



Endothelin Antagonism Normalizes VEGF Signaling And Cardiac Function In STZ-Induced Diabetic Rat Hearts

Authors:

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Abstract

Abnormal alterations in cardiac expression of vascular endothelial growth factor (VEGF) as well as its receptors and impairment in the development of coronary collaterals have recently been reported in diabetic subjects. However, the presence of pharmacological intervention on these defects in diabetes remains unsettled. Here, we studied the effect of endothelin (ET) receptor blockade on cardiac VEGF signaling pathways and cardiac function in Sprague-Dawley rats 5 wk after induction of type I diabetes with streptozotocin (65 mg/kg ip) in comparison with age-matched control rats. After streptozotocin (1 wk), some diabetic rats were treated with the ET receptor antagonist SB-209670 (1 mg/day) for 4 wk. VEGF, its receptors, and its angiogenic signaling molecules [phosphorylated Akt and endothelial nitric-oxide synthase (eNOS)] were analyzed by Western blot, ELISA, real-time PCR, and immunohistochemistry, and cardiac function was evaluated by echocardiography. Coronary capillary morphology was assessed by lectin and enzymatic double staining. We found significant decreases in cardiac expression of VEGF, its receptors, phosphorylation of Akt and eNOS, and coronary capillary density in diabetic rats compared with controls. Treatment of diabetic rats with SB-209670 reversed these alterations to the control levels and ameliorated impairment of cardiac function. From a molecular point of view, the present study is the first to indicate the potential usefulness of an ET receptor antagonist in the treatment of cardiac dysfunction in type I diabetes.

DIABETES MELLITUS is associated with macro- and microvascular complications in multiple organ systems (11). A large body of epidemiological and pathological data demonstrate that diabetes is an independent risk factor for cardiovascular disease in both men and women (11), and coronary artery disease is considered the leading cause of morbidity and mortality in patients with diabetes (12).

Vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogen that is important in neovascularization under both physiological and pathophysiological conditions, plays a crucial role in developmental blood vessel formation and regulation of hypoxia-induced tissue angiogenesis (2, 9).

The following two high-affinity VEGF tyrosine kinase receptors have been identified: fms-like tyrosine kinase 1 (Flt-1) and fetal liver kinase 1 (Flk-1), both of which are expressed almost exclusively in endothelial cells (39). A significant increase in VEGF levels can be observed after myocardial infarction in nondiabetic patients (46), consequently leading to the development of collateral vessels in the advanced stages of coronary atherosclerosis (1). In diabetic patients, however, there is inadequate collateral vascular formation in response to ischemia that ultimately results in increased cardiovascular morbidity and mortality rates (1). Thus diabetic patients suffering from coronary artery disease have a less favorable outcome compared with nondiabetic patients, including a three- to fourfold increase in mortality risk (48, 50). Interestingly, a previous study showed that myocardial expression levels of mRNAs for VEGF and its receptors, Flt-1 and Flk-1, were significantly decreased in nonischemic short-term streptozotocin-induced diabetic rats, and a twofold reduction in VEGF and Flk-1 was observed in autoptic ventricular specimens from diabetic patients compared with nondiabetic subjects who died from myocardial infarction (6).

Evidence is now strong for a role of endothelin (ET)-1, a potent vasoconstrictor with vasoproliferative activity (35), in diabetes. Plasma ET-1 levels have been shown to be elevated in animal models of diabetes mellitus (14, 30, 51) and in diabetic patients (8, 37). Furthermore, it has been reported that activity of endogenous ET-1 on ET_A receptors is enhanced in the resistance vessels of diabetic patients (3). Thus the ET system appears to play a key role in the development of cardiovascular complications associated with diabetes and, therefore, therapy with intervention designed to suppress the ET system may prevent the development of cardiovascular complications in diabetes.

In the present study, we found downregulated cardiac expression levels of VEGF and its receptors, impaired cardiac VEGF signaling, decreased coronary capillary density, and cardiac dysfunctions in rats with 5 wk of streptozotocin-induced diabetes. Diabetic rats exhibited a significant increase in ET-1 in left ventricular (LV) tissues compared with nondiabetic controls. The final goal of this study was to determine whether treatment with SB-209670, which is an antagonist against both ET_A and ET_B receptors, can improve deteriorations of the VEGF signaling pathways, coronary capillary morphology, and cardiac functions in diabetic rats.

MATERIALS AND METHODS

Experimental animals. Male Sprague-Dawley rats (10 wk and 200–250 g body wt) were used in this study. After an overnight fast, the animals received a single intraperitoneal injection of streptozotocin (65 mg/kg). Streptozotocin was dissolved in a citrate solution (0.1 M citric acid and 0.2 M sodium phosphate, pH 4.5). Control rats ($n = 26$) received an equivalent volume of citrate buffer alone. All animals injected with streptozotocin developed diabetes, as indicated by plasma glucose levels >250 mg/dl (Glucometer Elite; Bayer) at 48 h following the injection. After the streptozotocin injection (1 wk), diabetic animals were randomly divided into two groups. One group of diabetic rats ($n = 20$) was given SB-209670 (SmithKline Beecham Pharmaceuticals) at a dose of 1 mg/day for 4 wk subcutaneously through an osmotic pump that was implanted in the back. Another group ($n = 27$) was given the physiological saline alone. Treatment with SB-209670 for 4 wk was also conducted on some control rats. All rats were fed with the same diet and water until they were used 4 wk later. All animal procedures were reviewed and approved by the Animal Experiment Committee at the University of Tsukuba.

Morphometric analysis. Frozen sections of LV (8 μ m thick) were stained with lectin *Griffonia simplicifolia* (20). For visualization, 3,3-diaminobenzidine/ H_2O_2 was used as chromogen. Vascular endothelium was stained with lectin with capillaries appearing as black or dark brown dots. Sections were examined with an Olympus microscope, and counts of capillaries were made in 30 square fields/sample at a final magnification of X400.

Double staining of sections was carried out to discriminate arteriolar and venular capillaries, as previously described (22). Sections (16 μ m thick) cut from the frozen LV at the widest part were used.

Western blot analysis. LV tissues were homogenized with 10 vol of 20 mM Tris·HCl (pH 7.4), 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM Na_3VO_4 , 2 mM dithiothreitol, 20 mM glycerophosphate, 0.6% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 60 μ g/ml aprotinin, and 1 μ g/ml leupeptin on ice using a homogenizer. The homogenate was gently rotated for 30 min at 4°C and then centrifuged at 13,000 g for 10 min at 4°C, and the protein concentration of the resulting supernatant was determined. The samples (20 μ g of protein) were then subjected to heat denaturation at 96°C for 7 min with Laemmli buffer. Western blot analysis for detection of phosphorylated and nonphosphorylated forms of different target molecules was performed according to the method described in previous studies with minor modification (15, 18, 25, 28, 45, 52). Briefly, each sample was separated on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (Millipore, Billerica, MA) membrane. The membrane was incubated in blocking buffer with 5% skim milk in PBS containing 0.1% Tween 20 for 12 h at 4°C, followed by incubation with primary antibody for 1 h at room temperature. The membrane was washed with PBS containing 0.1% Tween 20 three times and incubated with horseradish peroxidase-conjugated secondary antibody, which was an anti-rabbit, anti-mouse, or anti-goat IgG (1:2,000 dilution with blocking buffer; Cell Signaling, Beverly, MA), for 1 h at room temperature. After being washed, as described above, the expression level of each molecule was determined using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) followed by exposure to Hyperfilm (Amersham Biosciences).

The following antibodies, which are commercially available, were used: rabbit antihuman VEGF polyclonal antibody (Immunological Laboratories, Fujioka, Japan), rabbit antihuman Flk-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit antihuman Flt-1 polyclonal antibody (Santa Cruz Biotechnology), rabbit antihuman endothelial nitric oxide synthase (eNOS) polyclonal antibody (Affinity BioReagents, Golden, CO), rabbit polyclonal phosphorylated Akt (Ser⁴⁷³) antibody (Cell Signaling Technology), rabbit polyclonal Akt antibody (Cell Signaling), Ser¹¹⁷⁷-phospho-eNOS (Santa Cruz Biotechnology), rabbit polyclonal phospho-Flk-1 (Tyr⁹⁵¹) antibody (Santa Cruz Biotechnology), and rabbit polyclonal phospho-Flk-1 (Tyr⁹⁹⁶) antibody (Santa Cruz Biotechnology).

Immunoprecipitation and immunoblotting for phospho-Flk-1. Aliquots of protein lysates were obtained as described in previous studies (28, 45, 52). Protein extracts were incubated overnight at 4°C with 3 μ g of rabbit polyclonal anti-Flk-1 (Santa Cruz Biotechnology). Subsequently, 50 μ l of protein A-agarose were added. Immunoprecipitated proteins were separated on 8% sodium-dodecyl-(lauryl)-sulfate-PAGE, transferred to nitrocellulose filters, and exposed to rabbit polyclonal anti-phospho-Flk-1 (Santa Cruz Biotechnology; see Refs. 28, 45, and 52).

Immunoassay. Analysis of the levels of VEGF and eNOS in LV tissues was also performed using the ELISA-based immunoassay kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The level of Akt protein, which was phosphorylated at serine residue 473, in LV tissue extract, was quantitatively determined by an ELISA kit, AKT [pS473] (BioSource International). Concentrations of ET-1 in tissue extracts were determined using an ET-1 Enzyme Immuno Assay Kit (Immuno-Biological Laboratories).

Immunofluorescence and confocal analysis. For immunohistochemical determination of target molecules, the tissue sections were exposed to the fluorescent secondary antibody after overnight incubation with each primary antibody, as fully described in our previous reports (24, 25). Immunofluorescent images were observed under Laser Scanning Confocal Imaging System (24, 25). Phosphorylated eNOS and phosphorylated Akt were purchased from Santa Cruz Biotechnology and Cell Signaling Technology, respectively.

RNA preparation and real-time quantitative PCR. Total RNA was extracted from LV tissues by the guanidinium thiocyanate-phenol-chloroform single-step extraction method with ISOGEN (Nippon Gene). The single-stranded cDNA was used in real-time quantitative PCR for evaluation of relative expression levels of target genes. We have previously described the primers and conditions used for real-time quantitative PCR analyses (24, 25).

Echocardiography. Rats were anesthetized with pentobarbital sodium (40 mg/kg ip), and LV fractional shortening (FS) and stroke volume (SV) was measured using a Doppler-echocardiographic system (SONOS 4500; Koninklijke Philips Electronics), as described in our previous report with minor modification (16). We determined the heart rate, LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), LVFS, which was calculated according to the following formula: LVFS (%) = [(LVEDD - LVESD)/LVEDD] X 100, and diameter of the ascending aorta at end-diastole with a 12-MHz transducer using M-mode echocardiography. Doppler ultrasonographic flow velocity curves in the ascending aorta were simultaneously recorded, and mean blood velocity throughout a cardiac cycle was determined by planimetry (Image J; National Institutes of Health). SV was calculated as a product of the velocity integral and aortic cross-sectional area. Furthermore, cardiac output (CO) was calculated as the product of the SV and heart rate. After the echocardiographic measurements, a microtip pressure transducer catheter (SPC-320; Millar Instruments) was inserted in the left carotid artery. Arterial blood pressure was monitored with a pressure transducer (model SCK-590; Gould) and was recorded with the use of a polygraph system (Nihon Kohden, Tokyo, Japan). After measurement, the catheter was advanced in the LV for the determination of LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP), as previously described (42).

Statistical analysis. Data are reported as means \pm SD. Statistical analyses were performed using one-way ANOVA, and post hoc comparisons were made with Fisher's protected least-significance t -test for multiple comparisons. A P value of <0.05 was considered significant.

RESULTS

Characteristics of experimental animals. The general features of diabetic rats treated with SB-209670 or its vehicle and age-matched control animals are summarized in Table 1. Body

weight was significantly decreased in diabetic rats, and treatment with SB-209670 had no effect on the diabetes-induced weight loss. The fed plasma glucose levels in diabetic rats were elevated ~4.5-fold compared with controls. On the other hand, their plasma insulin levels were extremely lower than controls. Treatment of diabetic rats with SB-209670 for 4 wk did not alter hyperglycemia and hypoinsulinemia. LV weight was significantly decreased in diabetic rats compared with controls and was unaffected by SB-209670 treatment. The LV weight-to-body weight ratio was significantly higher in diabetic rats, which was significantly reversed by SB-209670 treatment. Blood pressure was not significantly different among the groups of animals.

These experimental diabetic rats exhibited severe glycosuria (250–500 mg/dl) that was not improved by SB-209670, whereas no study group showed urinary protein excretion. A 10-fold increase in the 24-h urinary volume was observed in diabetic rats, which was not affected by SB-209670. All diabetic animals survived throughout the study period regardless of whether SB-209670 was used.

The ET-1 level in LV tissues as determined by ELISA was significantly elevated in diabetic rats (2.1 ± 0.3 pg/mg, $n = 17$) compared with controls (1.4 ± 0.2 pg/mg, $n = 17$).

Expression of VEGF and its receptors. ELISA and Western blot analysis showed a 40% decrease in VEGF protein after 5 wk of diabetes compared with nondiabetic controls (Fig. 1, A and B). The ELISA kit applied in the present study recognizes both the 165- and 121-amino acid residue forms of VEGF. The present study used the following two antibodies for immunodetection of VEGF: anti-human VEGF mouse monoclonal antibody (Santa Cruz Biotechnology) and anti-human VEGF rabbit polyclonal antibody (Immunobiological Laboratories, Fujioka, Japan). Both of the antibodies could detect VEGF at 46 kDa and VEGF monomer at 23 kDa. We thus revealed the data obtained with anti-human VEGF rabbit polyclonal antibody, which is specific for human, rat, and mouse. In fact,

Table 1. General and hemodynamic characteristics of experimental rats

Parameters	Control ($n = 10$)	Diabetic ($n = 13$)	Diabetic with SB-209670 ($n = 13$)
Body wt, g	503±55	350±42*	361±38*
Plasma glucose, mg/dl	120±10	534±78*	535±79*
Plasma insulin, ng/ml	7.94±2.44	0.32±0.30*	0.24±0.17*
LV wt, mg	851±81	687±143*	716±66*
I.V wt-to-body wt, mg/g	1.59±0.11	2.31±0.35*	1.83±0.14†
SBP, mmHg	121±13	116±13	118±9
DBP, mmHg	93±11	86±10	86±26
LVSP, mmHg	112±21	106±12	108±6
LVEDP, mmHg	2.1±1.8	7.2±2.4*	4.4±1.7†
Heart rate, beats/min	360±39	337±18	336±22
LVEDD, mm	6.47±0.32	6.58±0.41	6.67±0.45
LVESD, mm	3.76±0.14	4.18±0.35*	3.97±0.31
IVST, mm	1.1±0.1	1.0±0.1	1.1±0.1
PWT, mm	1.3±0.2	1.2±0.2	1.2±0.1
Stroke volume, ml	0.48±0.02	0.41±0.08*	0.48±0.06†

Values are means \pm SD; n , no. of rats. LV, left ventricular; SBP and DBP, systolic and diastolic blood pressure, respectively; LVSP and LVEDP, mean LV systolic and LV end-diastolic pressure, respectively; LVEDD and LVESD, LV end-diastolic and LV end-systolic diameter, respectively; IVST and PWT, mean interventricular septal thickness and posterior wall thickness, respectively. $P < 0.05$ vs. control (*) and vs. diabetic (†).

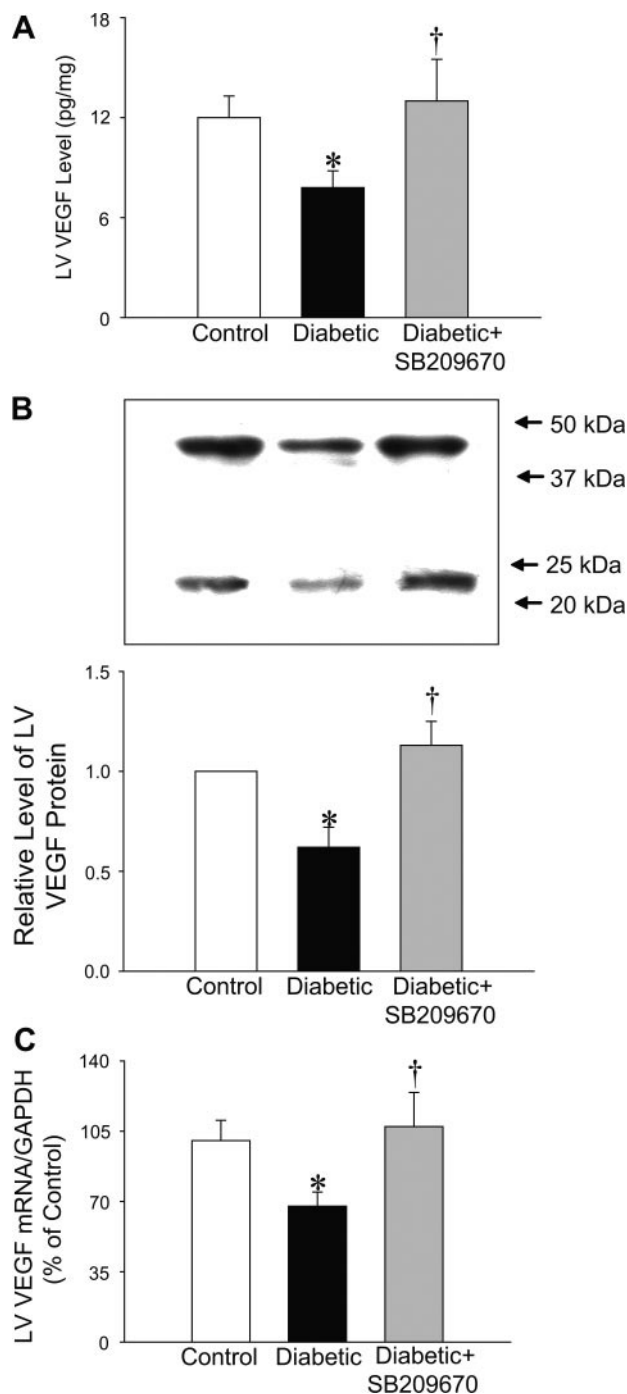


Fig. 1. Protein and gene expression levels of vascular endothelial growth factor (VEGF) in left ventricular (LV) tissues of nondiabetic control, diabetic, and SB-209670-treated diabetic rats. ELISA (A) and Western blot analysis (B) were performed for protein expression. For immunoblot analysis, in each of the experiments, the band obtained in the nondiabetic control is normalized to 1.0. C: total RNA isolated from LV was analyzed by real-time quantitative PCR. Values are means \pm SD. $P < 0.05$ vs. nondiabetic control (*) and vs. diabetic (†); $n = 17$ rats.

human VEGF₁₆₅ is typically expressed as a 46-kDa homodimer of 23-kDa monomers and is the major, most abundant, and most biologically active form of VEGF (39, 40). A similar reduction was also observed at its gene level in LV of diabetic rats, which was obtained from real-time quantitative

PCR (Fig. 1C). Treatment with SB-209670 for 4 wk normalized LV expression of VEGF at both protein and mRNA levels.

On immunoblots, LV expression levels of Flk-1 and Flt-1 proteins were reduced by 42 and 33%, respectively, in diabetic rats (Fig. 2, A and C). The decrease in protein expression of these VEGF receptors in LV of diabetic rats correlated with their mRNAs (Fig. 2, B and D). SB-209670 treatment greatly ameliorated downregulation of the two VEGF receptors in diabetes.

VEGF signaling pathways. As shown in Fig. 3, the phosphorylation level of the VEGF angiogenic receptor, Flk-1, was significantly lower in LV tissues of diabetic rats than in controls. The decrease in the phosphorylated Flk-1 level was completely reversed by SB-209670 treatment. In the present study, both expression levels of phosphorylated and nonphosphorylated Flk-1 were decreased in the diabetic heart, which was consistent with the previous report (45). In addition, when we calculated the ratio of phospho-Flk-1 to Flk-1, this ratio was also significantly decreased in diabetic heart compared with the nondiabetic control and was significantly reversed by

SB-209670 (Fig. 3). Two rabbit polyclonal antibodies raised against COOH terminus and against amino acids 931–997 of Flk-1 were used in this study, and the combined data from these antibodies are presented in Fig. 2A. Flk-1 is activated through ligand-stimulated receptor dimerization and trans(au-to)phosphorylation of at least six tyrosine residues as follows: Tyr⁹⁵¹ and Tyr⁹⁹⁶ in the kinase insert; Tyr¹⁰⁵⁴ and Tyr¹⁰⁵⁹ in the kinase domain; and Tyr¹¹⁷⁵ and Tyr¹²¹⁴ in the COOH-terminal tail (27, 39, 58). The present study detected the phosphorylation level at Tyr⁹⁵¹ and Tyr⁹⁹⁶ sites, and the data presented in Fig. 3 were the combined data from both of the antibodies.

The results of immunoblot analysis revealed that total protein expression of Akt, a downstream effector of VEGF, was unchanged by diabetes. However, the phosphorylated level of Akt was significantly diminished in diabetic rats. The decrease in Akt phosphorylation in diabetic LV tissues was also confirmed by analysis using the ELISA method (data not shown). We found a substantial reduction in the phosphorylated level of eNOS in diabetic LV tissues without any significant change in

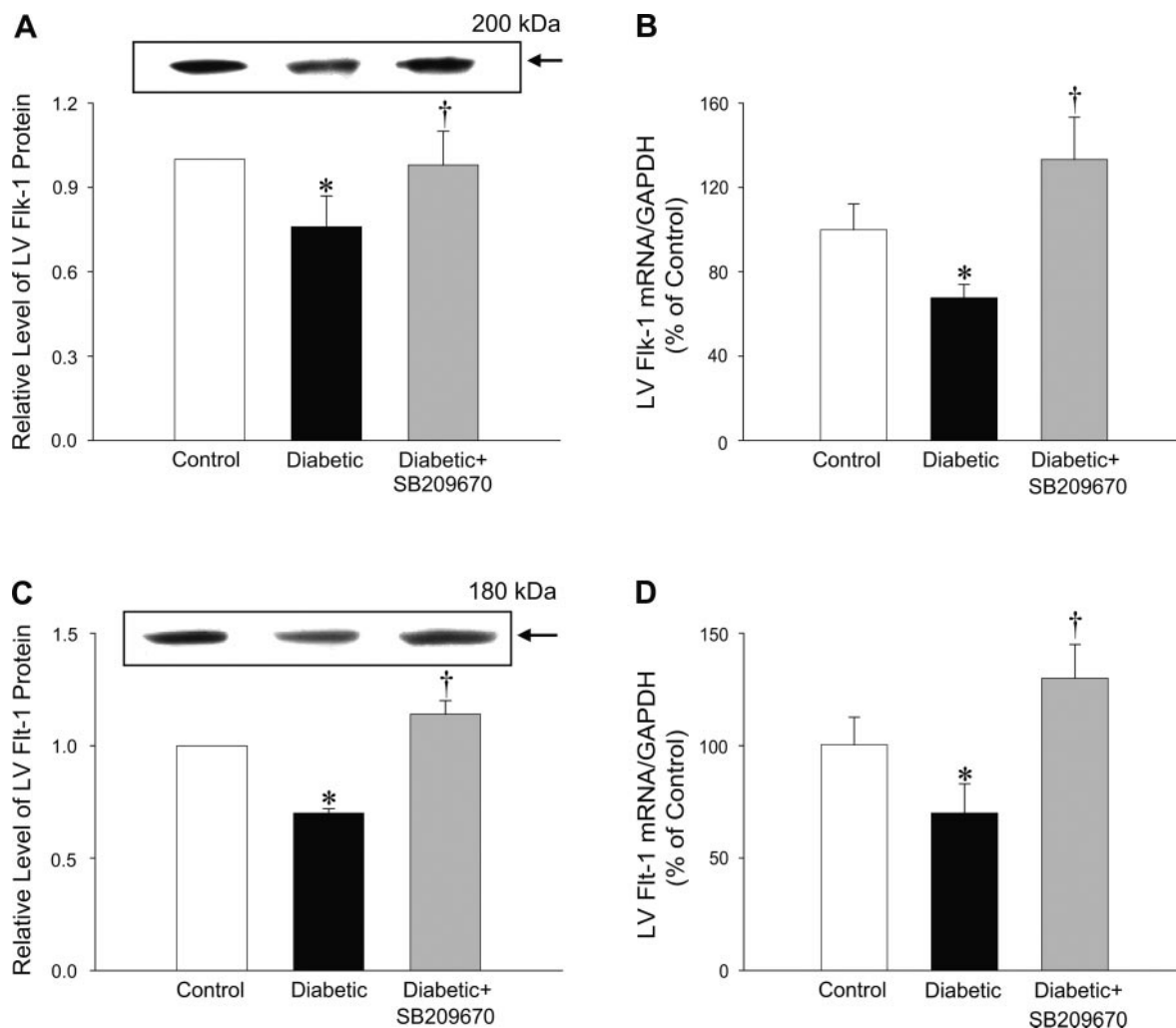


Fig. 2. Protein and gene expression levels of fetal liver kinase 1 (Flk-1; A and B) and fms-like tyrosine kinase 1 (Flt-1; C and D) in LV tissues of nondiabetic control, diabetic, and SB-209670-treated diabetic rats. Western blot analysis was performed for protein expression, and immunoreactive Flk-1 (A) and Flt-1 (C) were detected as a single band with a molecular mass of 200 and 180 kDa, respectively. For immunoblot analysis, in each of the experiments, the band obtained in the nondiabetic control is normalized to 1.0. Total RNA isolated from LV was analyzed by real-time quantitative PCR (B and D). Values are means \pm SD. $P < 0.05$ vs. nondiabetic control (*) and vs. diabetic (†); $n = 17$.

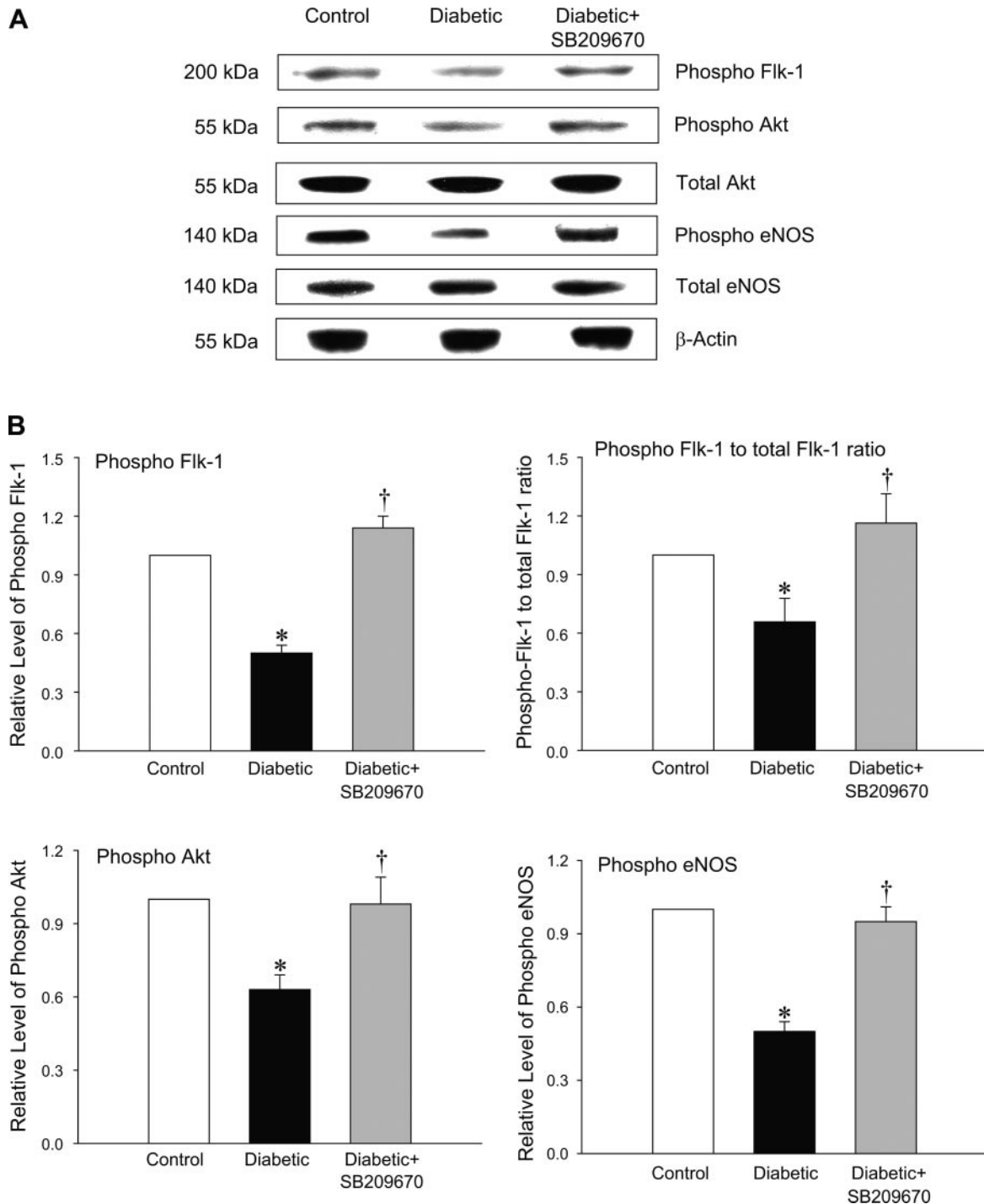


Fig. 3. Analysis of Flk-1, Akt, and endothelial nitric oxide synthase (eNOS) phosphorylation in LV tissues of nondiabetic control, diabetic, and SB-209670-treated diabetic rats. Protein phosphorylation was analyzed by Western blot using phosphospecific antibodies. *A*: representative blots. Protein loading was verified using anti- β -actin antibodies. *B*: densitometric results. In each of the experiments, the band obtained in the nondiabetic control is normalized to 1.0. Values are means \pm SD. * $P < 0.05$ vs. nondiabetic control; † $P < 0.001$ vs. diabetic; $n = 17$.

total eNOS expression. Treatment with SB-209670 normalized the decreased phosphorylation levels of Akt and eNOS observed in diabetic LV tissues. Immunofluorescence studies showed that phosphorylated Akt and phosphorylated eNOS were evidently reduced in endothelial cells of coronary vessels from diabetic rats compared with controls and the reductions were sharply ameliorated by SB-209670 treatment (Fig. 4).

Morphological data. The coronary capillary morphology obtained from lectin staining represented that streptozotocin-induced diabetes resulted in an evident reduction in coronary capillaries in LV sections (Fig. 5A). The total capillary density determined by the lectin method showed a 25% reduction in diabetes (Fig. 5B). Treatment of diabetic rats with SB-209670 significantly improved the capillary density to the control level.

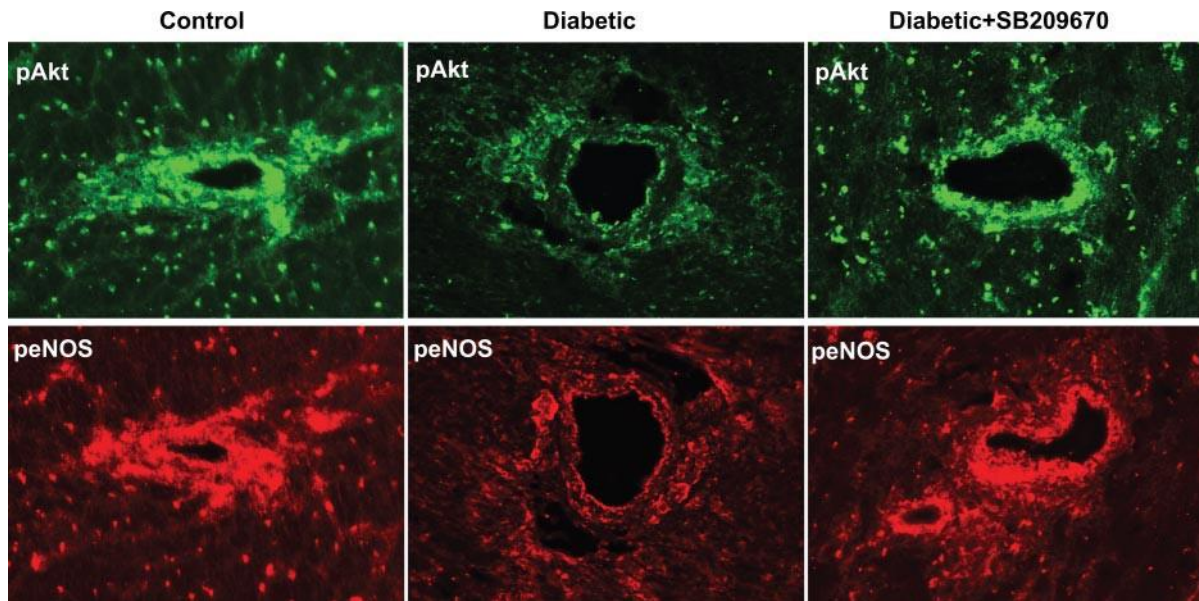


Fig. 4. Representative immunofluorescence images for phosphorylated Akt (pAkt) and phosphorylated eNOS (peNOS) in LV cross sections from nondiabetic control, diabetic, and SB-209670-treated diabetic rats from immunofluorescent staining. Positive staining was focused on coronary vessels. Magnification X200.

Figure 4C shows representative micrographs of coronary capillaries of LV sections in control, diabetic, and SB-209670-treated diabetic rats obtained by the double-staining method. The venular capillary portion, which stained red, was evidently remarkable in control rats, whereas the intermediate and arteriolar capillary portions, which stained violet and blue, respectively, were much pronounced in diabetic rats. Treatment of diabetic rats with SB-209670 evidently reversed the decreased portion of venular capillaries in diabetic rats.

Cardiac functions assessed by echocardiography. Representative M-mode echocardiograms of control, diabetic, and SB-209670-treated diabetic rats are shown in Fig. 6. LVFS and CO were significantly lower in diabetic rats compared with controls (Fig. 6, B and C). Treatment of diabetic rats with SB-209670 significantly ameliorated these cardiac function parameters. Although no significant difference was found in LVEDD and LVSP between control and diabetic rats, LVESD and LVEDP were significantly higher in diabetic animals and were improved to the control level by SB-209670 treatment (Table 1). Interventricular septal thickness and posterior wall thickness were almost the same among different experimental rats (Table 1). Treatment of nondiabetic control rats with SB-209670 did not significantly affect any parameters of cardiac functions ($n = 5$, data not shown).

DISCUSSION

The present study demonstrated that cardiac expression of VEGF and its receptors was downregulated, cardiac VEGF signaling was impaired, the coronary capillary density was decreased, and cardiac function went down in rats with 5 wk of streptozotocin-induced diabetes compared with age-matched nondiabetic control animals. The present study for the first time provides a comprehensive investigation of the VEGF, VEGF receptors, and the downstream molecules of VEGF angiogenic signaling (Akt and eNOS) with the morphometric evaluation of coronary capillary network in the early streptozotocin-induced diabetic heart, although some studies have already investigated

the VEGF and its receptor expression in streptozotocin-induced diabetic hearts of various durations. In addition, the key data from this study provide substantial evidence as to the importance of the ET system in the pathogenesis of these cardiac deteriorations in diabetes. We thus showed that altered VEGF signaling pathways and reduced coronary capillaries as well as cardiac dysfunctions were nearly completely prevented by treatment of diabetic animals with the mixed ET_A and ET_B receptor antagonist SB-209670.

There has been increasing research interest in understanding the impact of diabetes on coronary vascular function, and diabetes is identified as one of the first negative predictors of collateral vessel formation (1). The presence and extent of coronary collateral circulation may have prognostic relevance for individual patients, in terms of the outcome of coronary events (1). For instance, adequate collateral vessels in myocardium at risk may limit the size of infarct following coronary occlusion and, consequently, improve the prognosis (1). Because VEGF is known to play an essential role in angiogenesis (9), VEGF may participate in promoting the formation of new capillaries from preexisting coronary vessels under some pathological conditions. We found a significant downregulation of VEGF and its receptors, Flk-1 and Flt-1, in LV tissues of streptozotocin-induced diabetic rats. Previous work from another laboratory has also shown that, with the use of RT-PCR or Northern blot, cardiac gene expression of VEGF and its receptors is significantly decreased in streptozotocin-induced diabetic rats as well as established diabetic patients with hypertension and/or myocardial infarction (6). Moreover, the present study represents the first report that Flk-1 phosphorylation and Akt phosphorylation, both of which are an important event in VEGF signaling, were significantly impaired in streptozotocin-induced diabetic cardiac tissues. VEGF activates Akt via a phosphatidylinositol 3'-kinase (PI 3-kinase)-dependent pathway, and this downstream effector may play a key role in the "hallmark" biological processes of VEGF, such as endothelial cell proliferation, migration, and survival (7, 47). Al-

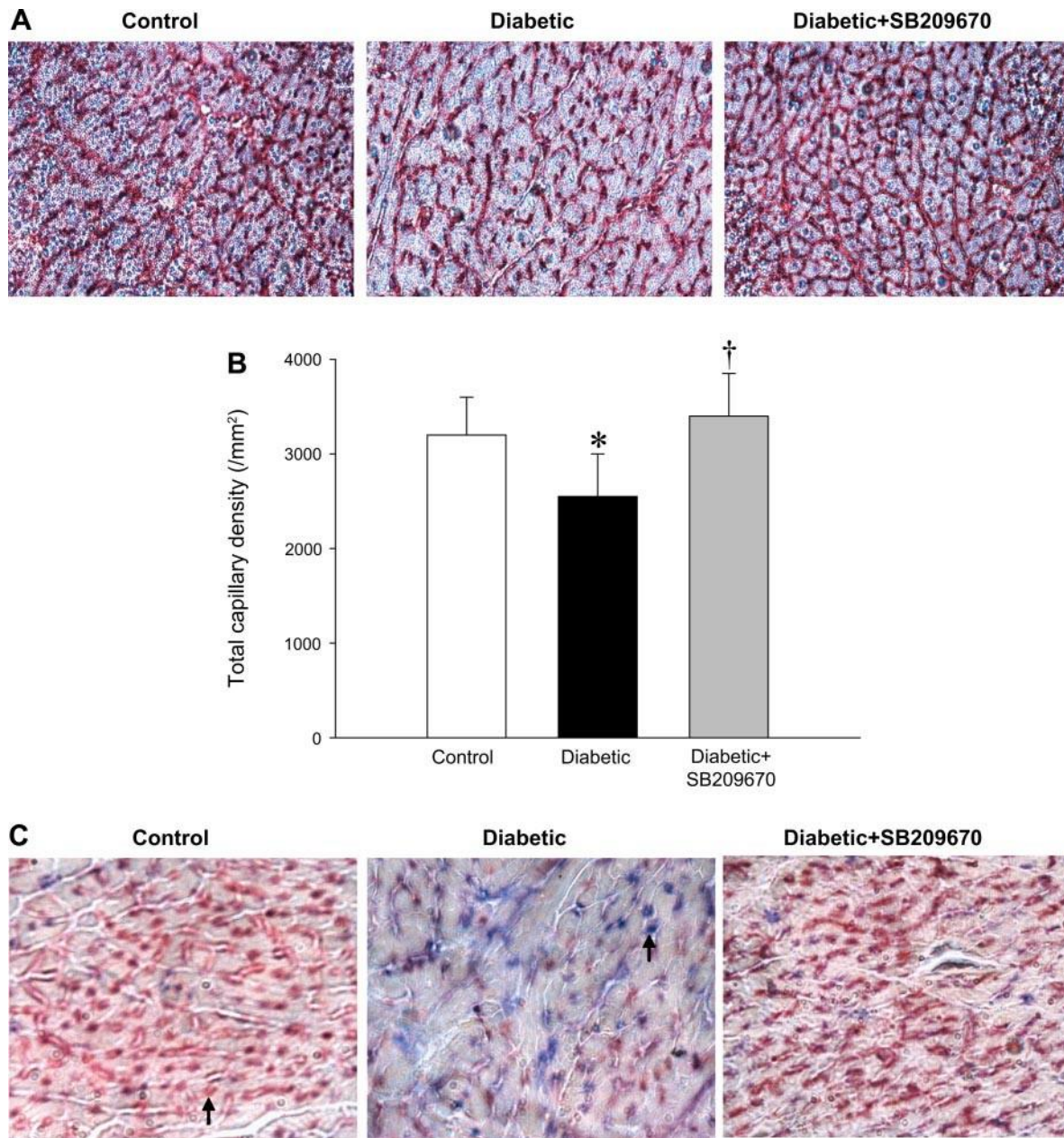


Fig. 5. Evaluations of morphologically existing coronary capillaries in LV sections of nondiabetic control, diabetic, and SB-209670-treated diabetic rats. **A**: photomicrographs showing capillaries detected by staining the endothelium with lectin. Magnification X400. **B**: bar graph summarizing the total capillary density, obtained by the lectin staining method. Values are means \pm SD (30 fields X 17 samples). $P < 0.05$ vs. nondiabetic control (*) and vs. diabetic (†). **C**: photomicrographs of subendocardial LV sections. Arteriolar, intermediate, and venular capillaries were stained blue, violet, and red, respectively (see arrows). Magnification X400.

though the PI 3-kinase/Akt signal transduction pathway regulates angiogenesis, Akt may be important in vascular homeostasis through eNOS activation (38). Thus Akt-dependent phosphorylation of eNOS is indicated to mediate the effects of VEGF on endothelial cells (10). We found a significant reduction in the phosphorylation level of eNOS in diabetic rat hearts. Various distribution patterns of eNOS may be seen in heart tissues from diabetic subjects in different studies. In the previous study, we have shown that, in a type 2 insulin-resistant diabetic rat model that exhibits modest hyperglycemia but strong insulin resistance, eNOS expression was increased in coronary vessels by immunohistochemistry compared with the

nondiabetic control (21). In the present study, the ratio of phospho-eNOS to total eNOS was decreased in streptozotocin-induced diabetic hearts although the total eNOS protein expression was almost unchanged. In another study, at 12 and 24 wk after streptozotocin-induced diabetes, the eNOS level in the heart tissue was significantly decreased (49). Thus we speculate that eNOS protein expression in the heart may be diminished with the progression of type 1 diabetes.

The exact mechanisms triggering the alterations in cardiac VEGF signaling in diabetes remains an open question. However, insulin deficiency may be suggested as one of the potential mechanisms because insulin can induce VEGF expression

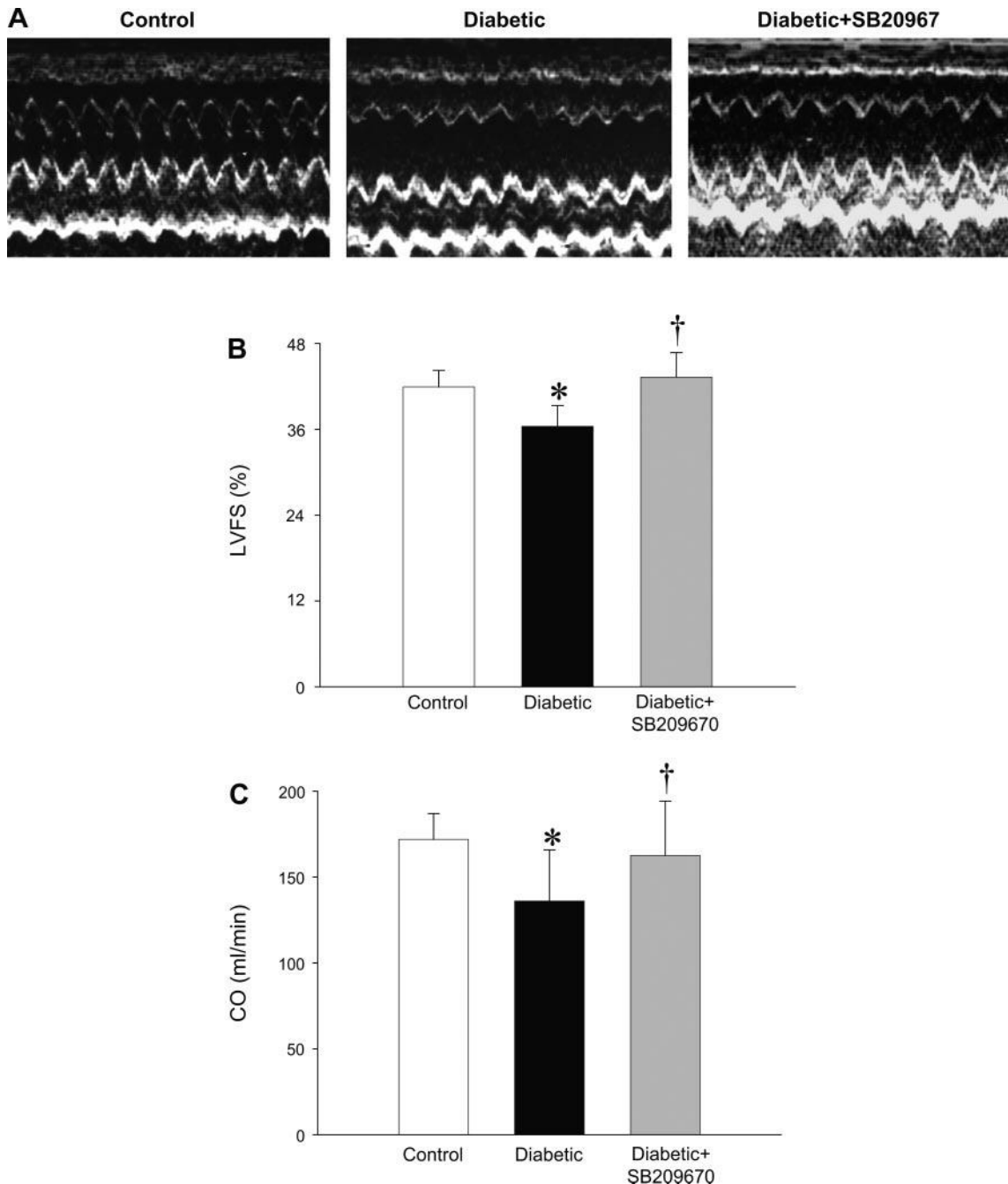


Fig. 6. A: representative M-mode echocardiograms of nondiabetic control, diabetic, and SB-209670-treated diabetic rats. A two-dimensional short-axis view was recorded at the level of papillary muscle. B and C: summarized data for LV fractional shortening (LVFS) and cardiac output (CO) measured by echocardiography. Values are means \pm SD. $P < 0.05$ vs. nondiabetic control (*) and vs. diabetic (†); $n = 10-13$.

(34). In different cell types, insulin has been shown to increase VEGF mRNA expression by activating the PI 3-kinase/Akt pathway (34). Intriguingly, contrary to the results obtained in streptozotocin-induced diabetic rats, a very recent study showed increased expression of VEGF but decreased expression of its receptors and signaling pathway in cardiac tissues of type 2 diabetic patients with chronic coronary artery disease (45). The reason for this apparent discrepancy is not clear but may be related to the possibility that plasma and tissue insulin levels may have been greatly elevated in type 2 diabetic patients in association with their insulin resistance.

The morphometric analysis showed arterIALIZATION of the venular capillary portion. Because capillary angiogenesis usually initiates from the venular site, the lower proportion of venular capillaries resulted in decreased capillary density in streptozotocin-induced rat hearts. Cardiac VEGF signaling may be involved in the angiogenic changes in the coronary capillary network. Therefore, in streptozotocin-induced diabetic rats, diminished VEGF signaling in the heart may be responsible for the decreased capillary density.

Generally, in diabetic rat models, cardiac function appears to deteriorate in proportion to the duration of diabetes. However,

cardiac dysfunction may be observed even at the prediabetic stage. Indeed, abnormal LV diastolic function has been found from the prediabetic state in Otsuka Long-Evans Tokushima Fatty rats, a model of human type 2 diabetes (36). In the present study, both systolic and diastolic dysfunction were evident in rats with 5 wk of streptozotocin-induced diabetes, although cardiac function appears to be depressed from a more early term in this diabetic model (19). The relationship between VEGF/VEGF receptor expression, microvascular integrity, and cardiac function has been demonstrated most vividly in experiments using transgenic mice lacking VEGF isoforms, VEGF₁₆₄ and VEGF₁₈₈ (4). These transgenic animals exhibit impaired myocardial angiogenesis, leading to the development of severe LV dysfunction (4). Direct intramyocardial gene transfer of plasmid DNA encoding human VEGF₁₆₅, which can replenish myocardial VEGF expression, could result in increased capillary density and thus improved myocardial function in the streptozotocin-induced diabetic rat model (57). However, to date, no pharmacological approach has been known to restore microvascular homeostasis and to recover cardiac function in diabetic cardiomyopathy. The present study represents the first evidence that ET receptor antagonist therapy can normalize VEGF signaling, coronary capillary density, and cardiac functions in diabetic rats.

We showed that the ET-1 level in LV tissues was 1.5-fold higher in diabetic than in control rats. In diabetes, the change in balance of endothelial mediators released by endothelium may shift to ANG II and ET, substances that enhance proliferation of smooth muscle cells and limit the coronary reserve of myocardium (41). The expression levels of ET-1 and its receptors have been documented to be augmented in the hearts of rats with both short and long terms of streptozotocin-induced diabetes (5, 29, 54). Finally, chronic ET receptor blockade with bosentan has been shown to improve functional cardiac performance in streptozotocin diabetic rats of more prolonged duration (53). A growing body of evidence thus has suggested that alterations in the ET system have a significant role in the pathogenesis of diabetic heart disease (5, 54).

The interaction between VEGF and ET-1 in vascular endothelial and smooth muscle cells is well documented (33). ET-1 and VEGF apparently play a complementary and coordinated role during neovascularization and malignant ascite formation in ovarian carcinoma (44). The endothelial autocrine regulatory role of ET-1 as a putative angiogenic factor in the process of neovascularization has been recently reported (43). In contrast with those reports, ET receptor blockade with bosentan shows a marked proangiogenic effect in an ischemic leg after femoral occlusion, and this effect appears to be directly dependent on the VEGF/eNOS pathways (17). Bosentan was considered to modulate ischemia-induced angiogenesis by increasing the speed of revascularization and maintaining sustained activation of the process (17). However, several lines of evidence suggest cell mitogen and proliferating effects of ET-1 in *in vitro* studies (13, 55, 56). We clearly demonstrate that ET receptor blockade with SB-209670 normalizes declined cardiac VEGF angiogenic signaling and thus impaired coronary capillary morphology in diabetic rats. In diabetes, plural stimuli may be involved in the angiogenic response, depending on the tissue type or organ investigated. Future studies should aim to shed light to the mechanistic insight underlying the improvement of the declined cardiac VEGF signaling by ET receptor

antagonism in diabetes. In the present study, blood pressure was unchanged after ET receptor blockade with SB-209670 in diabetic rats, implying the blood pressure-independent mechanism for the regulation of cardiac VEGF signaling. We confirmed that no significant change was seen in cardiac function or LV VEGF expression in nondiabetic control rats treated with SB-209670. Furthermore, the specific effect of SB-209670 on ET receptors was supported by our preliminary experiments showing no difference in VEGF levels between cultured neonatal rat ventricular cardiomyocytes with or without treatment with SB-209670.

In the present study, total myocardial protein extracts were used for determination of target molecules, which is similar to other studies addressing VEGF signaling in diabetic animal models (6, 45, 57). To gain more information on changes in the downstream molecules of VEGF angiogenic signaling, phosphorylated Akt and phosphorylated eNOS, in the diabetic heart, we performed immunofluorescence staining and found that phosphorylated Akt and phosphorylated eNOS were markedly downregulated in diabetic rat coronary endothelial cells and this downregulation was greatly improved by SB-209670. From the practical view point, it is difficult to create an environment in coronary endothelial cells simulating the present *in vivo* study condition to directly assess downregulation of the downstream molecules of VEGF angiogenic signaling. In addition, future studies should focus on exploring the upstream molecules that contribute to downregulation of the cardiac VEGF signaling cascade in diabetic rats. Further studies would be also required to shed light to the mechanistic insights into ET antagonist-mediated improvement of diabetic cardiomyopathy.

For the clinical assessment of our diabetic rat model, we observed the retina, kidney, and penile tissue in the current experimental setting. It should be noted that, at this diabetic stage, there was no morphological abnormality in retina. However, retinal VEGF expression was increased ~35%, which was significantly normalized by the blockade of ET receptors (31, 32). Although the VEGF expression level in the whole kidney was not significantly upregulated, renal NO levels declined but were reversed by SB-209670 (unpublished observation). Similar to our previous report, we found a significant decrease in VEGF and NO levels in the penile tissues (23), which was greatly improved by SB-209670 (unpublished observation). In light of the findings presented in this study, together with the above unpublished observations, we suggest that the ET-1 blockade may be a potential therapeutic approach to prevent diverse diabetic complications seen from the early stage.

Recently, we have demonstrated in a conference proceeding that, in the 2-wk streptozotocin-induced diabetic heart without any significant impairment in cardiac function, the downregulated gene expression of VEGF was normalized by a selective ET_A receptor blocker, whereas the downregulated VEGF receptor gene expression was not recovered (26). These observations have led us to use a dual ET receptor antagonist in the present study. We showed that both ET_A and ET_B receptor antagonism is effective in normalizing the defects in VEGF angiogenic signaling and the accompanied cardiac dysfunction in early streptozotocin-induced diabetic rats. Thus it seems to be important to block both ET receptors for the normalization

of the impairment in VEGF signaling in early type I diabetic heart.

Finally, our present study cannot directly point out the defect and downregulation of VEGF angiogenic signaling cascade to be the crucial cause to initiate and aggravate diabetic cardiomyopathy. As a result, the present findings cannot make any cause and effect conclusions about VEGF preventing the cardiomyopathy. These findings are corollary. This limitation of interpretation should be clearly taken into consideration before making any solid conclusion from the current findings.

In conclusion, data presented herein may imply that, in type 1 diabetes, downregulation and deficiency of cardiac VEGF and its signaling pathways may play a key role in the initiation and aggravation of diabetic cardiomyopathy resulting from impaired cardiac microvasculature, that ET antagonist can confer protection against type 1 diabetic cardiomyopathy, and that it also protects VEGF levels. From a molecular point of view, we showed for the first time the potential usefulness of the ET receptor blocker to prevent the development of diabetic cardiovascular complications.

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