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Involvement of NAD(P)H Oxidase in a Potential Link between Diabetes and Vascular Smooth Muscle Cell Proliferation

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The cellular mechanisms that contribute to the acceleration of atherosclerosis in diabetes are poorly understood. Therefore, the potential mechanisms involved in the diabetes-dependent increase in vascular smooth muscle cell (VSMC) proliferation was investigated. Using primary culture of VSMC from streptozotocin-induced diabetic rat aorta, cell proliferation assay showed two-fold increase in cell number accompanied with enhanced superoxide generation compared to normal VSMC, 2 days after plating. Both the increased superoxide production and cell proliferation in diabetic VSMC were significantly attenuated by not only tiron (1 mM), a superoxide scavenger, but also by diphenyleneiodonium (DPI; 10 μM), an NAD(P)H oxidase inhibitor. NAD(P)H oxidase activity in diabetic VSMC was significantly higher than that in control cell, accompanied with increased mRNA expression of p22phox, a membrane subunit of oxidase. Furthermore, inhibition of p22phox expression by transfection of antisense p22phox oligonucleotides into diabetic VSMC resulted in a decrease in superoxide production, which was accompanied by a significant inhibition of cell proliferation. Based on these results, it is suggested that diabetes-associated increase in NAD(P)H oxidase activity via enhanced expression of p22phox contributes to augmented VSMC proliferation in diabetic rats.

Key Words: Diabetes, NAD(P)H oxidase, Superoxide, VSMC proliferation

INTRODUCTION

Atherosclerotic disease is a primary cause of death in diabetic patients (Garcia et al, 1974), and diabetes is a well-established risk factor for peripheral vascular disease caused by atherosclerosis (Melton et al, 1980). In the process of atherosclerotic lesion formation, abnormal proliferation of vascular smooth muscle cell (VSMC) is recognized as a key event (Ross, 1995). Several studies have shown that cultured VSMC from spontaneous autoimmune and streptozotocin (STZ)-induced diabetic rats grow faster than control cell (Larson et al, 1988; Kawano et al, 1993).

It is well known that diabetes is associated with increased oxidative stress (Giugliano et al, 1996), and accumulation of reactive oxygen species has also been reported in human and rodent diabetic vessels (Karasu et al, 1997; Inoguchi et al, 2000; Karasu, 2000). Increasing evidences suggest that reactive oxygen species may participate in signaling pathways stimulating VSMC growth and DNA synthesis (Griendling & Alexander, 1997). In particular, the importance of superoxide as a mediator involved in the regulation of VSMC growth has been outlined by several reports (Lee et al, 1998). However, the precise cellular mechanisms underlying the enhanced VSMC proliferation in diabetes remain unclear.

Among various potential sources of vascular superoxide production such as NAD(P)H-dependent oxidases (Ushio-Fukai et al, 1996), xanthine oxidase (White et al, 1996), lipoxygenase, mitochondrial oxidases, and NO synthases (Vasquez-Vivar et al, 1998), NAD(P)H oxidase is known as an important source of vascular superoxide production in animal models of diabetes (Kim et al, 2002). The activity of the NAD(P)H oxidase in vascular cell is modulated by extracellular signals, known to influence vascular remodeling and lesion development (Ushio-Fukai et al 1996). Furthermore, gene polymorphism affecting at least one of the subunits (p22phox) has been linked to the development of atherosclerosis in humans (Innoue et al, 1998; Cahilly et al, 2000).

Despite the importance of NAD(P)H oxidase in vascular pathobiology, its role in VSMC proliferation in diabetes has not yet been elucidated. Thus, we evaluated the role of superoxide in diabetes-associated VSMC proliferation as well as the characteristics and mechanisms of superoxide production in diabetic VSMC. Furthermore, the potential role of NAD(P)H oxidase, in particular p22phox, in the enhanced proliferative capacity of diabetic VSMC was investigated.

METHODS

Animals and induction of diabetes

Diabetes was induced with a single intraperitoneal

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ABBREVIATIONS: VSMC, Vascular smooth muscle cell; STZ, streptozotocin; NBT, nitroblue tetrazolium.

injection of streptozotocin (STZ, 55 mg/kg) in Sprague-Dawley male rats, weighing 250~300 g. Five weeks after STZ administration, blood glucose level was measured with a blood glucose test meter (Accutrend sensor, Roche Inc., Nutley, NJ). The rats were killed by injection of pentobarbital sodium (50 mg/kg, i.p.) and thoracic aorta was harvested for smooth muscle cell preparation.

Cell culture

Vascular smooth muscle cell (VSMC) from explants of thoracic aorta from control and STZ-induced diabetic rats were grown in DMEM containing 10% FBS, and cells between passages 2 and 5 were used for experiments. Cells were identified as VSMC on the basis of their morphological and growth characteristics. Briefly, VSMC exhibited a typical hill-and-valley growth pattern and also exhibited positive staining with antibody against α-smooth muscle actin, but no staining with antibody against factor VIII antigen.

Cell proliferation assay

VSMC was seeded on 24-well plates at 2×10^4 cells/well in 400 μl of DMEM containing 10% FBS. After 24 hrs, G0/early G1 synchronization was achieved by serum deprivation. To stimulate cell growth, media were switched to DMEM containing 10% FBS for 48 hrs. For the measurement of cell proliferation, MTT (0.5 μg/ml) was added and incubated for 4 hrs. After then, 400 μl of DMSO was added to dissolve the formazan crystals formed, and optical density was measured with an ELISA plate reader (Powerwave $\times$340 Bio-Tek instrument Inc., Winooski, VT) using test and reference wavelengths of 570 and 630 nm, respectively. Cell number was also counted from a parallel set of cultures after trypsinization of the cells by using a hemocytometer.

Measurement of superoxide production

VSMC was incubated with nitroblue tetrazolium (NBT) in order to allow superoxide, generated by the cell, to reduce NBT to blue formazan. In the present experiment, after NBT (1 mg/ml) was added to the growth medium, the generation of insoluble formazan was verified by microscopic examination. After removal of the medium, the cells were lysed and formazan was dissolved with 1.4 volumes of 2 M KOH and DMSO. Absorbance of formazan was measured at 654 nm with an ELISA plate reader (Powerwave $\times$340 Bio-Tek Instrument Inc.). The quantity of formazan was calculated by using extinction coefficient of blue formazan (0.72 L/mmol/mm).

Measurement of NAD(P)H oxidase activity

VSMC was washed twice with ice-cold phosphate-buffered saline (PBS), and collected by scrapping with 1ml PBS. The cells were centrifuged at 3,000 rpm for 10 min, and the pellets were homogenized with equal volume of lysis buffer containing various protease inhibitors [20 mM monobasic potassium phosphate (pH 7.0), 1 mM EGTA, 10 μM aprotinin, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.5 mM phenylmethlysulfonyl fluoride] and incubated for 30 min on ice. The homogenate was centrifuged at 12,000 rpm for 20 min to remove unbroken cells and debris. Protein content was determined by using a bicinchoninic acid protein assay kit (Sigma Chemical Co., St. Louis, MO). NAD(P)H oxidase activity was measured by measuring NAD(P)H-induced superoxide production with lucigenin-enhanced chemiluminescence. This assay was performed in Krebs/HEPES buffer with 25 μg protein and 25 μM lucigenin as the electron acceptor. The reaction was started by addition of various substrates such as NADH (100 μM), NADPH (100 μM), xanthine (100 nM), arachidonic acid (100 μM), and succinate (5 mM). The photon emission was measured every 15 sec for 10 min in a microtiterplate luminometer (Microlumat LB96P, EG&G Berthold, Germany). A buffer blank containing lucigenin was subtracted (<5% of the cell signal) from each reading before transformation of the data by comparison with standard curve generated with xanthine/xanthine oxidase. In some experiments, inhibitors were added to samples 10 min before readings.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol regent (Gibco-BRL), as described in the product protocol. The PCR primers for amplification of p22phox mRNA were based on the published rat aortic smooth muscle cell sequence: sense; 5'-GACGCTTCACGCAGTGGTACT-3' and antisense; 5'-CAC GACCTCATCTGTCACTGG-3'. Reaction products were separated by 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide.

Transfection of antisense oligonucleotides

To transfect oligonucleotides, VSMC was seeded in 24-well plates and grown in DMEM containing 10% FBS. After 24 hrs cells were washed with serum-free medium and then transfected with sense or antisense oligonucleotides (1 μg) with the aid of Lipofectin reagent (Gibco-BRL) in serum free medium for 24 hrs. The sequences of oligonucleotides were as follows: sense p22phox: 5'-GGTCCTCA CCATGGGGCAGATC-3' and antisense p22 phox: 5'-GATCT GCCCCATGGTGAGGACC-3'. After washing, 10% FBS-containing DMEM was applied for 24 hrs, and then assayed for superoxide production or cell proliferation.

Statistical analysis

Data were expressed as means$\pm$SEM. Statistical comparisons between two groups were performed by Student's t-test, whereas comparisons among multiple groups were analyzed with one-way ANOVA. When the p value was $<$ 0.05 with ANOVA, the Bonferroni's correction for multiple comparisons was used to evaluate the significance of difference between groups.

RESULTS

At the time of death, blood glucose levels were 145$\pm$7 mg/dL and 593$\pm$40 mg/dL in control and STZ-induced diabetic rats, respectively.

Enhanced proliferative capacity of diabetic VSMC

By using cultured VSMC from the aorta of control and STZ-induced diabetic rats, the kinetics of proliferation was assessed. Thus, cells were plated at a density of 2×10^4 cells/well, maintained in serum-rich medium and the number of cells was counted every day over a period of 2 days. As seen in Fig. 1A, VSMC from diabetic rat showed approximately two-fold increase in cell number, 2 days after plating compared to the control (diabetic VSMC = 8.2×10^4 cells/well vs. control VSMC=4.6×10^4 cells/well, $p<0.01$). Consistent with these findings, in cultures maintained for 2 days in 10% FBS medium, the MTT assay also showed approximately two-fold increase in activity in diabetic VSMC compared to the control VSMC (Fig. 1B). These results suggest that diabetic VSMC proliferates faster than control cells, when stimulated with serum-rich medium.

Pharmacological modulation of VSMC proliferation

To assess whether alteration of reactive oxygen species generation could modulate VSMC proliferation, growth of 10% FBS-stimulated cells was determined by either inhibiting the generation of reactive oxygen species (DPI) or facilitating their removal (superoxide: tiron and SOD).

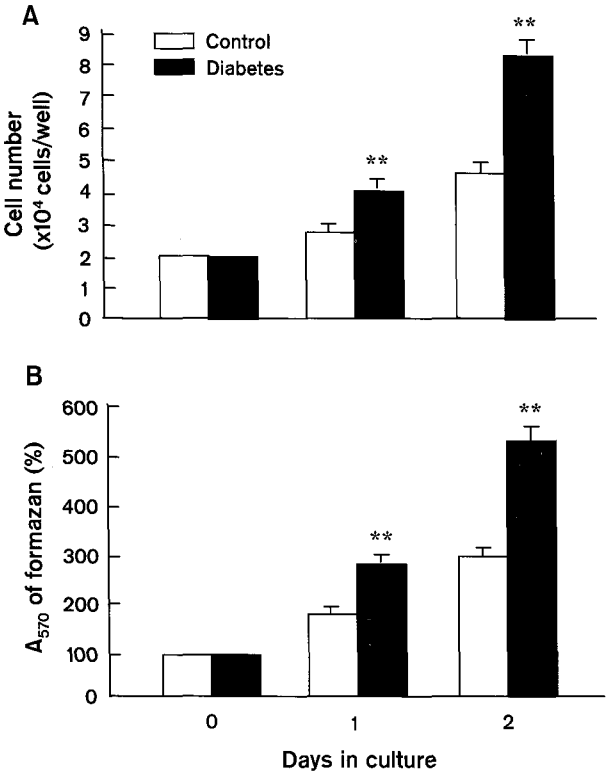

Fig. 1. Comparison of FBS-induced cell proliferation in control and diabetic VSMC. Cells were plated in the 24-well plates and synchronized, and then the cell cycle was initiated with 10% FBS. A) Cultures were counted with a hemocytometer. B) Cell proliferation was assayed by using MTT method, and data show percent of the formazan absorbance in day 0. Data are mean±SEM from triplicate determinations repeated in 4 separate experiments. **$p<0.01$ vs. corresponding value in control.

As shown in Fig. 2, while tiron (1 mM) itself, a cell membrane permeable superoxide scavenger, had a weak effect (18% of vehicle level) on control VSMC proliferation, it markedly attenuated the enhanced proliferation of diabetic VSMC (approximately 75% of vehicle level). In contrast to tiron, SOD (500 units/ml), which is not permeable to cell membrane, failed to prevent the enhanced cell proliferation in both control and diabetic VSMC, suggesting a role of intracellular superoxide in VSMC proliferation.

Since NAD(P)H oxidase is known as the most important source of superoxide in vascular cells, the effect of DPI, an inhibitor of flavin containing enzyme such as NAD(P)H oxidase, was assessed on the 10% FBS-stimulated cell proliferation in both control and diabetic VSMC. DPI (10 μM) markedly attenuated 10% FBS-stimulated cell proliferation in diabetic VSMC, whereas it caused a slight decrease in control VSMC proliferation. These results suggest that NAD(P)H oxidase is more important in proliferative signaling of diabetic VSMC than control cells.

Quantification of superoxide generation by NBT reduction

Since NBT reduction is very sensitive to low levels of superoxide because of accumulation of blue formazan over time in the cell, we used this indicator in the present study. Incubation of cells with NBT resulted in blue formazan staining, and the NBT reduction accumulated in VSMC is shown in Fig. 3. The NBT reduction in diabetic VSMC (0.42 ±0.02 μmol/min/10^5 cells) was significantly higher than that in control cells (0.27 ± 0.01 μmol/min/10^5 cells). While tiron (1 mM) had a weak inhibitory effect on NBT reduction in control VSMC, the enhanced NBT reduction in diabetic cells was markedly attenuated by treatment with tiron (1 mM), a cell membrane permeable superoxide scavenger, but not by treatment with SOD (500 units/ml), which is not permeable to cell membrane.

The effects of inhibitors on superoxide generated by different enzymatic sources are also shown in Fig. 3. DPI

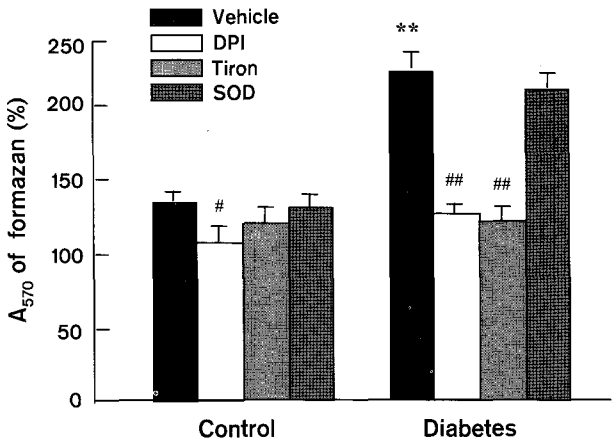

Fig. 2. Effect of DPI (10 μM), tiron (1 mM), and SOD (500 units/ml) on 10% FBS-stimulated cell proliferation in control and diabetic VSMC. Cell proliferation was measured for 24 hrs and expressed as percentage of the value in day 0. Values are means±SEM from 4 independent experiments. **$p<0.01$ vs. corresponding value in control. $^{\#}p<0.05$; $^{\#\#}p<0.01$ vs. vehicle.

(10 μM), an NAD(P)H oxidase inhibitor, significantly inhibited NBT reduction in diabetic VSMC from 0.42± 0.02 μmol/min/10^5 cells to 0.26±0.02 μmol/min/10^5 cells, but not in control cell. Interestingly, neither L-NAME (10 μM), a nitric oxide synthase inhibitor, nor allopurinol (10 μM), a xanthine oxidase inhibitor, significantly decreased NBT reduction in diabetic VSMC. These results indicate that a flavoprotein-dependent oxidase is more important than nitric oxide synthase or xanthine oxidase, but not is exclusive pathway for intracellular superoxide generation in diabetic VSMC.

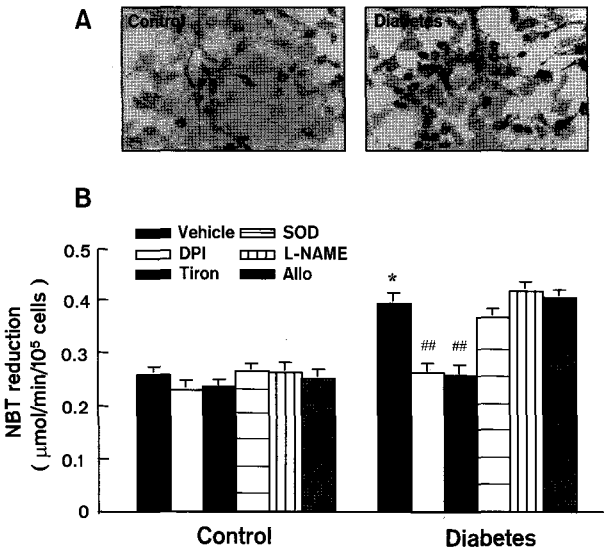

Fig. 3. A: Representative photographs of control and diabetic VSMC stained with NBT. B: Effects of various inhibitors such as DPI (10 μM), tiron (1 mM), SOD (500 units/ml), L-NAME (10 μM), and allopurinol (Allo; 10 μM) on superoxide generation in control and diabetic VSMC. Data are shown as mean±SEM from 4 experiments in each group. *$p<0.05$ vs. corresponding value in control. ##$p<0.01$ vs. vehicle.

Changes in NAD(P)H oxidase activity

To ascertain that NAD(P)H oxidase is the major pathway responsible for oxidative stress in the diabetic vasculature, NAD(P)H-induced superoxide generation of cell homogenates from control and diabetic VSMC was measured with the use of lucigenin chemiluminescence. As shown in Fig. 4, lucigenin chemiluminescence was strongly stimulated with NADH in both control and diabetic VSMC, but not by other substrates such as NADPH, xanthine, arachidonic acid, and succinate. In the present experiment, NADH-stimulated superoxide production in diabetic VSMC (24.11±1.2 nmol/min/mg protein) was significantly greater than that in control VSMC (5.51±2.1 nmol/min/mg protein). Furthermore, NADH-stimulated superoxide generation in diabetic VSMC was attenuated by treatment with DPI, but not by other inhibitors such as allopurinol (xanthine oxidase inhibitor; 100 μM), rotenone (inhibitor of mitochondrial electron transport; 100 μM), L-NAME (nitric oxide synthase inhibitor; 10 μM), and indomethacin (cyclooxygenase inhibitor; 10 μM)(Fig. 5).

Effect of p22phox antisense oligonucleotides on NBT reduction

Since it has previously been demonstrated in VSMC that up-regulation of p22phox is a pivotal mechanism for NAD(P)H oxidase activation (Griendling et al, 1994), we investigated the relative abundance of p22phox mRNA in VSMC by using RT-PCR assay. As shown in Fig 6A, relative quantification of mRNA band intensity, normalized to β-actin, revealed that level of p22phox mRNA expression was higher in diabetic VSMC than control cells. The expression of p22phox mRNA in both control and diabetic VSMC was substantially reduced in cells transfected with p22phox antisense oligonucleotides, compared to cells treated with vehicle or transfected with sense oligonucleotides.

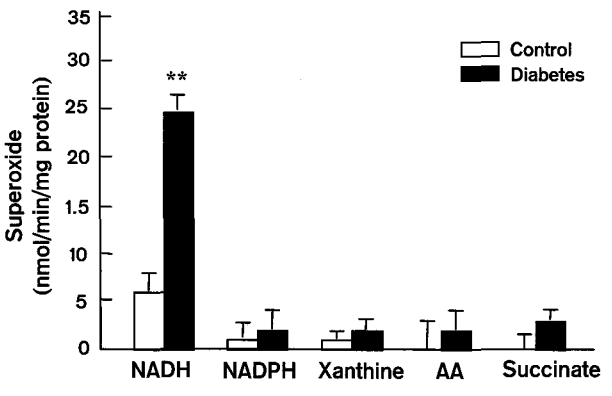

Fig. 4. Superoxide generation from VSMC homogenates was determined by lucigenin-enhanced chemiluminescence in response to potential oxidase enzyme substrates, such as NADH (100 μM), NADPH (100 μM), xanthine (100 nM), arachidonic acid (AA; 100 μM), and succinate (5 mM). Bars represent mean±SEM from 5 measurements. **$p<0.01$ vs. corresponding value in control.

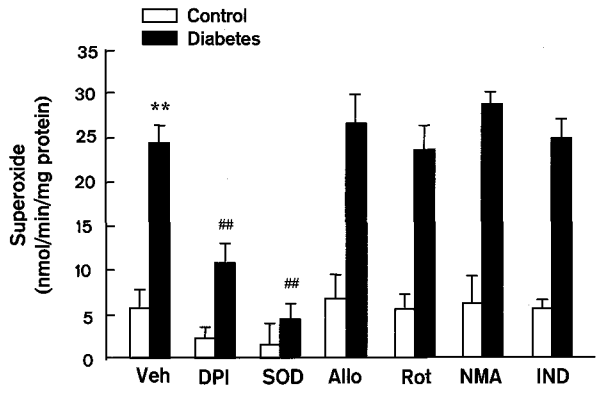

Fig. 5. Effect of various inhibitors, such as DPI (10 μM), SOD (500 units/ml), allopurinol (Allo; 100 μM), rotenone (Rot; 100 μM), L-NAME (NMA; 10 μM), and indomethacin (IND; 10 M), on NADH-stimulated superoxide production in control and diabetic VSMC. Data are shown as mean±SEM from 5 experiments in each group. **$p<0.01$ vs. corresponding value in control. ##$p<0.01$ vs. vehicle.

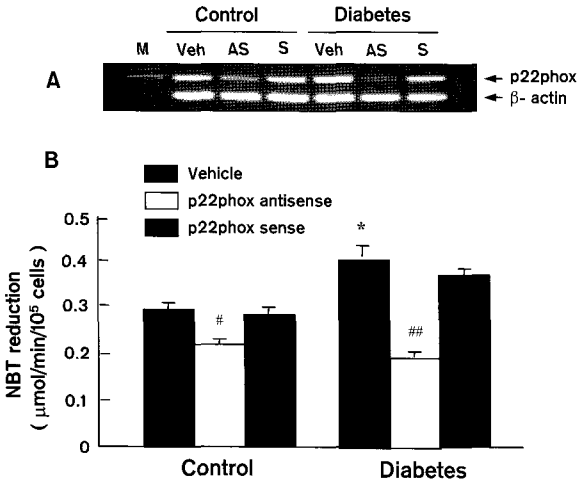

Fig. 6. Effect of transfection of p22phox antisense or sense oligonucleotides on cellular superoxide production in control and diabetic VSMC. A: Expression of p22phox mRNA by RT-PCR. M, molecular marker; Veh, vehicle; AS, antisense oligonucleotides; S, sense oligonucleotides. B: Cellular superoxide production measured by NBT reduction. Data are shown as mean±SEM from 5 experiments in each group. *$p<0.05$ vs. corresponding value in control. #$p<0.05$; ##$p<0.01$ vs. vehicle.

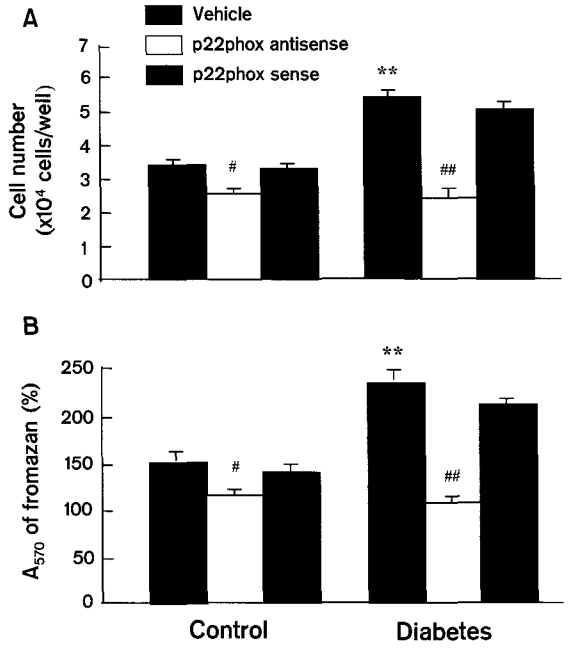

Fig. 7. Effect of transfection of antisense or sense p22phox oligonucleotides on FBS-induced cell proliferation in control and diabetic VSMC. A: Cultures were counted with a hemocytometer. B: Cell proliferation was assayed by using MTT assay and expressed as the percentage of the value in day 0. Results are presented as means±SEM for 5 independent experiments. **$p<0.01$ vs. corresponding value in control. #$p<0.05$; ##$p<0.01$ vs. vehicle.

As shown in Fig. 6B, although inhibition of p22phox expression by antisense oligonucleotides was associated with the attenuated cellular production of superoxide in both control and diabetic VSMC, the inhibitory effect of antisense oligonucleotides on superoxide production in diabetic VSMC was significantly higher than that in control cells, confirming the functional role of p22phox in the overproduction of superoxide in diabetic vasculature.

Effect of p22phox antisense oligonucleotides on VSMC proliferation

As shown in Fig. 7, analysis of the cell proliferation data revealed that inhibition of p22phox expression with antisense oligonucleotides was associated with a significant down-regulation of VSMC proliferation. The inhibitory effect of antisense oligonucleotides on cell proliferation was significantly higher in diabetic VSMC than that in control cell.

DISCUSSION

The major findings of this study were that (1) VSMC from diabetic rat aorta grew at a higher rate than cells from non-diabetic rats, (2) superoxide production was higher in diabetic VSMC compared with that in control cell, (3) NADH oxidase activity and p22phox expression were higher in diabetic VSMC, and (4) both the increased superoxide production and cell proliferation in diabetic VSMC were ameliorated by transfection with p22phox antisense oligonucleotides. The data presented here provide evidences defining the molecular pathways that lead to diabetes-associated regulation of intracellular redox state in VSMC and establish a sequential link between diabetes-associated NAD(P)H oxidase activity, an increase in intracellular superoxide, and VSMC proliferation.

Diabetes frequently complicates atherosclerosis (Kannel et al, 1979). Various risk factors associated with diabetes, such as abnormal metabolism of lipoprotein (Kannel et al, 1971), hyperinsulinemia (Stout, 1968), advanced glycated end products (Brownlee et al, 1988) and enhanced platelet aggregation (Bern, 1978), may cause atherosclerosis, but any single risk factor by itself does not explain the relationship between diabetes and atherosclerosis. Thus, it is suggested that the mechanism of diabetic macroangiopathy should be studied from the view points of characteristic changes in diabetic arterial cells.

VSMC exist in a diverse range of phenotypes, and the transition of VSMC from a contractile to a synthetic, proliferative state (phenotypic modulation) appears to be an early event in the pathogenesis of atherosclerosis (Owens, 1995; Karin & Bornfeldt, 1996). Numerous studies using VSMC from diabetic animals and human have demonstrated an increased migratory and proliferative phenotype (Kimura et al, 1998; Absher et al, 1999). In accordance with the previous reports, in the present experiment, VSMC from the aorta of STZ-induced diabetic rats grew at a higher rate and to higher densities than cells from control rats. Furthermore, while tiron (1 mM) itself, a cell membrane permeable superoxide scavenger, had a weak effect on FBS-induced cell proliferation in control VSMC, it markedly enhanced proliferation of diabetic VSMC, suggesting that enhanced superoxide production may be an important modulator of diabetic VSMC proliferation. However, in contrast to tiron, SOD (500 units/ml), which is not permeable to cell membrane, failed to prevent the enhanced cell proliferation in both control

and diabetic VSMC, suggesting a role of intracellular superoxide in VSMC proliferation.

Since NAD(P)H oxidase is known as the most important source of superoxide in vascular cells, the effect of DPI, an inhibitor of flavin containing enzymes such as NAD(P)H oxidase, was assessed on the FBS-stimulated cell proliferation in both control and diabetic VSMC. DPI (10 μM) markedly attenuated FBS-stimulated cell proliferation in diabetic VSMC, whereas it had a slight effect on cell proliferation of control cell. These results suggest that NAD(P)H oxidase is more important for the proliferative signaling in diabetic VSMC than that in control cells.

In animal models and human diabetes, evidences suggest that increased superoxide production is a feature of systemic vascular disease states and may promote atherogenesis. Potential sources of vascular superoxide production include NAD(P)H-dependent oxidases (Ushio- Fukai et al, 1996), xanthine oxidase, lipoxygenase, mitochondrial oxidases, and NO synthases (Vasquez-Vivar et al, 1998). NAD(P)H oxidase appears to be the principal source of superoxide production in several animal models of vascular diseases, including diabetes (Kojda & Harrison, 1999; Warnholtz et al, 1999). In accordance with the previous reports, our results clearly showed that superoxide production in diabetic VSMC was more prominent than that in control cells. The enhanced superoxide production in diabetic VSMC was markedly reduced by treatment with DPI, an NAD(P)H oxidase inhibitor, but not by inhibitors for other oxidases such as xanthine oxidase and nitric oxide synthase, suggesting that NAD(P)H oxidase is involved in the enhanced production of superoxide in diabetic VSMC. This suggestion is based on measurement of oxidase activity in VSMC, in which cellular superoxide production was strongly stimulated with NADH in both control and diabetic VSMC, but not by other substrates such as NADPH, xanthine, arachidonic acid, and succinate. Furthermore, in the present experiment, NADH-induced superoxide production in diabetic VSMC was significantly greater than that in control cells.

NAD(P)H oxidase is a multisubunit complex with 2 membrane-associated subunits (p22phox and gp91phox) and 3 cytosolic subunits (p40phox, p47phox, and p67phox), regulated by Rac G proteins, and this oxidase has been previously identified in cultured VSMC and endothelial cells (Bayraktutan et al, 1998; Mohazzab et al, 1994). Although its role in signal transduction is, to date, only partially understood, p22phox, one of the electron transfer elements of NADH oxidase, is absolutely required for enzyme activity (Ushio-Fukai et al, 1996). By using Northern blot analysis of p22phox mRNA expression in the aorta from type 2 diabetic rats, we previously demonstrated that its expression in diabetic rat was higher than that in control rat (Kim et al, 2002). Consistent with the previous results, we demonstrated in the present experiment that the expression of p22phox in diabetic VSMC was increased, accompanied with enhanced superoxide production. The enhanced superoxide production in diabetic VSMC was reduced by transfection of p22phox antisense oligonucleotides but not by sense oligonucleotides, suggesting that p22phox is directly involved in NADH oxidase enzyme activity in diabetic VSMC. However, novel homologues of the gp91phox subunit, such as Mox-1, have been described in a variety of cell subtypes, including VSMC (Suh et al, 1999), and studies in knockout mice suggest redundancy among the NAD(P)H oxidase subunits (Hsich et al, 2000). Therefore, future work is required to assess the importance of homologous NAD(P)H oxidase subunits in VSMC superoxide production and to identify how expression and activity of the NAD(P)H oxidase components are regulated in diabetes.

In addition to providing insights into the mechanisms involved in enhanced vascular production of superoxide in diabetes, our present data demonstrated identify that inhibition of NADH oxidase by DPI attenuated the enhanced cell proliferation of diabetic VSMC. Since nonspecific effects of DPI on other flavoproteins by direct binding could not be excluded (Majander et al, 1994), we further confirmed the finding in p22phox-deficient cells where FBS-stimulated VSMC proliferation was markedly decreased, thus providing additional support for a central role of p22phox in VSMC proliferation. In accordance with the effect of antisense oligonucleotides on superoxide production, the inhibitory effect of antisense oligonucleotides on cell proliferation was bound to be significantly greater in diabetic VSMC than that in control cell, further supporting the involvement of p22phox subunit in superoxide production in diabetic vasculature and it's necessity for VSMC proliferation. Taken together, although NAD(P)H oxidase is a normal component of signaling machinery that might have been parasitized to serve VSMC proliferation, the present study demonstrates that activation of NAD(P)H oxidase via the enhanced p22phox expression is a link between diabetes, superoxide production and enhanced VSMC proliferation.

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