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Enhanced Expression of Cell Adhesion Molecules in the Aorta of Diabetic Mice is Mediated by gp91phox-derived Superoxide

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Endothelial activation and subsequent recruitment of inflammatory cells are important steps in atherogenesis. The increased levels of cell adhesion molecules (CAM) have been identified in diabetic vasculatures, but the underlying mechanisms remain unclear. To determine the relationship among vascular production of superoxide, expression of CAM and diabetes, superoxide generation and expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E- and P-selectin in the aorta from control (C57BL/6J) and diabetic mice (ob/ob) were measured. In situ staining for superoxide using dihydroethidium showed an increased superoxide production in diabetic aorta, accompanied with an enhanced NAD(P)H oxidase activity. Immunohistochemical analysis revealed that the endothelial expression of ICAM-1 (3.5±0.4) and VCAM-1 (3.8±0.3) in diabetic aorta was significantly higher than those in control aorta (0.9±0.5 and 1.6±0.3, respectively), accompanied with the enhanced expression of gp91phox, a membrane subunit of NAD(P)H oixdase. Furthermore, there was a strong positive correlation ($r=0.89$, $P<0.01$ in ICAM-1 and $r=0.88$, $P<0.01$ in VCAM-1) between ICAM-1/VCAM-1 expression and vascular production of superoxide. The present data indicate that the increased production of superoxide via NAD(P)H oxidase may explain the enhanced expression of CAM in diabetic vasculatures.

Key Words: Diabetes, ICAM-1, VCAM-1, E-selectin, NAD(P)H oxidase

INTRODUCTION

Macrovascular complications in diabetes are responsible for the majority of the mortality associated with diabetes, irrespective of whether the patient has type 1 or type 2 diabetes (Garber, 1992). Of these macrovascular complications, 75% of all cases involve coronary atherosclerosis (Levin & Sicard, 1990; Garber, 1992), but the classical risk factors such as hyperlipidemia and hypertension do not account for the increased incidence of atherosclerosis in diabetes (Wagner & Jilma, 1997). Recently, a putative role of cell adhesion molecules (CAM) in the development of diabetic vascular complications has been suggested, because both increased oxidative stress and low-grade inflammation are hallmarks of diabetes (Davi et al, 1999; Haffner et al, 2002; Zhang et al, 2003).

The expression of endothelial CAM leads to attachment and migration of leukocytes into the vessel wall (Osborn, 1990; Ross, 1993; Haller et al, 1995), and this process is generally considered to be an initial event in the pathogenesis of the early lesion of atherosclerosis (Ross, 1993). Elevated levels of CAM expression have also been demonstrated in the vasculatures of diabetic patients (Ribau et al, 1999) and experimental alloxan-diabetic rabbits (Haddcock et al, 1991). However, to date, there have been few reports concerning the molecular mechanisms involved in the enhanced expression of CAM in diabetic vasculatures.

The relationship between oxidative stress and an augmented expression of inflammatory mediators in coronary arteries has been demonstrated in a porcine model of diabetes (Zhang et al, 2003). Reportedly, superoxide may function as a second messenger by activating certain gene transcription factors, notably the nuclear transcription factor-kappa B (NF-κB) (Schmidt et al, 1995; Cominacini et al, 1997). The induction of certain CAM [e.g. intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin] and cytokines is mediated by NF-κB (Ghosh et al, 1998), therefore, vascular cell-derived superoxide may promote the activation of the above proinflammatory factors in diabetes. However, the precise source and role of superoxide in diabetic vasculatures are the subject of continued debate.

Thus, the present study was aimed to determine the characteristics of the expression of ICAM-1, VCAM-1, E- and P-selectin with special attention to their potential correlation with vascular production of superoxide in aorta from diabetic mice. Furthermore, the role of NAD(P)H oxidase in the vascular production of superoxide in diabetic mice was also determined.

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ABBREVIATIONS: CAM, cell adhesion molecules; ICAM-1, intercelluar adhesion molecule-1; VCAM-1, vascular adhesion molecule-1; DPI, diphenyleneiodonium.

METHODS

Animals

All animal studies were reviewed and approved by Institutional Animal Care and Use Committee and the Animal Research Committee, in accordance with Public Health Policy regarding the use and care of laboratory animals. The genetically obese Lep^{ob}/Lep^{ob} (ob/ob) mice and their lean littermates (C57BL/6J) of 12 weeks of age (Jackson Laboratories, Bar Harbor, ME) were used. Animals were maintained in microisolation cages with autoclaved food and bedding to minimize exposure to viral and microbial pathogens. After body weight was determined, plasma glucose and insulin levels were measured by a blood glucose test meter (Accutrend sensor, Roche Inc., Nutley, NJ) and the mouse insulin enzyme-linked immunosorbent assay kit (Wako Chemicals, Tokyo), respectively. Mice were sacrificed under anesthesia with diethyl ether for isolation of thoracic aorta.

Measurement of superoxide production

To quantify vascular production of superoxide, aortic sections were labeled with the oxidative dye dihydroethidium (Molecular Probes, Eugene, OR), which is oxidized in the presence of superoxide to ethidium bromide and gives red fluorescecnce (Kim et al, 2002a; Miller et al, 1998). Briefly, unfixed frozen rings of aortic segments were cut into 5μm thick sections and placed on a glass slide. Samples were incubated with dihydroethidium (2μmol/L) in a light-protected humidified chamber at 37°C for 30 min. To determine the enzymatic source of superoxide production, aortic sections were preincubated with various inhibitors of oxidant enzymes for 30 min, followed by addition of dihydroethidium. The images were obtained with a laser scanning confocal microscope (LSM 510, Carl Zeiss Inc., Germany). For ethidium bromide detection, a 543-nm He-Ne laser combined with a 585-nm long-pass filter was used. The average fluorescence intensity at the maximal response period (25 min) was presented as units per square millimeter of aorta.

Measurement of NAD(P)H oxidase activity

NAD(P)H oxidase activity was measured with the lucigenin assay which is specific for superoxide. Briefly, aortic segments were isolated and homogenized with a motor-driven tissue homogenizer for 2 min in 50 mM phosphate buffer containing 0.01 mM EDTA and protease inhibitors. The homogenate was centrifuged at 1,000×g for 10 min to remove unbroken cells and debris. Protein content was determined by using Bicinchoninic acid protein assay kit (Sigma Chemical Co., St. Louis, MO). The assay was performed in 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5μM lucigenin as the detector and NAD(P)H as the substrates (final volume 150μl). NADH and NADPH were used at a final concentration of 100μM, and the reaction was started by the addition of 25 g of protein. The photon emission was measured every 15 sec for 10 min in a microtiterplate luminometer (Microlumat LB96P, EG&G Berthold, Germany).

Immunohistochemistry

Aortic segements (5 mm in length) were embedded in Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetech Co., Tokyo) and snap-frozen in liquid nitrogen. Serial cryosections (5μm) were mounted on 3-aminopropylmethoxysilane (APES)-coated slides (Marienfeld, Bad Mergentheim, Germany), and immunohistochemistry was performed with standard techniques. Briefly, sections were blocked in 10% donkey serum and then incubated overnight at 4°C with goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) directed against ICAM-1 (1 : 200), VCAM-1 (1 : 200), E-selectin (1 : 500), P-selectin (1 : 100), gp91phox (1 : 1,000), p22phox (1 : 200), and p47phox (1 : 200), respectively. For negative control, the primary antibody was replaced with an irrelevant antibody. The sections were washed and incubated with a biotinylated donkey anti-goat IgG (1 : 100) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 60 min, and then incubated with avidin-biotin horseradish peroxidase complex (ABC Vectastain Elite Kit, Burlingame, CA). Color was developed by the addition of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co.). The sections were lightly stained in hematoxylin and then dehydrated through alcohol and xylene.

Quantification of immunohistochemical staining

Five serial sections were immunostained and quantitatively analyzed for each animal by two operators who had no knowledge of the origin of the tissues. All stained sections were examined with light microscope (Leica, Wetzlar, Germany) and photomicrographs were prepared. The staining intensity for each antibody was evaluated using a 5-grade system: 4=strong and complete staining; 3=strong staining; 2=moderate staining; 1=weak staining, and 0= negative staining in comparison with negative control.

Statistical analysis

Data are expressed as means±SEM or percentage of control. Two-tailed Student's *t*-test was used for 2-group comparisons. Relationships between superoxide production and ICAM-1/VCAM-1 expression, gp91phox expression and superoxide production, and levels of blood glucose and gp91phox expression were determined by analysis of regression line. $P < 0.05$ was considered statistically significant.

RESULTS

Male ob/ob mice showed a significant increase in body weight throughout the study period (until 12 weeks of age) (Table 1). In addition, the plasma glucose and insulin concentrations in ob/ob mice were significantly higher than those in the control mice.

Superoxide production and NAD(P)H oxidase activity

After loading with the oxidation-sensitive dye dihydroethidium, a marked increase in ethidium bromide fluorescence was found in the aortic sections of ob/ob mice, which reflected an increase in superoxide production, compared with that in control mice. The enhanced fluorescence

Table 1. Mean body weight and levels of plasma glucose and insulin in control and ob/ob mice

	Control (15)	ob/ob (15)
Body weight (g)	26.0±1.8	47.0±3.4*
Plasma glucose (mg/dl)	184.0±42.0	347.0±85.3*
Plasma insulin (ng/ml)	2.0±0.3	165.0±21.0*

Each value represents mean±SEM. *P＜0.01 vs. corresponding value in control. Numbers in parentheses represent the number of animals used.

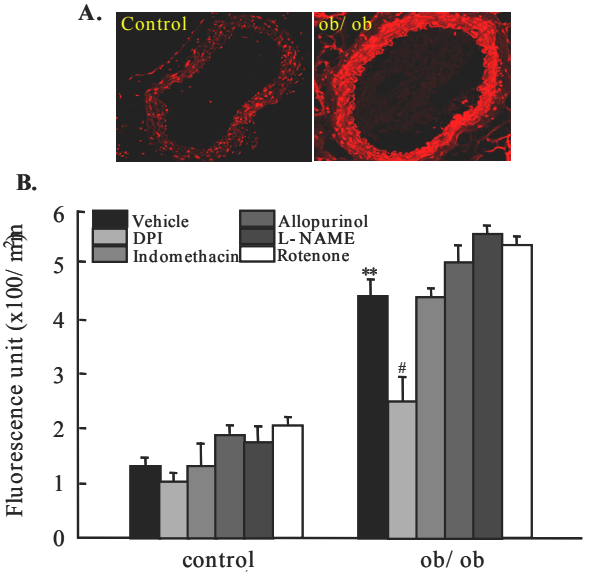

Fig. 1. (A) Representative photographs (×200) of dihydroethidium -stained aortic sections from control and ob/ob mice. The intensity of red fluorescence in sections represents the amount of superoxide generated. (B) Effects of various inhibitors such as DPI (10μM), indomethacin (10μM), allopurinol (100μM), L-NAME (100μM), and rotenone (100μM) on superoxide production in control and diabetic aorta. Data are presented as means±SEM from 6 experiments in each group. **P＜0.01 vs. corresponding value in control. #P＜0.05 vs. corresponding vehicle.

intensity in diabetic aorta was attenuated by treatment with diphenyleneiodonium (DPI), an NAD(P)H oxidase inhibitor, but not by other inhibitors such as indomethacin (cyclooxygenase), allopurinol (xanthine oxidase), L-nitro-L-arginine methyl ester (L-NAME, endothelial nitric oxide synthase), and rotenone (mitochondrial dehydrogenase) (Fig. 1).

Consistent with the above results, significantly higher levels of NAD(P)H oxidase activity, as measured by NAD(P)H-induced superoxide production, were observed in diabetic aorta than in non-diabetic aorta (15.3±2.5 versus 5.2±0.9 nmol/min/mg protein, P＜0.01) (Fig. 2).

Expression of CAM

The location of each antigen was indicated by the dark brown reaction product generated by DAB system. In the

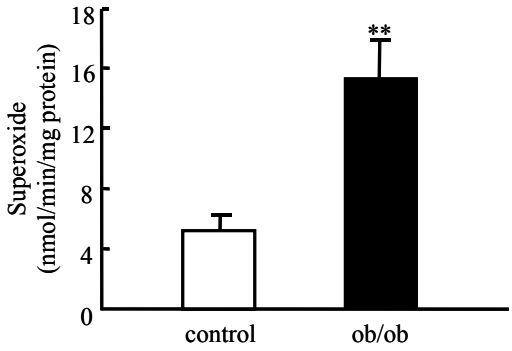

Fig. 2. Superoxide generation from aortic homogenates was determined by lucigenin-enhanced chemiluminescence in response to NADH/NADPH (100μM) as potential substrates. Bars represent mean±SEM from 5 experiments. **P＜0.01 vs. control.

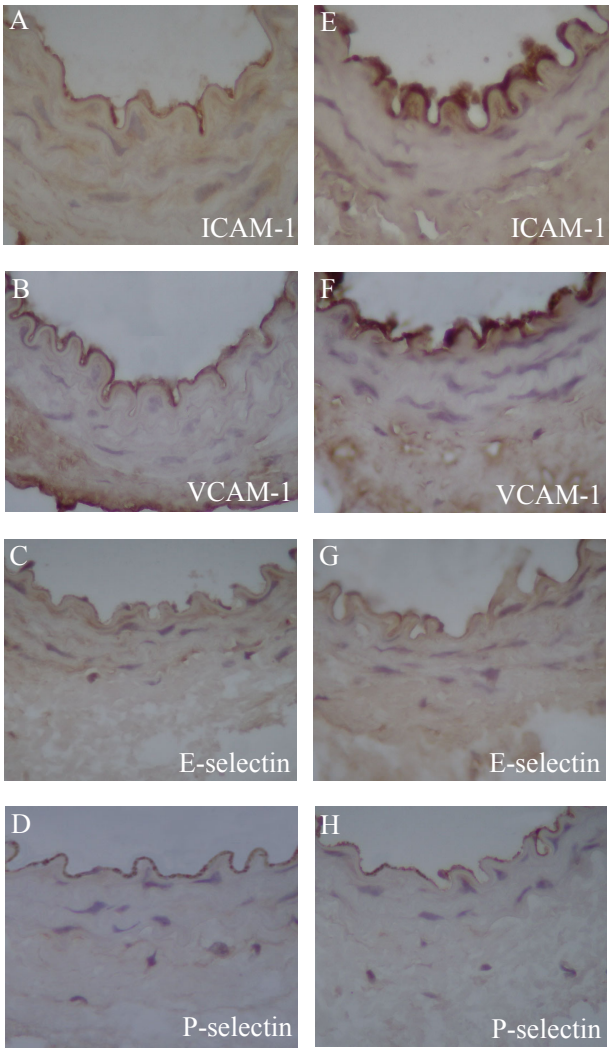

Fig. 3. Photomicrographs (×400) showing expression of ICAM-1, VCAM-1, E- and P-selectin in frozen sections of aorta from control (A through D) and ob/ob mice (E through H). The location of each antigen was indicated by the dark brown reaction product generated by DAB system. The nuclei were counterstained with Mayer's hematoxylin.

aorta from control and diabetic mice, expression of ICAM-1, VCAM-1 and P-selectin, but not of E-selectin, was detected by immunohistochemistry predominantly in endothelial layer. The endothelial expression of ICAM-1 (3.5±0.4) and VCAM-1 (3.8±0.3) in diabetic aorta was significantly higher than that in control aorta (0.9±0.5 and 1.6±0.3, respectively) (Fig. 3 and Table 2). However, there was no difference in the endothelial expression of E- and P-selectin between diabetic and control aorta. In the present study, no nonspecific reactions were observed in any sections processed as negative controls in which the primary antibody was replaced with an irrelevant antibody.

Correlation between superoxide production and CAM expression

To determine whether vascular production of superoxide would influence the expression of CAM including ICAM-1 and VCAM-1 in control and diabetic aorta, the relationship between superoxide production and endothelial expression of ICAM-1/VCAM-1 was assessed using analysis of regression line. As shown in Fig. 4, there was a strong positive correlation ($r=0.89$, $P<0.01$ in ICAM-1 and $r=0.88$, $P<0.01$ in VCAM-1) between these two parameters.

Table 2. Endothelial expression of cell adhesion molecules in the aorta from control and ob/ob mice

	Control	ob/ob
ICAM-1	0.9±0.5	3.5±0.4*
VCAM-1	1.6±0.3	3.8±0.3*
E-selectin	0.4±0.3	0.7±0.3
P-selectin	0.8±0.7	0.9±0.6

The data are presented as mean staining intensity±SEM from 6 experiments. *$P<0.01$ vs. corresponding value in control.

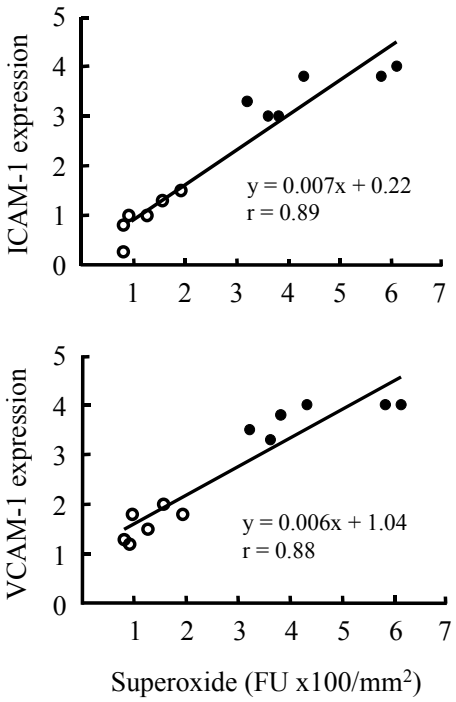

Fig. 4. Endothelial expression of ICAM-1/VCAM-1 was plotted as a function of superoxide production in the aorta from control (open circle) and ob/ob mice (filled circle). FU represents fluorescence unit.

Expression of NAD(P)H oxidase subunits

To further elucidate the individual contribution of NAD(P)H oxidase subunits in vascular production of superoxide in diabetes, its subunit expression was examined in control and diabetic aorta. As shown in Fig. 5, positive endothelial, medial and adventitial staining for gp91phox was observed in aorta from control and diabetic mice. The endothelial (3.8±0.3), medial (2.0±0.8) and advential (3.0±0.5) staining for gp91phox were significantly greater in diabetic aorta than in control (1.3±0.7, 0.8±0.5, and 0.5±0.2, respectively) (Table 3). However, the staining intensity for p22phox in the aorta from control and diabetic mice was similar. In contrast to membrane-bound subunits (gp91phox and

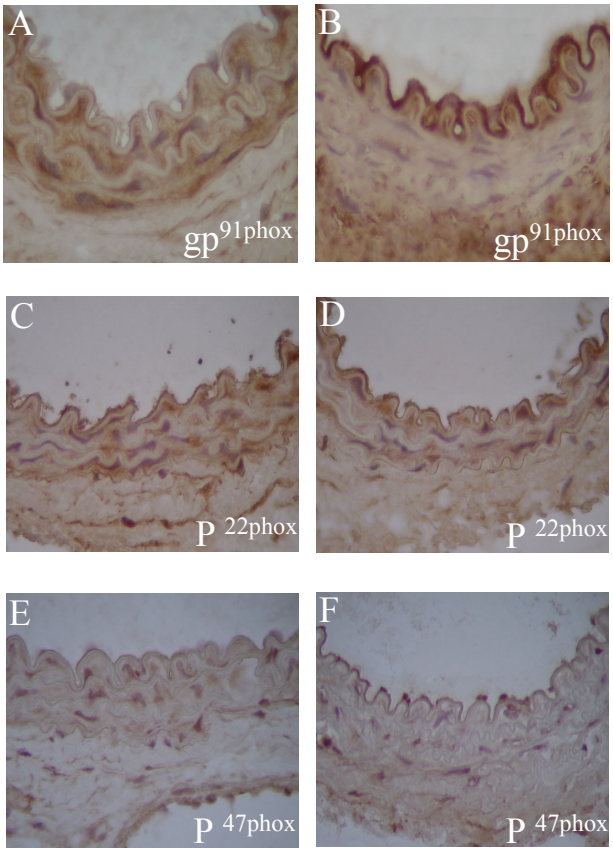

Fig. 5. Photomicrographs (×400) showing expression of gp91phox, p22phox and p47phox in frozen sections of aorta from control (A through C) and ob/ob mice (D through F). The location of each antigen was indicated by the dark brown reaction product generated by DAB system. The nuclei were counterstained with Mayer's hematoxylin.

Table 3. Expression of NAD(P)H oxidase subunits in the aorta from control and ob/ob mice

	Control			ob/ob		
	ED	SMC	AD	ED	SMC	AD
gp91phox	1.3±0.7	0.8±0.5	0.5±0.2	3.8±0.3**	2.0±0.8*	3.0±0.5**
p22phox	1.8±0.6	1.5±0.8	1.0±0.5	1.5±0.5	1.0±0.4	0.5±0.2
p47phox	0.5±0.5	0.2±0.2	0.2±0.3	0.2±0.2	0.5±0.5	0.2±0.4

The data are presented as mean staining intensity±SEM from 6 experiments. ED, SMC and AD represent endothelial cell, smooth muscle cell and adventitial layer, respectively. *P＜0.05; **P＜0.01 vs. corresponding value in control.

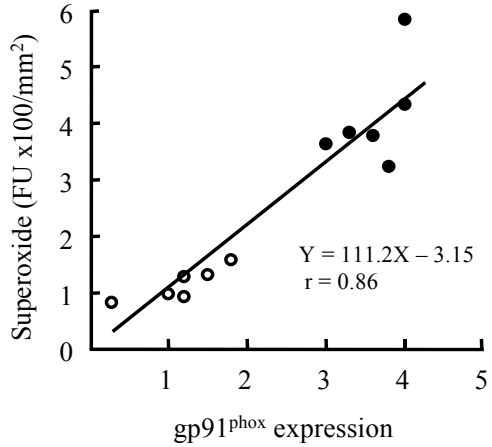

Fig. 6. Superoxide production was plotted as a function of endothelial expression of gp91phox in the aorta from control (open circle) and ob/ob mice (filled circle). FU represents fluorescence unit.

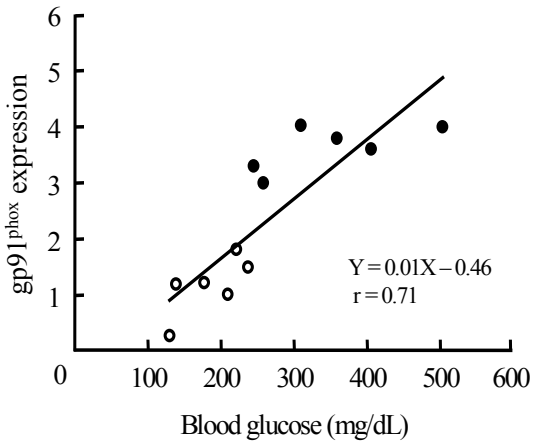

Fig. 7. Endothelial expression of gp91phox was plotted as a function of levels of blood glucose from control (open circle) and ob/ob mice (filled circle).

p22phox) of NAD(P)H oxidase, immunostaining for p47phox, a cytosolic subunit of NAD(P)H oxidase, was very weak or absent in all of the samples.

Relationship between gp91phox expression and superoxide production

To elucidate the contribution of gp91phox in vascular production of superoxide in diabetic mice, superoxide production in the aorta from control and diabetic mice was plotted as a function of the levels of endothelial gp91phox expression. As shown in Fig. 6, the amount of vascular production of superoxide correlated well ($r=0.86$, $p<0.01$) with the endothelial expression levels of gp91phox.

Relationship between levels of blood glucose and gp91phox expression

To elucidate the contribution of hyperglycemia to gp91phox expression in diabetic mice, endothelial gp91phox expression in the aorta from control and diabetic mice was plotted as a function of the levels of blood glucose. As shown in Fig. 7, the level of gp91phox expression correlated well ($r=0.85$, $p<0.01$) with the level of blood glucose.

DISCUSSION

The present study demonstrated the enhanced endothelial expression of ICAM-1 and VCAM-1 as well as an augmented activity of NAD(P)H oxidase in the aorta from ob/ob mouse, a spontaneous diabetic strain. In situ staining for superoxide using dihydroethidium revealed a marked increase of superoxide production in aortic segments of diabetic mice, compared with control mice, accompanied with the enhanced expression of gp91phox, a membrane subunit of NAD(P)H oxidase. Furthermore, the above results together revealed a strong correlation between vascular production of superoxide and expression of ICAM-1/VCAM-1.

Atherosclerosis-related macrovascular complications in diabetes are responsible for the majority of the mortality associated with diabetes (Garber, 1992). Of these macrovascular complications, the majority of cases involve coronary atherosclerosis (Garber, 1992; Levin & Sicard, 1990). Although a precise mechanism by which development of atherosclerosis is enhanced in diabetic patients has not been fully identified, several investigators have reported that the elevated levels of CAM play an important role in atherosclerotic vascular diasease in diabetes (Hwang et al, 1997; Jager et al, 2000; Matsumoto et al, 2002).

The expression of CAM in vascular endothelial cells is an early and critical step in adherence of circulating inflammatory cells to endothelial cells, and in facilitating their

migration across the endothelial layer, ultimately leading to the formation of atherosclerotic lesions (Fruebis et al, 1997; Scalia et al, 1998). In experimental alloxan-diabetic rabbits, the number of aortic endothelial cell which expressed CAM was increased, when compared to normoglycemic rabbits (Hadcock et al, 1991). Furthermore, studies in diabetic patients have also shown that both the enhanced aortic expression of CAM (Ribau et al, 1999) and elevated circulating levels of soluble forms of ICAM-1 and VCAM-1 have been demonstrated (Cominacini et al, 1995; Schmidt et al, 1996; Jude et al, 2002). In agreement with these previous results, the present study clearly demonstrated the enhanced expression of ICAM-1 and VCAM-1, but not E- and P-selectin in the endothelial layer of aorta from ob/ob mouse, a spontaneous diabetic strain. Therefore, it was speculated that the enhanced expression of ICAM-1 and VCAM-1 in aortic endothelium of diabetic mice might provide a milieu for atherosclerotic lesion formation.

The mechanisms underlying the diabetes-related increase in CAM expression are not clear. Recent evidences suggest that oxygen free radicals may function as second messengers by activating certain gene transcription factors, notably the nuclear transcription factor-kappa B (NF-κB) (Schmidt et al, 1995; Cominacini et al, 1997). Since the induction of certain adhesion molecules is mediated by NF-κB (Ghosh et al, 1998), vascular cell-derived oxygen free radicals may promote the expression of CAM in diabetes. In this study, in situ staining for superoxide using dihydroethidium revealed a marked increase of superoxide production in endothelial, vascular smooth muscle and adventitial layers of diabetic aorta, compared with control vessel. Furthermore, the vascular production of superoxide correlated well with the expression of ICAM-1 and VCAM-1, suggesting the potential role of oxygen free radicals in the accelerated expression of CAM in diabetic aorta.

However, the endothelial expression of E- and P-selectin in diabetic aorta in the present experiment was not different from that in control aorta. These data are consistent with other reports in which the increased mRNA levels of ICAM-1 and VCAM-1, but not E-selectin, were demonstrated in rabbit and mouse atherosclerotic lesions (Sakai et al, 1997; Iiyama et al, 1999) and P-selectin immunoreactivity was not enhanced in diabetic retina in human (McLeod et al, 1995). Considering the fact that selectin gene expression transiently appears on the endothelial cell surface after acute events such as inflammation and rapidly declines even in the continuous presence of cytokines (Blankenberg et al, 2003; Collins et al, 1995), it could be speculated that a minor difference in the promoters of the selectin genes with ICAM-1 and VCAM-1 genes could result in different regulation of expression. However, the exact mechanism for this difference in selectin gene expression is not obvious.

In animal models and human diabetes, evidences from experimental studies suggest that increased vascular production of superoxide is a feature of systemic vascular disease states. Among various sources of vascular superoxide such as NAD(P)H oxidases, xanthine oxidase, lipoxygenase, mitochondrial oxidases, and nitric oxide synthase (Ushio-Fukai et al, 1996; White et al, 1996; Vasquez-Vivar et al, 1998), NAD(P)H oxidase appears to be the principal source of superoxide in several animal models of vascular diseases, including diabetes (Kojda et al, 1999; Warnholtz et al, 1999; Kim et al, 2002b). In accordance with the previous reports, our experimental results clearly showed that superoxide production in diabetic aorta was more prominent than that in control aorta. The enhanced superoxide production in diabetic aorta was markedly reduced by treatment with DPI, an NADPH oxidase inhibitor, but not by inhibitors for other oxidases such as xanthine oxidase, nitric oxide synthase, and mitochondrial electron transporters, suggesting that NAD(P)H oxidase is involved in the enhanced production of superoxide in diabetic aorta. This suggestion has also been demonstrated by measuring NAD(P)H oxidase activity in aortic homogenates, in which NAD(P)H-induced superoxide production was significantly greater in diabetic aorta than that in control aorta.

Although increased oxidative stress is hallmark of diabetes, the precise source of oxidative stress in diabetic vasculature is the subject of continued debate. Among various sources of reactive oxygen species (ROS), NAD(P)H oxidase has been considered to be an important source of ROS in the vasculature (Zalba et al, 2000). The vascular NAD(P)H oxidase shares some similarities with the multisubunit enzyme complex that comprises the respiratory burst oxidase of neutrophils (Chanock et al, 1994; Babior, 1999). It has recently been observed that overproduction of superoxide via the enhanced activity of vascular NADH oxidase contributes to the impaired endothelium-dependent vasodilation in OLETF rat, an animal model of type 2 diabetes (Kim et al, 2002b). However, the precise mechanisms implicated in the NAD(P)H oxidase activation remains unclarified.

Reportedly, the membrane-bound subunits of NAD(P)H oxidase such as gp91phox and p22phox are essential for the stability of oxidase and its catalytic activity (Parkos et al, 1988; Thrasher et al, 1994). Thus, to clarify the mechanism involved in the NADH oxidase activation in ob/ob mice, we determined protein expression of gp91phox and p22phox by immunohistochemical analysis. The experimental results showed that the endothelial expression of gp91phox in the aorta from ob/ob mice was significantly higher than those from control mice, suggesting that the translational regulation of these oxidase subunit was upregulated in the diabetic vasculature. Furthermore, there was a significant correlationship between the levels of gp91phox expression and superoxide generation, suggesting the functional role of gp91phox in the enhanced production of superoxide in diabetic aorta.

Taken together, the present study demonstrated a positive correlation between the expression of ICAM-1/VCAM-1 and vascular production of superoxide as well as vascular production of superoxide and endothelial expression of gp91phox in association with an enhanced superoxide production in diabetic aorta, and suggested that NAD(P)H oxidase induced ICAM-1/VCAM-1 expression in the aorta in a redox-sensitive manner with the involvement of the upregulated expression of gp91phox.

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