Evidence for Inducible Nitric-Oxide Synthase Expression and Activity in Vascular Smooth Muscle of Streptozotocin-Diabetic Rats

ANDREA L. BARDELL and KATHLEEN M. MACLEOD

Division of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada

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ABSTRACT

Experiments were performed to investigate whether nitric-oxide synthase (NOS) activity can be detected in vascular smooth muscle (VSM) from 12- to 14-week streptozotocin (STZ)-diabetic rats. Concentration-response curves to norepinephrine (NE) of superior mesenteric arteries from diabetic and age- and gender-matched control rats were obtained in the presence of dexamethasone (0.1 μM) to prevent in vitro induction of iNOS. Incubation of endothelium-intact arteries from diabetic rats with the nonselective NOS inhibitor, Nω-(1-iminoethyl)-ornithine (L-NIO) (300 μM), increased the NE sensitivity (expressed as the pD2 or log EC50) from 6.58 ± 0.05 to 8.39 ± 0.12 (mean ± S.E.M., n = 8). L-NIO produced a significantly smaller increase in control arteries. However, significant calcium-independent (iNOS) activity in control or diabetic arteries, or calcium-independent (iNOS) activity was detected in diabetic arteries. These data suggest that iNOS is functionally expressed in VSM of arteries from 12- to 14-week STZ-diabetic rats. The possible causes and consequences of the iNOS induction are discussed.

The diabetic state is associated with an increased incidence of cardiovascular complications. Hyperglycemia has been recently identified as an independent risk factor for the development of cardiovascular disease (Diabetes Control and Complications Trial Research Group, 1993). Endothelial dysfunction (as reflected as an imbalance in the release of, or sensitivity to, endothelial-derived vasoconstrictors and vasodilators) has been proposed as an important contributor to diabetes-induced VSM dysfunction (Taylor et al., 1992).

Nitric oxide (NO) derived from the endothelial subtype of NOS (eNOS) is an important mediator of vasodilation (Furchgott, 1999), and abnormal release of or response to NO has been proposed as a contributing factor to VSM dysfunction in the diabetic state (Cohen, 1995; Huszka et al., 1997). Although NO from eNOS may be of primary importance under normal conditions, another constitutive subtype of NOS, nNOS, and the inducible subtype of NOS, iNOS, may be expressed in VSM under pathological conditions (Boulanger et al., 1998; Gonzalez-Fernandez et al., 1998). Recently, induction of iNOS has been demonstrated in cardiomyocytes from rats with streptozotocin-induced diabetes (Smith et al., 1997) and in platelets from patients with both type 1 and type 2 diabetes (Tannous et al., 1999). In VSMC, iNOS may be induced by various cytokines including IL-1β, tumor necrosis factor-α, interferon-γ, nuclear factor-κB, and IL-6 (De Vera et al., 1996). Activation of protein kinase C (PKC) has also been shown to result in enhanced expression of iNOS in VSMC (Paul et al., 1997). There is some evidence that the chronic diabetic state is associated with changes in the expression of various cytokines, which may be due to in part to advanced glycosylation endproducts (AGEs) (Campbell and Harrison, 1990; Vlassara

**ABBREVIATIONS:** VSM, vascular smooth muscle; NO, nitric oxide; iNOS, inducible nitric-oxide synthase (NOS2); nNOS, neuronal nitric-oxide synthase (NOS1); eNOS, endothelial nitric-oxide synthase (NOS3); L-NIO, Nω-(1-iminoethyl)-ornithine; EIT, S-ethylisothiourea; 7-NINA, 7-nitroindazole; L-NMMA, Nω-monomethyl-L-arginine acetate; STZ, streptozotocin; PKC, protein kinase C; AGE, advanced glycosylation endproduct; ET-1, endothelin-1; NE, norepinephrine; VSMC, vascular smooth muscle cells; IL, interleukin; TBS, Tris-buffered saline; DAB, 3,3-diaminobenzidine.

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et al., 1994; Festa et al., 1998). In addition, enhanced activation of various PKC isoforms has been reported in diabetic VSM (Inoguchi et al., 1992). Therefore, in long-term diabetes, iNOS may be induced in VSM synergistically due to AGE-mediated alterations of cytokine production and/or enhanced PKC activation.

Abnormal NOS expression and NO production in vascular smooth muscle may result in various effects. Induction of iNOS in VSM may be particularly detrimental, as iNOS synthesizes 10- to 50-fold more NO than the constitutive NOS subtypes (Moncada and Higgs, 1995). Any increase in production of NO has potential for adverse effects, since the free radical NO can interact with oxygen-derived free radicals to produce peroxynitrite, which is thought to be the major mediator of the cytotoxic effects of NO (Snyder and Bredt, 1992). In addition, alteration of NO levels may result in an imbalance in the release of other endothelium-derived factors, contributing to endothelial dysfunction (Warner, 1999). Therefore, investigation of NOS activity in VSM in the diabetic state may be of particular importance in understanding the etiology of endothelial and vascular dysfunction associated with chronic diabetes mellitus.

The present study was undertaken to investigate whether NOS activity can be detected in VSM of superior mesenteric arteries from control and 12- to 14-week streptozotocin (STZ)-diabetic rats. To this end, cumulative concentration-response curves to norepinephrine (NE) of isolated mesenteric arterial rings from diabetic and control rats were obtained in the absence and presence of L-arginine (L-NIO, a nonselective NOS inhibitor), S-ethylisothiourea (EIT, a selective iNOS inhibitor), or 7-NINA (the water-soluble salt of the prototypical nNOS inhibitor 7-nitroindazole) (Moore et al., 1993; Nakane et al., 1995). Immunohistochemical analysis with selective antibodies to eNOS, nNOS, and iNOS, and quantification of nNOS and iNOS activity of mesenteric arteries from control and diabetic rats were also performed.

### Materials and Methods

**Experimental Animals.** Male Wistar rats weighing 190 to 220 g were obtained from the Animal Care Center, University of British Columbia. Rats were treated according to the Guidelines of the Canadian Council for Animal Care. Bolus injection of STZ (55 mg/kg i.v.) was administered via the tail vein under light halothane anesthesia 12 to 14 weeks before use. Control rats received citrate buffer vehicle (0.1 µM, pH 4.5). Both diabetic and control rats were allowed access to food and water ad libitum. STZ-treated animals were considered diabetic and retained for experiments if their blood glucose was greater than 200 mg/dl, 7 days following STZ-injection.

**Preparation of Isolated Mesenteric Arteries.** Rats were deeply anesthetized with an i.p. injection of pentobarbital (65 mg/kg). The chest cavity was opened, and blood was taken by cardiac puncture. The superior mesenteric artery was then carefully removed and placed in a Petri dish containing cold Krebs' solution of composition (mM): NaCl 113, KCl 4.7, NaHCO₃ 25.0, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, and dextrose 11.5, pH 7.4, continuously aerated with 95% O₂, 5% CO₂. Water-soluble dexamethasone (0.1 µM) was added to the Krebs' solution to prevent iNOS induction in vitro during the course of the experiment. Tissues were cleaned of excess fat and connective tissue and cut into two 4-mm rings. The endothelium was either kept intact or removed by careful rubbing of the vessel lumen. Ring preparations of mesenteric arteries were placed individually in isolated tissue baths containing 20 ml of Krebs' solution continuously aerated with 95% O₂, 5% CO₂ and maintained at 37°C. Isometric contractions were measured with a force-displacement transducer connected to a Grass model 7E polygraph (Grass Instruments, Quincy, MA) as previously described (MacLeod, 1985). Tissue preparations were equilibrated for 90 min under a resting tension of 1 g, which was previously found to be optimal for both control and diabetic arteries (MacLeod, 1985). During the equilibration period, the Krebs' solution was replaced every 20 min.

**Cumulative Concentration-Response Curves to NE.** Endothelial status was first assessed by determining the ability of acetylcholine (10⁻⁵ M) to relax a precontracted to phenylephrine (3 × 10⁻⁶ M). Arteries were then washed three times with Krebs' solution and allowed to re-equilibrate for 60 min before a concentration-response curve to NE was obtained. The tissues were again washed three times and allowed to re-equilibrate for 45 min, following which one arterial ring of each diabetic and control pair was incubated with one of the following antagonists: L-NIO (300 µM), 7-NINA (100 µM), or EIT (10 µM). In some experiments, L-arginine (1 mM) or D-arginine (1 mM) was added with the antagonist. Subsequently, a second concentration-response curve to NE was obtained. The other arterial ring of the pair remained untreated and served as a control to determine whether any changes in reactivity occurred during the course of the experiment. No significant time-dependent changes in the NE response were detected (data not shown). After the second NE concentration-response curve, the tissues were washed and allowed to re-equilibrate for 30 min. Finally, the maximum response to KCl was determined in the presence of phenolamine (10⁻⁵ M).

**Table 1**

<table>
<thead>
<tr>
<th>General characteristics of rats 12 to 14 weeks after STZ or vehicle injection</th>
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<tbody>
<tr>
<td>Results are expressed as mean ± S.E.M. of values obtained in 39 control and 39 diabetic rats.</td>
</tr>
<tr>
<td>Body Weight Plasma Glucose Plasma Insulin</td>
</tr>
<tr>
<td>g mmol/l ng/ml</td>
</tr>
<tr>
<td>Control 556 ± 5 8.65 ± 0.10 8.9 ± 0.7</td>
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<tr>
<td>Diabetic 343 ± 7* 23.65 ± 0.39* 0.51 ± 0.09*</td>
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* p < 0.05 compared with corresponding control values.

**Table 2**

<table>
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<tr>
<th>Sensitivity (pD₂) and maximum responses (R₃₃₋max) to NE of endothelium-intact and endothelium-denuded control and diabetic mesenteric arteries in the absence and presence of 300 µM L-NIO</th>
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</thead>
<tbody>
<tr>
<td>Results are expressed as mean ± S.E.M. of values obtained in arteries from eight control and eight diabetic animals.</td>
</tr>
<tr>
<td>Endothelial Status R₃₃₋max (Untreated) R₃₃₋max (L-NIO) pD₂ (Untreated) pD₂ (L-NIO)</td>
</tr>
<tr>
<td>Control Intact 151.6 ± 2.1 150.5 ± 1.8 6.51 ± 0.03 7.08 ± 0.03***</td>
</tr>
<tr>
<td>Control Denuded 157.8 ± 1.3 161.5 ± 2.0 7.27 ± 0.08** 7.29 ± 0.09</td>
</tr>
<tr>
<td>Diabetic Intact 194.6 ± 4.5* 207.7 ± 2.1* 6.58 ± 0.05 8.39 ± 0.12***</td>
</tr>
<tr>
<td>Diabetic Denuded 198.6 ± 1.7* 199.9 ± 2.5* 7.48 ± 0.03** 8.38 ± 0.15***</td>
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</table>

* p < 0.05 compared with corresponding control. 
** p < 0.05 compared with endothelium-intact values. 
*** p < 0.05 compared with untreated values (two-way ANOVA followed by Neuman-Keuls test).
Contractile responses to NE of each arterial ring were expressed as a percentage of the maximum response of the same ring to KCl.

**Measurement of NOS Activity.** Control and diabetic mesenteric arteries were excised and cleaned as described above and flash frozen in liquid nitrogen. Isolated arteries were stored at $-70^\circ$C until assayed. Because the assay procedure requires 80 mg of tissue/sample, four to six cleaned mesenteric arteries were pooled for each sample. Tissues were crushed with a mortar and pestle under liquid nitrogen. The frozen dry weight was obtained, homogenization buffer was added, and the sample was homogenized by sonication. The sample was then centrifuged at 16,000g for 20 min at $4^\circ$C, and the supernatant (consisting of the cytosolic fraction containing iNOS and nNOS) was retained on ice. NOS activity of the supernatant was quantitated by measuring the formation of radiolabeled $[^{14}\text{C}]$-citrulline from $[^{14}\text{C}]$-arginine as previously described (Schulz et al., 1995). For each sample, incubations at $37^\circ$C for 30 min were performed in duplicate in the presence or absence of either EGTA (1 mM) or EGTA plus L-NMMA (1 mM each) to determine the level of calcium-dependent and calcium-independent NOS activity. $[^{14}\text{C}]$-citrulline was separated from $[^{14}\text{C}]$-arginine by cation-exchange chromatography using activated AG 50W-X8 resin and quantified by liquid-scintillation counting. Protein content of the cytosolic fraction

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**Fig. 1.** Cumulative concentration-response curves to NE of endothelium-intact (closed symbols) and endothelium-denuded (open symbols) control (A) and 12- to 14-week STZ-diabetic (B) superior mesenteric arteries in the absence (squares) and presence (triangles) of 300 μM L-NIO. Each point represents the mean ± S.E.M. ($n = 8$ control and 8 diabetic animals).

**Fig. 2.** Cumulative concentration-response curves to NE of endothelium-intact superior mesenteric arteries from control rats in the absence (.), 300 μM L-NIO (▲) (A), 300 μM L-NIO + 1 mM L-arginine (●) (B), or 300 μM L-NIO + 1 mM d-arginine (□) (C). Each point represents the mean ± S.E.M. ($n = 5$ control and 5 diabetic animals).
was measured with the Bio-Rad (Richmond, CA) protein reagent with bovine serum albumin used as a standard.

**Immunohistochemistry.** Superior mesenteric arteries were excised and cleaned as described above. Arteries were then fixed in 10% neutral buffered formalin followed by paraffin processing through increasing grades of ethyl alcohol, xylene, and Paraplast (Fisher Scientific, Nepean, Ontario, Canada). Tissue blocks were sectioned at 3 μm, and the luminal artery cross sections were mounted on positively charged slides.

Endogenous peroxidase activity was quenched with 3% (w/v) aqueous hydrogen peroxide for 10 min, and slides were rinsed with water. Background staining was minimized with 2% normal goat serum in Tris-buffered saline (TBS). Sections were incubated with the primary antibody [polyclonal anti-iNOS, -nNOS, or monoclonal anti-eNOS 1:2500 dilution in TBS with 1% (w/v) BSA or monoclonal anti-macrophage ED2 1:1000 dilution] overnight in a humid chamber. The primary antibody was rinsed off with TBS, and sections were incubated with a biotinylated species-specific secondary antibody (1:150 dilution in TBS) for 1 h at room temperature. The secondary antibody was rinsed off with TBS, and the streptavidin-biotin peroxidase complex (ABC kit, Vector Laboratories, Inc., Burlingame, CA) was applied for 1 h at room temperature. The ABC reagent was rinsed off with TBS, and sections were stained with DAB reagent (60 mg/100 ml of TBS, 500 μl of DAB intensifier, 100 μl of 30% hydrogen peroxide) for 10 min. Sections were rinsed with tap water and counterstained with 0.1% (w/v) nuclear fast red in 5% (w/v) aluminum sulfate. Slides were rinsed with tap water, dehydrated in alcohol, cleared in xylene, and mounted in resinous mounting medium. Paraffin-embedded sections of rat pituitary and spleen were also processed and served as positive controls for detection of nNOS and macrophages, respectively. Photographs were taken with a photomicroscope at 50× magnification.

**Plasma Glucose and Insulin Determination.** Plasma glucose levels were measured by colorimetric enzyme assay using the Peridochrom glucose assay kit obtained from Boehringer Mannheim (Mannheim, Germany). Plasma insulin was measured by radioimmunoassay using a rat insulin radioimmunoassay kit obtained from Linco Research Inc. (St. Charles, MO).

**Statistical Analysis.** NE concentration-response curves were analyzed by nonlinear regression analysis using GraphPad (San Diego, CA) Prism software for the determination of pD2 (−log EC50) values and maximum contractile responses (Rmax). All values are expressed as mean ± S.E.M. Statistical significance was evaluated by two-way ANOVA followed by Newman-Keuls post hoc tests for multiple comparisons and considered significantly different if p < 0.05.

**Drugs and Chemicals.** L-NIO, EIT, and 7-NINA were obtained from Tocris Ltd. (Ballwin, MO), and polyclonal anti-iNOS antibody and L-NMMA were obtained from Calbiochem (La Jolla, CA). Polyclonal anti-nNOS and monoclonal anti-eNOS antibodies were obtained from Transduction Laboratories (Franklin Lakes, NJ). Monoclonal anti-macrophage (ED2) antibody was obtained from Serotec Inc. (Raleigh, NC). [14C]L-Arginine was obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). The Bio-Rad protein reagent was obtained from Bio-Rad. All other chemicals were obtained from Sigma Chemical (Oakville, Ontario, Canada). Stock solutions of NE were made with ascorbic acid (4 mg/ml) to prevent oxidation.

**Results**

**General Characteristics of Control and Diabetic Rats.** Twelve to 14 weeks after injection, STZ-diabetic rats had significantly lower body weights, increased plasma glucose levels, and decreased plasma insulin levels compared with their age- and gender-matched vehicle-treated controls (Table 1). The STZ-diabetic rats also exhibited other symptoms associated with diabetes including osmotic diarrhea, polyuria, and cataracts.

**Pharmacological Investigation of the Effects of Inhibition of NOS.** In untreated arteries, the maximum contractile response to NE of diabetic rat mesenteric arteries was found to be significantly greater than that of control arteries,
although no significant difference in the NE pD₂ values could be detected (Table 2). Neither endothelial-denudation nor pharmacological inhibition of NOS had any significant effect on maximal contractile responses to NE of either control or diabetic arteries (Table 2, Figs. 1–3).

Preincubation of endothelium-intact vessels with L-NIO resulted in a leftward shift in the NE concentration-response curve in both control and diabetic arteries (Fig. 1, A and B) associated with a significant increase in the NE pD₂ values (Table 2). However, the NE pD₂ value in diabetic arteries in the presence of L-NIO was significantly greater than that in control arteries (Table 2). Removal of the endothelium also resulted in a leftward shift in the NE concentration-response curve and a significant increase in NE pD₂ values in both control and diabetic arteries (Fig. 1, Table 2). Incubation of endothelium-denuded control arteries with L-NIO had no further effect on the NE response. However, L-NIO produced a further leftward shift in the NE response and a significant increase in NE pD₂ values of endothelium-denuded diabetic arteries (Fig. 1B, Table 2).

The subtype of NOS contributing to the effect of L-NIO in endothelium-denuded diabetic mesenteric arteries was investigated by obtaining cumulative concentration-response curves to NE in endothelium-denuded vessels in the absence and presence of the nNOS inhibitor 7-NINA (100 μM) or the iNOS inhibitor EIT (10 μM). Preincubation with 7-NINA had no effect on NE responses of either control or diabetic mesenteric arteries (Table 3). Similarly, no significant increase in sensitivity to NE was obtained in endothelium-denuded control vessels following preincubation with 10 μM EIT (Table 3). However, 10 μM EIT produced a leftward shift in the NE concentration-response curve associated with a significant increase in NE pD₂ values in endothelium-denuded diabetic mesenteric arteries (Table 3).

**Reversibility of NOS Inhibition.** To determine whether the increase in NE sensitivity seen with L-NIO was due to competitive inhibition of NOS, cumulative concentration-response curves to NE were obtained in endothelium-intact control mesenteric arteries treated with L-NIO alone or in the presence of L-arginine or D-arginine (1 mM each). L-Arginine abolished the leftward shift in the NE concentration-response curve and the increase in NE pD₂ values due to L-NIO, while D-arginine had no effect on the NE response in the presence of L-NIO (Fig. 2, Table 4).

**TABLE 3**

<table>
<thead>
<tr>
<th>7-NINA (100 μM)</th>
<th>EIT (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>Control</td>
<td>7.34 ± 0.09</td>
</tr>
<tr>
<td>Diabetic</td>
<td>7.69 ± 0.20</td>
</tr>
</tbody>
</table>

* p < 0.05 compared with all other values in the group (two-way ANOVA followed by Neuman-Keuls test).

NE concentration response curves were also obtained in endothelium-denuded diabetic arteries treated with EIT alone or in the presence of L-arginine or D-arginine to verify that the increase in NE pD₂ values with EIT in diabetic vessels was due to competitive inhibition of NOS. As was found with L-NIO, the effect of EIT on the NE response was abolished by coincubation with L-arginine but was not affected by D-arginine (Fig. 3, Table 4).

**Immunohistochemistry.** Immunohistochemical analysis was performed to investigate whether NOS protein could be detected in VSM from control or diabetic rats. Immunostaining of mesenteric arteries with specific antibodies for eNOS indicated that eNOS was expressed only in the endothelial cell monolayer of both control and diabetic arteries (data not shown). Furthermore, immunostaining for NOS produced no positive signal in either control or diabetic arteries, although the antibody used produced positive staining in sections of mouse brain at the same dilution (data not shown).

There was a striking difference between control and STZ-diabetic vessels in immunostaining for iNOS. A strong positive signal for iNOS was observed in the medial and adventitial layers of the superior mesenteric artery of STZ-diabetic rat but not control rat arteries (Fig. 4). To determine whether macrophage infiltration is the source of iNOS protein in the diabetic mesenteric arteries, control and diabetic arteries were incubated with a specific antibody to rat macrophage (clone ED2). No positive staining for macrophage was obtained in control or diabetic vessels, although the antibody used provided a positive signal in rat spleen sections at the same dilution (data not shown).

**Quantitative Measurement of NOS Activity.** To investigate NOS activity in control and 12- to 14-week STZ-diabetic rat mesenteric arteries, the citrulline assay for quantitative analysis of cytosolic calcium-dependent (nNOS) and calcium-independent (iNOS) activity was performed (Fig. 5). Calcium-dependent (nNOS) activity in control (0.24 ± 0.39 pmol/min/mg of protein) and diabetic arteries (0.25 ± 0.46 pmol/min/mg of protein) was not significantly elevated above background levels. Similarly, almost no calcium-independent activity (1.43 ± 0.39 pmol/min/mg of protein) was detected in control mesenteric arteries. However, a marked elevation in calcium-independent (iNOS) activity, to 18.06 ± 4.11 pmol/min/mg of protein, was detected in mesenteric arteries from diabetic rats.

**TABLE 4**

<table>
<thead>
<tr>
<th>Mesenteric Arteries</th>
<th>Inhibitor</th>
<th>pD₂ (Untreated)</th>
<th>pD₂ (Inhibitor)</th>
<th>pD₂ (Inhibitor + L-Arginine)</th>
<th>pD₂ (Inhibitor + D-Arginine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (endothelium-intact)</td>
<td>L-NIO</td>
<td>6.29 ± 0.08</td>
<td>7.11 ± 0.07*</td>
<td>6.28 ± 0.11</td>
<td>7.13 ± 0.09*</td>
</tr>
<tr>
<td>Diabetic (endothelium-denuded)</td>
<td>EIT</td>
<td>7.22 ± 0.08</td>
<td>8.07 ± 0.12*</td>
<td>7.46 ± 0.32</td>
<td>8.13 ± 0.16*</td>
</tr>
</tbody>
</table>

* p < 0.05 compared with untreated values.
Discussion

The results of the present investigation provide evidence that iNOS is functionally expressed in VSM of superior mesenteric arteries from 12- to 14-week STZ-diabetic rats but not in their age- and gender-matched controls. The increase in sensitivity of endothelium-denuded diabetic arteries to NE in the presence of EIT, positive immunostaining for iNOS, and the presence of high levels of calcium-independent NOS activity in diabetic arteries all indicate the presence of iNOS in diabetic VSM. The elevated NO levels that result may be implicated in the cardiovascular dysfunction associated with diabetes mellitus.

In the present study, endothelium-intact mesenteric arteries from diabetic rats exhibited an increased maximum responsiveness (when normalized for the maximum response of the same preparation to KCl) with no change in sensitivity to NE compared with responses of age- and gender-matched control rats. These data are consistent with previous reports from this laboratory, which has found increased maximum responses of diabetic arteries to NE but not KCl, with little or no change in the NE pD$_2$ value (reviewed in Subramanian and MacLeod, 1999). Nonselective inhibition of NOS with L-NIO resulted in a leftward shift, with no change in maximum response, of the concentration-response curves to NE in both control and diabetic endothelium-intact arteries, suggesting that NO release in these arteries normally limits NE
sensitivity. However, the magnitude of the shift produced by L-NIO was significantly greater in diabetic arteries. It is unlikely that the observed increase in NE sensitivity produced by L-NIO is caused by a nonspecific effect of the compound, since the stereospecificity of the interaction of L-NIO with nNOS was confirmed by reversal of the leftward shift of the concentration-response curve with L-arginine but not D-arginine. The L-NIO-induced increase in sensitivity to NE in control arteries is likely due primarily to inhibition of eNOS from the endothelial cell layer, since L-NIO had no effect on NE sensitivity in endothelium-denuded control arteries. In contrast, the presence of a leftward shift in the NE concentration-response curve with L-NIO in endothelium-denuded diabetic arteries suggests the presence of NOS activity in diabetic VSM.

The two major classes of NOS are the constitutive and the inducible subtypes. The constitutive NOS subtypes, particularly eNOS and cytosolic nNOS (also known as NOS3 and NOS1, respectively), are calcium-dependent and are subject to regulation by phosphorylation. The inducible subtype, iNOS (or NOS2), is calcium-independent and is regulated primarily at the transcriptional level (Morris and Billiar, 1994). Although endothelial-derived NO (from eNOS) may be of primary importance in VSM under normal conditions, both nNOS and iNOS may also be expressed under pathological conditions. nNOS was originally purified from peripheral neurons but is now known to be expressed in VSM under certain conditions (Michel and Feron, 1997). iNOS was originally isolated from an immunologically activated macrophage cell line but is now known to be induced in a wide variety of cell types including cardiac myocytes, glial cells, endothelial cells, and VSM cells (Marin and Rodriguez-Martinez, 1997).

The expression of nNOS in VSM from spontaneously hypertensive rats has recently been reported (Boulanger et al., 1998). However, in the present study, incubation of endothelium-denuded control and diabetic arteries with 100 μM 7-NINA had no effect on NE responses. At this concentration, 7-NINA has been reported to be a selective inhibitor of nNOS in arterial ring preparations (Moore et al., 1993). In support of the pharmacological experiments, no positive immunostaining for nNOS was observed in either control or diabetic arteries. Furthermore, quantitative measurement of NOS activity of the cytosolic fraction (containing nNOS and iNOS) of control and diabetic arteries revealed no significant calcium-dependent activity. Therefore, it seems unlikely that nNOS is the subtype of NOS present in diabetic mesenteric arteries.

iNOS has also been reported to be expressed in VSM under various pathological conditions (Gonzalez-Fernandez et al., 1998), and the results of the present study provide substantial evidence that it is this subtype that is expressed in diabetic mesenteric arteries. First, EIT (10 μM), which has been reported to be 40- to 50-fold more selective for iNOS than for nNOS or eNOS (Nakane et al., 1995), mimicked the increase in NE sensitivity seen with L-NIO in endothelium-denuded diabetic mesenteric arteries but had no effect on NE responses in endothelium-denuded control arteries. L-Arginine, but not D-arginine, reversed the leftward shift in the concentration-response curve to NE, confirming that EIT acts as a stereoselective competitive inhibitor of NOS. Second, immunohistochemical analysis demonstrated a strong positive signal for iNOS expression in mesenteric arteries from diabetic but not control rats. Finally, quantitative measurement of cytosolic NOS activity indicated that calcium-independent (iNOS) activity in diabetic arteries was significantly increased above both background and levels in control arteries, further suggesting that iNOS is functionally expressed in diabetic VSM.

The possibility that the presence of iNOS in the diabetic arteries was due to its induction in vitro seems unlikely, as all experiments were conducted in the presence of dexamethasone at a concentration (0.1 μM) that has been reported to inhibit iNOS induction in vitro (Knowles et al., 1990). Furthermore, iNOS induction in vitro has been reported to require a time period of hours (Zheng et al., 1997), whereas isolated arteries obtained for immunohistochemical analysis or for quantitative NOS assay were fixed in formalin or flash frozen in liquid nitrogen, respectively, within minutes of excision. It is not likely that the iNOS detected in VSM of diabetic arteries in the present study is due to macrophage infiltration as no positive staining above background was detected for the macrophage-specific ED2 antibody in either control or diabetic arteries.

The mechanism by which iNOS is induced in diabetic VSM is uncertain. However, both AGE-mediated alterations in cytokine production and/or enhanced PKC activation could be implicated (De Vera et al., 1996; Paul et al., 1997). Recent studies have demonstrated increased levels of transforming growth factor-β1 and IL-6, which may be due to the formation of AGES, in long-term diabetes (Yamamoto et al., 1993; Vlassara et al., 1994). Evidence for increased activation of the β2 isoform of PKC has also been reported in aorta and hearts from diabetic rats (Inoguchi et al., 1992). Studies in vitro suggest that hyperglycemia itself may contribute to PKC activation, since high ambient glucose concentrations have been reported to activate PKC in cultured VSMC (Williams and Schrier, 1992).

Once induced, iNOS synthesizes a prolonged and increased release of NO as compared with eNOS and nNOS (Moncada and Higgs, 1995). Any increase in NO production has potential for free radical-mediated damage, particularly under conditions of oxidative stress where peroxynitrite is formed more readily (Snyder and Breit, 1992). There has been considerable recent evidence that there is an increase in the
generation of oxygen-derived free radicals in diabetes (Giugliano et al., 1996). Therefore, increased NO production in diabetic VSM has potential for considerable damage.

Abnormal production of NO may also contribute to endothelial dysfunction due to an imbalance in the release of other endothelium-derived factors. Endothelin-1 (ET-1) is a potent endothelium-derived vasoconstrictor whose production may be altered in diabetes (Takeda et al., 1991). NO may be involved in the regulation of ET-1 release, since inhibition of NOS increases ET-1 release (Kiff et al., 1991), while ET-1 may stimulate the release of NO (Warner et al., 1989). Therefore, any alterations in NO or ET-1 production in the diabetic state may contribute to a complex response as there is a likely in vivo interplay between these two factors.

On the other hand, the consequences of iNOS induction in diabetic VSM do not have to be detrimental. Increased NO production could act in a protective manner by limiting the enhancement of vasoconstrictor responses of diabetic arteries. This is supported by the observation that the sensitivity of diabetic arteries to NE is not significantly different from control in the absence of L-NIO but is enhanced in its presence. Furthermore, induction of iNOS may help to compensate for the decreased release of or responsiveness to endothelial-derived NO, which has been commonly reported in diabetic arteries (Cohen, 1995; Marin and Rodriguez-Martinez, 1997).

As demonstrated in the Introduction, induction of iNOS has been observed in cardiomyocytes from STZ-diabetic rats (Smith et al., 1997) and in platelets from diabetic patients (Tannous et al., 1999). The present demonstration of iNOS expression in VSM suggests that induction of iNOS may be a widespread phenomenon in diabetes. Although the consequences of iNOS expression are not fully understood, increased NO production has been implicated in ventricular dysfunction (Smith et al., 1997) and renal hyperfiltration (Bank and Aynedjian, 1993) in STZ-diabetic rats. Furthermore, the induction of iNOS is associated with increased production of peroxynitrite in platelets from diabetic individuals (Tannous et al., 1999).

In conclusion, the results of this investigation demonstrate that iNOS is functionally expressed in VSM from rats with chronic STZ-induced diabetes at a time when vasoconstrictor responsiveness is also enhanced. The relationship between the duration of hyperglycemia and the induction of iNOS as well as the factor(s) responsible for its induction are presently under investigation.

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Send reprint requests to: Dr. K. M. MacLeod, Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall, Vancouver, BC V6T 1Z3 Canada. E-mail: kmm@interchange.ubc.ca