidant stress has been implicated in the pathogenesis of diabetic angiopathies (7).

From the Department of Internal Medicine and Pharmacology, College of Medicine, Pusan National University, Pusan, South Korea.

Address correspondence and reprint requests to Chi D. Kim, Department of Pharmacology, College of Medicine, Pusan National University, Ami-dong 1-Ga, Seo-gu, Busan 602–739, Korea. E-mail: chidkim@pusan.ac.kr.

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ACh, acetylcholine; DPI, diphenyleneiodonium; eNOS, endothelial nitric oxide synthase; L-NMMA, N^G -monomethyl-L-arginine; NO, nitric oxide; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase.

Convincing data have been presented to show that production of reactive oxygen species (ROS) occurs in nonphagocytic cells, including endothelial cells (8), vascular smooth muscle cells (9), and aortic adventitial fibroblasts (10), and superoxide anion has a variety of biological functions that accompany induction of gene expression (11), cellular proliferation (12), apoptosis (13,14), and hypertrophy (15). However, the enzymatic source of ROS responsible for various vascular-associated diseases in diabetes remains unknown.

Among various sources for ROS, such as NAD(P)H oxidase, cyclooxygenase, xanthine oxidase, nitric oxide (NO) synthase, and mitochondrial electron transport, NAD(P)H oxidase has been considered to be a major source of ROS in the vasculature (16). Vascular NAD(P)H oxidase is similar in structure to the neutrophil NADPH oxidase, which consists of four major subunits: a membrane-associated cytochrome b_{558} , comprising gp91phox and p22phox and two cytosolic components, p47phox and p67phox. During stimulation, cytosolic components translocate to the plasma membrane to form a catalytically active oxidase (17,18).

On the other hand, the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, established by Kawano et al. (19), is a distinct model of type 2 diabetes. This animal model displays some characteristic features, such as late onset of hyperglycemia (after 18 weeks of age), hyperinsulinemia, obesity, and insulin deficiency. Considering the fact that diabetes is a major public health issue because of its increasing prevalence and the severity of its secondary vascular complications, OLETF rats are regarded as an important animal model to understand human type 2 diabetes.

In the present study, we attempted to clarify whether NAD(P)H oxidase contributes to impaired endothelium-dependent vasodilation in type 2 diabetic rats. We found enhanced vascular production of superoxide anion in association with increased activity of NADH oxidase with enhanced vascular p22phox mRNA expression in OLETF rats, suggesting the possible role of NADH oxidase in vascular complications in type 2 diabetes.

RESEARCH DESIGN AND METHODS

Animals. All procedures were in accordance with institutional guidelines for animal research. Male OLETF rats and LETO rats (genetic control for OLETF rats) were supplied by the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). All rats were kept under controlled temperature ($23 \pm 2^\circ\text{C}$) and humidity ($55 \pm 5\%$) with a 12-h light/dark cycle. They were

provided with lab food (Oriental Kobo) and autoclaved tap water ad libitum. At the time of the study, the rats were 30 weeks old.

Vessel preparation. The aorta was placed in modified Krebs/HEPES buffer (composition [in mmol/l]: NaCl, 99.01; KCl, 4.69; CaCl₂, 1.87; MgSO₄, 1.2; K₂HPO₄, 1.03; NaHCO₃, 25.0; Na-HEPES, 20.0; glucose, 11.1; pH 7.4). Part of the cleaned aorta was cut into 2-mm ring segments for measurement of vascular superoxide production and examination of vascular relaxation, and the remainder was snap-frozen in liquid nitrogen and stored at -70°C.

Vascular relaxation. Aortic rings were connected to isometric force transducers in organ chambers as described earlier (20). The rings were then submaximally contracted with U46619 (30 nmol/l), and after a plateau had been reached, the relaxations to acetylcholine (ACh; 10⁻⁸ to 10⁻⁶ mol/l) or sodium nitroprusside (SNP; 3 × 10⁻¹⁰ to 10⁻⁶ mol/l) in the presence or absence of superoxide dismutase (SOD; 500 units/ml) were recorded.

Immunohistochemistry for endothelial NO synthase. For immunohistochemical staining of endothelial NO synthase (eNOS), after immersion fixation in acetone (4°C), aortic sections were incubated with 5% goat serum/PBS/Tween-20 to block nonspecific protein binding sites. A monoclonal antibody to eNOS (Oncogene, Boston, MA) was applied for 60 min, followed by incubations with peroxidase-conjugated secondary antibody (Oncogene) and ABC reagent (Vector Laboratories, Burlingame, CA) for 1 h. After a 30-s immersion in 0.1 mol/l sodium acetate buffer (pH 5.2), eNOS immunoreactivity was made visible with diaminobenzidine solution (Vector Laboratories, Burlingame, CA) and hematoxylin counterstaining. Immunoreactivity for eNOS was quantitated by Image-Pro Plus Imaging software (Media Cybernetics, Silver Spring, MD), and the stain density was expressed as pixels per micrometers squared of tissue.

Vascular superoxide production. Lucigenin, an acridylum dinitrate compound that emits light on reduction and interaction with superoxide anion, was used to measure vascular superoxide production (21,22). Briefly, the vessels were placed in a modified Krebs/HEPES buffer and allowed to equilibrate for 30 min at 37°C. The Krebs/HEPES buffer with 250 μmol/l lucigenin was placed into a luminometer (EG & G, Berthold, Germany). The chemiluminescence was measured continuously at 37°C for 60 min. The vessels were then dried in a 90°C oven for 24 h for determination of dry weight. The amount of superoxide anion was calculated by comparison with a standard curve generated from known quantities of xanthine and xanthine oxidase (23). In some experiments, diphenyleneiodinium (DPI; 10 μmol/l) and SOD (500 units/ml) were added to the samples 10 min before readings.

Enzymatic source of superoxide anion. Aortic segments (2–3 cm) were homogenized with a motor-driven tissue homogenizer for 2 min in 50 mmol/l phosphate buffer containing 0.01 mmol/l EDTA. The homogenate was centrifuged at 1,000g for 10 min to remove unbroken cells and debris. Protein content was determined by using a bicinchoninic acid protein assay kit (Sigma, St. Louis, MO). Protein (25 μg) was used to measure the production of superoxide anion by lucigenin-enhanced chemiluminescence. NADH (100 μmol/l), NADPH (100 μmol/l), xanthine (100 nmol/l), arachidonic acid (100 μmol/l), and succinate (5 mmol/l) were added to discriminate the role of each substrate in superoxide production in this fraction.

In additional experiments, vessels were initially homogenized in Tris-HCl buffer containing protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride and 1 μg/ml each of antipain, aprotinin, bestatin, leupeptin, soybean trypsin inhibitors, pepstatin A, and 0.1% 2-mercaptoethanol). The supernatant from the low-speed centrifugation was subjected to 21,000g centrifugation for 45 min at 4°C to separate membrane and cytosolic fractions. The membrane and cytosolic fractions containing 25 μg protein were used to examine oxidase activity of these cellular subfractions. Furthermore, superoxide production in response to NADH was examined after the addition of DPI (10 μmol/l), SOD (500 units/ml), allopurinol (100 μmol/l), rotenone (100 μmol/l), N^G-monomethyl-L-arginine (L-NMMA; 10 μmol/l), or indomethacin (10 μmol/l) in the membrane fraction.

Northern blot hybridization. Total RNA was extracted from aortas using TRIzol reagent (Gibco/BRL, Life Technologies, Rockville, MD). RNA (30 μg) was separated by electrophoresis on 1.5% agarose-formaldehyde gel, visualized by ethidium bromide staining, and transferred to ζ-probe blotting membranes. After immobilization by ultraviolet cross-linking, blots were prehybridized for 4 h at 65°C. A full-length cDNA for rat p22phox (provided by Kathy K. Griending) was labeled with [³²P-α]dCTP using the rediprime II random prime labeling system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, U.K.). After hybridization with radiolabeled probes, blots were exposed to Kodak film for 24–48 h at -70°C, developed, and photographed. The density of individual bands was normalized to 28S ribosomal RNA.

Data analysis and statistics. Data are presented as means ± SE or percentage of control. Statistical comparisons between groups were per-

TABLE 1
Levels of blood glucose and body weight

	LETO	OLETF
Body weight (g)	518 ± 13	618 ± 63
Plasma glucose (mg/dl)	125.9 ± 15.7	194.7 ± 11.3*

Data are means ± SE; n = 8 per group. *P < 0.01 vs. LETO rats.

formed with two-tailed Student's *t* test for unpaired data. A value of *P* < 0.05 was considered statistically significant.

RESULTS

As shown in Table 1, 30-week-old OLETF rats (618 ± 63 g) weighed more than LETO rats (518 ± 13 g), and the plasma glucose concentrations in fasting animals were significantly greater in OLETF rats than in LETO rats (194.7 ± 11.3 mg/dl vs. 125.9 ± 15.7 mg/dl, *P* < 0.01).

Vascular relaxations. During U46619-induced submaximum contraction, the relaxation after ACh treatment was significantly impaired in endothelium-intact aortic rings derived from OLETF rats compared with those from nondiabetic control rats (Fig. 1A). The reduced vascular reactivity to ACh in OLETF rats was significantly enhanced by pretreatment with SOD (500 units/ml), suggest-

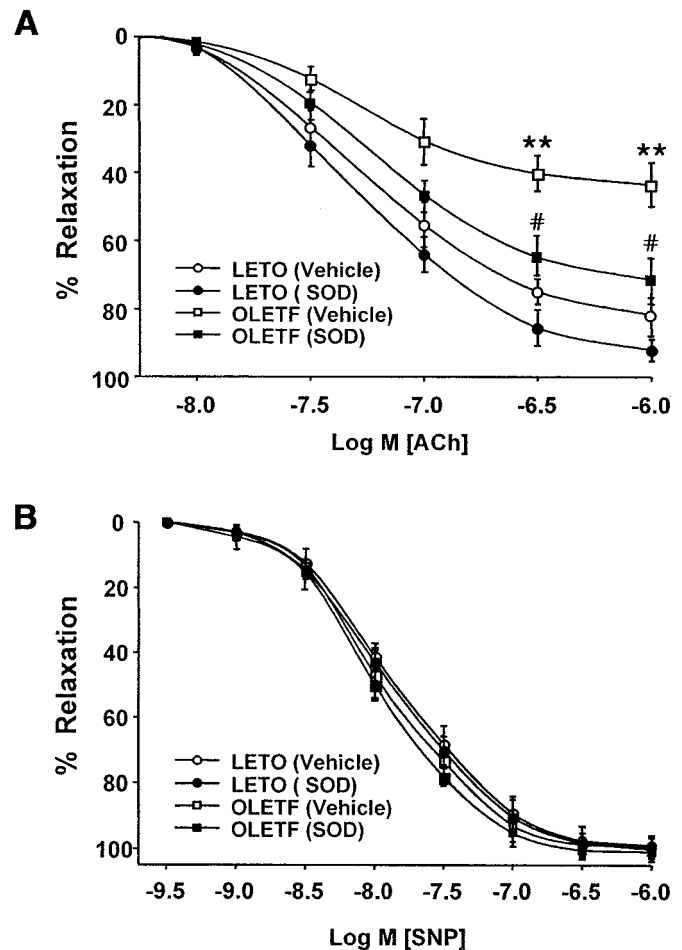


FIG. 1. Vascular relaxations after treatment with ACh (A) or SNP (B) in the precontracted aortic rings from LETO and OLETF rats were recorded in the presence (●, ■) or absence (○, □) of SOD (500 units/ml). Data are means ± SE of eight experiments. **P* < 0.05, ***P* < 0.01 compared with LETO (Vehicle) rats. #*P* < 0.05 compared with corresponding values of OLETF (Vehicle) rats.

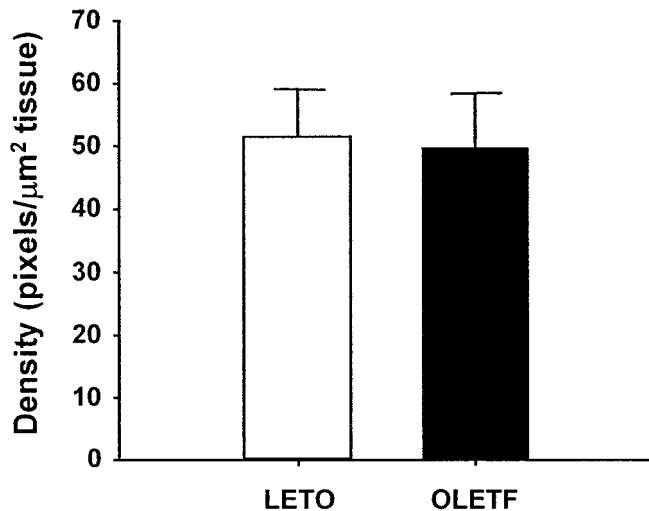


FIG. 2. Comparison of densities of immunohistochemical stain for eNOS in aortas from LETO and OLETF rats. The density of eNOS staining in aortic cross sections was expressed as pixels per micrometers squared of tissue.

ing the role of superoxide anion in the impaired ACh-mediated vascular relaxation in OLETF rats. Because OLETF rats showed decreased vascular relaxation after ACh treatment, vasodilatory responses to the NO donor SNP were assessed to determine whether ACh-induced abnormal relaxation in OLETF rats was due to decreased vascular reactivity to NO. Vascular relaxation after SNP treatment was not different between groups, suggesting that the impaired relaxation reaction to ACh in aorta from OLETF rats was not due to alterations in vascular sensitivity to NO (Fig. 1B).

Immunohistochemistry for eNOS. Immunohistochemical results showed no difference in the density of eNOS staining between aortas from LETO and OLETF rats (Fig. 2). In addition, the staining was only present on the endothelial layer of thoracic aorta in both animals.

Vascular superoxide production. To determine whether

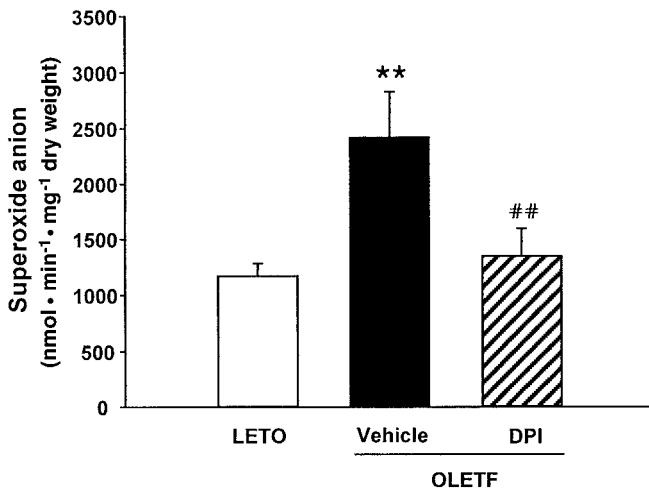


FIG. 3. Vascular superoxide production in aortic segments from LETO and OLETF rats was assessed by lucigenin chemiluminescence assay in the absence (Vehicle) or presence of DPI (10 μmol/l). Data are expressed as nanomoles per minute per milligram dry weight of vessels. Bars are means ± SE of four experiments in each group. ***P* < 0.01 compared with LETO rats; ##*P* < 0.01 compared with vehicle of OLETF rats.

the impaired vasodilation was accompanied by an increase in the vascular production of superoxide anion, we measured vascular production of superoxide anion using aortas from LETO and OLETF rats. As shown in Fig. 3, superoxide production in the thoracic aorta from OLETF rats was markedly increased compared with that from LETO rats ($2,421.39 \pm 407.01 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ dry wt}$ vs. $1,345.44 \pm 249.60 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ dry wt}$; *P* < 0.01). In addition, the enhanced lucigenin signal in OLETF rats was significantly inhibited by treatment with the NAD(P)H oxidase inhibitor DPI (10 μmol/l).

Enzymatic source for superoxide anion. Superoxide production in response to a variety of substrates was investigated using aortic homogenates. Superoxide production derived from NADH was greater than that of the other potential substrates, NADPH, arachidonic acid, xanthine, or succinate (Fig. 4A). This observation demonstrated that NADH is a major substrate to produce superoxide anion in the vascular homogenates of LETO and OLETF rats. Furthermore, in aortic homogenates from OLETF rats, the superoxide generation in response to NADH ($15.41 \pm 2.12 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ protein}$) was ~3-fold higher than that of LETO rats ($5.73 \pm 2.12 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ protein}$).

Membrane-bound versus cytosolic oxidase activity. To assess the subcellular localization of oxidase activities, vascular homogenates were separated into membrane and cytosolic fractions. As shown in Fig. 4B and C, NADH-dependent oxidase activity was predominantly located in the particulate fraction, and NADH-driven superoxide production measured in the particulate fraction was higher in OLETF ($16.55 \pm 1.28 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ protein}$) than in LETO ($8.9 \pm 1.33 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ protein}$) rats.

Superoxide production under several inhibitors in particulate fractions. Enhanced NADH oxidase activity in OLETF rats was confirmed by measuring superoxide level under treatment with several interventions, such as DPI (10 μmol/l), SOD (500 units/ml), allopurinol (100 μmol/l), rotenone (100 μmol/l), L-NMMA (10 μmol/l), and indomethacin (10 μmol/l). NADH-evoked oxidase activity in the particulate fraction of LETO and OLETF rats was decreased by the addition of 10 μmol/l DPI. In addition, treatment with SOD at high concentration was more effective in reducing the NADH-stimulated lucigenin chemiluminescence signal. However, stimulated NADH oxidase activity was insensitive to allopurinol (inhibitor of xanthine oxidase), rotenone (inhibitor of mitochondrial electron transport chain), L-NMMA (nitric oxide synthase inhibitor), and indomethacin (cyclooxygenase inhibitor) (Fig. 5).

Expression of vascular p22phox mRNA. Because p22phox, one of the membrane-associated components of vascular NAD(P)H oxidase, has been shown to be present and functional in the vasculature (24–27), we investigated p22phox mRNA expression in LETO and OLETF rats by Northern blot analysis. As shown in Fig. 6, the level of p22phox mRNA expression was greater in OLETF than in LETO rats. These data suggest that the upregulated expression of p22phox mRNA may be responsible for the overproduction of superoxide anion in aorta from OLETF rats.

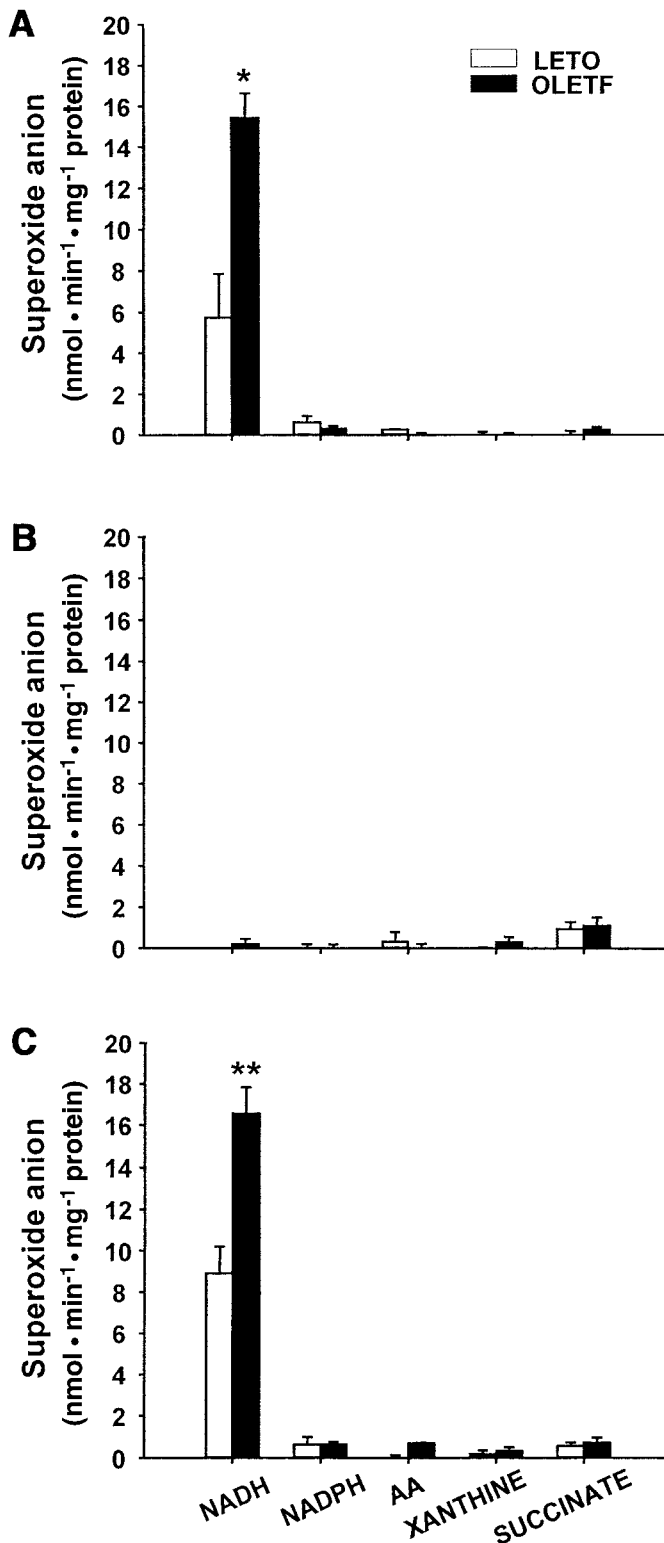


FIG. 4. Production of superoxide anion in vascular homogenates (A), and cytosolic (B) and particulate (C) fractions in response to several potential substrates, NADH (100 $\mu\text{mol/l}$), NADPH (100 $\mu\text{mol/l}$), arachidonic acid (AA; 100 $\mu\text{mol/l}$), xanthine (100 nmol/l), and succinate (5 mmol/l). Bars are means \pm SE of four experiments in each group. * $P < 0.05$, ** $P < 0.01$ compared with LETO rats.

DISCUSSION

Vascular complication is a major cause of morbidity and mortality in diabetic patients. However, the precise mech-

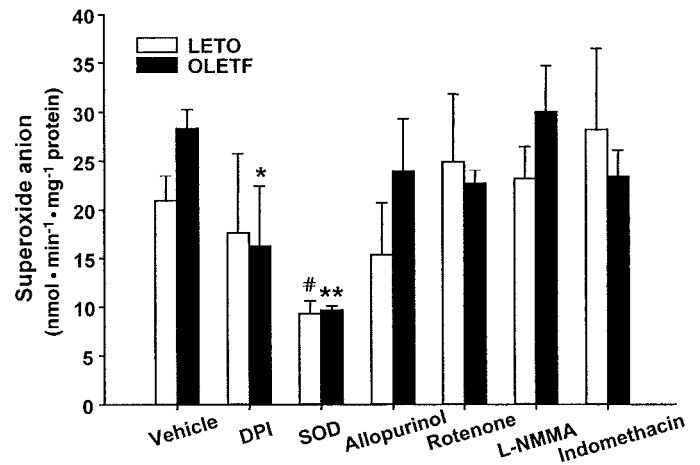


FIG. 5. Effects of various inhibitors, such as DPI (10 $\mu\text{mol/l}$), SOD (500 units/ml), allopurinol (100 $\mu\text{mol/l}$), rotenone (100 $\mu\text{mol/l}$), L-NMMA (10 $\mu\text{mol/l}$), and indomethacin (10 $\mu\text{mol/l}$), on NADH-stimulated superoxide production in particulate fractions. Bars are means \pm SE of four experiments in each group. * $P < 0.05$, ** $P < 0.01$, # $P < 0.05$ compared with corresponding values in vehicle.

anisms of abnormal vascular reactivity in diabetes remain unclear. In the present experiments, we demonstrated that enhanced superoxide production via vascular NADH oxidase is associated with abnormal vascular reactivity in an animal model of type 2 diabetes.

Diabetes-induced abnormality in endothelium-dependent vasodilation could be caused by either diminished production of endothelial NO or promoted inactivation of NO. In the present experiment, aortic segments from OLETF rats exhibited impaired endothelium-dependent vasodilation that was partially normalized by pretreatment with SOD (Fig. 1). Furthermore, there was no significant difference in eNOS expression in the aortic endothelium of OLETF and control rats (Fig. 2). These observations suggest that reduced NO availability through the interaction between NO and superoxide anion is a causal factor in the impaired vasodilation in OLETF rats.

It has become evident that cells in the vascular wall produce superoxide anion using NAD(P)H as a major substrate (28). Thus, using a lucigenin chemiluminescence assay, we determined whether superoxide anion is highly produced in aorta from type 2 diabetic rats. Superoxide production was significantly accelerated in the aortic rings derived from OLETF rats, and the enhanced production of superoxide anion was inhibited to the level of control rats by treatment with DPI (Fig. 3). From this result, we noted that production of superoxide anion in diabetic vessels is achieved via the flavoprotein-containing enzymes, such as NAD(P)H oxidase, xanthine oxidase, cyclooxygenase, and NO synthase. In studies using vascular homogenates, NADPH, arachidonic acid, xanthine, and succinate showed no effects on superoxide production (Fig. 4). These findings excluded an important role for cyclooxygenase, xanthine oxidase, NO synthase, and mitochondrial electron transport as a potential mechanism for superoxide production, indicating that the targeted inhibition of elevated superoxide production by DPI is NADH oxidase.

The NAD(P)H oxidase expressed in the vascular tissues exhibits several differences from that expressed in phagocytes. The preferred substrate for oxidase in phagocytes is NADPH rather than NADH, while vascular oxidase favors

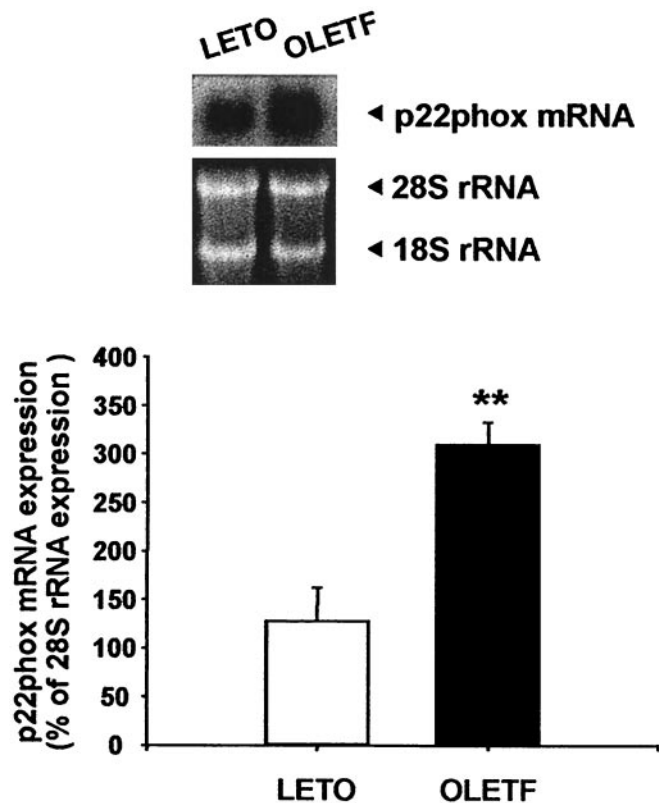


FIG. 6. The expression of p22phox mRNA in the thoracic aorta derived from LETO and OLETF rats. The top panel shows a representative Northern blot with the p22phox mRNA band at 0.8 kb. Ethidium bromide staining of the 28S and 18S ribosomal RNA is shown in the lower panel. This was used to normalize small differences in loading of total RNA or the efficiency of mRNA transfer to the membrane in each lane. ** $P < 0.01$ compared with LETO rats.

NADH (9,29,30). Previous studies showed that vascular NADH oxidase is a potent membrane-bound extramitochondrial oxidase for electron transfer to molecular oxygen (21,31). In accordance with those reports, our results clearly show that vascular NAD(P)H oxidase used NADH as a substrate for superoxide production, and no lucigenin signal was captured in the cytosolic fraction of the vasculature (Fig. 4). Furthermore, in the particulate fractions, the accelerated activity of NAD(P)H oxidase in OLETF rats was partially inhibited by DPI and blocked by SOD (Fig. 5). However, other oxidase inhibitors did not affect the NADH-derived superoxide production in particulate fractions of OLETF rats. By performing these assays, we concluded that OLETF rats have distinct characteristics—impaired endothelium-dependent vasodilation and profound superoxide production triggered by membrane-bound NAD(P)H oxidase using NADH predominantly as a substrate.

The vascular NAD(P)H oxidase is composed of several cytosolic and membrane-bound subunits. In particular, p22phox forming cytochrome b_{558} by making a complex with gp91phox is integral for the oxidase activation to produce superoxide anion. In a previous study, p22phox-mediated expression of cytochrome b_{558} in the absence of gp91phox was investigated (32); the importance of p22phox was increased. Therefore, there is a possibility that promoted superoxide production by NADH oxidase is influenced by increased mRNA expression of p22phox in

diabetic rats. After Northern blot analysis, we found that p22phox mRNA expression was markedly increased in diabetic vessels (Fig. 6). From these results, it is suggested that high production of superoxide anion in OLETF rats is the consequence of either stimulated NADH oxidase activity or elevated p22phox mRNA expression, or both.

To our knowledge, the precise mechanisms involved in the activation of vascular NAD(P)H oxidase in diabetes are still unknown, but hyperglycemia, a key clinical manifestation of diabetes, has been suggested to be an important determinant. Koya and King (33) have shown that high glucose levels activate protein kinase C in various vascular cells. Furthermore, it has been reported that high glucose levels stimulate the production of ROS through protein kinase C-dependent activation of NAD(P)H oxidase in vascular smooth muscle cells (34). According to the previous reports, protein kinase C may play an important role in the process of NAD(P)H oxidase activation; however, further studies are required to elucidate the precise molecular mechanisms involved in the activation of NAD(P)H oxidase in type 2 diabetes.

In conclusion, it is suggested that impaired endothelium-dependent vasodilation in OLETF rats is, in part, a result of increased NADH oxidase-dependent superoxide production via enhanced vascular expression of p22phox.

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