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The Role of Janus Kinase in Superoxide-mediated Proliferation of Diabetic Vascular Smooth Muscle Cells

Ji Young Lee, Ji Young Park, and Chi Dae Kim

Department of Pharmacology, College of Medicine, and Medical Research Center for Ischemic Tissue Regeneration, Pusan National Universit Busan 602-739, Korea

To elucidate a potential molecular link between diabetes and atherosclerosis, we investigated the role of Janus tyrosine kinase (JAK) for NAD(P)H oxidase-derived superoxide generation in the enhanced proliferative capacity of vascular smooth muscle cells (VSMC) of Otsuka Long-Evans Tokushima Fatty (OLETF) rat, an animal model of type 2 diabetes. An enhanced proliferative response to 10% fetal bovine serum (FBS) and superoxide generation with an increased NAD(P)H oxidase activity were observed in diabetic (OLETF) VSMC. Both the enhanced proliferation and superoxide generation in diabetic VSMC were significantly attenuated by AG490, JAK2 inhibitor, and PP2, Src kinase inhibitor. Tyrosine phosphorylation of proteins in diabetic VSMC, especially JAK2, was increased compared to control VSMC. Furthermore, the enhanced NAD(P)H oxidase activity in diabetic VSMC was significantly attenuated by AG490 in a dose-dependent manner. Together, these results indicate that the signal pathway which leads to diabetes-associated activation of Src kinase/JAK is critically involved in the diabetic VSMC proliferation through NAD(P)H oxidase activation and superoxide generation.

Key Words: JAK2, VSMC, Superoxide, Proliferation, NAD(P)H oxidase

INTRODUCTION

Diabetes mellitus complicates frequently atherosclerosis (Kannel & Mcgree, 1979), and atherosclerotic disease is a primary cause of death in diabetic patients (Garcia et al, 1974). In the process of atherosclerotic lesion formation, abnormal proliferation of vascular smooth muscle cell (VSMC) has been recognized as a key event (Ross, 1995; Faries et al, 2001). The progression of atherosclerotic lesions is accelerated by diabetes (Ruderman & Haudenschild, 1984), but the cellular mechanisms underlying the accelerated progression of atherosclerotic lesions in diabetic arteries are not well understood. Nevertheless, reactive oxygen species (ROS) has recently been implicated (Vendrov et al, 2006; Ruef et al, 2000).

Recent evidence suggests that ROS are essential mediators of cell signaling, initiated by growth factors and cytokines (Griendling & Alexander, 1997). Stimulation of VSMC by growth factors such as platelet-derived growth factor, fibroblast growth factor (Sundaresan et al, 1995) and thrombin (Ruef et al, 1999) enhances ROS generation. Thus, oxidative stress, which represents a consequence and a cause of vascular dysfunction, appears to be involved in mediating and sustaining abnormal VSMC growth during atherosclerosis. However, the mechanisms by which ROS mediate cell growth remain unclear.

Tyrosine phosphorylation of cellular proteins is key determinants of cell growth and differentiation in response to mitogenic signaling. The Janus tyrosine kinase (JAK)/signal transducer and activators of transcription (STAT) pathway are important links between activation of cell surface receptors and nuclear transcriptional events leading to cell growth (Darnell, 1998). Once tyrosine-phosphorylated by JAK, activated STAT dimer translocates to the nucleus to transactivate target gene expression (Darnell, 1998). The activation of the JAK/STAT pathway has recently been observed in response to generation of intracellular ROS (Modesti et al, 2005; Madamanchi et al, 2001). Especially, p47 phox and small G protein rac, which is a cytosolic component of NADPH oxidase, regulate the JAK2 pathway through activation of the NADPH oxidase and production of ROS (Schieffer et al, 2000; Pelletler et al, 2003).

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 the 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 cells is modulated by extracellular signals which are known to influence vascular remodeling and lesion development (Ushio-Fukai et al

Corresponding to: Chi Dae Kim, Department of Pharmacology, College of Medicine, Pusan National University, 10, Ami-dong 1-ga, Seo-gu, Busan 602-739, Korea. (Tel) 82-51-240-7730, (Fax) 82-51-244-1036, (E-mail) chidkim@pusan.ac.kr

ABBREVIATIONS: VSMC, vascular smooth muscle cells; ROS, reactive oxygen species; OLETF, Otsuka Long-Evans Tokushima Fatty; LETO, Long-Evans Tokushima Otsuka.

1996). Furthermore, gene polymorphism which affects 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 tyrosin kinase in vascular pathobiology, its role in VSMC proliferation in diabetes has not yet been elucidated. Furthermore, it is not known whether NAD(P)H oxidases are affected by JAK/STAT signal in diabetic VSMC. Thus, we evaluated the involvement of JAK in NAD(P)H oxidase activation leading to superoxide production in diabetes-associated VSMC proliferation. For a model of spontaneous type II diabetes, the Otsuka Long-Evans Tokushima Fatty (OLETF) rat was used.

METHODS

Animals

All procedures were in accordance with Institutional Guideline for Animal Research. Otsuka Long-Evans Tokushima Fatty (OLETF) male rats, animal model of type 2 diabetes, and LETO (Long-Evans Tokushima Otsuka, a genetic control for OLETF rats) rats were generously donated by the Tokushima Research Institute (Otsuka Pharmaceutical Co., Tokushima, Japan). All rats were kept in a pathogen-free facility under controlled temperature (23± 2°C) and humidity (55±5%). Blood glucose and insulin levels were determined by a blood glucose test meter (Accutrend sensor, Roche Inc., Nutley, NJ) and an enzyme-linked immunosorbent assay kit (Wako Chemicals, Tokyo), respectively. At the time of the study, the rats were 50 wks old.

Cell culture

VSMC were grown from explants of thoracic aorta from LETO and OLETF rats. 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. VSMC were grown in DMEM containing 10% fetal bovine serum (FBS), and cells between passages 2 and 5 were used for experiments.

Cell proliferation assay

VSMC at 1×10^4 cells/well were seeded on 24-well plates in 400 μl of DMEM containing 10% FBS. After 24 hrs, G_0/early G_1 synchronization was achieved by serum deprivation. To stimulate cell growth, media were switched to DMEM containing 10% FBS in the absence or presence of various inhibitors. For the measurement of cell proliferation, methylthiazoletetrazolium (MTT, 0.5 μg/ml) was added and incubated for 4 hrs. Subsequently, 400 μl of dimethyl sulfoxide was added to dissolve the formazan crystals formed, and optical density was measured with an ELISA plate reader (Powerwave×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.

Protein analysis by Western blot

Western blot analysis was performed as described previously (Habib et al, 1993). Cells were harvested, washed twice with ice-cold PBS, and lysed in a TNN buffer (50 mM Tris-HCl, pH 8.0, 120 mM sodium chloride, 0.5% NP-40) that was supplemented with protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 100 μg/ml PMSF, 5 μg/ml of pepstatin, and 1 mM DTT) and phosphatases inhibitors (20 mM NaF and 2 mM Na_3VO_4) for 1 hr on ice by vortexing every 10 min. Lysates were centrifuged at 12,000 rpm for 30 min to remove insoluble material. The protein concentration was determined by Lowry's method (Sigma, USA) using bovine serum albumin (BSA) as a standard. Equal amounts of proteins were resolved on SDS-PAGE gels. The proteins on the gels were subsequently transferred onto a nitrocellulose membrane (Hybond C, Amersham Corp.). Antibodies to phosphotyrosine and donkey anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies were used at 1 : 1,000 dilution. Proteins were detected by an enhanced chemiluminescence (ECL) reagent using a commercial kit (Amersham Life Science).

Measurement of superoxide production

VSMC were incubated with nitroblue tetrazolium (NBT) in order to allow superoxide, generated by the cells, to reduce NBT to blue formazan. In the present experiment, the generation of insoluble formazan was verified by microscopic examination after NBT (1 mg/ml) was added to the growth medium. After removal of the medium, the cells were lysed and formazan was dissolved with 1.4 volumes of 2 M KOH and dimethyl sulfoxide. Absorbance of formazan was measured at 654 nm with an ELISA plate reader. 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

NAD(P)H oxidase activity was measured as described previously (Griendling et al, 1994). Briefly, VSMC that had been exposed to inhibitors for 4 hrs in the presence of 10% FBS were washed and were scraped from the plate. The cells were centrifuged at 1,000 g for 5 min, and the pellets were homogenized with an 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 20 min on ice. The homogenate was centrifuged at 1,000 g for 10 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 using lucigenin chemiluminescence. This assay was performed in Krebs/HEPES buffer with 25 μM lucigenin as the electron acceptor, and NADH/NADPH (100 μM) as substrates. The reaction was started by the addition of 25 μg of protein, and photon emission was measured every 15 sec for 10 min in a microtiterplate luminometer (Microlumat LB96P, EG and G Berthold, Germany). A buffer blank containing lucigenin was subtracted from each reading before transformation of the data by comparison with standard curve generated with

xanthine/xanthine oxidase.

Statistical analysis

Data were expressed as means±SE. 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

Enhanced proliferative capacity of diabetic VSMC

To compare the VSMC proliferative capacity from diabetic rats (OLETF) with wild type rats (LETO), cell growth was assessed by cell counting and MTT assay. VSMC from diabetic rats (OLETF) showed approximately two-fold increase in cell number 2 days after plating, compared to the control (LETO) cells (Fig. 1). In the cultures maintained for 3 days in 10% FBS medium, the MTT assay also showed approximately 2.5 fold increase of activity in diabetic VSMC, compared to the control cells. These results suggest that diabetic VSMC proliferate faster than control cells, when stimulated with serum-rich medium.

Involvement of JAK2 in enhanced proliferation o diabetic VSMC

Increased tyrosine phosphorylation of cellular proteins occurs by activation of tyrosine kinases and has been shown to mediate the effects of growth factors (DaSilva et al, 1994), therefore. we examined tyrosine phosphorylation of proteins by Western blot with anti-phosphotyrosine antibody in VSMC from LETO and OLETF rats as an early event in signaling cascade activation in type 2 diabetes. Fig. 2 illustrates that tyrosine phosphorylation of 130 kDa protein was significantly increased in OLETF VSMC, this band most likely corresponding to JAK2. To further ascertain this band, we investigated the effects of AG 490, which is a specific JAK2 specific inhibitor, on tyrosine phosphorylation. As shown in lane 2 of Fig. 2A, AG 490 inhibited the 10% FBS-induced tyrosine phosphorylation of 130 kDa, JAK2, expression in diabetic VSMC. In contrast, however, it did not suppress the phospho-JAK2 protein level in the control VSMC. Since Src kinase can be upstream of JAK (Wang et al, 2000), we analyzed the level of phospho-JAK2 after treatment of the cells with Src kinase inhibitor, PP2. This inhibitor blocked the tyrosine phosphorylation of JAK2, similar to the inhibitory effect of AG490 in OLETF rat (Fig. 2B, lane 4).

Because of increased JAK activation in VSMC proliferation (Marrero et al, 1997), we examined the effect of AG490 on cell proliferation. In diabetic VSMC, AG490 suppressed the cell proliferation to the level of control VSMC (Fig. 3A). These findings indicate the JAK2 activation is involved in growth signal of diabetic VSMC proliferation.

Modulation of superoxide production by JAK2

Recent studies have shown that JAK/STAT signaling pathway was involved in oxidative stress (Modesti el al, 2005; Yu et al, 2006). To assess whether alteration of ROS generation could be modulated by JAK2, the growth of 10% FBS-stimulated diabetic cells was determined by either facilitating or inhibiting the ROS generation with JAK2 inhibitor. Since NBT reduction is very sensitive to low levels of superoxide because of accumulation of blue for-

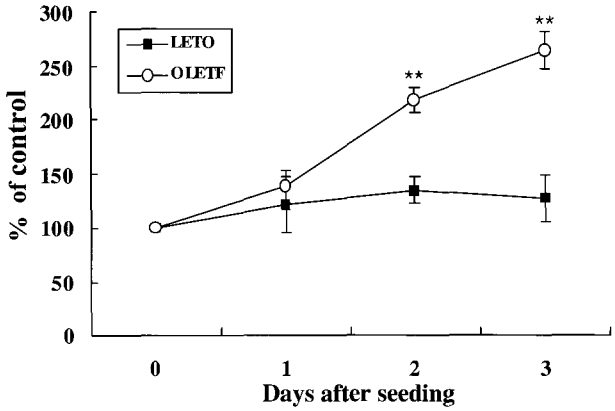

Fig. 1. Comparative study on 10% FBS-stimulated cell proliferation in LETO and OLETF VSMC. Cells were seeded in 24-well plates, synchronized, and then the cell cycle was initiated with 10% FBS. Cell proliferation was also measured by MTT assay, and data show percent of formazan absorbance on day 0. Data represent mean± SE from triplicate determinations repeated in 4 separate experiments. **$p<0.01$ vs. each vehicle.

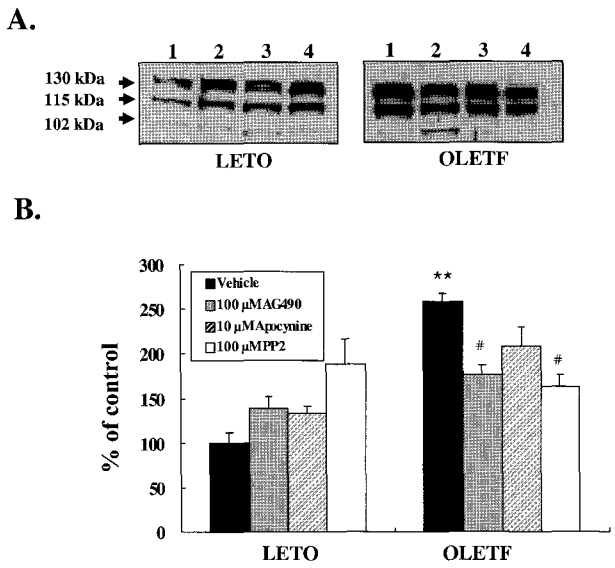

Fig. 2. Enhanced tyrosine phosphorylation of proteins in diabetic VSMC. Tyrosine phosphorylations of proteins were analyzed by Western blot. (A) Phosphotyrosine-proteins in 10% FBS-stimulated LETO and OLETF VSMC. Lane 1, control with 10 % FBS; lane 2, treatment of 100 μM AG490; lane 3, treatment of 100 μM apocynine; lane 4, treatment of 100 μM PP2. (B) Quantization of tyrosine phosphorylation was performed by densitometric analysis. **$p<0.01$ vs. vehicle of LETO rats, $^{\#}p<0.05$ vs. vehicle of OLETF rats.

mazan in cells, this indicator was used in the present study. Incubation of cells with NBT resulted in the formazan staining. As shown in Fig. 3B, the NBT reduction in the diabetic VSMC was significantly higher than that in the control cells. On the other hand, the enhanced NBT reduction in diabetic VSMC was significantly attenuated by AG 490 (maximally to 40.3% of the vehicle level). These results demonstrate that tyrosine phosphorylation of JAK2 plays important roles in superoxide generation of diabetic VSMC.

Effects of PP2 on the superoxide-mediated VSMC proliferation

It has been found that Src family kinase is required for cell proliferation by JAK activation (Simon et al, 2002; Proietti et al, 2005). To test whether the redox imbalance in enhanced diabetic VSMC proliferation is modulated by Src kinase, the growth of 10% FBS-stimulated diabetic cells was determined by inhibiting the ROS generation and cells proliferation with PP2, Src kinase inhibitor. As shown in Fig. 4, PP2 had an inhibitory effect on enhanced diabetic VSMC proliferation (Fig. 4A). Also, it markedly attenuated the enhanced ROS production of diabetic VSMC (Fig. 4B). These results indicate that Src kinase, cytosolic tyrosine kinase, plays an important role in ROS-facilitated diabetic VSMC proliferations via phospholylation of JAK2.

Effect of AG490 on NAD(P)H consumption

Since NAD(P)H oxidase is known as the most important source of superoxide in vascular cells, the effect of AG490 to decrease ROS generation and proliferation of diabetic VSMC was assessed by determining the NAD(P)H oxidase activity. NAD(P)H oxidase activity in the control and diabetic VSMC was determined by using lucigenin enhanced chemiluminescence assay. As seen in Fig. 5, AG490 had a weak inhibitory effect on NAD(P)H consumption in the control VSMC. However, 100 μM AG490 significantly inhibited the NAD(P)H consumption in the diabetic VSMC, suggesting that JAK2 has a critical role played in the proliferative signaling of diabetic VSMC through NAD(P)H activation.

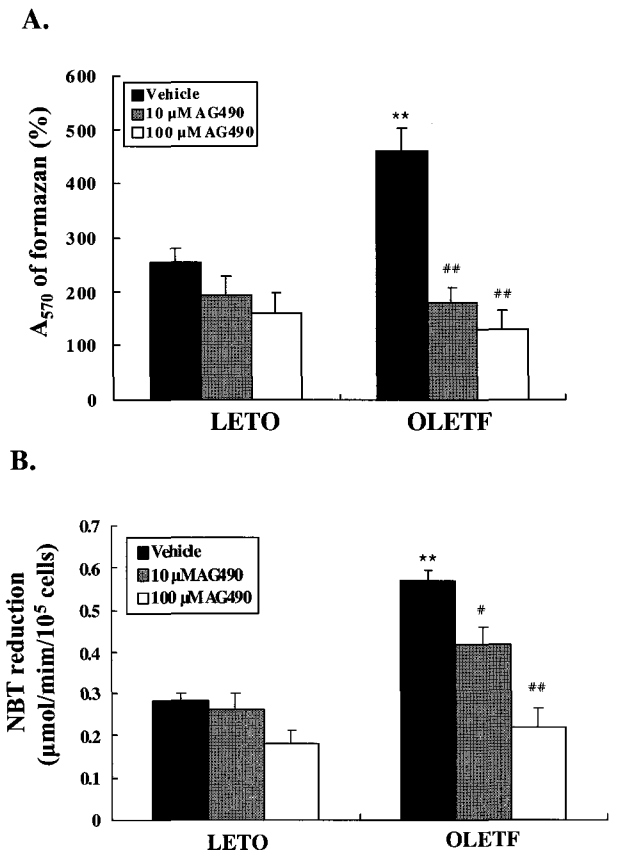

Fig. 3. Involvement of AG490 in superoxide-mediated proliferation of diabetic VSMC. (A) Cells were treated with 10%-FBS for 24 hr, and data show percent of formazan absorbance on day 0. (B) Superoxide generation from LETO and OLETF rats. After pretreatment with inhibitors for 30 min, the cells were then incubated with NBT for 4 hrs. Data are shown as mean±SE from 7 experiments in each group. **p<0.01 vs. vehicle of LETO rats, #p<0.05, ##p<0.01 vs. vehicle of OLETF rats.

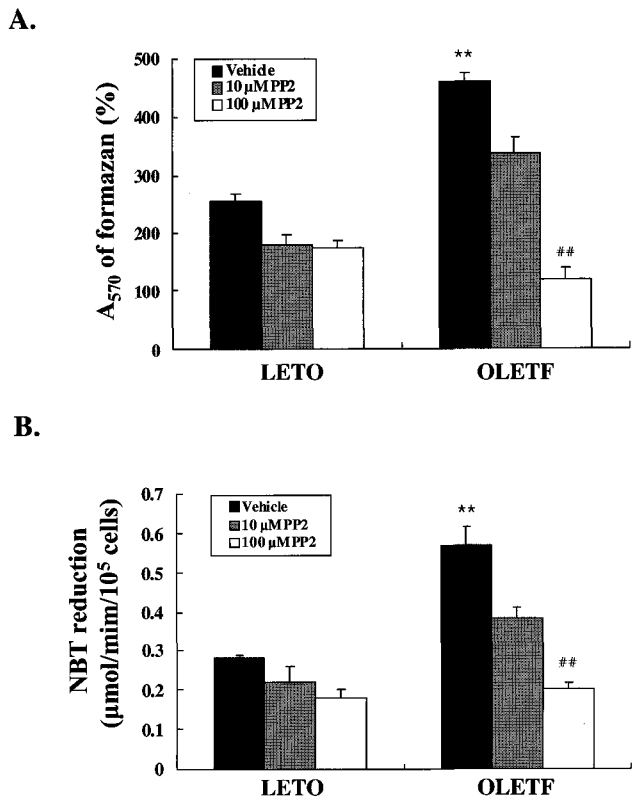

Fig. 4. Effects of PP2 on superoxide generation and proliferation of VSMC. (A) MTT assay. Cells were treated with 10%-FBS for 24 hr, and data show percent of formazan absorbance on day 0. (B) Superoxide generation from LETO and OLETF rats. After pretreatment with PP2 for 30 min, the cells were then incubated with NBT for 4 hrs. Data are shown as mean±SE from 5 experiments in each group. **p<0.01 vs. vehicle of LETO rats, ##p<0.01 vs. vehicle of OLETF rats.

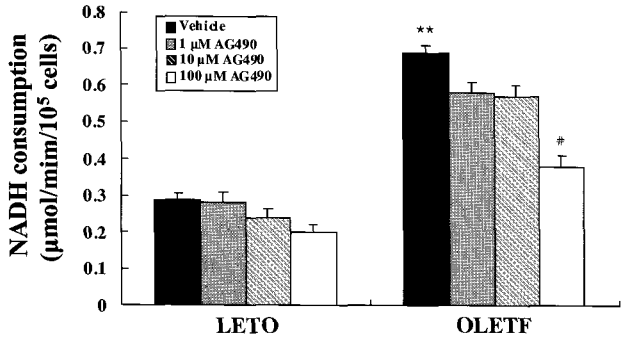

Fig. 5. Inhibition of NAD(P)H oxidase activity by AG490. NAD(P)H oxidase activity was measured which cellular homogenates by lucigenin-enhanced chemiluminescence assay. Data are shown as mean±SE from 5 experiments in each group. $^{**}p<0.01$ vs. vehicle of LETO rats, $^{\#}p<0.05$ vs. vehicle of OLETF rats.

DISCUSSION

The present results show that diabetic VSMC proliferation is enhanced by oxidative stress, especially superoxide, which is mediated through NAD(P)H oxidase activation. Tyrosine phosphorylation of JAK2 is increased in diabetic VSMC proliferation and NAD(P)H-derived superoxide production is decreased by JAK2 and Src kinase inhibitors. These data indicate that the signal pathway which leads to diabetes-associated activation of Src kinase/JAK is critically involved in the diabetic VSMC proliferation through NAD(P)H oxidase activation and superoxide generation.

The proliferation of VSMC is the major step in the development of atherosclerotic lesions (Ross, 1993). The accumulation of VSMC in the neointima is believed to be due to a combination of cell proliferation and directed migration of arterial cells in response to vascular injury. Recent report indicates that JAK is involved in neointima formation (Seki et al, 2000). However, the question of how JAK activates diabetic VSMC proliferation, resulting in neointima formation remains poorly understood. In the present study we demonstrated that activation of JAK regulated the enhanced proliferation of diabetic VSMC. Furthermore, our data suggest that the Src kinase is an upstream of JAK for mitogenic signal transduction in diabetic VSMC. This is consistent with data by other workers, showing that overexpression of v-Src or c-Src kinase activity resulted in constitutive JAK activation (Campbell et al, 1997; Wang et al, 2000).

The excessive ROS burden in insulin resistance and diabetes has been thought to promote atherogenesis by affecting impaired vascular cells. Among various enzymatic sources of ROS such as NAD(P)H oxidase, cyclooxygenase, xanthine oxidase, nitric oxide synthase, and mitochondrial electron transport, NAD(P)H oxidase has been considered as the major source of ROS in the vasculature (Zalba el al, 2000) and implicated in numerous cellular processes and vascular diseases (Griendling et al, 2000). Our experimental results clearly showed that superoxide production in diabetic VSMC was more prominent than that in the control cells. The enhanced superoxide production in diabetic VSMC was markedly reduced by treatment with JAK2 and Src kinase inhibitors such as AG490 and PP2, suggesting that JAK2 and Src kinase signal is involved in the enhanced production of superoxide in diabetic VSMC. This possibility was further confirmed in the present study by measuring oxidase activity: NAD(P)H oxidase activity in diabetic VSMC was significantly higher than that in the control cells, and NAD(P)H oxidase activity was completely blocked by treatment with AG490.

Cytoplasmic JAK are crucial components of diverse signal transduction pathways that govern cellular survival, proliferation, differentiation and apoptosis. In cultured VSMC, it has been demonstrated that JAK and STAT proteins are constitutively expressed and activated. After binding of growth factor, cytoplasmic STAT are recruited and activated by either tyrosine kinase receptor (RTKs) (Ren & Schaefer, 2002), or by non soluble tyrosine kinase of JAK and Src kinase families after binding of cytokines to their receptors (Darnell et al, 1994). We found that tyrosine phosphorylation was increased in diabetic proliferation induced by serum, that tyrosine phospho-protein, especially JAK2, in diabetic VSMC was decreased by AG490 and PP2, and that these inhibitors blocked the diabetic VSMC growth and superoxide generation. These results together suggest that, in addition to JAK2-mediated simulation, another possible mechanism for the activation of VSMC involves the generation of intracellular ROS.

In summary, we report here that the Src kinase and JAK2 play an important role in superoxide-mediated diabetic VSMC proliferation. In addition, enhanced activation of NAD(P)H in diabetic VSMC is decreased with JAK inhibitor, indicating that this pathway modulates cellular responses to the generation of ROS. Our results suggest that this pathway plays a significant role in the progression of pathophysiologic diabetic vascular diseases such as atherosclerosis. The relative importance of these effects of JAK for diabetic VSMC proliferation and NAD(P)H-derived superoxide generation remains to be an issue for further study.

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