Acute Endothelium-Mediated Vasodilation Is Not Impaired at the Onset of Diabetes

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Abstract—Vascular injury and impaired vascular function are central to the increased mortality associated with diabetes. Hyperglycemia in diabetes has been suggested to play a role in this process, in part by impairing the function of the vascular endothelium. It has been difficult, however, to isolate the direct effect of glucose in both humans and in animal models of diabetes. This was evaluated in the present study in 7 rats that were chronically instrumented with a Transonic flow probe at the iliac bifurcation of the abdominal aorta, a nonoccluding catheter inserted immediately anterior to the flow probe, and a femoral vein catheter. Acute infusions of acetylcholine and sodium nitroprusside (1 and 10 μg/min IA) increased hindquarter blood flow significantly by approximately 27 and 10 mL/min over baseline, respectively, at the high dose. Streptozotocin (70 mg/kg IV) was administered, but normoglycemia was maintained with continuous intravenous insulin infusion to control for potential streptozotocin side effects. Diabetes was induced 5 to 7 days later by stopping the insulin infusion. Hindlimb blood flow (measured 24 hours per day) decreased during the diabetic period and was accompanied by an increase in mean arterial pressure, suggesting a vasoconstrictor response. However, the responses to acetylcholine and sodium nitroprusside were not altered significantly on either day 2 or day 6 of the diabetic period. This suggests that neither endothelium-mediated vasorelaxation nor responsiveness to nitric oxide is impaired during the initial phase of diabetes and that diabetic hyperglycemia does not have a significant, direct effect to impair endothelium-mediated relaxation in insulin-dependent diabetes mellitus. The mechanism for the change in baseline blood flow and its potential influence on endothelial function, however, are not known. (Hypertension. 1998;32:541-547.)

Key Words: endothelium-derived relaxing factor • blood flow • diabetes • glucose • vasodilation • nitric oxide

Cardiovascular dysfunction and vascular injury are the leading causes of morbidity and mortality for diabetic patients,1,3 yet the underlying mechanisms remain unclear. There is good evidence that cumulative actions of glucose mediated at the tissue level can markedly alter vascular structure and function,4–6 and other hormonal and metabolic factors likely exacerbate those actions. However, it is unclear what direct actions glucose has that are important in the development and progression of the vascular disease process in diabetes.

One potential action that has received considerable interest is an interaction between glucose and the vascular endothelium. Endothelium-mediated vasodilation has been reported to be impaired in the early stages of insulin-dependent diabetes mellitus (IDDM) in humans, with no other major complications.2,5 Those findings suggest that the hyperglycemia in diabetes may have a direct effect on the vascular endothelium to impair its ability to mediate vasodilation; however, many other studies have found no impairment in endothelium-mediated vasodilation in diabetes.10–12

One possible explanation for the opposing findings may be the stage of diabetes during which function is assessed. Cumulative actions of hyperglycemia in IDDM might lead to structural changes that are the actual cause of impaired endothelial function, and it is virtually impossible in humans at this time to determine when these changes begin and at what point they may be great enough to affect function significantly. Thus, it is not known in IDDM whether the hyperglycemia can impair endothelial function directly or whether that impairment is the result of some cumulative, secondary effect such as a change in structure.

In the present study, we used a unique model of streptozotocin (STZ) diabetes in rats13 to evaluate endothelial function immediately after induction of diabetes, before sufficient time was allowed for development of structural changes in vascular tissues. The unique feature of this model is that potential side effects of STZ were controlled for with an intravenous insulin-replacement regimen and by induction of the diabetic period by withdrawal of the insulin-replacement therapy.13 This allowed independent assessment of the direct effect of diabetic hyperglycemia on endothelium-mediated dilation in vivo. This study therefore evaluated endothelial function during the first 6 days of chronic IDDM while controlling for potential side effects of STZ.

Methods

The experiments were conducted in 7 male Sprague-Dawley rats (~350 g, Harlan Sprague-Dawley, Madison, Wis), and the proto-
cols were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. Anesthesia was induced with pentobarbital (50 mg/kg), and atropine was administered (40 µg per rat, IP) to ensure an unobstructed airway. Under aseptic conditions, a laparotomy was performed, and a 2-mm SB Transonic (Transonic Systems Inc) flow probe was placed on the abdominal aorta at the iliac bifurcation. A nonocclusive polyvinyl catheter then was implanted in the abdominal aorta, immediately anterior to the flow probe, by inserting the 4-mm catheter tip through a puncture wound in the aortic wall made with the tip of an L-shaped 18-gauge needle. The insertion point was sealed with cyanoacrylate adhesive, and the catheter and flow probe cable were exteriorized through the lateral abdominal wall. A femoral vein catheter was implanted through a separate incision, and the tip was maneuvered into the vena cava. All incisions were infiltrated with penicillin G procaine (300,000 U/mL) and bupivacaine (0.25%) at closure, and the flow probe leads and catheters were routed subcutaneously to the scapular region and exteriorized through a Dacron-covered plastic button sutured subcutaneously over the scapulae.

The rats were allowed to recover from surgery and then were placed in individual metabolic cages in a quiet, air-conditioned room with a 12-hour light cycle. The catheters and flow probe leads were passed through a stainless steel spring that was attached to the button, and the opposite end of the spring was connected to a customized adapter on an electrical swivel (Airflyte Electronics) mounted above the cage. The 4 flow probe leads were soldered to the swivel wires, and the artery and vein catheters were passed through a hole in the center of the electrical swivel and through a 3-cm section of spring to a dual-channel hydraulic swivel (Instech) above. The short spring section served to connect the 2 swivels so that both would turn synchronously with rat movement.

The venous catheter was connected, via the hydraulic swivel, to a syringe pump (Harvard Apparatus) that ran continuously throughout the study. All solutions contained antibiotic (penicillin G 25,000 U/d per rat and mezlocillin 0.03 g/d per rat) and were infused through a Millipore filter (0.22 mm, Cathivex, Millipore Corp). The arterial catheter was filled with heparin solution (1000 USP U/mL) and reconnected to the swivel, and a T-connector was inserted, also via the hydraulic swivel, to a pressure transducer for continuous measurement of arterial pressure. The flow probe connector was soldered to the electrical swivel wires and connected to a Transonic model T101 flowmeter (Transonic Systems Inc) for continuous measurement of hindquarter blood flow. The pulsatile flow signals from the flowmeter and the amplified pulsatile arterial pressure signals were sent to an analog-digital converter and analyzed by computer using customized software. The analog signals were sampled at 500 samples per second for 4 seconds every 60 seconds continuously throughout the experiment. Total sodium intake throughout the experiment was maintained constant at approximately 3.1 mmol/d by continuous intravenous infusion of 20 mL/d sterile 0.9% saline combined with sodium-deficient rat chow (0.066 mmol sodium/g; Teklad). A sodium-deficient diet ensured that the daily sodium intake could be controlled precisely at normal levels by the infusion. This infusion was begun immediately after placement of the rat in the metabolic cage, and 5 to 7 days were allowed for acclimation.

**Experimental Protocol**

A 3-day precontrol period followed acclimation, and then STZ was administered (70 mg/kg IV). The following day, after rats were determined to be hyperglycemic, a continuous intravenous infusion of regular insulin (porcine, Novo Nordisk) was begun at 4 U/d by adding the insulin to the daily saline infusion. The insulin dose was titrated per rat on an individual basis, based on daily blood glucose measurement, to maintain glycemic control for the next 5 to 7 days, which was termed the control period. We have demonstrated previously the ability of this method to maintain good glycemic control in STZ-treated rats. After normal stable blood glucose concentrations were established, the insulin dose was lowered to 0 to induce diabetes; the diabetic period lasted 6 days. To control for potential time-dependent effects, the diabetic period was followed by a recovery period in which the insulin dose was raised to restore good glycemic control. This is a powerful design because each animal serves as its own control. The acute infusion studies were performed on days 2 and 6 of the diabetic period and once during each of the other periods.

**Acetylcholine and Sodium Nitroprusside Infusions**

Acetylcholine (ACh) was used to estimate endothelium-mediated vasorelaxation, and sodium nitroprusside (SNP) was used to estimate vascular responsiveness to nitric oxide (NO). The acute infusion procedure was begun at approximately 8:30 AM on scheduled days, and the rats were left undisturbed in their metabolic cages throughout the infusion. The abdominal aortic catheter was cleared, flushed with saline, and reconnected to the swivel, and a T-connector was inserted into the line leading from the swivel to the pressure transducer. An infusion pump was connected to the T-connector, and a 0.9% saline infusion at 0.903 mL/min was started through the arterial catheter.

After 20 minutes, the saline vehicle was exchanged for a solution of ACh in saline that delivered ACh at a dose of 1 µg/min. This was infused for 20 minutes and exchanged for a solution that delivered ACh at 10 µg/min. After 20 minutes at that dose, a 20-minute saline vehicle infusion was resumed and then followed by 20-minute infusions of SNP at 1 and 10 µg/min and then by saline vehicle again.

**Analytic Methods**

Blood glucose was measured daily during normal catheter flushing procedures, which was immediately before the infusion studies on scheduled days. After catheter dead space was cleared, 0.5 mL arterial blood was withdrawn, and 1 drop was placed on a test strip for analysis with an Accucheck III blood glucose analyzer. The remainder of the blood was returned to the rat. During the acute infusion studies, the analog pressure and flow signals were sampled at 500 samples per second for 4 seconds every 15 seconds, rather than every 60 seconds as with the 24-hour daily measurements. The last 10 minutes of each infusion period were used for data analysis.

Daily hemodynamic data were analyzed by ANOVA with repeated measures, and results from the acute infusions were analyzed by 2-factor ANOVA with repeated measures on 1 factor. Supplemental testing with Scheffé’s test was used to determine specific within-group differences, and completely randomized F tests with Scheffé’s test were used to determine specific between-group differences. A value of *P*<0.05 was accepted as statistically significant, and data are presented as mean±SEM.

**Results**

Blood glucose increased significantly from an average of 5.6±0.1 mmol/L (≈101 mg/dL) during the precontrol period to 23.6±0.8 mmol/L (≈424 mg/dL) the morning after STZ administration. The average blood glucose level for the remainder of the control period was 4.5±0.5 mmol/L, and the insulin dose averaged 3.7±0.1 U/d. The insulin infusion dose was decreased to 0 U/d for the 6-day diabetic period, and blood glucose averaged 22.2±1.1 mmol/L, ranging from 17.6±2.0 mmol/L on day 1 to 24.2±0.9 mmol/L on day 4. Blood glucose averaged 7.8±1.8 mmol/L by day 4 after the insulin infusion was restored in the recovery period.

The acute ACh and SNP infusions caused dose-dependent, statistically significant increases in blood flow during each experimental period. Figure 1 shows hindquarter blood flow during the acute ACh infusions in each experimental period. In the precontrol period, blood flow measured during the baseline saline-infusion period (Saline) averaged 31±7 mL/min, increased significantly by approximately 13 mL/min during infusion of ACh at 1 µg/min (ACh I), and increased an additional 14 mL/min when the dose was increased to 10 µg/min (ACh II).
Blood flow returned to 32±3 mL/min during the saline recovery. Two-factor ANOVA revealed that the overall responses to ACh were not different between the experimental periods, but there were significant increases in blood flow within in each experimental period for both doses, except for dose I during the control period.

Figure 2 shows the hindquarter blood flow responses to the acute SNP infusions during each experimental period. Note that the saline-infusion period that followed the dose II ACh infusion served both as the recovery for the ACh infusions as well as the baseline control period for the subsequent SNP infusions, and those data are plotted again in Figure 2 as the first Saline data set. At the precontrol period, hindquarter blood flow increased, although not significantly, by approximately 8 mL/min during SNP infusion at 1 μg/min (SNP I) and by an additional 2 mL/min at the higher dose (SNP II), which was significantly above the Saline level. Blood flow again returned to baseline levels when the infusion was switched back to saline, averaging 34±6 mL/min. Similar to the response to ACh, there were no significant differences in the overall response to SNP infusion between experimental periods, but there were significant increases in blood flow within in each experimental period for both SNP doses, except for SNP I in the control and recovery periods.

Although Figures 1 and 2 show that the overall responses to ACh and SNP were not altered significantly during the diabetic period, it also appears that baseline blood flows were different between periods. The first Saline data points in Figure 1 represent baseline blood flow before infusion of ACh and SNP, and those data are presented with the corresponding mean arterial pressure (MAP) values in the Table. There were no statistical differences, however, among baseline blood flows using ANOVA, whereas baseline MAP was significantly higher in the diabetic period. On the other hand, the 24-h/d measurements of hindquarter blood flow shown in Figure 3 revealed a significant decrease in flow by day 4 of the diabetic period, from an average of 29±5 mL/min during the precontrol period to 20±5 mL/min. MAP averaged 104±2 mm Hg during the precontrol period and tended to run higher in the diabetic period, averaging 109±3 mm Hg on day 5; however, unlike findings of our previous report, the increase in MAP was not statistically significant.

Examination of the baseline data collected before the acute ACh and SNP infusions together with the 24-h–averaged data suggests that blood pressure may have been more labile without a significant sustained increase and that baseline hindquarter blood flow was decreased significantly. Because of this decrease, the acute flow data also are presented as a percentage of control (baseline) in Figures 4 and 5. For the ACh infusions (Figure 4), the percent increase in blood flow was not statistically significant in the precontrol, control, or
recovery periods except for dose II during the precontrol period. However, the percent increase in blood flow during the diabetic period was statistically significant for both doses on both days of testing (days 2 and 6), and ANOVA showed that the 258% increase in flow at dose II on diabetes-day 6 was significantly greater. Note that this significant percent increase in blood flow during ACh infusion in the diabetic period no longer was apparent after glycemic control was restored in the recovery period.

The responses to SNP expressed as percentage of control (Figure 5) were different from those during ACh infusion in that the effects of both doses were statistically significant during all periods, except for dose I during the control period. Day 6 of the diabetic period again showed a significantly enhanced response.

**Discussion**

This study showed that at the onset of IDDM, the acute blood flow responses to ACh or SNP were not impaired, and the blood flow changes expressed as a percentage of baseline (control) in Figures 4 and 5 show further that there was no trend or tendency for impairment of the blood flow response. This suggests that neither endothelium-mediated vasorelaxation nor responsiveness to NO is impaired during the initial phase of diabetes. In addition, these results suggest that neither hyperglycemia nor decreased circulating insulin has significant direct effects on endothelium-mediated relaxation in IDDM.

Excessive cardiovascular morbidity and mortality in diabetes are caused primarily by vascular disease, as in the eyes and kidneys, for example, and impaired endothelial function has been implicated in the development and progression of the vascular injury. Impaired endothelial function has been postulated to participate in the injury process through several mechanisms, such as by contributing to the premature development of atherosclerosis that is characteristic of IDDM, preventing thrombosis, and modulating vascular smooth muscle growth and function. However, there is considerable controversy regarding whether endothelial dysfunction occurs in IDDM. Possible explanations for discrepancies between studies include the significant between-study differences in blood glucose levels at the time of testing in many of the human studies, whether studies incorporate IDDM or NIDDM patients, and potential differences related to in vitro versus in vivo experiments. In addition, much of this controversy may be related to the stage of diabetes in which endothelial function is assessed, an especially important consideration if it is proposed that endothelial dysfunction may help initiate vascular dysfunction and injury. In humans, this is because even when attempts are made to study only young normoalbuminuric patients, evaluation of IDDM always occurs years after initiation of the disease. Therefore, it cannot be determined whether the study variable already has been influenced to some extent by episodes of poor glycemic control in the months or years preceding diagnosis, or even after diagnosis but during the time before study. Studies in animals with experimentally induced diabetes may be influenced similarly, because few hemodynamic studies have been initiated earlier...
than 2 to 4 weeks after induction of diabetes. For example, endothelium-mediated vasodilation was impaired in STZ-diabetic rats and in alloxan-diabetic dogs studied 4 weeks after induction of diabetes, and a report by Dai et al in STZ-diabetic rats provides evidence that the degree of impairment increases with duration of diabetes in that model. However, attempts to study cardiovascular function immediately after induction of diabetes face the potential confounding influence of side effects of STZ.

The experimental model in this study was designed to address the issue of time by evaluating endothelial function immediately after induction of diabetes, before sufficient time was allowed for development of structural changes in vascular tissues. Potential side effects of STZ were controlled for by the insulin-replacement regimen and by the method for actually inducing poor glycemic control, ie, by withdrawing the insulin replacement therapy. All variables measured in this study indicate that the STZ-treated rats were not significantly different from their pre-STZ condition during the period of intravenous insulin replacement. This is in agreement with our previous observations in the same experimental model, in which sodium balance also was measured and found to be not different. Thus, changes associated with the induction of diabetes in this model can be assumed to be independent of potential STZ side effects not related to its insulin-lowering action. In addition, responses to induction of diabetes in this model are more likely related directly to decreased insulin or increased glucose per se, rather than to secondary effects, such as structural changes, resulting from sustained actions of those variables.

As shown in Figures 1 and 2, the overall responses to both ACh and SNP were not affected significantly by diabetes, with each agonist causing similar dose-dependent increases in blood flow during all experimental periods. Plotting of the data as a percentage of control blood flow to account for changes in baseline flow, however, suggested that the vasodilatory response was significantly greater by day 6 of diabetes. A greater response during diabetes is consistent with recent reports by Cosentino et al and Graier et al that high glucose increases NO release by endothelial cells. However, it is possible that the greater percent increase in blood flow during diabetes may have been due in part to the more vasoconstricted baseline condition. Because of this, the physiological significance of the enhanced response is not clear from this experiment. On the other hand, the greater response at the very least strengthens our conclusion that vasodilation was not impaired during diabetes.

It is important to note that the ACh and SNP tests were not conducted during cyclooxygenase inhibition. This is important because ACh may induce the release of vasodilatory prostaglandins in addition to NO in some vascular beds. However, diabetes, particularly in rats, is associated with enhanced production of vasoconstrictor prostanoids, such as thromboxane. Thus, if our present results had been obtained during cyclooxygenase inhibition, they might have been due, at least to some extent, to the removal of vasoconstrictive factors. More importantly, however, impairment of ACh-mediated vasodilation in other diabetic studies has been reported both with and without concurrent cyclooxygenase inhibition. This suggests that ACh-mediated release of vasodilatory prostaglandins is not a significant confounder of these acute testing procedures for endothelium- and NO-mediated vasodilation in diabetes.

By showing that neither ACh- nor SNP-mediated vasodilation was impaired during the first 6 days of diabetes, the results from this study suggest that hyperglycemia alone does not impair endothelium-mediated vasodilation. In vitro studies have demonstrated direct impairment of endothelium-mediated vasodilation by hyperglycemia; however, our in vivo results are in agreement with a report by Houben et al which showed that 24 hours of local hyperglycemia in normal patients, induced by brachial artery infusion of glucose to yield a local concentration of ≈ 15 mmol/L, did not affect ACh- or SNP-mediated vasodilation. Thus, the impairment measured in other diabetic studies may be a consequence of cumulative effects of hyperglycemia rather than of direct interactions of glucose with the NO system.

Because no decreases in the dilatory responses to ACh or SNP were measured during the diabetic period, these results also suggest that the decreased insulin in IDDM does not have a direct effect on endothelium- or NO-mediated vasodilation. This finding at first may appear contradictory to the considerable evidence from Baron’s laboratory (Steinberg et al) that hyperinsulinemia increases skeletal muscle blood flow.
flow via an NO-dependent mechanism. However, the results from the present study merely indicate that normal insulin levels are not required for endothelium-mediated vasodilation to occur in IDDM. Thus, while insulin appears to require a normally functioning NO system to induce vasodilation,24–36 NO-mediated vasodilation induced by other mechanisms does not appear to require insulin.

An interesting and unexpected finding from this study is that baseline hindquarter blood flow decreased significantly during the diabetic period. With the rise in blood pressure, this indicates that vascular resistance increased. This experiment was not designed to study the mechanisms for chronic blood flow control, and it is not clear whether the increased vascular resistance represents a primary vasoconstrictor response, perhaps due to hyperglycemia, or whether it is a secondary response. One potential mechanism, for example, based on Baron’s findings that increased insulin increases skeletal muscle blood flow via an NO-dependent mechanism,34,35 is that that insulinopenia in IDDM could reduce blood flow by withdrawal of that effect. However, additional studies including chronic NO synthase inhibition and L-arginine infusion protocols will be necessary to more accurately examine that possibility. In addition, Utriainen et al.37,38 have suggested that plasma insulin concentrations in the range of normal circulating levels have only a mild vasodilator effect, and the importance of tissue metabolic rate in long-term tissue blood flow control also must be considered. Therefore, the role of insulin and NO in mediating the changes in baseline hindquarter blood flow in IDDM remains unclear.

The decrease in hindquarter blood flow during the diabetic period was unexpected because it is difficult to reconcile with observations that the onset of poor glycemic control in diabetic patients is associated with increased blood flow.39 In addition, it is well appreciated that renal blood flow is increased during the early phases of IDDM in rough proportion to the degree of glycemic control.40,41 However, Kiff et al.42 measured a similar decrease in hindquarter blood flow in conscious Wistar rats 28 days after STZ-induced diabetes, and they also measured increases in renal and mesenteric blood flow. Those results suggest therefore that there is significant regional variation in the blood flow response to diabetic hyperglycemia.

The mechanism for the vasoconstriction and changes in blood flow distribution are not known, but with the sodium and volume loss that invariably accompany diabetic hyperglycemia, some redistribution may be necessary to account for the increases in blood flow to other tissues such as the kidneys. Sodium balance was not measured in the present study because of the acute infusion experiments, but our previous study with this model demonstrated that the amount of sodium lost in the urine over 4 days of poor glycemic control exceeded total sodium intake of 1 day.43 In addition, cumulative water balance decreased by nearly 30 mL despite water being available for drinking ad libitum.17 Thus, the reduction in hindquarter blood flow in the present study may have been the result of general volume depletion. However, more detailed long-term studies that include cardiac output and body fluid volume measurements will be needed to accurately evaluate this.

In conclusion, the first 6 days of hyperglycemia in rats with IDDM are not associated with impaired endothelium-mediated or NO-mediated vasodilation, suggesting that impairment in those vasodilatory responses in diabetes may require a more prolonged hyperglycemic period. In addition, because these observations were made at the onset of diabetes and were independent of any measurable STZ side effect, this suggests that hyperglycemia in diabetes does not have a significant direct effect to impair endothelial mechanisms for vasodilation. However, additional studies will be required to investigate the mechanisms for the decrease in baseline hindquarter blood flow and evaluate any potential influence that this may have on endothelial function.

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