Diabetes is associated with a perturbation of signaling pathways in vascular tissue, which causes vasomotor dysfunction such as hypertension and accelerated atherosclerosis. In the present study, the mechanisms of vasomotor dysfunction, Akt (Thr\textsuperscript{308} and Ser\textsuperscript{473}) phosphorylation and expression of endothelial NO (nitric oxide) synthase, and inducible NO synthase were investigated in human diabetic internal mammary arteries. The phospho-Akt (Thr\textsuperscript{308}) level in arteries from diabetic patients was reduced to about one-half of the level in nondiabetic patients, suggesting impaired insulin signaling in human diabetic vascular tissue. Augmented vasoconstriction was observed in diabetic arteries, due in part to deficiency of basal and stimulated NO production. This correlated with decreased endothelial NO synthase expression and activity in diabetic vessels. The sensitivity of diabetic vessels to the NO donor, sodium nitroprusside, was reduced as well, suggesting that NO breakdown and/or decreased sensitivity of smooth muscle to NO are also responsible for abnormal vasoconstriction. In addition, the abnormal vasoconstriction in diabetic vessels was not completely abolished in the presence of N\textsubscript{o}-nitro-L-arginine methyl ester, revealing that NO-independent mechanisms also contribute to vasomotor dysfunction in diabetes. In conclusion, diabetes downregulates the Akt-signaling pathway and compromises human arterial function through a decrease in NO availability as well as through NO-independent mechanisms. *Diabetes* 54:2415–2423, 2005

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**Original Article**

**Compromised Arterial Function in Human Type 2 Diabetic Patients**

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**Diabetes is associated with hypertension, accelerated development of atherosclerosis, and microcirculatory disorders (1–3).** The latter conditions may lead to retinopathy, nephropathy, and gangrene (3,4), which are common complications of diabetes. Although blood vessels are not classic targets of insulin signaling (5), insulin is known as a vasodilator (6,7) and an apoptosis (8) and mitogenic (9–11) regulator. Insulin signals through the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, which increases activity (10) and expression (12) of endothelial nitric oxide synthase (eNOS) and regulates a variety of cellular activities in endothelial and smooth muscle cells (9,13). For this reason, chronic insulin PI3K/Akt signaling deficiency could affect vasomotor mechanisms of blood vessels. During the last 10–15 years, the investigation of vasomotor dysfunction in diabetes has been carried out extensively in animal models, primarily streptozotocin-treated rats and alloxan-treated rabbits. Although the data on contractile responsiveness are controversial (14–16) and depend on the type and duration (17) of the disease, endothelial dysfunction based on nitric oxide (NO) deficiency (14,18,19) has been well established. Exaggerated production of vasoconstrictors (14,18,19) and elevated myogenic response of small arteries (20) have also been shown. The results obtained in animal studies, however, need to be validated in humans, especially since the recent discovery of significant divergence in vasomotor mechanisms between human and animal blood vessels (21,22).

Studies on the effects of hyperglycemia on eNOS and NO production in human endothelial cells have produced conflicting data. In some studies of human aortic endothelial cells, long-term exposure to high glucose (22–30 mmol/l) increased eNOS mRNA transcription (23) and basal NO production (24). In another study, the basal level of NO production and Ca\textsuperscript{2+}-stimulated NO production were found unchanged after similar high-glucose exposure in the same model, but insulin-stimulated NO synthesis decreased (25). The phosphorylated eNOS level was unchanged in this study but attenuated eNOS protein expression has been found in coronary artery endothelial cells (26). In addition, protein glycosylation resulted in reduction of eNOS phosphorylation by Akt (10), and glycated-oxidized HDL caused the attenuation of eNOS expression (27).
Due to controversial results, the above experimental models cannot faithfully predict the altered in vivo vasoresponsiveness in patients. In vivo studies have shown that an increase of skin blood flow in response to the endothelial cells agonists serotonin (28) and acetylcholine and the endothelial-independent NO donors (30) resulted in generation of 30 and 25% higher force, respectively. The present study was designed with the purpose of filling the gap in knowledge related to alterations of vascular regulation in human diabetes. We investigated the contributions of endothelial dysfunction as well as augmented smooth muscle contractility in abnormal vasoconstriction in diabetic patients and examined whether these defects are associated with alterations in the insulin signaling pathway.

**RESEARCH DESIGN AND METHODS**

**Tissue preparation.** Internal mammary arteries (IMAs) were collected from a total of 126 patients (aged 35–80 years) undergoing coronary artery bypass grafting at St. Paul's Hospital (Vancouver) with the approval of the institutional research ethics board. Diabetes status was accepted as diagnosed in patient medical records. Consent forms were obtained from the patients after the nature of the procedure was explained. Isolated segments of human mammary arteries were placed in vials with cold sterile RPMI-1640 medium, stored at 4°C, and used fresh for vasomotor function studies on the same day or the next day if obtained in the afternoon. For the vasomotor function studies, vessels were carefully cleaned of blood, adipose, and connective tissue, taking care to preserve the endothelium, and cut into 4-mm segments. For immunohistochemical study, vessels were fixed in 10% buffered (pH 7.4) formalin phosphate for 12–24 h and embedded in paraffin blocks. Investigation of molecular mechanisms was conducted in samples obtained from different groups of diabetic and nondiabetic patients. For this purpose, vessels were cleansed of cellular conditions and flash-frozen in liquid nitrogen within a few minutes after dissection. The flash-frozen samples were stored at −80°C until RNA and protein extraction.

**Force measurements.** For measurement of force generation by the vessels, segments were suspended in glass-jacketed tissue baths between two stainless steel clips, one of which was connected to a force transducer. Vessels were equilibrated under conditions as previously described (21) at a resting tension of 4 g. After two 80-mmol/l K+ challenges, phenylephrine concentration–response curves were generated in two adjacent rings, one of them being pretreated for 30 min with 200 μmol/l of an eNOS blocker Nω-nitro-arginine methyl ester (l-NAME).

To explore endothelium-dependent and -independent relaxation, vessels were precontracted with 50 μmol/l phenylephrine, after which cumulative applications of acetylcholine or sodium nitroprusside were made to generate concentration–response curves. All chemicals were purchased from Sigma.

Computer recording was accomplished through force-displacement transducers (FT03E; Grass Instrument Division, Astro-Med) connected with an ADInstruments OCTAL Bridge Amplifier and MacLab 8/s system of ADInstruments (Castle Hill, Australia). Chart recording software (ADInstruments, Mountain View, CA) was used for data acquisition.

**RT-PCR.** Total RNA and protein were extracted from IMA specimens by using TRIzol reagent (Invitrogen Life Technologies). RNA was reverse-transcribed into cDNA in a Gene Amp thermocycler (Gene Amp PCR System 9700; Applied Biosystems) and 5-μl first-strand cDNA was used in the PCR experiments with the addition of gene-specific forward and reverse primers. The sequences of each primer are presented in Supplementary Table S1. The standard curve was generated using each primer. PCR products (10 μl) were electrophoresed in agarose gel staining with ethidium bromide. Gels were photographed in ultraviolet light using Digi Doc-It software (BioRad). The intensities of bands were analyzed by Quantity One Image software (BioRad). The eNOS and iNOS mRNA levels were normalized to the β-actin mRNA signal (40).

**Western blotting and NO synthase activity.** Protein concentration in the extracts was determined by Bradford reagent using BSA as the standard (BioRad). Activity of NO synthase (eNOS and iNOS together) in protein extract (30 μg) was determined colorimetrically by using a NO synthase assay kit (Calbiochem) according to the manufacturer’s instructions. After denaturation, protein samples were separated on SDS-PAGE and transferred to polyvinylidenefluoride membranes (BioRad). Non-specific binding sites were blocked, then membranes were incubated with the primary mouse monoclonal antibodies against human eNOS and iNOS (BD Biosciences) and primary rabbit polyclonal antibodies against Akt, phospho-Akt (Thr308), phospho-Akt- (Ser177), and phospho-eNOS(Ser177) (Cell Signaling). Afterward, membranes were incubated with peroxidase-conjugated secondary antibodies (Sigma), and then immunoreactive bands were detected with the Enhanced Chemiluminescent Western Blotting Detection Kit (Amersham Life Sciences). For each protein level, two membranes were reprobed with anti-β-actin antibodies (Sigma). The expression level of each protein was presented as a ratio of the density of the target protein band versus that of the β-actin from the same sample or as indicated in RESULTS.

**Statistical analysis.** The data are presented as means ± SE. Relaxation is presented as a percentage of the initial contraction. Half-maximal effective concentration (EC50) values for concentration response curves were determined for each curve, and mean log EC50 values were calculated thereafter as an average for each group of patients. The Student’s t test was used for comparison of the two groups. The two-way ANOVA for the concentration response curve comparison and correlations were tested in JMP (SAS Institute).

**RESULTS**

**Patient statistics.** Patients with type 2 diabetes ranged in age from 35 to 78 years, with an average age of 63.35 ± 2.02 years. In the control group, ages ranged from 35 to 80 years with an average age of 63.69 ± 1.28 years. The portion of patients with both types of diabetes was 32.61% of the total. The portion of diabetic females in the total number of diabetic patients (36.4%) was higher than the portion of nondiabetic females in the total number of nondiabetic patients (22.5%). In all, 30% of the total number of diabetic patients had type 1 diabetes. All measured parameters from the type 1 diabetic patients were within the range of data from type 2 diabetic patients. However, the number of patients with type 1 diabetes (three to four for each type of experiment) was not sufficient for statistical treatment, so data on type 1 diabetes are not presented.

**Smooth muscle and endothelial function.** α-Adrenergic stimulation with 50 μmol/l phenylephrine and depolarization in 80 mmol/l K+ buffer solution of diabetic IMAs resulted in generation of 30 and 25% higher force, respectively, than that in nondiabetic arteries (Table 1). Comparison of phenylephrine concentration response curves for diabetic and nondiabetic arteries revealed that the former was more sensitive to low concentrations of the agonist (Fig. 1).

To examine the role of endogenous NO in the increased vasoconstriction of diabetic vessels, phenylephrine concentration–response curves were also generated after pretreatment with the NOS inhibitor l-NAME. Figure 2A illustrates that inhibition of endogenous NO synthesis
results in a notably higher contractile response in nondiabetic arteries. In nondiabetic arteries precontracted with 50 μmol/l phenylephrine, addition of L-NAME caused increases in tension (data not shown) comparable with the difference observed between the curves in Fig. 2A. This demonstrates a significant level of NO production in vessels of nondiabetic patients. L-NAME, however, did not significantly increase the sensitivity of nondiabetic vessels to phenylephrine, as can be seen from the EC50 values for phenylephrine in the absence and presence of L-NAME (Table 1 and Fig. 1).

In contrast to nondiabetic arteries, the IMAs from diabetic patients demonstrated neither increased response to phenylephrine after L-NAME pretreatment (Fig. 2B) nor changes in tension if L-NAME was added at the plateau after phenylephrine precontraction. This reveals a lack of endogenous NO.

Stimulated endothelium-dependent relaxation is also impaired in diabetic vessels (Fig. 3A). Acetylcholine, an agonist of endothelial cells, induced ~80% relaxation of phenylephrine-precontracted arteries of nondiabetic individuals, whereas the maximal relaxation of arteries of diabetic patients was <60%. Moreover, EC50 values for acetylcholine that represent the sensitivity of endothelium to this agonist (Table 1) indicate that the arteries of diabetic patients were >10 times less sensitive to acetylcholine than nondiabetic patients. Maximal levels of acetylcholine-induced relaxation in the nondiabetic group were inversely correlated with the age of the patients (Table 1). EC50 values tended to associate with the age. This association of endothelial function with the age was weakened in diabetic patients.

It is possible that the NO produced by endothelial cells in diabetic vessels is less efficacious. To estimate the component of relaxation, which is lost in the diabetic IMAs because of decreased sensitivity of smooth muscle to NO, the relaxation induced by sodium nitroprusside (SNP) was studied. SNP is a donor of NO and bypasses endogenous NO production by endothelial cells. Although SNP could completely dilate both types of phenylephrine-precontracted arteries, diabetic vessels required higher concentrations of SNP to achieve the same level of a relaxation than nondiabetic vessels (Fig. 3B and Table 1).

The augmented vasoconstriction of the diabetic artery is not solely due to NO-related factors. In fact, elimination of endogenous NO did not equalize the contractile responses of diabetic and nondiabetic arteries. Thus, despite the presence of L-NAME, diabetic arteries responded to lower concentrations of phenylephrine than nondiabetic arteries (Fig. 1), and the amplitude of the contraction was higher. This suggests that the intrinsic contractility of smooth muscle cells is enhanced in diabetes.

**NO synthase enzyme activity and expression.** To analyze the causes of functional NO deficiency in diabetic arteries, NO synthase activities, the integrity of the endothelial layer, eNOS and iNOS expression, and eNOS phosphorylation levels were investigated. The total activity of NO synthase was 26.1% downregulated in diabetic arteries (Fig. 4A). Staining of arterial cross-sections with an endothelial cell marker, von Willebrand factor, did not reveal endothelial denudation in either diabetic or nondiabetic patients. The average thickness of endothelial layer in diabetic and nondiabetic IMAs was similar (Fig. 4B and C). Hence, the decreased NO production in diabetic arteries did not result from denudation of the endothelium.

RT-PCR and Western blotting revealed a 50% decrease in transcriptional level and ~30% decrease in protein expression of eNOS (Fig. 5A and B) in the diabetic arteries compared with the tissue from nondiabetic patients. The phospho-eNOS-Ser1177 level normalized to the total eNOS protein was not changed (Fig. 5C), which means that the absolute amount of phospho-eNOS-Ser1177 was decreased proportionally with the decreased expression of the eNOS.
In contrast to eNOS, the transcriptional level of iNOS tended to be increased and the protein expression was almost doubled in diabetic arteries (Fig. 6A and B).

Akt signaling. Akt phosphorylation is a key step in insulin signaling and may contribute to functional alterations in diabetic cells. Therefore, the overall Akt expression and the phospho-Akt levels were measured. Vessels from diabetic patients had a dramatic reduction in phospho-Akt-Thr308 (Fig. 4B), whereas neither phospho-Akt-Ser473 nor total Akt expression were significantly different between diabetic and nondiabetic groups. There was no association found between phosphorylation of the Thr308 and the Ser473 site.

DISCUSSION

The striking result of this study is that vascular dysfunction associated with diabetes can be demonstrated in isolated IMAs from a population of cardiac patients during coronary bypass surgery. Thus, despite considerable heterogeneity due to differences in sex, age, and disease status, it appears possible to study disease mechanisms in human diabetic arteries, which represent a more relevant experimental model than blood vessels from rodents.

Based on the sample population of patients involved in this study and data from other studies (41), the portion of diabetic patients among all bypass patients is ~33%, which is greater than the portion of people with diabetes in the general population. The proportion of type 1 diabetes patients in bypass surgery is also greater than the proportion in the general population of diabetic patients. Diabetes disrupted the inverse relationship between age and endothelial function. Finally, diabetes appears to increase the proportion of female patients among all bypass patients. These observations are in agreement with the recognized importance of diabetes as a risk factor in cardiovascular disease.

Our data show that diabetes adversely affects multiple cellular mechanisms in human arteries, thus causing vaso-motor dysfunction. Stimulation of diabetic arteries resulted in augmented vasoconstriction, due to both endothelial dysfunction and increased contractility of smooth muscle cells. First, the basal level of NO in diabetic vessels was not sufficient to regulate vascular tone (15,19). Furthermore, we demonstrated attenuation of stimulated endothelium-dependent relaxation in response to the endothelial cell agonist, acetylcholine, in the diabetic arteries (Fig. 3A), which supports previous findings for these vessels (35,36). The reduced responsiveness in diabetic individuals to endothelial cell agonists has also been observed in vivo (28,29,42,43). Thus, the endothelium of human diabetic arteries is not able to generate the level of NO required to regulate blood flow and pressure through alterations in lumen diameter.

One of the causes of the failure of relaxation is the attenuation of the overall NO production (Fig. 4A) due to reduction in eNOS mRNA and protein expression (Fig. 5).
This is in contrast to the mouse diabetic model in which eNOS expression was not decreased (19), which emphasizes the difference between the diabetic alterations in humans and in animal models. It should also be noted that an increase in iNOS expression observed in the human diabetic tissue (Fig. 6) did not compensate for the loss of eNOS activity.

The second cause for impaired relaxation appears to be enhanced breakdown of NO in diabetic vessels, as the relaxation in response to the NO donor SNP was also affected in the diabetic arteries (Fig. 3B). This is different from a previous study on human vessels (36), in which no difference in SNP effects was found between diabetic and nondiabetic patients. NO breakdown could be due to reactive oxygen species, which are elevated in human diabetic vessels (31,32). Altered smooth muscle sensitivity to NO could also play a role in the decreased effect of NO donors. Our data suggest that diabetic patients may be less sensitive to nitrovasodilators. Indeed, reduced responses to SNP and glycerol trinitrate have been observed in vivo in type 2 diabetic patients (29,43). All together, a weakened NO control over vasomotion is one of the causes of augmented vascular tone in diabetic arteries. The loss of NO in human diabetic vascular tissue could also contribute to the accelerated development of atherosclerosis.

Although the endothelium seems to be the first target of diabetes, the vasomotor dysfunction in human diabetes is not limited to a reduction of NO levels. The second

FIG. 4. A: Overall NO synthase (NOS) activities in diabetic and nondiabetic vessels (control, n = 18; diabetic, n = 14) *P < 0.05. B: Average thickness of the endothelial layer in cross-sections of IMAs from diabetic and nondiabetic patients stained with von Willebrand factor (n = 6). C: Examples of stained cross-sections of diabetic and nondiabetic arteries.

FIG. 5. A: eNOS mRNA expression in IMAs of diabetic and nondiabetic patients (n = 9) with representative mRNA bands. *P < 0.05. B: Western immunoblotting of eNOS in IMAs of diabetic (n = 13) and nondiabetic patients (n = 22) with a representative immunoblot. **P < 0.01. C: Phospho-eNOS (Ser1177) normalized to total eNOS in the same IMAs with a representative immunoblot.
mechanism is independent of NO and represents the increased sensitivity of diabetic smooth muscle cells to agonist-induced stimulation because the difference in the phenylephrine response between diabetic and nondiabetic arteries is not abolished by addition of l-NAME (Fig. 1). The augmented vasoconstriction is especially marked at the lower to moderate agonist concentrations, which are within the physiological range. The mechanisms by which smooth-muscle responsiveness is enhanced in diabetic human vessels should be further investigated. Increased contractile activity may be related to abnormal intracellular Ca\(^{2+}\) metabolism (32), which is associated with the diabetic state. Alternatively, sensitization to Ca\(^{2+}\) through a Rho-kinase pathway, which is a feature of excitation-contraction coupling in human vessels (21,22), may contribute to elevated vasoconstriction in diabetes. Endogenous prostaglandins or endothelin-1 may also be factors in abnormal vasoconstriction (14,18,19).

In further exploring the alterations in signaling mechanisms in human diabetic vessels, we found that phosphorylation of Akt, the key intermediate of the insulin signaling pathway, was attenuated in diabetic arteries to ~50% of that in nondiabetic vessels (Fig. 7). More specifically, Akt phosphorylation was impaired at the Thr\(^{308}\) site, which is a downstream target of PI3K through 3-phosphoinositide-dependent kinase 1, whereas neither Akt protein expression nor its phosphorylation at Ser\(^{473}\) were affected. This indicates that Akt protein itself was present in the tissue but PI3K signaling to Akt was impaired. Interestingly, that experimental insulin resistance in adipose cells could be induced by inhibition of Akt Thr\(^{308}\) phosphorylation with practically unchanged Ser\(^{473}\) phosphorylation (44). As the Akt signaling pathway regulates a variety of mechanisms in both endothelial and smooth muscle cells, it could participate in alterations of vascular function in diabetes. In endothelial cells, it could lead to downregulation of eNOS activity because failure of endothelial function occurred without denudation of endothelium (Fig. 4). Akt normally activates eNOS through phosphorylation at Ser\(^{1177}\), and a deficit of Akt phosphorylation could result in lowered eNOS activation. In this context, inhibition of PI3K-Akt signaling pathway downregulated eNOS expression induced by insulin (12). Although we found the same ratio of the phospho-eNOS-Ser\(^{1177}\) to total eNOS in both groups, the absolute level of phospho-eNOS\(^{1177}\) was decreased with the reduction in expression of the protein, which could explain the observed reduction in NO level in diabetes.

Thus, the decreased level of Thr\(^{308}\)-phosphorylated Akt protein and the reduced eNOS expression indicate that the
insulin signaling pathway is compromised in human diabetic vascular tissue. This could contribute to the endothelial dysfunction, which was (at least in part) responsible for the augmented vasoconstriction in diabetic individuals. The upregulated contractile state of blood vessels contributes to hypertension and diminishes blood flow, thus accelerating development of atherosclerosis associated with diabetes. Reduced Akt phosphorylation in diabetic patients could also underlie some other alterations coupled with diabetes.

Upregulated iNOS expression neither compensated for the reduced production of NO by eNOS nor led to NO-mediated vasodilation in the diabetic IMAs (Fig. 2B). Although Akt signaling, which is known to induce iNOS, was downregulated, we found increased iNOS expression in diabetic arteries. iNOS is known to be induced in diabetes (45), and iNOS induction was associated with impairment in Akt activation in muscle in the mouse model, whereas disruption of iNOS protected against insulin resistance (46). This is consistent with the well-recognized detrimental role of iNOS in diabetes complications and is possibly related to high levels of superoxide, which with NO forms peroxynitrite. In fact, iNOS was identified as a source of high levels of peroxynitrite in diabetic platelets (47).

The herein described abnormal vasoconstriction of human diabetic arteries was observed in vitro in the absence of insulin and with equal glucose concentrations in the media for diabetic and nondiabetic vessels. This indicates the presence of inherent alterations in cell structure or signaling pathways in diabetic vascular tissue, although these alterations could be the result of long-term exposure of vascular tissues to high glucose and insulin. One of the causes of change in cell function could be glycation of proteins (10,48). Alternatively, cellular malfunction could be a manifestation of insulin resistance primarily developed in vascular tissue of type 2 diabetic patients. Exposure to insulin and glucose should further magnify the difference between diabetic and nondiabetic tissue. Insulin at physiological concentrations increases the eNOS activity (10) and expression in endothelial cells (12). Insulin added in vitro during the experiment is expected to have lower relaxing effects in diabetic vessels because its signaling is downregulated in diabetic tissue. Hyperglycemia inhibits eNOS phosphorylation at the Akt site (49). The overall effect of diabetes on in vivo vascular function is a combination of inherent alterations in vascular cells and the influence of in situ concentrations of glucose and insulin, either of which could vary with age, type of diabetes, and duration of disease state.

In conclusion, the present study demonstrates the feasibility of using human vessels obtained during coronary...
artery bypass surgery for investigation of the mechanisms of vascular disorders in human diabetes. We showed that diabetes compromises the insulin signaling pathway in the IMAs obtained from diabetic patients. Augmented vascular constrictor observed in the diabetic vessels is caused by multiple cellular mechanisms. One mechanism is endothelial dysfunction, which was confirmed by the decreased basal NO levels and impaired relaxation in response to endothelial cell stimulation, as well as by reduced eNOS expression and activity. The second mechanism is NO breakdown or decreased sensitivity of smooth muscle cells to NO, which is evident from the impaired relaxation in response to the NO donor, SNP. Finally, increased smooth muscle sensitivity to stimulation is another contributing factor. Altered diabetic vascular tissue reactivity should be taken into consideration in therapeutic care of diabetic patients.

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