EVIDENCE FOR THE FUNCTIONAL EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN VASCULAR SMOOTH MUSCLE FROM DIABETIC RATS

by

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Abstract

Nitric oxide (NO) has important physiological and pathophysiological functions in VSM. The purpose of the present research was to investigate the hypothesis that NOS activity is altered in arteries from diabetic rats.

Concentration-response curves to NA, in the presence of dexamethasone (0.1μM) to prevent in vitro induction of iNOS, were obtained in superior mesenteric arteries from rats 12-14 weeks following STZ-injection and their age and gender-matched controls. Endothelial denudation or preincubation with non-selective NOS inhibitors, LNMMA or L-NIO (300μM), produced an increase in NA sensitivity (as reflected by the NA pD_2 value) in arteries from both control and STZ-diabetic rats. Following endothelial denudation, LNMMA or L-NIO still increased the NA pD_2 value in diabetic but not control arteries. The selective nNOS inhibitor 7-NINA had no effect on NA responses of endothelium-denuded control or STZ-diabetic arteries. However, the selective iNOS inhibitor, EIT (10μM), produced an increase in the NA pD_2 value in endothelium-denuded diabetic but not control arteries.

Immunohistochemistry revealed that eNOS is present in the endothelial cell monolayer of both control and diabetic arteries. No positive signal for nNOS was obtained in either control or diabetic arteries. However, immunostaining for iNOS indicated the presence of iNOS throughout all layers of diabetic but not control arteries.

Quantitative measurement of cytosolic NOS activity indicated no significant calcium-dependent (nNOS) activity in control or diabetic arteries at any time following STZ-injection. Similarly, no calcium-independent (iNOS) activity was detected in control arteries. However, significant iNOS activity was detected in 12-14 week STZ-

diabetic arteries.

These data suggest the novel finding that iNOS is functionally expressed in VSM of arteries from 12-14 week STZ-diabetic rats. Time course studies indicate that the induction of iNOS occurs sometime after 8 weeks of diabetes. Further studies will be required to establish the significance of iNOS induction in diabetic VSM.

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ACh	acetylcholine
AG	aminoguanidine
AGEs	advanced glycosylation end-products
BH_4	(6R)-5,6,7,8-tetrahydrobiopterin
cGMP	guanosine 3',5' cyclic monophosphate
DNA	deoxyribonucleic acid
EDRF	endothelium derived relaxing factor
EIT	S-ethylisothiourea
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
HDL	high-density lipoproteins
IFN-γ	interferon gamma
IL-1	interleukin-1
iNOS	inducible nitric oxide synthase
KCl	potassium chloride
LDL	low-density lipoproteins
L-NIO	N^5-(1-iminoethyl)L-ornithine
LNMMA	N^G-monomethyl-L-arginine
LPS	lipopolysaccharide
μM	micromolar

mg	milligram
mM	millimolar
mmol	millimoles
NA	noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor – kappa B
7-NINA	7-nitroindazole
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
PE	phenylephrine
PKC	protein kinase C
pmol	picomoles
RAGE	receptor for advanced glycosylation end-products
ROS	reactive oxygen species
s.e.m.	standard error of the mean
SOD	superoxide dismutase
STZ	streptozotocin
TNF-α	tumour necrosis factor alpha
VSM	vascular smooth muscle
VSMC	vascular smooth muscle cells

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Dedicated to my mother,

who has been the strongest influence in my life,

for all of her love and endless support.

Introduction

A. Overview

Diabetes mellitus can be considered as a cluster of syndromes, characterized by insulin deficiency (type 1 diabetes mellitus) or insulin resistance (type 2 diabetes mellitus). The diabetic state is associated with hyperglycemia and altered metabolism, including increased utilization of free fatty acids (Rodrigues et al, 1997). Diabetic patients are at greater risk of developing long-term pathophysiological complications including peripheral neuropathy, nephropathy, and cardiovascular complications, resulting in an increased risk of premature morbidity and mortality. Hyperglycemia has been recently identified as an independent risk factor for the development of cardiovascular disease (Diabetes Control and Complications Trial Research Group, 1993).

Diabetes has been reported to be associated with altered vascular reactivity to neurotransmitters and circulating hormones (Christlieb et al, 1976). 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 vascular smooth muscle (VSM) dysfunction (Taylor et al., 1992). Endothelium-derived relaxing factor (EDRF), which is nitric oxide (NO) produced by the endothelial subtype of nitric oxide synthase (eNOS), is an important vasodilator (Furchgott, 1999). Abnormal release of or response to NO has been proposed as a contributor to vascular and endothelial dysfunction in the diabetic state (Huszka et al., 1997; Cohen, 1995). Previous studies of NOS activity in diabetic

VSM have focused on eNOS. Although EDRF 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 vascular smooth muscle cells (VSMC) under pathological conditions (Boulanger et al., 1998; Gonzalez-Fernandez et al., 1998). The present study was undertaken to investigate NOS activity in diabetic VSM.

B. Nitric oxide

Since its discovery as EDRF, a great deal of research has been conducted on the physiologic and pathophysiologic roles of NO in the cardiovascular system (Palmer et al, 1987; Ignarro, 1999). NO diffuses readily across cell membranes (Kerwin et al, 1995) and mediates vasodilation by binding to soluble guanylate cyclase, resulting in increased production of cGMP (Nakatsu and Diamond, 1989). An increase in intracellular cGMP levels leads to decreased intracellular calcium levels, resulting in vasorelaxation (Chabaud et al, 1994). NO also reacts with sulfhydryl groups, forming S-nitrosothiols, which may serve as cellular NO carriers or active intermediates in NO cell signaling (Ignarro et al, 1981).

NO plays important roles in the control of vascular tone, platelet aggregation, and VSM proliferation (Marin and Rodriguez-Martinez, 1997). However, if there is a high concentration of reactive oxygen species (ROS) present in the local environment of NO production, overproduction of NO can result in tissue dysfunction and damage (Bhagat, 1996).

C. Nitric oxide synthase enzymes

NO is synthesized from the terminal guanidino nitrogen atom of L-arginine and molecular oxygen by the nitric oxide synthase (NOS) family of enzymes, with NADPH, tetrahydrobiopterin (BH_4), FMN, FAD, and calmodulin as cofactors. The NOS enzymes are cytochrome P450 enzymes, which differ from all other mammalian cytochrome P450 mono-oxygenases in that they contain both the reductase and oxygenase domain (Nathan and Xie, 1994). All of the NOS isoforms have two binding domains. The N-terminal domain contains the oxygenase activity and binds heme, BH_4, and the substrate, L-arginine. The C-terminal domain contains the reductase activity and binds NADPH, FAD, and FMN. The calcium regulatory protein calmodulin binds between the two domains and may be involved in electron transfer between the domains (Abu-Soud et al, 1994).

The two major classes of NOS are the constitutive subtypes and the inducible subtype. The constitutive NOS subtypes, eNOS and nNOS (also known as NOS3 and NOS1 respectively) are calcium-dependent and are subject to regulation by phosphorylation by protein kinase C (PKC) (Nakane et al, 1991). The inducible subtype, iNOS (or NOS2), is calcium-independent and is regulated primarily at the transcriptional level (Morris and Billiar, 1994).

<u>eNOS</u>

eNOS is a calcium-dependent constitutive subtype of NOS which was originally purified and cloned from vascular endothelial cells but may also be expressed in platelets, neutrophils, and cardiac myocytes under certain conditions (Michel and

Feron, 1997). The eNOS subypte of NOS has a N-myristoylation site that causes its localization to the cell membrane, thus eNOS is present predominantly in the particulate fraction (Forstermann et al, 1991; Busconi and Michel, 1993).

Previous studies of the role of nitric oxide in the cardiovascular system in diabetes have focused on NO derived from eNOS (EDRF). Numerous studies have suggested that the diabetic state is associated with a decreased release of or decreased responsiveness to endothelial-derived NO (Huszka et al, 1997; Cohen, 1995). Proposed explanations for the decreased responsiveness to EDRF in diabetes include accelerated inactivation of NO due to increased oxidative stress, enhanced activation of PKC (resulting in phosphorylation and inhibition of eNOS), and increased production of vasoconstrictor prostanoids or ET-1 that counteract the effect of NO (Tesfamariam and Cohen, 1992; Takeda et al, 1991).

nNOS

Another calcium-dependent subtype of NOS is nNOS. nNOS was originally purified from peripheral neurons but is now known to be expressed in the brain, sympathetic ganglia, peripheral nitrergic nerves (nonadrenergic, noncholinergic nerves), adrenal glands, skeletal muscle, and, under certain conditions in VSM (Gath et al, 1996; Marin and Rodriguez-Martinez, 1997; Brophy et al, 2000). nNOS activity in the peripheral nervous system is associated with relaxation of smooth muscle and, in the brain, is associated with NO-mediated synaptic plasticity (Kerwin et al, 1995). The expression of nNOS in the uterine artery has been reported during pregnancy and is thought to be involved in the re-distribution of cardiac output with pregnancy (Garvey et al, 1994). Further evidence for the expression of nNOS has been reported

in bovine carotid arteries (Brophy et al, 2000) and in carotid arteries from spontaneously hypertensive rats (Boulanger et al, 1998). nNOS is predominantly a cytosolic enzyme (Forstermann et al, 1991).

iNOS

iNOS was originally isolated from an immunoactivated 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). iNOS is similar to the constitutive NOS isoforms in that it contains a calmodulin regulatory sequence. However, calmodulin is tightly bound to iNOS, thus its activity is independent of stimulation by calcium (reviewed in Schulz and Triggle, 1994). Unlike the constitutive isoforms, iNOS is not subject to regulation by phosphorylation (Marin and Rodriguez-Martinez, 1997). Once induced, iNOS synthesizes a prolonged and increased release of NO as compared to eNOS and nNOS (Moncada and Higgs, 1995).

Although it is widely accepted that cytokine-stimulated induction of iNOS in macrophages is an important element of the inflammatory response (Wei et al, 1995), iNOS may be functional in other tissue types under pathological conditions, including disease states such as diabetes. Induction of iNOS has been recently demonstrated in cardiac myocytes from rats with streptozotocin-induced diabetes (Smith et al, 1997). In addition, evidence for iNOS activity has recently been reported in platelets from type 1 and type 2 diabetic patients (Tannous et al., 1999). It is possible that induction of iNOS occurs in diabetic tissues as a result of pathophysiological changes associated with chronic diabetes.

D. Mechanism of iNOS induction in VSM

Apart from potential limitations of substrate and cofactor availability, iNOS activity does not appear to be regulated once the enzyme is expressed. Therefore, iNOS activity is predominantly regulated at the level of DNA transcription. Gene transcription and induction of iNOS is inhibited by glucocorticoids, including dexamethasone (Knowles et al, 1990). Regulation of iNOS expression in VSMC appears to be very complex, involving several factors acting alone or synergistically. Distinct species differences have been reported for the transcriptional control of iNOS expression in VSMC (Paul et al, 1997). Furthermore, significant differences in regulation of iNOS induction have been reported for VSMC from the same species but different vascular origin (Kolyada et al, 1996).

The iNOS gene has been cloned and sequenced from the mouse, rat, and human. A sequence homology of 47-66% of the promoter region has been reported between species (DeVera et al, 1996). The promoter region of all three genes (mouse, rat, and human) includes several putative elements including NF-κB, IFN-γ, IL-1β, and TNF-responsive elements (DeVera et al, 1996). Induction of iNOS in VSM may occur synergistically in response to various stimuli including IL-1β, TNF-α, INF-γ, IL-6, and/or LPS or oxidative stress induced activation of NF-κB (Hecker et al, 1999).

Transcriptional control of iNOS in vascular smooth muscle may also be regulated by protein kinase C (PKC). Individual PKC isoforms may regulate iNOS expression in both a positive and negative manner with distinct cell-type specific differences (Hecker et al, 1999). In a recent study, activation of PKC-α and PKC-ε

was associated with enhanced LPS-induced iNOS expression in RAW 264.7 macrophages and attenuated iNOS induction in rat aortic smooth muscle cells (Paul et al, 1997). In contrast, in the same study, it was suggested that activation of PKC-ζ may contribute to iNOS induction in rat aorta, but may inhibit iNOS induction in the macrophage cell line (Paul et al, 1997). The mechanism by which iNOS gene expression in VSM is regulated by individual PKC isoforms in different cell types remains to be investigated.

E. Potential contributors to iNOS induction in diabetes

Formation of Advanced Glycosylation End-products (AGEs)

Glucose can form glucosylation products with amino groups from proteins or amino acids by a non-enzymatic process. Early glucosylation products may undergo a complex series of irreversible chemical arrangements, including autoxidation of the Amadori product (a 1-amino-1-deoxyketose formed by the reaction of reducing sugars with protein amino groups), to form advanced glycosylation end-products (Brownlee, 1994). Chronic diabetes is associated with an increased accumulation of AGEs (Schleicher and Nerlich, 1996). A highly significant correlation exists between AGE accumulation and severity of diabetic microvascular damage in the retina, kidney, and peripheral nerve (Beisswenger et al, 1995).

Vascular endothelial cells express an AGE-specific receptor, designated receptor for AGEs (RAGE), which appears to be a member of the Ig receptor superfamily (Neeper et al, 1992). The RAGE receptor appears to mediate signal transduction through the generation of reactive oxygen species (ROS) (Brownlee, 2000). ROS are generated by AGE binding and are reported to activate NF-κB (Bierhaus et al, 1997). Interaction of AGEs with RAGE induces changes in gene expression, including upregulation of various cytokines including TGF-β and IL-1 (Vlassara et al, 1988). Thus, AGE-mediated upregulation of cytokines may contribute to iNOS induction in VSM.

Oxidative stress

The diabetic state has been reported to be associated with increased levels of oxidative stress. Oxidative stress occurs when antioxidant defenses, including superoxide dismutase (SOD) in a cell are overwhelmed due to increased exposure to oxidants (Darley-Usmar and White, 1997).

Increased generation of oxygen-derived free radicals has been reported in diabetes (Giugliano et al, 1996). Increased lipid peroxidation and elevated levels of superoxide anion have been reported in aorta from STZ-induced diabetic rats (Chang et al, 1993). Elevation of levels of reactive oxygen species (ROS) may also occur due to the formation of AGEs with long-term hyperglycemia and poor metabolic control. The oxidant, peroxynitrite, which is the product of the interaction of NO with ROS, may also contribute to oxidative stress.

The consequences of oxidative stress may include modulation of antioxidant and free radical scavenger levels and activation of stress responsive transcription factors, including NF-κB (Li and Karen, 1999). Therefore, activation of NF-κB with increased oxidative stress may contribute to iNOS induction in VSM. It has also been suggested that increased production of free radicals in aorta from STZ-induced diabetic rats leads to accelerated inactivation of NO (Hattori et al, 1991).

Altered activation of PKC isoforms

There is some evidence for enhanced PKC activation with chronic diabetes although this remains an area of controversy. Previous studies from our laboratory have demonstrated enhanced PKC-mediated contractile responses to noradrenaline

(NA) in mesenteric arteries from male rats with STZ-induced diabetes of 12 weeks duration (Harris and MacLeod, 1988), providing indirect evidence for enhanced PKC activation with chronic diabetes. Direct evidence for diabetes-induced PKC activation has also been reported. Activation of the β2 isoform of PKC is enhanced in the aorta and heart from diabetic rats (Inoguchi et al, 1992). In addition, prolonged high ambient glucose concentrations activate PKC in cultured VSMC in vitro (Williams and Schrier, 1992). Thus, enhanced activation of PKC with long-term diabetes may also potentiate iNOS induction in VSM.

F. Potential consequences of iNOS induction in VSM

Induction of iNOS in VSM would result in an overproduction of NO, as iNOS synthesizes 10-50 fold more NO than the constitutive NOS subtypes (Moncada and Higgs, 1995). Any increase in production of NO has potential for adverse effects, particularly under conditions of oxidative stress. NO itself is not cytotoxic although highly chemically reactive, possessing a very short half-life (5-10 seconds in vitro) (Ignarro, 1990). NO may react rapidly with oxygen derived free radicals to produce peroxynitrite ($ONOO^-$). Peroxynitrite can result in oxidation of lipids and the modification of proteins in the cell by nitration of tyrosine residues (Koppenol et al, 1992). The oxidation of lipoproteins, including low-density lipoproteins (LDLs) by peroxynitrite has been implicated in the formation of atherosclerotic lesions (Leeuwenburgh et al, 1997). Peroxynitrite may disrupt signal transduction events in the cell by modification of tyrosine residues and carbohydrates (Darley-Usmar and White, 1997). Furthermore, peroxynitrite has been reported to generate lipid products with vasoconstrictive properties (Elliot et al, 1998).

Other consequences of iNOS induction in VSM are possible. For instance, alteration of NO levels may result in an imbalance in the release of other endothelium-derived factors including endothelin-1 (ET-1) as there is a likely in vivo interplay between ET-1 and NO (Warner, 1999). Induction of iNOS in VSM may also act to compensate for a decrease in the release of, or responsiveness to, endothelial derived NO, which has been suggested to occur in diabetes in experimental animals (Durante et al, 1988).

G. Streptozotocin (STZ)-induced diabetes

STZ-model of diabetes

The present study was aimed at investigating the effects of chronic diabetes on NOS activity in VSM. The STZ model of type I diabetes was utilized, since a single bolus injection of STZ consistently results in chronic hyperglycemia and hypoinsulinemia (Rakieten et al, 1963).

STZ [2-deoxy-2-(3-methyl-3-nitrosourea) 1-D-glucopyranose] binds with high affinity to membrane receptors on pancreatic-β cells. The mechanism of β-cell destruction by STZ involves free radical generation, methylation, and nitric oxide production (reviewed in Rodrigues, 1999). Destruction of pancreatic β-cells results in permanent hypoinsulinemia in these animals and produces characteristics associated with the diabetic state including hyperglycemia, polydypsia, polyuria, cataracts, and decreased body weight gain. The severity of the diabetic state induced by STZ is dose-dependent and may vary between species, route of administration (intravenous or intraperitoneal), and animal age and weight. However, numerous studies have indicated that a single bolus injection of at least 40mg/kg administered intravenously to male rats consistently results in a permanent hypoinsulinemic and hyperglycemic state (reviewed in Rodriguez, 1999).

Altered vascular reactivity associated with long-term STZ-induced diabetes

Changes in vascular reactivity that occur in the STZ-model of diabetes have been investigated in various arteries and vascular beds. Numerous investigations, including studies from our laboratory, have demonstrated that maximum responsiveness to α-adrenoceptor stimulation is enhanced in aorta and mesenteric arteries from STZ-diabetic rats of 12-14 week duration (MacLeod 1985, Abebe et al, 1990). Enhanced contractile responses of 12-14 week STZ-diabetic arteries are thought to arise from direct stimulation of α_1-adrenoceptors (Abebe et al, 1990). Furthermore, the enhanced responsiveness to α_1-adrenergic receptor stimulation is not thought to be due to a nonspecific increase in contractile responsiveness of STZ-diabetic arteries, since no significant difference has been reported between control and diabetic arteries in response to potassium-induced depolarization (MacLeod 1985; Abebe et al, 1994). Additional studies from this laboratory have indicated that the enhanced contractile responses to NA of STZ-diabetic aorta and mesenteric arteries are associated with an increase in phosphoinosotide turnover (Abebe and MacLeod, 1992). Altered phosphoinositide metabolism may result in overproduction of the second messengers, inositol 1,4,5-trisphosphate (IP_3) and diaglycerol (DAG), which may in turn result in increased calcium release from intracellular stores and/or enhanced activation of PKC (Abebe and MacLeod, 1990).

It has been suggested that in addition to increased contractile responsiveness, NO production and release may be altered in arteries from STZ-diabetic rats. A number of studies have shown that endothelial production of NO or vascular relaxation in response to ACh is impaired in arteries from long-term STZ-diabetic rats

(Fortes et al, 1983; Durante et al, 1988). However, studies from this laboratory have reported no differences in rat aorta between control and 12 week STZ-diabetic in cGMP levels in the absence and presence of ACh (Harris and MacLeod, 1988). There is further controversy regarding NO production in diabetes since increased release of NO has also been proposed to occur (Bhardwaj and Moore, 1988; Langenstroer and Pieper, 1992). An increased release of NO may be masked by inactivation of NO in a local environment of elevated free radicals (Langenstroer and Pieper, 1992).

H. Rationale and hypothesis

Rationale

Long-term hyperglycemia that occurs in chronic diabetes is associated with changes in vascular reactivity, endothelial and vascular dysfunction, and vascular deterioration. Other changes that are associated with long-term hyperglycemia, including cytokine production resulting from AGE formation and increased oxidative stress, and activation of PKC, may result in induction of iNOS gene transcription. Since alterations in NOS expression and NO production may result in various effects, investigation of NOS activity in vascular smooth muscle in the diabetic state may be of particular importance in understanding the etiology of endothelial and vascular dysfunction associated with chronic diabetes mellitus.

Hypothesis

It is hypothesized that iNOS is functionally expressed in VSM of rats with chronic diabetes.

Specific objectives of the present investigation

1. To investigate pharmacologically NOS activity of isolated endothelium-intact and endothelium-denuded superior mesenteric arterial rings from control and 12-14 week STZ-diabetic rats by obtaining cumulative concentration-response curves to NA in the absence and presence of the non-selective NOS inhibitors LNMMA and L-NIO.

2. To determine which subtype of NOS is active in diabetic VSM by obtaining cumulative concentration response curves to NA in endothelium-denuded arterial rings in the absence and presence of the selective nNOS inhibitor 7-nitroindazole or the selective iNOS inhibitors aminoguanidine or S-ethylisothiourea.

3. To investigate the time course of iNOS induction by obtaining cumulative-concentration response curves to NA of endothelium-denuded arteries in the absence and presence of EIT, 2-3 weeks and 6-8 weeks following STZ-injection.

4. To perform immunohistochemical analysis for the detection of the individual NOS subtypes, eNOS, nNOS, and iNOS, in control and 12-14 week STZ-diabetic rat superior mesenteric arteries. Furthermore, to investigate whether macrophage infiltration is the source of iNOS in diabetic VSM by immunohistochemical staining for macrophage specific protein.

5. To quantitatively measure nNOS and iNOS activity of control and STZ-diabetic arteries 2-3 weeks, 6-8 weeks, and 12-14 weeks following injection.

Materials and Methods

A. Materials

Male Wistar rats weighing 190-220g were obtained from the Animal Care Center, UBC. The Periodochrom® glucose assay kit was obtained from Boehringer Mannheim (Mannheim, Germany). The rat insulin radioimmunoassay kit was obtained from Linco Research Inc. (St. Charles, MO). L-NIO, EIT, and 7-NINA were obtained from Tocris Ltd. (Ballwin, MO). The polyclonal anti-iNOS antibody and LNMMA 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 (clone ED2) antibody was obtained from Serotec Inc. (Raleigh, NC). The ABC Vector kit and the DAB reagent were obtained from Vector Inc. (Burlingame, CA). [^{14}C]-L-Arginine was obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). The BioRad protein reagent was obtained from BioRad (Richmond, CA). All other chemicals were obtained from Sigma Chemical (Oakville, Ontario, Canada).

B. Methods

1. Experimental Animals

Male Wistar rats weighing 190-220g were obtained from the Animal Care Centre, UBC, and were treated according to the Guidelines of the Canadian Council for Animal Care. Bolus injection of streptozotocin (STZ) (55mg/kg IV) was administered via the tail vein under light halothane anaesthesia 2-3 weeks, 6-8 weeks or 12-14 weeks prior to use. Control rats received citrate buffer vehicle (0.1μM, 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 16mmol/L, 7 days following STZ-injection.

2. Preparation of isolated mesenteric arterial rings

Rats were deeply anaesthetized with an intraperitoneal injection of pentobarbitol (65mg/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 Kreb's solution of composition (mM): NaCl 113, KCl 4.7, $NaHCO_3$, 25.0, $CaCl_2$, KH_2PO_4 1.2, $MgSO_4$ 1.2, and dextrose 11.5, pH 7.4, continuously aerated with 95% O_2-5% CO_2. Water-soluble dexamethasone (0.1μM) was added to the Kreb's 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 4mm 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 20ml Kreb's solution continuously aerated with 95% O_2-5% CO_2 and maintained at 37°C. Isometric contractions were measured with force-displacement transducers connected to a Grass model 7E polygraph. Tissue preparations were equilibrated for 90 minutes under a resting tension of 1g, which was previously found to be optimal for both control and diabetic arteries (MacLeod 1985). During the equilibration period, the Kreb's solution was replaced every 20 minutes.

3. Preparation of solutions for pharmacological experiments

The Kreb's solution and solutions of NA, acetylcholine (ACh), phenylephrine (PE), water-soluble dexamethasone, aminoguanidine (AG), S-ethylisothiorea (EIT), 7-nitroindazole (7-NINA), N^G-monomethyl-L-arginine (LNMMA), N^5-(1-iminoethyl)L-ornithine (L-NIO), L-arginine, L-Citrulline, and potassium chloride (KCl) were prepared in distilled water. Ascorbic acid (4mg/ml) was added to NA stock solutions to reduce oxidation.

4. Cumulative concentation-response curves to NA

Endothelial status was assessed by determining the ability of acetylcholine (10^{-5}M) to relax a precontraction to phenylephrine ($3x10^{-6}$M). Arteries were then washed 3 times with Kreb's solution and allowed to re-equilibrate for 60 minutes before a concentration-response curve to NA was obtained. The tissues were again washed 3 times and allowed to re-equilibrate for 45 minutes, following which one

arterial ring of each diabetic and control pair was incubated with one of the following antagonists (L-NIO (300μM), aminoguanidine (1mM), 7-NINA (100μM), or EIT (5μM or 10μM)). In some experiments, L-arginine (1mM) or D-arginine (1mM) was added with the antagonist. Subsequently, a second concentration-response curve to NA 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 NA response were detected (data not shown). After the second NA concentration-response curve, the tissues were washed and allowed to re-equilibrate for 30 minutes. Finally, the maximum response to KCl was determined in the presence of phentolamine (10^{-5}M). Contractile responses to NA of each arterial ring were expressed as a percent of the maximum response of the same ring to KCl.

5. 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. Tissue blocks were sectioned at 3μm 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 ten minutes 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 hour at room temperature. The secondary antibody was rinsed off with TBS and the streptavidin-biotin peroxidase complex (ABC Kit, Vector Inc) was applied for 1 hour at room temperature. The ABC reagent was rinsed off with TBS and sections were stained with DAB reagent (60mg/100ml TBS, 500µl DAB intensifier, 100µl 30% hydrogen peroxide) for 10 minutes. 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 mouse brain and rat spleen were also processed and served as positive controls for detection of nNOS and macrophages respectively. Photographs were taken with a photomicroscope at 50X magnification.

6. Quantitative 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°C until assayed. The assay procedure requires 80mg tissue/sample, so 4-6 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 and homogenization buffer containing HEPES (10mM), Na_2EDTA (0.1mM), DL-

dithiothreitol (DTT) (1mM), type-S trypsin inhibitor (10μg/ml), leupeptin hemisulfate (10μg/ml), and aprotinin (2μg/ml) was added at a ratio of 1mg tissue: 4μl homogenization buffer. Phenylmethylsulfonyl fluoride (PMSF) was added at a ratio of 1μL PMSF: 100μl homogenization buffer and samples were homogenized by sonication. The sample was then centrifuged at 16,000xg for 20min at 4°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 radiolabelled [^{14}C]-L-citrulline from [^{14}C]- L-arginine as previously described (Knowles et al., 1990a). For each sample, the supernatant was incubated in cocktail buffer of composition (mM): L-Valine 50, BH_4 0.01. NADPH 0.1, L-arginine 0.018, L-citrulline 1, DTT 1.05, [^{14}C]- L-arginine 0.002, for 30 minutes at 37°C. Samples were incubated in duplicate in cocktail buffer alone or in the presence of either EGTA (1mM) or EGTA plus LNMMA (1mM each) to determine the level of calcium-dependent and calcium-independent NOS activity. [^{14}C]-L-citrulline was separated from [^{14}C]- L-arginine by cation-exchange chromatography using activated AG 50W-X8 resin and quantified by liquid-scintillation counting. Protein content of the cytosolic fraction was measured with the BioRad protein reagent with bovine serum albumin used as a standard.

7. Blood Glucose and Insulin Determination

Plasma glucose levels were measured by colorimetric enzyme assay, while plasma insulin was measured by radioimmunoassay, both using commercially available kits (Periodochrom® glucose assay kit and Linco rat insulin radioimmunoassay kit respectively).

8. Statistical Analysis

NA concentration-response curves were analyzed by non-linear regression analysis using Graphpad Prism software for the determination of pD_2 (-log EC_{50}) values and maximum contractile responses (Rmax). All values are expressed as mean $\pm$ standard error of the mean (s.e.m.). Except where indicated, statistical significance was evaluated by two-way ANOVA followed by Newman-Keuls post-hoc tests for multiple comparisons and considered significantly different if $p<0.05$.

Results

A. General characteristics of control and diabetic rats

Two-3 weeks, 6-8 weeks, and 12-14 weeks following injection, STZ-treated rats had significantly increased plasma glucose levels and decreased plasma insulin levels compared to their age and gender-matched vehicle-injected controls (Table1). 6-8 weeks and 12-14 weeks following injection, STZ-diabetic rats had significantly reduced body weight as compared to control (Table 1). The STZ-diabetic rats also exhibited other symptoms associated with the disease including polyuria and osmotic diarrhea. Detection of cataracts in STZ-diabetic rats was time-dependent, and most notable after 6-8 weeks following injection.

Table 1

Summary of general characteristics of control and STZ-diabetic rats

Rats	Weeks following injection	Body weight (g)	Plasma glucose (mmol/l)	Plasma insulin (ng/ml)
Control, N=20	2-3	285±8	8.71±0.06	3.6±0.4
Control, N=18	6-8	439±6	8.74±0.10	4.3±0.4
Control, N=39	12-14	556±5	8.65±0.10	8.9±0.7
STZ-diabetic, N=20	2-3	266±8	22.95±0.24*	0.5±0.1*
STZ-diabetic, N=18	6-8	344±9*	23.33±0.28*	0.6±0.1*
STZ-diabetic, N=39	12-14	343±7*	23.65±0.39*	0.5±0.1*

All values are mean ± s.e.m.

*$p<0.05$ as compared to corresponding control values (One-way ANOVA)

B. Pharmacological investigation of the effects of the non-selective NOS inhibitors LNMMA and L-NIO

In order to investigate whether functional NOS activity could be detected in superior mesenteric arteries of control and 12-14 week STZ-diabetic rats, cumulative concentration-response curves to NA were obtained in endothelium-intact and endothelium-denuded vessels in the absence and presence of the non-selective NOS inhibitor LNMMA (300μM).

In untreated arteries, the maximum contractile response to NA of diabetic rat mesenteric arteries was found to be significantly greater than that of control arteries, although no significant difference in the NA pD_2 values could be detected (Table 2). Neither endothelial-denudation nor pharmacological inhibition of NOS had any significant effect on maximal contractile responses to NA of either control or diabetic arteries (Table 2, Fig.1-4).

Preincubation of endothelium-intact vessels with LNMMA resulted in a leftward shift in the NA concentration-response curve in both control and diabetic arteries (Fig.1) associated with a significant increase in the NA pD_2 values (Table 2). Removal of the endothelium also resulted in a significant increase in NA pD_2 values in both control and diabetic arteries (Table 2). Incubation of endothelium-denuded control arteries with LNMMA had no further effect on the NA response (Fig.2A, Table 2). However, LNMMA produced a further leftward shift in the NA response and a significant increase in NA pD_2 values of endothelium-denuded diabetic arteries (Fig.2B; Table 2).

A significant difference in NA pD_2 values was observed between endothelium-intact and endothelium-denuded STZ-diabetic arteries following preincubation with LNMMA (Table 2). It was hypothesized that the disparity in NA pD_2 values between treated endothelium-intact and endothelium-denuded diabetic arteries was due to insufficient diffusion of LNMMA to the VSM when the endothelium remained intact. In order to investigate this hypothesis, cumulative concentration-response curves to NA were obtained in endothelium-intact and endothelium-denuded control and 12-14 week STZ-diabetic mesenteric arteries in the absence and presence of L-NIO (300μM), another nonselective NOS inhibitor that has been reported to be transported intracellularly more efficiently (Kerwin et al, 1995).

As seen with LNMMA, preincubation of endothelium-intact vessels with L-NIO resulted in an increase in NA pD_2 values in both control and diabetic arteries (Fig 1; Table 2). However, the NA pD_2 value in diabetic arteries in the presence of LNIO was significantly greater than that in control arteries (Table 2). Incubation of endothelium-denuded control arteries with L-NIO produced no further effect on the NA responses (Fig 2A, Table 2). As observed with LNMMA, L-NIO produced a significant increase in NA pD_2 values of endothelium-denuded diabetic arteries (Fig 2B; Table 2). However, unlike LNMMA, no significant difference in NA pD_2 values was observed between endothelium-intact and endothelium-denuded STZ-diabetic arteries following preincubation with L-NIO (Table 2).

Table 2

Sensitivity (pD2) and maximum responses (Rmax) to NA of endothelium-intact and endothelium-denuded control and 12-14 week STZ-diabetic mesenteric arteries in the absence and presence of LNMMA or L-NIO (300μM)

Animals	Endo.	Rmax Untreated N=16	Rmax LNMMA N=10	Rmax L-NIO N=6	PD2 Untreated N=16	PD2 LNMMA N=10	PD2 L-NIO N=6
Control	Intact	151.6 ±2.1	148.5 ±2.8	150.5 ±1.8	6.51 ±0.04	7.01 ±0.11[c]	7.08 ±0.03[c]
Control	Denud.	157.8 ±1.1	157.4 ±2.4	163.5 ±2.0	7.31 ±0.07[b]	7.21 ±0.03	7.26 ±0.04
Diabetic	Intact	194.6 ±4.5[a]	195.2 ±3.0[a]	207.7 ±2.1[a]	6.58 ±0.05	7.18 ±0.10[c]	8.39 ±0.12[c]
Diabetic	Denud.	198.6 ±1.7[a]	196.3 ±2.3[a]	199.9 ±2.5[a]	7.32 ±0.04[b]	8.15 ±0.10[c,d]	8.38 ±0.15[c]

All values are mean ± s.e.m.

[a] $p<0.05$ compared to correspoding control values

[b] $p<0.05$ compared to endothelium-intact values

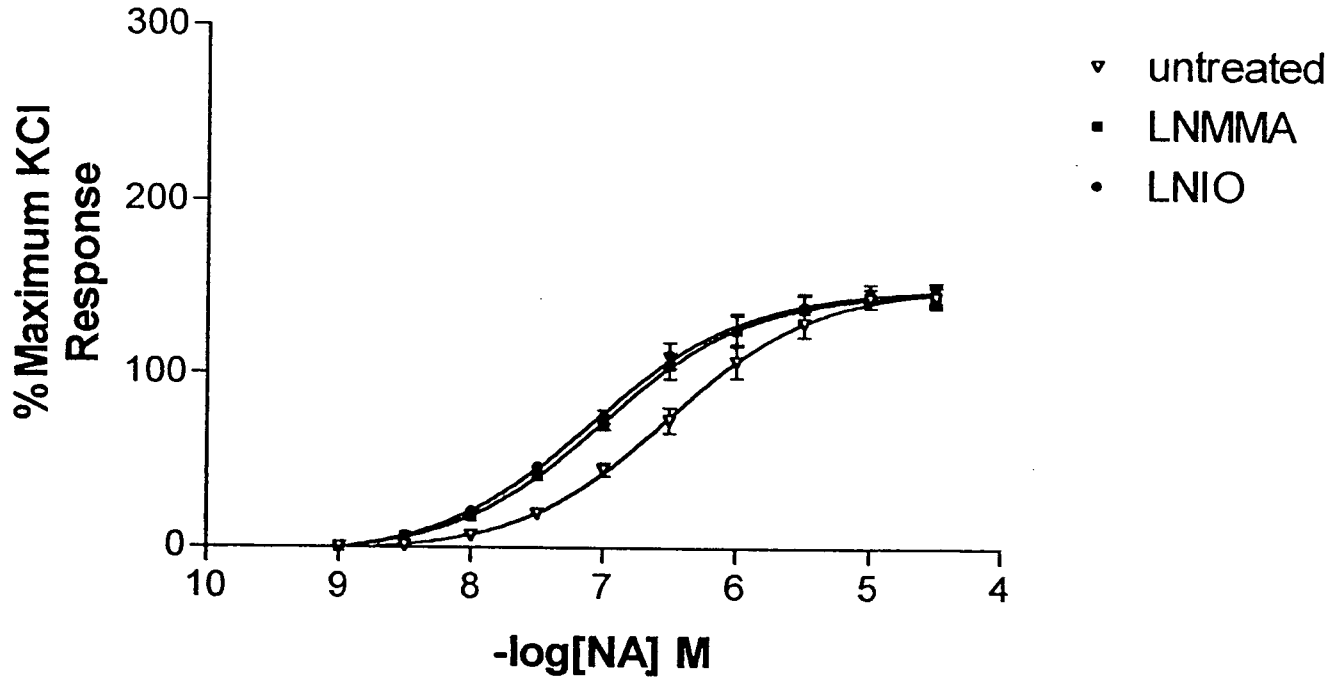
[c] $p<0.05$ compared to untreated values

[d] $p<0.05$ compared to treated endothelium-intact values

Figure 1

Cumulative concentration-response curves to NA of endothelium-intact control (**A**) and 12-14 week STZ-diabetic (**B**) superior mesenteric arteries in the absence (▽, N=16) and presence of 300μM LNMMA (■, n=10) or 300μM L-NIO (●, n=6). Each point represents the mean ± s.e.m.

A


300
200
100
0
% Maximum KCl Response
10
9
8
7
6
5
4
-log[NA] M
untreated
LNMMA
LNIO

B

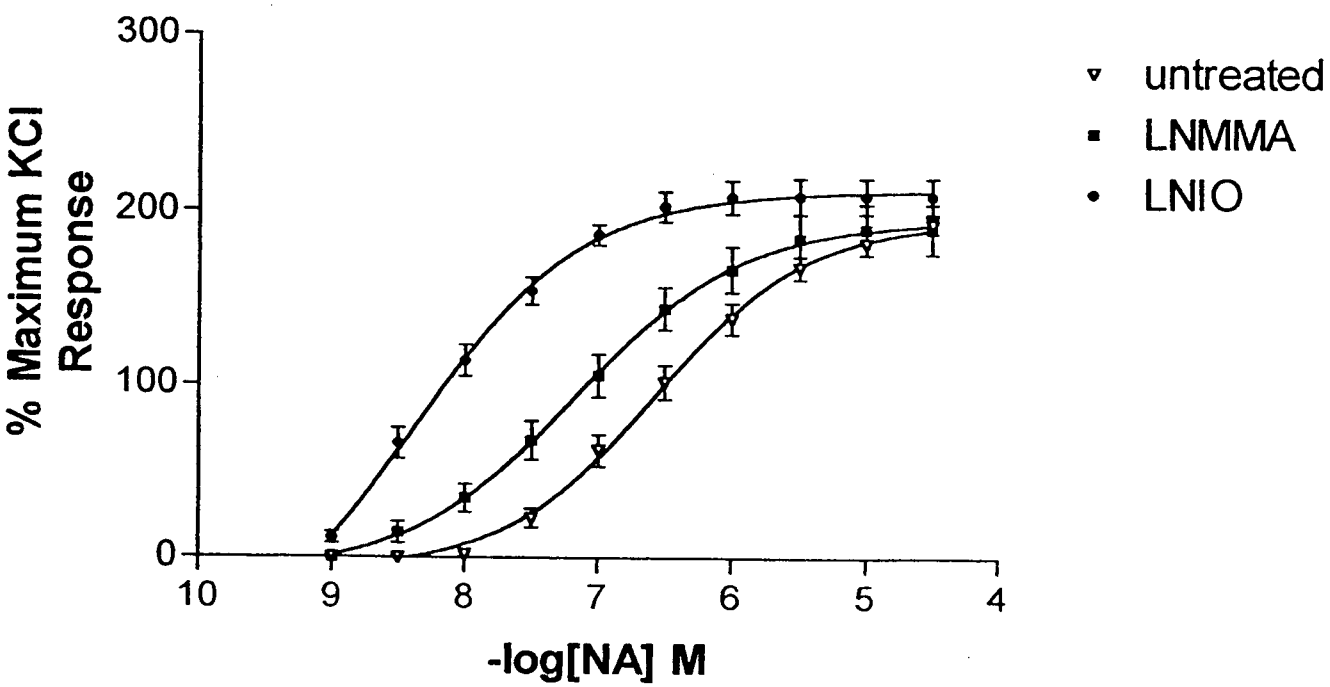
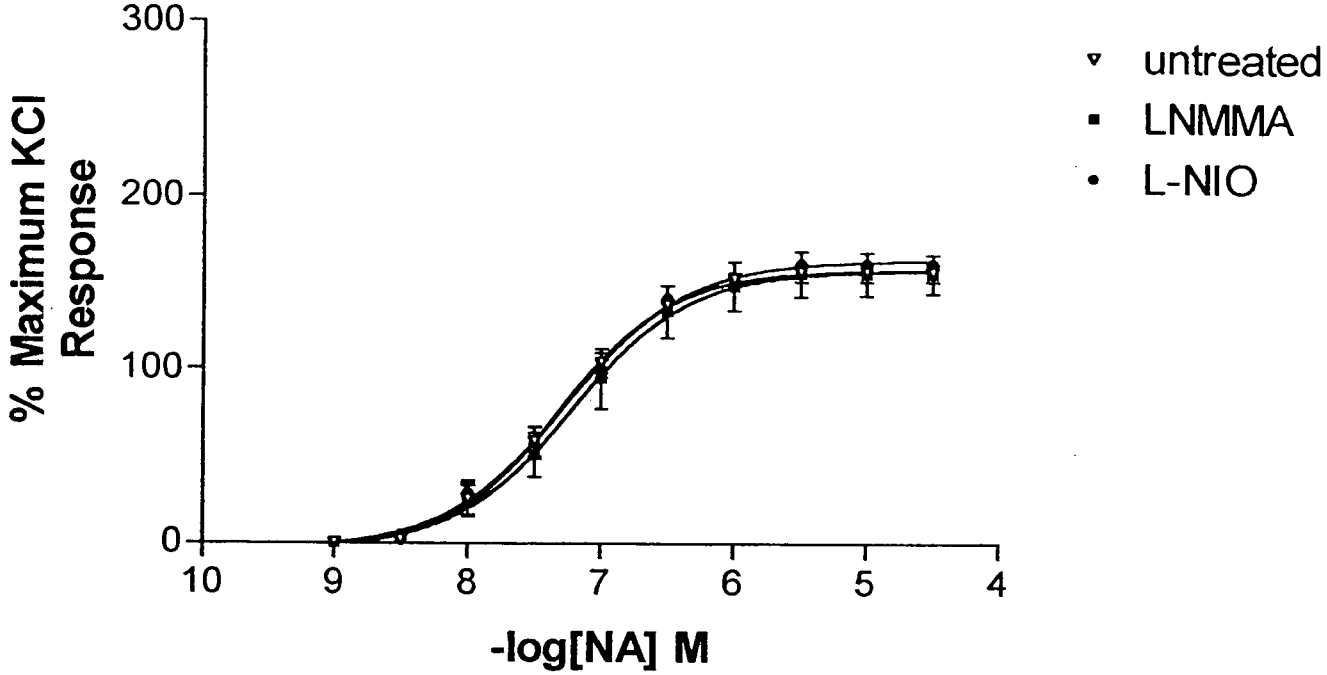

300
200
100
0
% Maximum KCl Response
10
9
8
7
6
5
4
-log[NA] M
untreated
LNMMA
LNIO

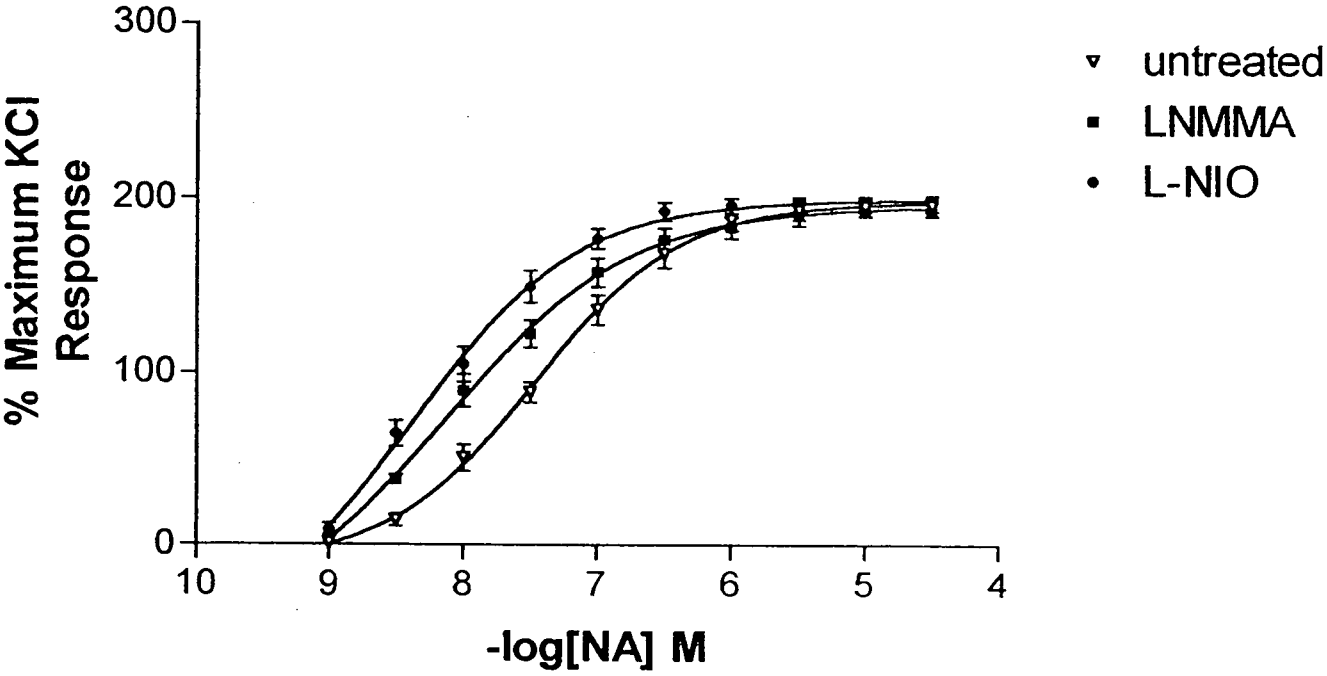
Figure 2

Cumulative concentration-response curves to NA of endothelium-denuded control (**A**) and 12-14 week STZ-diabetic (**B**) superior mesenteric arteries in the absence (▽, N=16) and presence of 300μM LNMMA (■, n=10) or 300μM L-NIO (●, n=6). Each point represents the mean ± s.e.m.

A


% Maximum KCl Response
300
200
100
0
10
9
8
7
6
5
4
-log[NA] M
untreated
LNMMA
L-NIO

B


% Maximum KCl Response
300
200
100
0
10
9
8
7
6
5
4
-log[NA] M
untreated
LNMMA
L-NIO

C. Effects of the selective nNOS inhibitor 7-NINA in endothelium-denuded arteries

In order to investigate whether nNOS is the subtype of NOS contributing to the observed increase in NA-sensitivity in endothelium-denuded 12-14 week STZ-diabetic arteries with LNMMA or L-NIO, cumulative concentration-response curves to NA were obtained in endothelium-denuded vessels in the absence and presence of the nNOS inhibitor 7-NINA (100μM). Pre-incubation with 7-NINA had no effect on NA responses of either control or diabetic endothelium-denuded mesenteric arteries (Fig.3, Table 3).

Table 3

Sensitivity (pD2) and maximum responses (Rmax) to NA of endothelium-denuded control and 12-14 week STZ-diabetic mesenteric arteries in the absence and presence of 7-NINA (100μM)

Animals	Rmax untreated	Rmax 7-NINA	pD2 value untreated	pD2 value 7-NINA
Control	166.1± 4.1	171.7±4.1	7.34± 0.09	7.28±0.07
Diabetic	225.8± 9.9*	223.5±5.3*	7.69± 0.20	7.56± 0.10

N=4

All values are mean ±s.e.m.

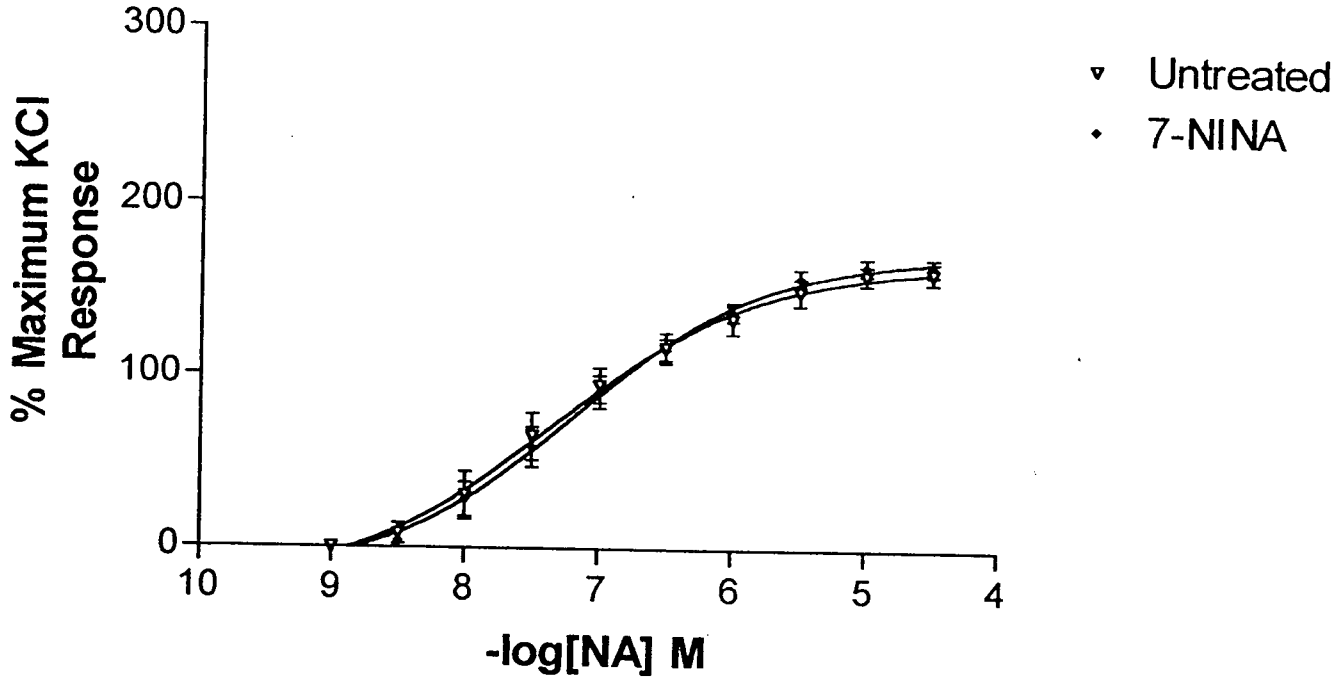
* P<0.05 as compared to corresponding control values

Figure 3

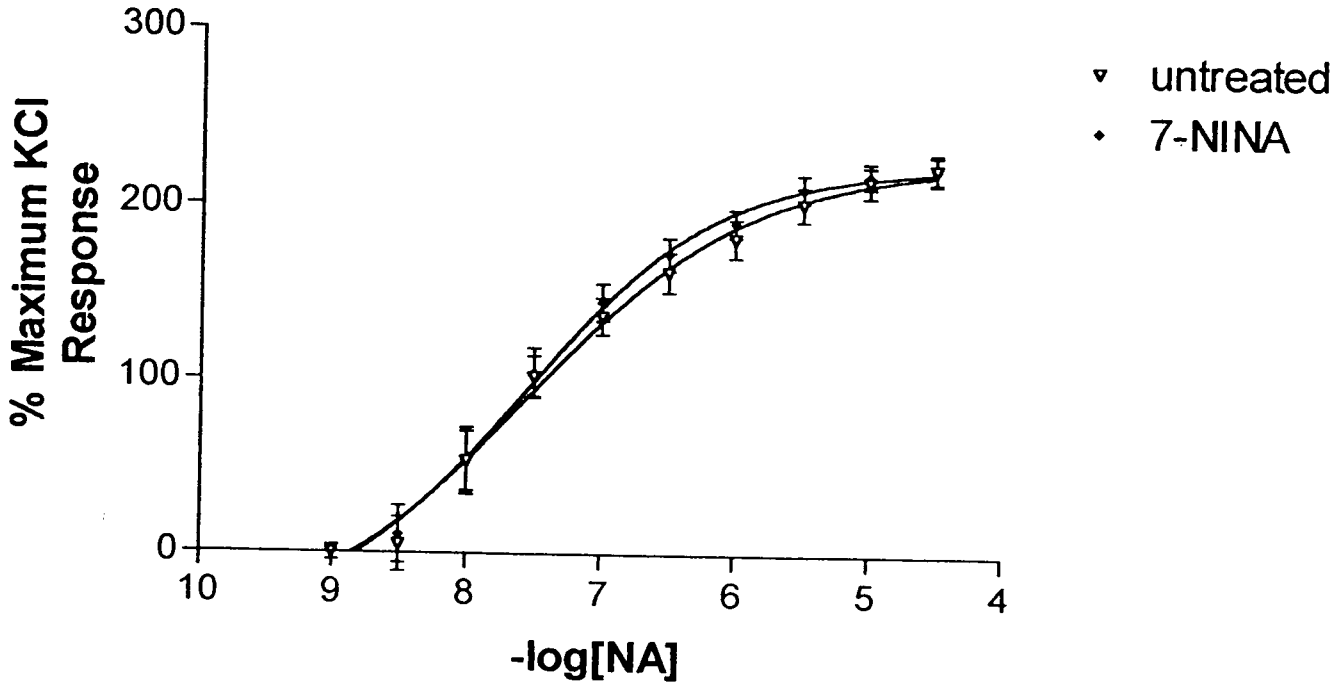
Cumulative concentration-response curves to NA of endothelium-denuded control (**A**) and 12-14 week STZ-diabetic (**B**) superior mesenteric arteries in the absence (∇, N=4) and presence of 100μM 7-NINA (◆, N=4).

Each point represents the mean ± s.e.m.

A


300
200
100
0
% Maximum KCl Response
10
9
8
7
6
5
4
-log[NA] M
Untreated
7-NINA

B


300
200
100
0
% Maximum KCl Response
10
9
8
7
6
5
4
-log[NA]
untreated
7-NINA

D. Effects of iNOS inhibitors in endothelium-denuded arteries

In order to investigate whether iNOS is the subtype of NOS contributing to the increase in NA sensitivity in endothelium-denuded 12-14 week STZ-diabetic arteries with LNMMA or L-NIO, cumulative concentration-response curves to NA were obtained in endothelium-denuded arteries in the absence and presence of the iNOS inhibitors, aminoguanidine (1mM) or EIT (5μM or 10μM).

1. *Aminoguanidine*

Aminoguanidine (1mM) had no effect on NA sensitivity in endothelium-denuded control arteries (Figure 4,Table 4). A significant increase in NA pD2 values was observed in endothelium-denuded and endothelium-intact STZ-diabetic arteries following preincubation with aminoguanidine (Figure 4,Table 4). However, aminoguanidine also produced a significant increase in NA pD2 values in endothelium-intact control arteries suggesting that aminoguanidine is also inhibiting eNOS at this concentration (Figure 4, Table 4).

Table 4

Sensitivity (pD2 values) to NA of endothelium-intact and endothelium-denuded control and 12-14 week STZ-diabetic mesenteric arteries in the absence and presence of aminoguanidine (AG) (1mM)

Animals	Endothelial-status	PD2 Untreated	pD2 AG
Control	Intact	5.92±0.19	6.89±0.26*
Control	Denuded	7.49±0.18**	7.59±0.20**
Diabetic	Intact	6.04±0.14	7.11±0.18*
Diabetic	Denuded	7.37±0.21**	8.32±0.38*,**

N=6

All values are mean ±s.e.m.

* P<0.05 as compared to untreated values

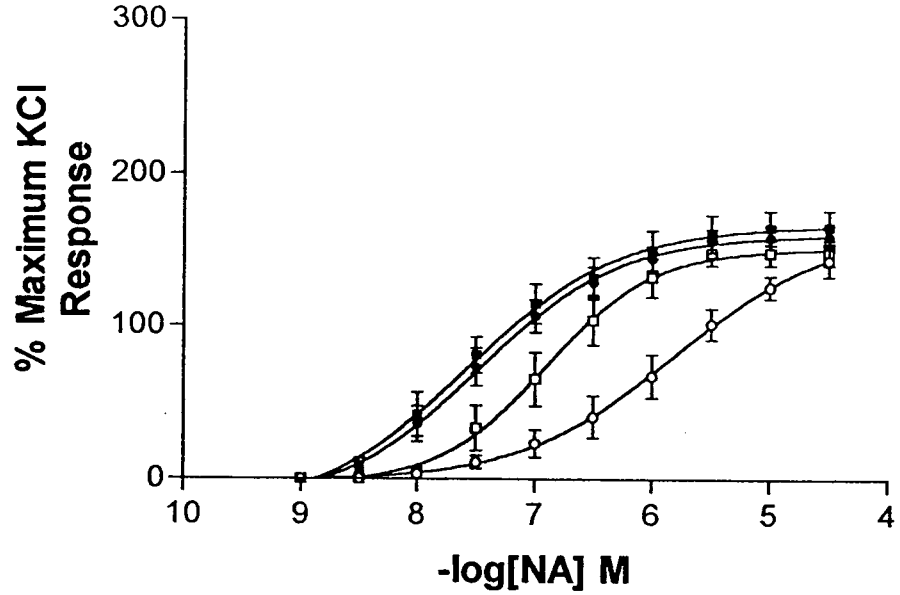
** P<0.05 as compared to corresponding endothelium-intact values

Figure 4

Cumulative concentration-response curves to NA of endothelium-intact (open symbols, N=4) and endothelium-denuded (closed-symbols, N=8) control (**A**) and 12-14 week STZ-diabetic (**B**) superior mesenteric arteries in the absence (circles) and presence (squares) of 1mM AG.

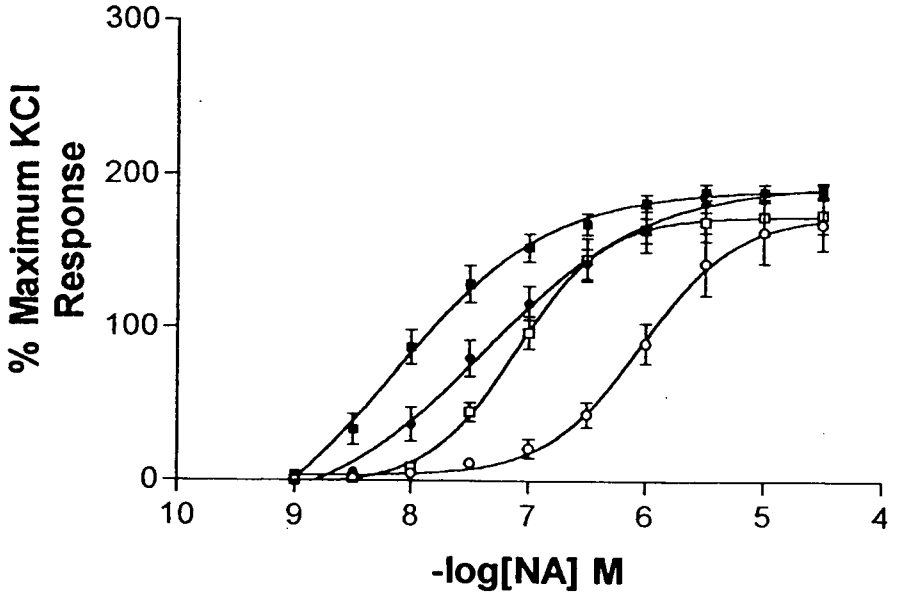
Each point represents the mean ± s.e.m.

A



- untreated,endo
- untreated, no endo
- AG,endo
- AG, no endo

B



- untreated, endo
- untreated, no endo
- AG, endo
- AG, no endo

2. *S-ethylisothiourea*

Since aminoguanidine did not appear to be acting as a selective inhibitor of iNOS, cumulative concentration-response curves to NA were obtained in endothelium-denuded arteries in the absence and presence of the highly selective iNOS inhibitor S-ethylisothiourea (EIT) (5μM or 10μM).

EIT (5μM) had no effect on NA sensitivity in either control or diabetic arteries. Similarly, no significant increase in sensitivity to NA was obtained in endothelium-denuded control vessels following preincubation with 10μM EIT (Figure 5,Table 5). However, 10μm EIT produced a leftward shift in the NA concentration-response curve, and a significant increase in NA pD_2 values in endothelium-denuded diabetic mesenteric arteries (Fig.5, Table 5).

To investigate whether EIT (10μM) also inhibits eNOS, cumulative concentration-response curves to NA were obtained in endothelium-intact control arteries. NA pD2 values in endothelium-intact arteries were 6.04±0.41 in the absence, and 6.14±0.33 in the presence of 10μM EIT (N=3, P>0.05).

Table 5

Sensitivity (pD2 values) to NA of endothelium-denuded control and 12-14 week STZ-diabetic mesenteric arteries in the absence and presence of EIT

Animals	EIT Concentration	pD2 untreated	PD2 EIT
Control, N=4	5μM	7.14±0.21	7.38±0.19
Control, N=5	10μM	7.40±0.09	7.39±0.19
Diabetic, N=4	5μM	7.42±0.21	7.38±0.13
Diabetic, N=11	10μM	7.43±0.23	8.40±0.13*

All values are mean ± s.e.m.

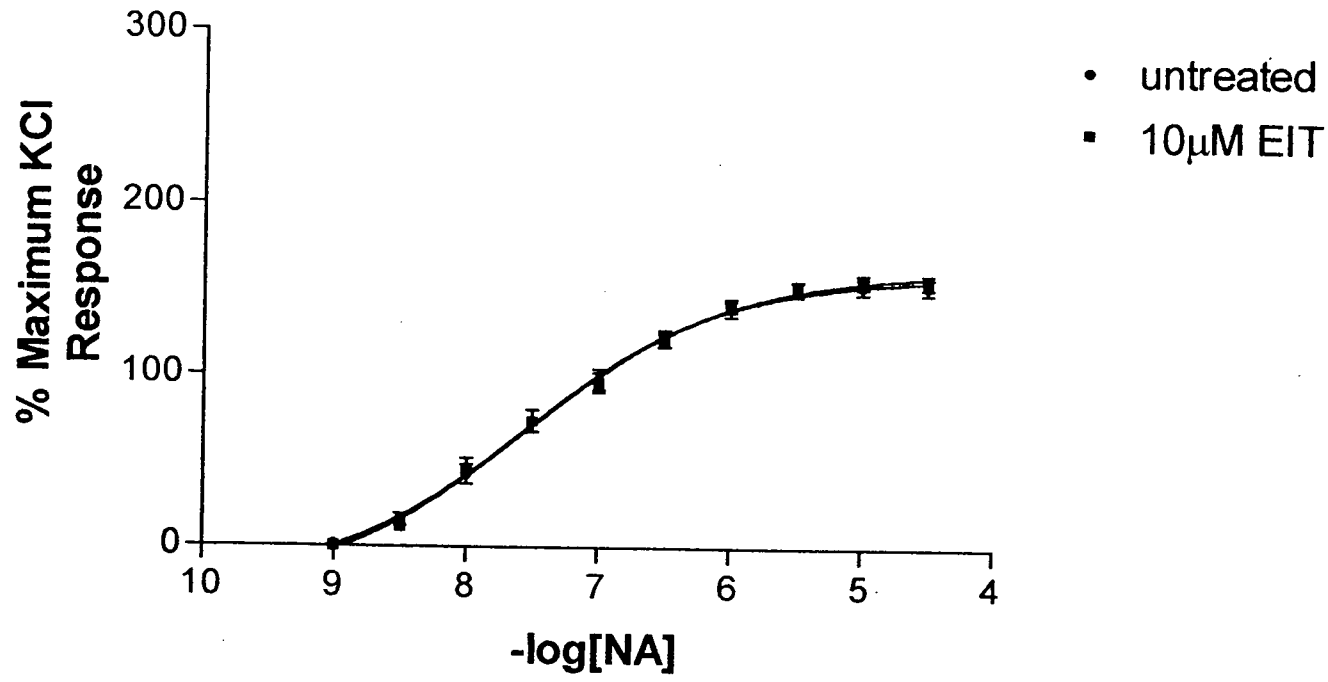
*P<0.05 as compared to untreated values

Figure 5

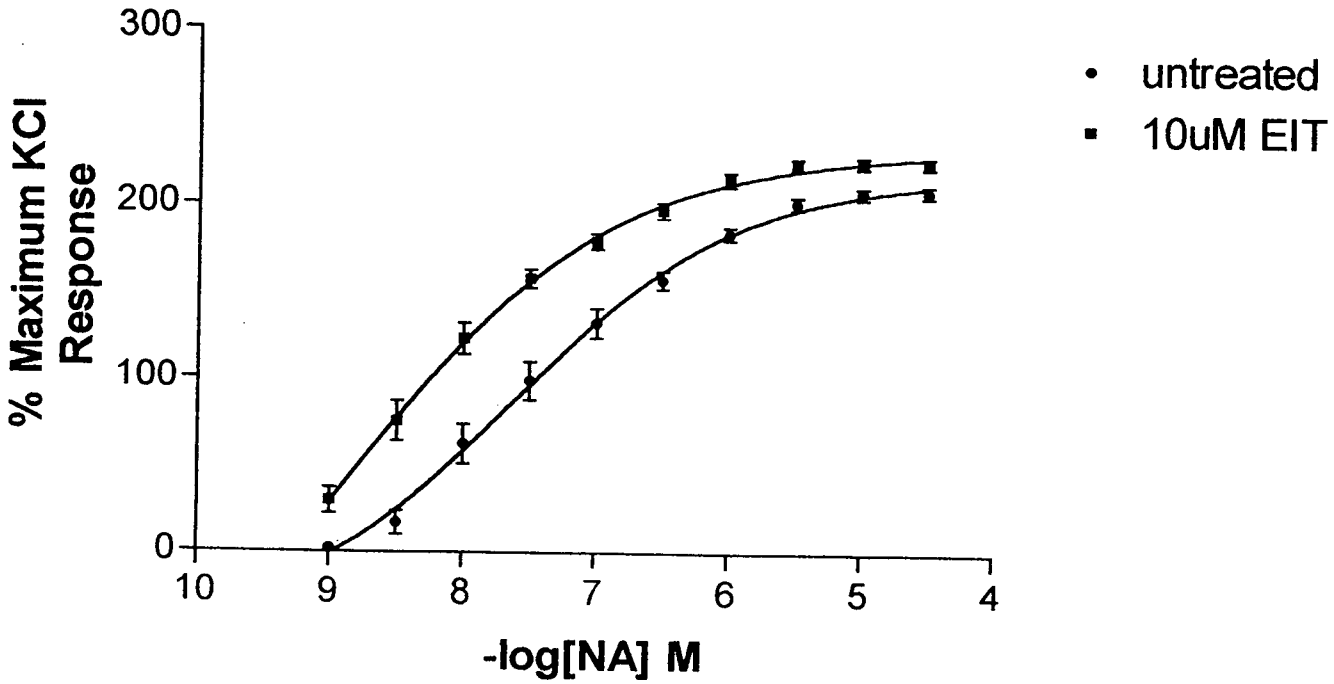
Cumulative concentration-response curves to NA of endothelium-denuded control (**A**, N=5) and 12-14 week STZ-diabetic (**B**, N=11) superior mesenteric arteries in the absence (●) and presence of 10μM EIT(■).

Each point represents the mean ± s.e.m.

A


300
200
100
0
% Maximum KCl Response
10
9
8
7
6
5
4
-log[NA]
untreated
10μM EIT

B


300
200
100
0
% Maximum KCl Response
10
9
8
7
6
5
4
-log[NA] M
untreated
10uM EIT

E. Investigation of reversibility of L-NIO and EIT

To determine if the increase in NA sensitivity seen with L-NIO was due to competitive inhibition of NOS, cumulative concentration-response curves to NA were obtained in endothelium-intact control mesenteric arteries treated with L-NIO alone or in the presence of L-arginine or D-arginine (1mM each). L-arginine abolished the leftward shift in the NA concentration-response curve and the increase in NA pD_2 values due to L-NIO, while D-arginine had no effect on the NA response in the presence of L-NIO (Table 6, Fig.6).

NA concentration response curves were also obtained in endothelium-denuded diabetic arteries treated with EIT (10μM) alone or in the presence of L-arginine or D-arginine to verify that the increase in NA pD_2 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 NA response was abolished by co-incubation with L-arginine but was not affected by D-arginine (Table 6, Fig.7).

Table 6

Effect of co-incubation of NOS inhibitors with L-arginine or D-arginine on NA pD_2 values in mesenteric arteries from control and 12-14 week STZ-diabetic rats.

Mesenteric Arteries	Inhibitor	pD_2 (untreated)	pD_2 (inhibitor)	pD_2 (inhibitor + L-arginine)	pD_2 (inhibitor + D-arginine)
Control (endothelium-intact)	L-NIO	6.29±0.08	7.11± 0.07*	6.28±0.11	7.13±0.09*
Diabetic (endothelium-denuded)	EIT	7.22±0.08	8.07± 0.12*	7.46±0.32	8.13±0.16*

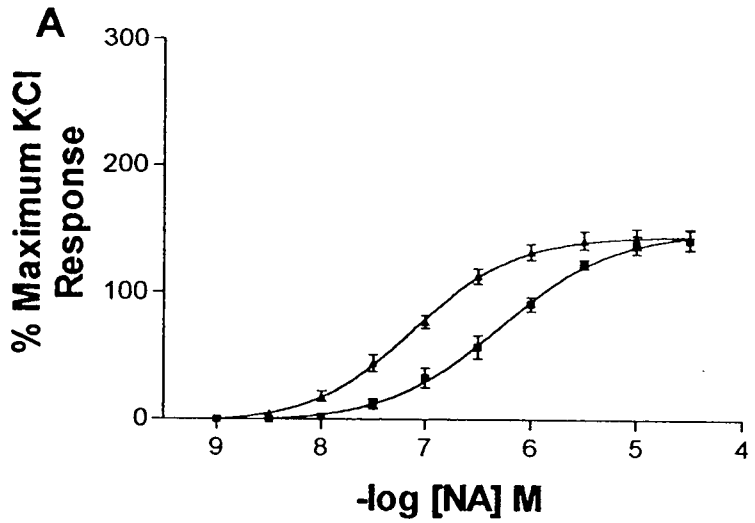
N=5 in each group.

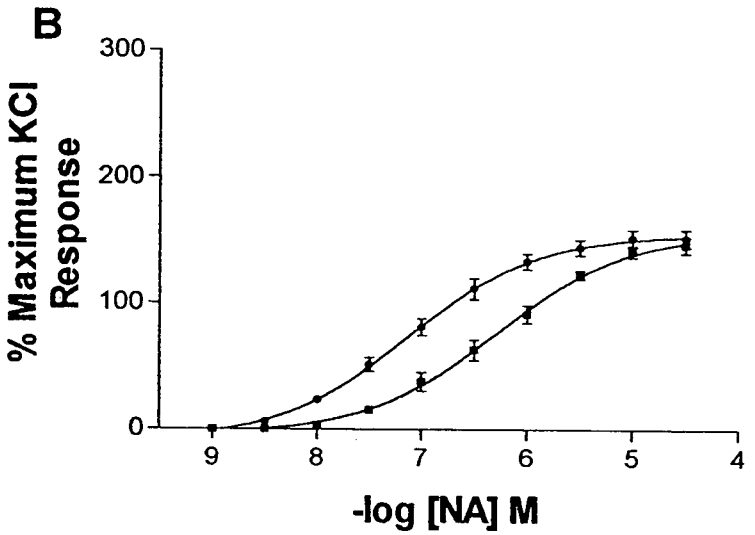
All values are mean ± s.e.m.

* $P<0.05$ compared to untreated values

Figure 6

Cumulative concentration-response curves to NA of endothelium-intact superior mesenteric arteries from control rats in the absence (■) and presence of 300μM L-NIO (▲) (**A**), 300μM L-NIO + 1mM D-arginine (●) (**B**), or 300μM L-NIO + 1mM L-arginine (□) (**C**). Each point represents the mean ± s.e.m. (N=5 animals)


A
% Maximum KCl Response
300
200
100
0
9
8
7
6
5
4
-log [NA] M
Untreated
L-NIO


B
% Maximum KCl Response
300
200
100
0
9
8
7
6
5
4
-log [NA] M
Untreated
L-NIO, D-Arg

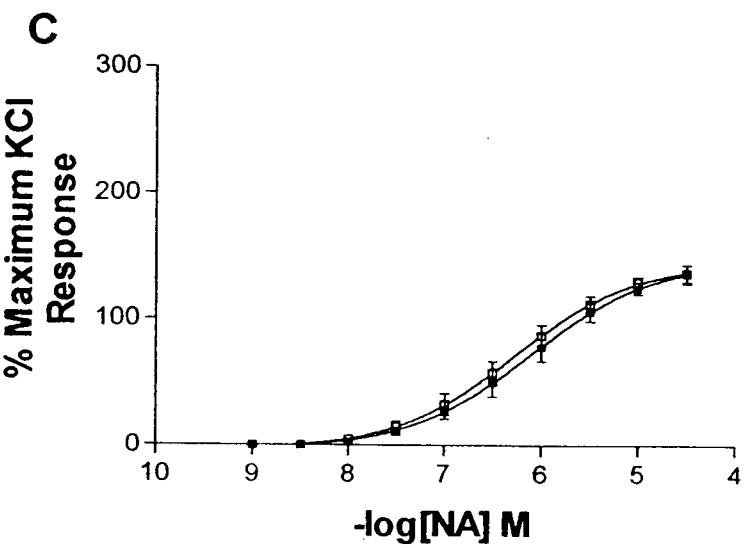
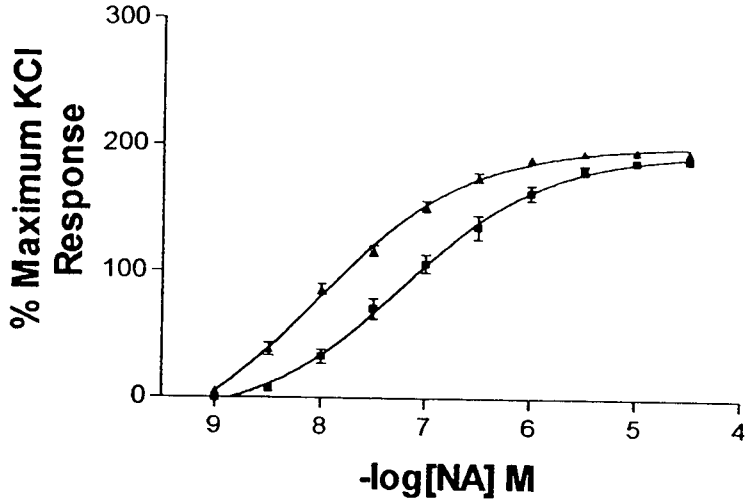

C
% Maximum KCl Response
300
200
100
0
10
9
8
7
6
5
4
-log[NA] M
untreated
L-NIO, L-Arg

Figure 7

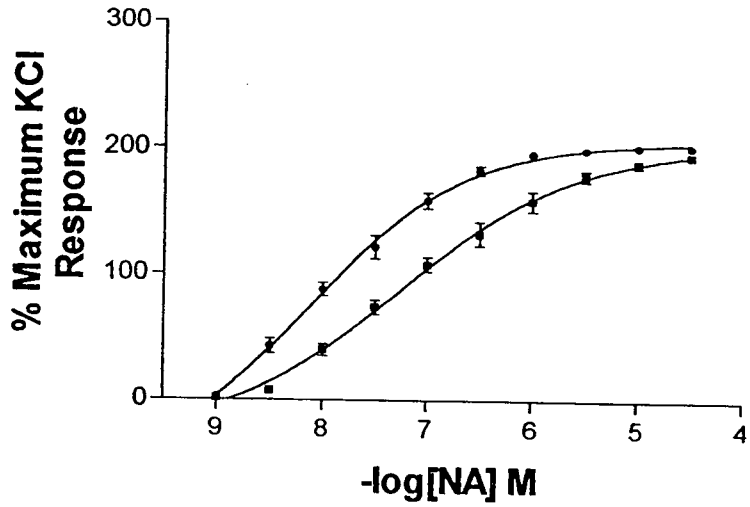
Cumulative concentration-response curves to NA of endothelium-denuded STZ-diabetic superior mesenteric arteries in the absence (■) and presence of 10μM EIT (▲) (**A**), 10μM EIT + 1mM D-arginine (●) (**B**), or 10μM EIT + 1mM L-arginine (□) (**C**). Each point represents the mean ± s.e.m. (**N**=5 animals)

A



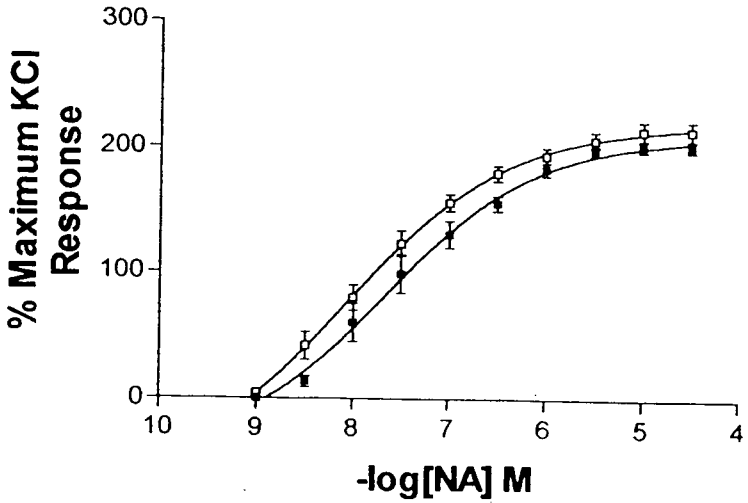
- Untreated
- EIT

B



- untreated
- EIT, D-Arg

C



- untreated
- EIT, L-Arg

F. Pharmacological investigation of the time-course of iNOS induction in diabetic VSM

To investigate the time course of iNOS induction in VSM of STZ-diabetic rats, cumulative concentration-response curves to NA in the absence and presence of EIT (10μM) were obtained in endothelium-denuded control and diabetic arteries 2-3 weeks and 6-8 weeks following STZ-injection.

No significant differences in sensitivity (pD2) or responsiveness (Rmax) were observed between untreated control and diabetic arteries 2-3 weeks following STZ injection (Table 7; Fig.8). NA pD2 values of 6-8 week STZ-diabetic arteries were also not significantly different from control (Table 7; Fig.9). As a group, 6-8 week STZ-diabetic arteries had significantly greater maximum responses to NA than control (2-way ANOVA). However, individual comparisons using Newman-Kuehl's post-hoc tests failed to show significant differences.

Preincubation with EIT had no significant effect on NA responses in endothelium-denuded 2-3 week or 6-8 week STZ-diabetic or control arteries (Table 7; Fig.8,9).

Table 7

Sensitivity (pD2) and maximum responses (Rmax) to NA of endothelium-denuded control and STZ-diabetic mesenteric arteries 2-3 weeks or 6-8 weeks following STZ-injection in the absence and presence of EIT (10μM)

Animals	Time-point	Rmax untreated	Rmax EIT	PD2 untreated	PD2 EIT
Control	2-3 weeks	164±9.4	167.6±7.4	7.24±0.15	7.33±0.12
Control	6-8 weeks	155.2±5.8	155.6±4.4	7.06±0.1	7.14±0.08
Diabetic	2-3 weeks	162.2±4.3	160.4±4.8	7.36±0.08	7.49±0.10
Diabetic	6-8 weeks	172.9±7.3	169.7±8.0	7.21±0.13	7.46±0.18

N=6

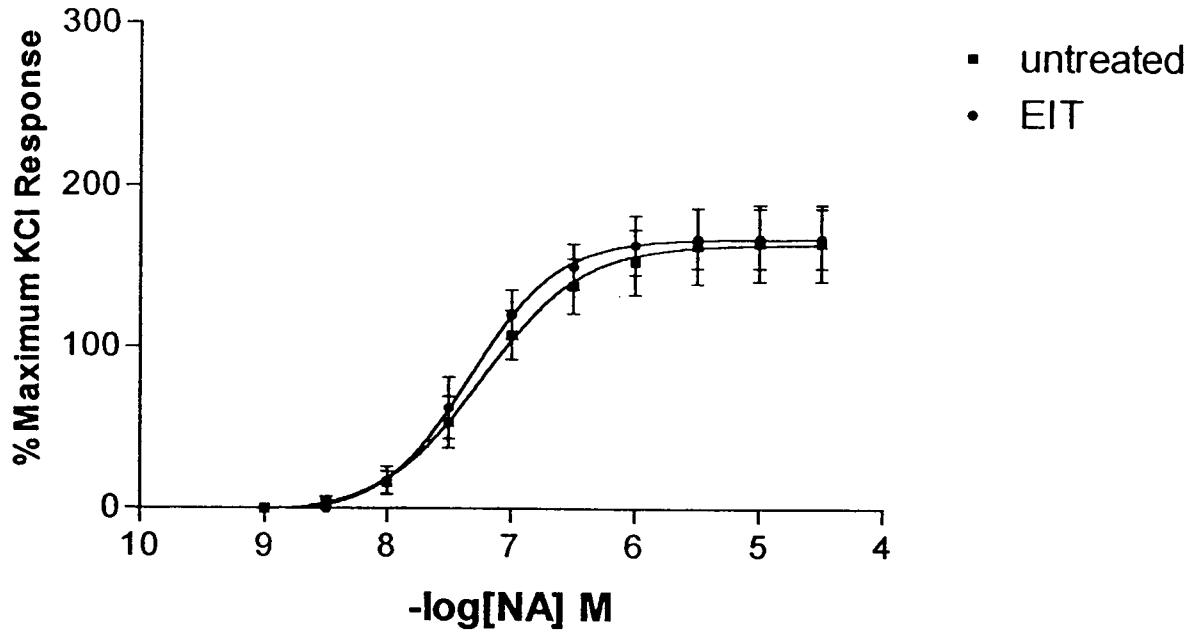
All values are mean ± s.e.m.

Figure 8

Cumulative concentration-response curves to NA of endothelium-denuded control (**A**) and 2-3 week STZ-diabetic (**B**) superior mesenteric arteries in the absence (●) and presence of 10μM EIT(■).

Each point represents the mean ± s.e.m., N=7

A


300
200
100
0
% Maximum KCl Response
10
9
8
7
6
5
4
-log[NA] M
untreated
EIT

B

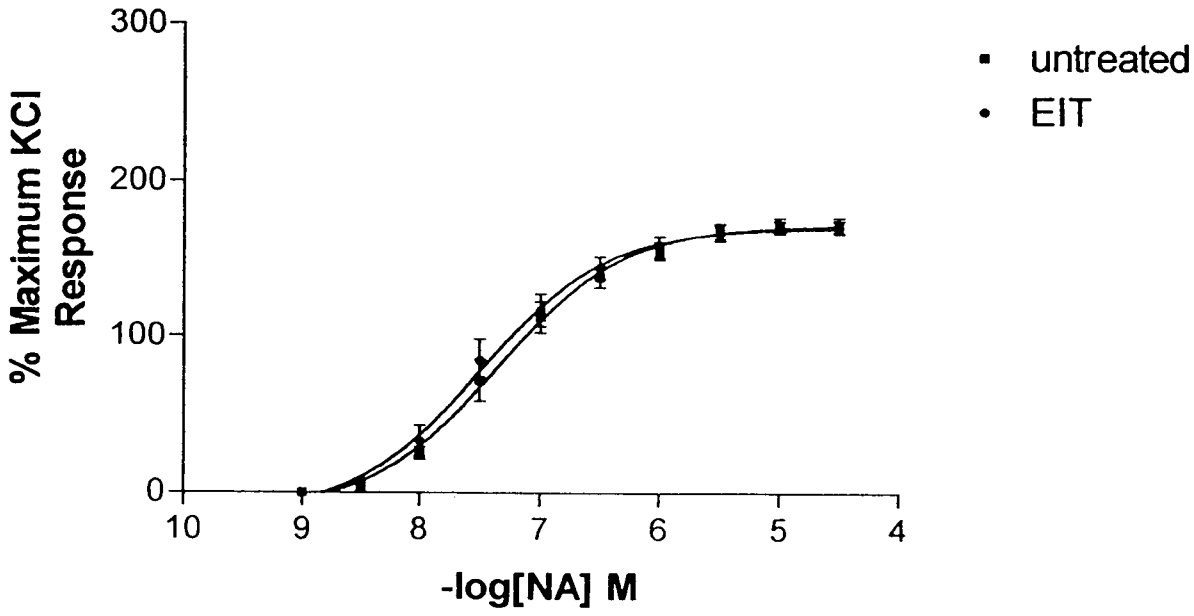
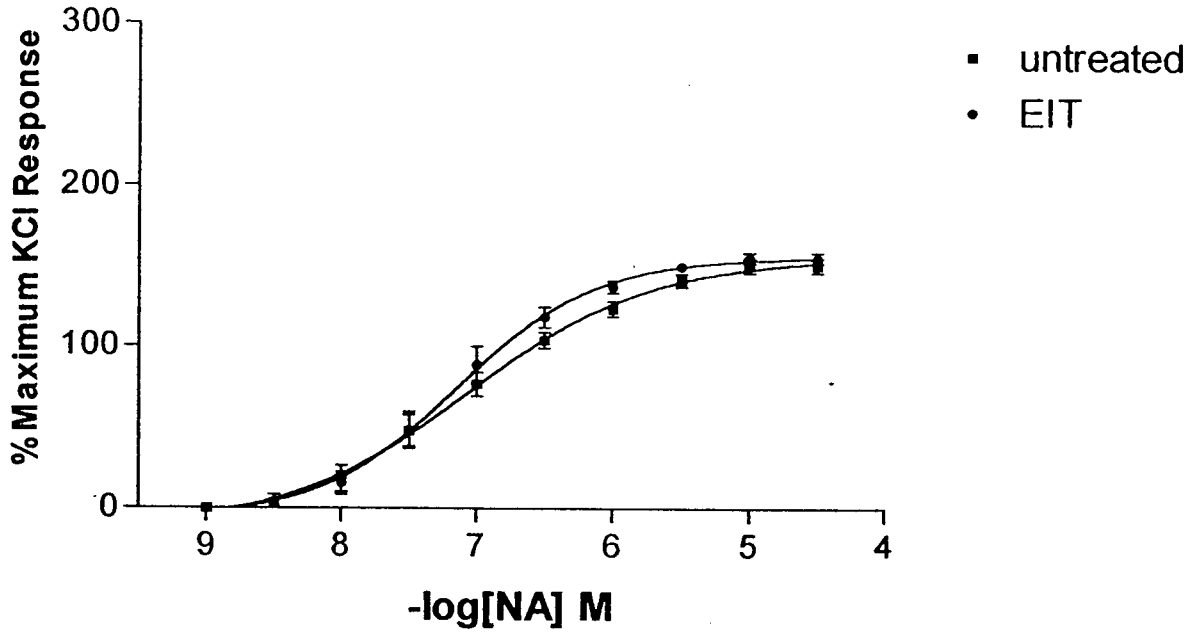

300
200
100
0
% Maximum KCl Response
10
9
8
7
6
5
4
-log[NA] M
untreated
EIT

Figure 9

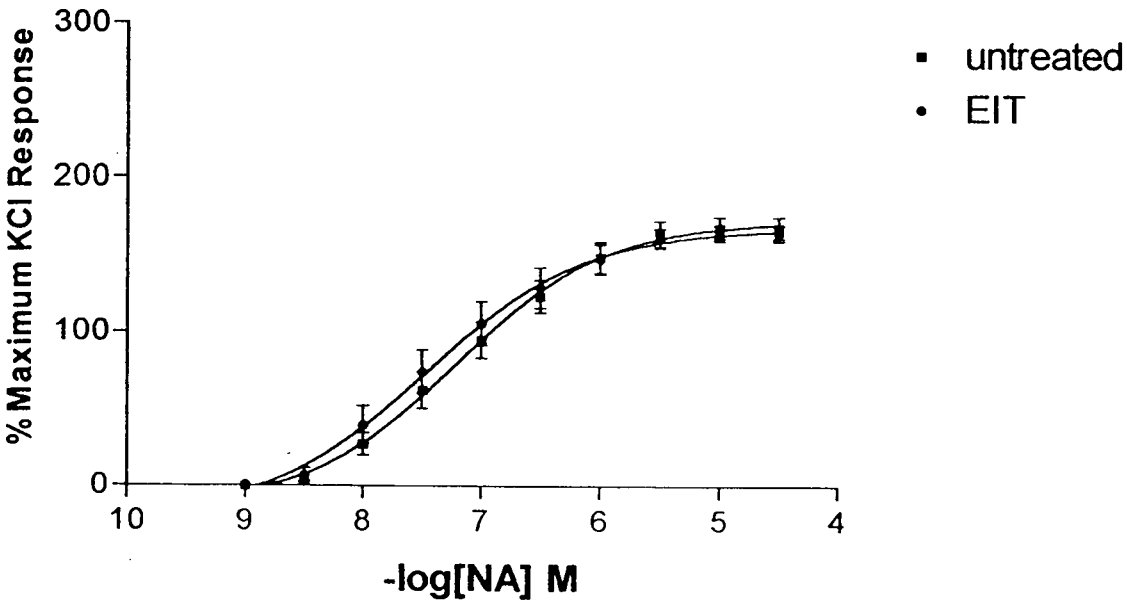
Cumulative concentration-response curves to NA of endothelium-denuded control (**A**) and 6-8 week STZ-diabetic (**B**) superior mesenteric arteries in the absence (●) and presence of 10μM EIT(■).

Each point represents the mean ± s.e.m., N=7

A


300
200
100
0
% Maximum KCl Response
9
8
7
6
5
4
-log[NA] M
untreated
EIT

B


300
200
100
0
% Maximum KCl Response
10
9
8
7
6
5
4
-log[NA] M
untreated
EIT

G. Immunohistochemical analysis of 12-14 week STZ-diabetic and control mesenteric arteries

Immunohistochemical analysis was performed in order to investigate whether the individual NOS subtypes could be detected in VSM from control or 12-14 week STZ-diabetic rats. In all immunostains, nuclei are indicated by dark pink dots and positive signals for antigen detection are indicated by dark black/purple dots.

eNOS

Immunostaining of mesenteric arteries for eNOS indicated that eNOS was expressed only in the endothelial cell monolayer of both control and STZ-diabetic arteries (Fig.10).

nNOS

Immunostaining for nNOS produced no positive signal in either control or diabetic arteries (Fig.11A-D). The anti-nNOS antibody used did produce a positive signal in the positive control (mouse brain sections) at the same dilution (Fig.11E).

iNOS

There was a striking difference between control and STZ-diabetic arteries in immunostaining for iNOS. A strong positive signal for iNOS was observed in the medial, adventitial, and intimal layers of the superior mesenteric arteries from STZ-diabetic but not control rats (Fig. 12).

Macrophage

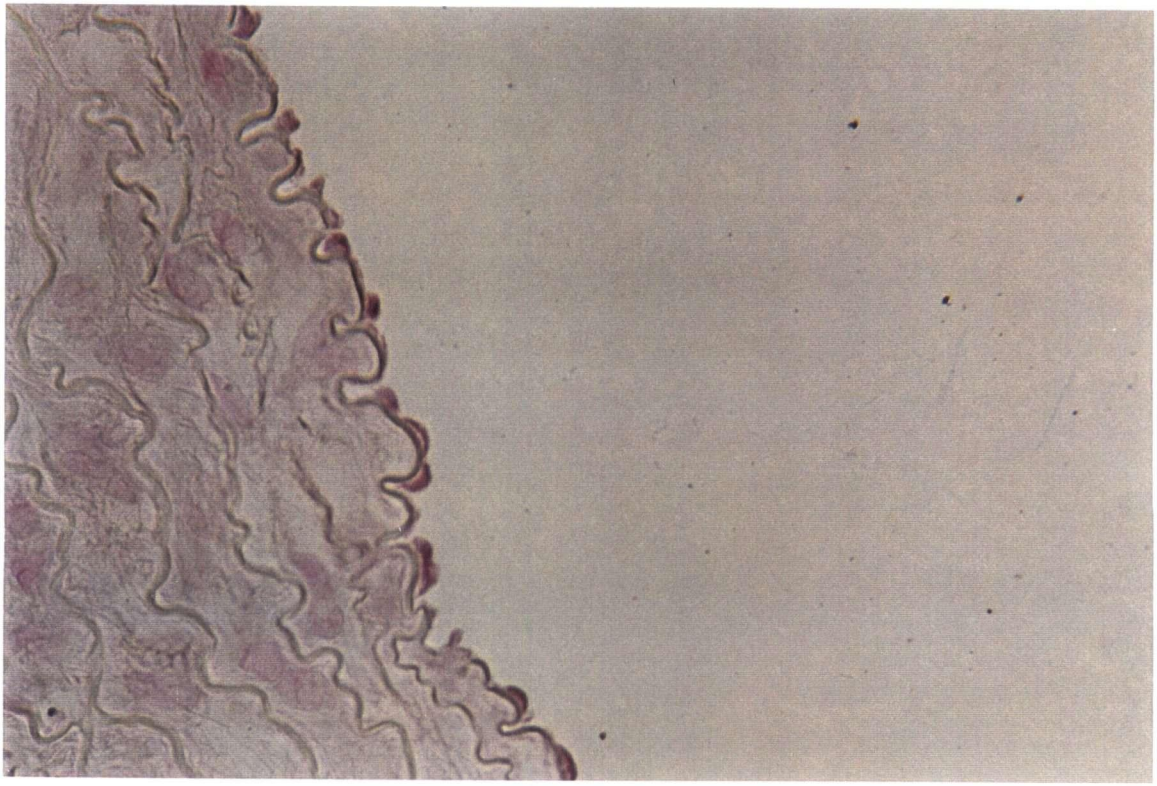
To determine whether macrophage infiltration is the source of iNOS expression in the diabetic mesenteric arteries, control and diabetic arteries were incubated with a specific antibody to rat macrophage (clone ED2). No positive staining for macrophages were obtained in control or diabetic arteries (Fig.13A,B). The anti-macrophage (clone ED2) antibody used did produce a positive signal in the positive control (rat spleen sections) at the same dilution (Fig.13C).

Figure 10

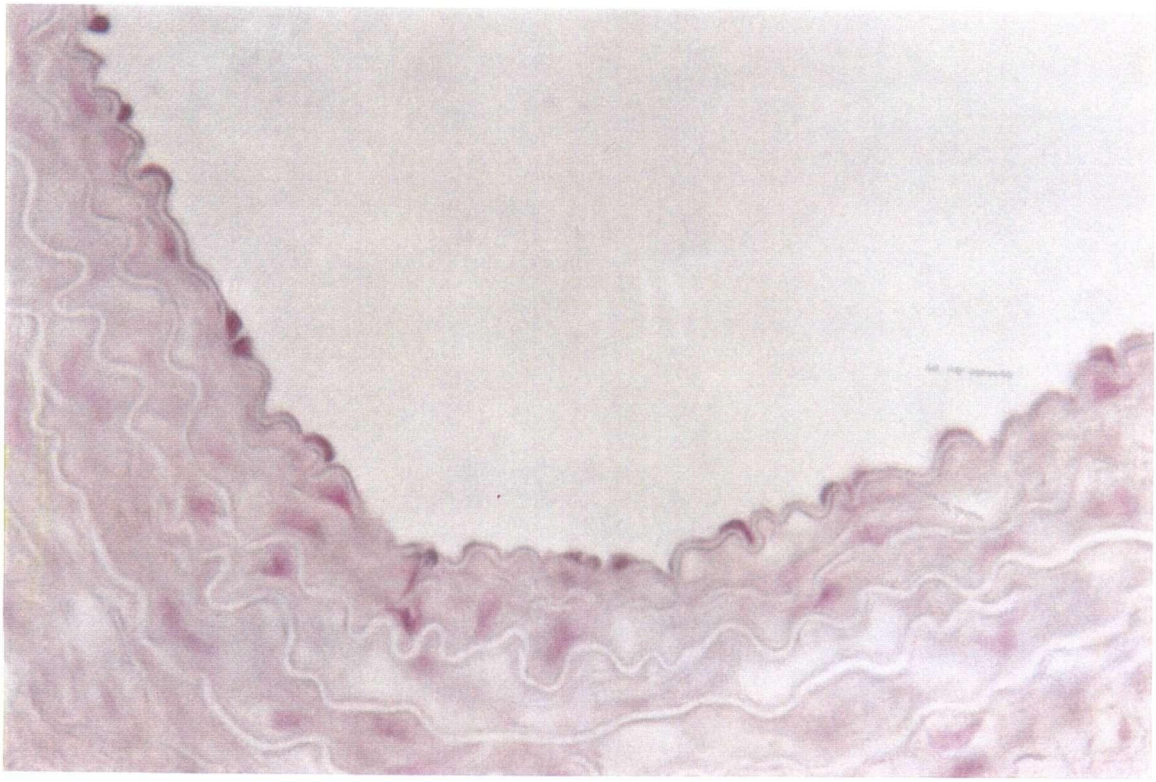
Immunostaining of endothelium-intact control (**A,B**) and 12-14 week STZ-diabetic (**C,D**) superior mesenteric arteries with the monoclonal anti-eNOS antibody.

Scale bar: 100μm

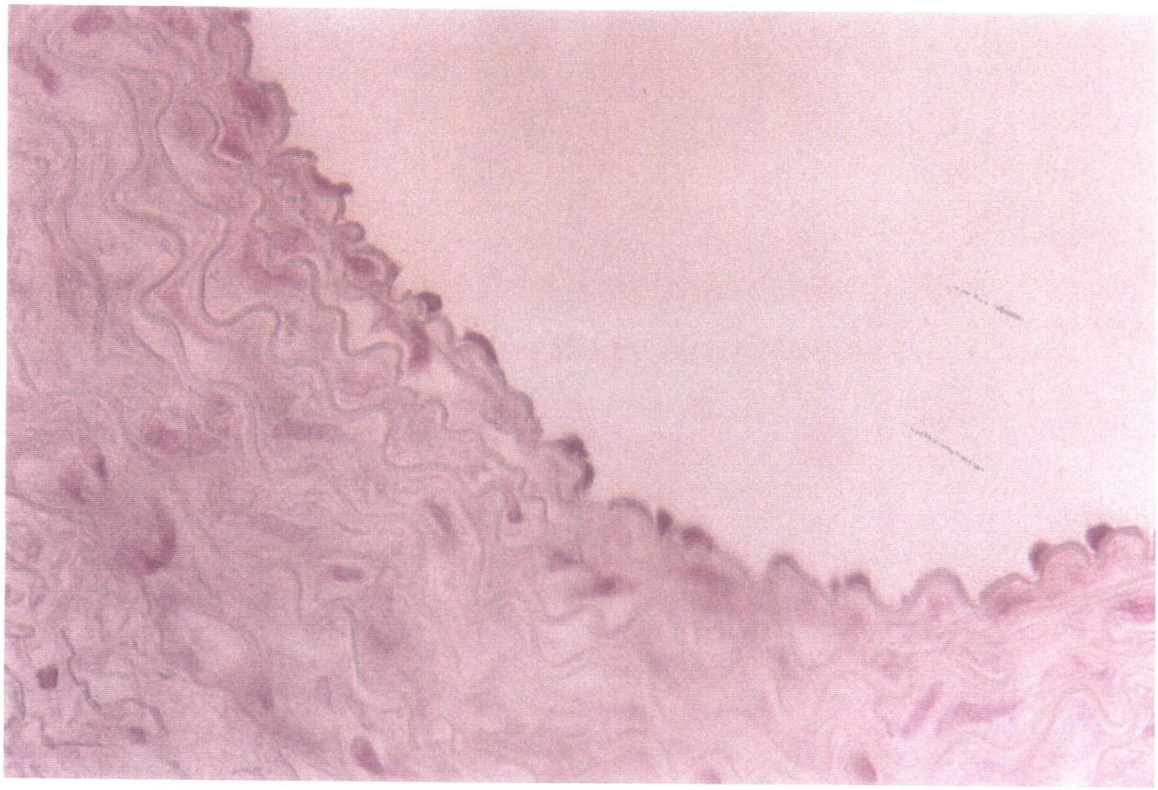
A



B



C



D

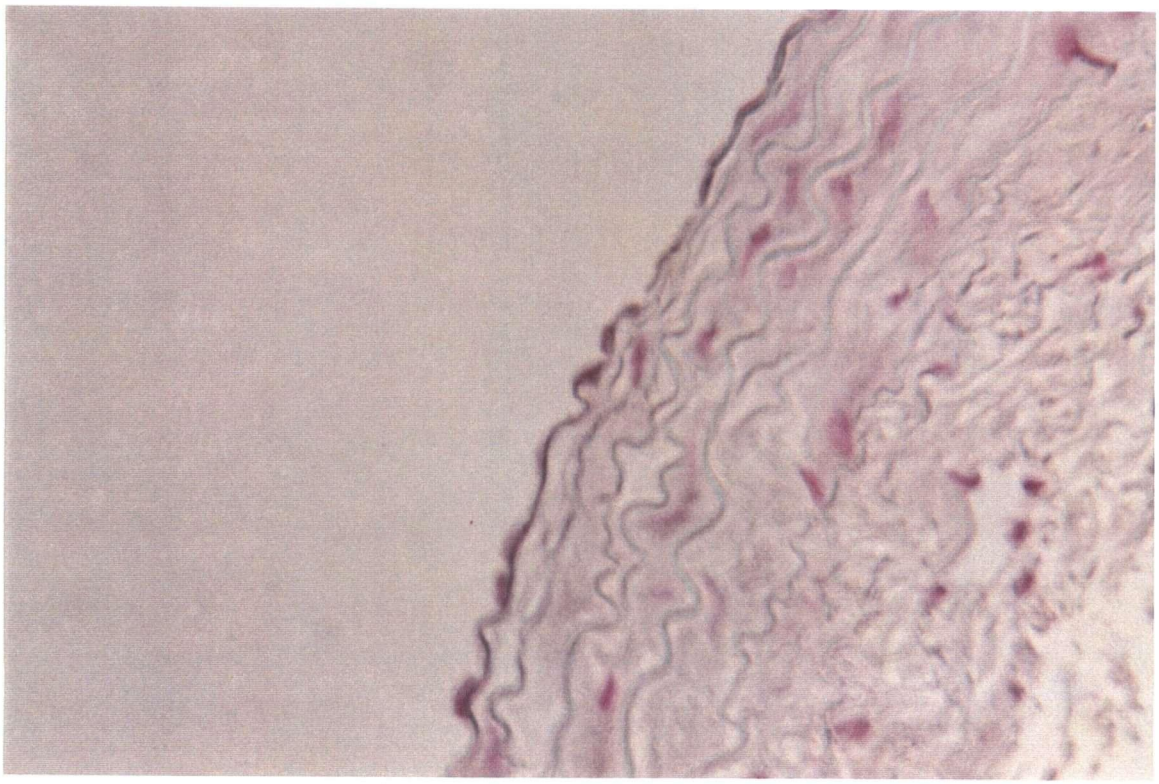
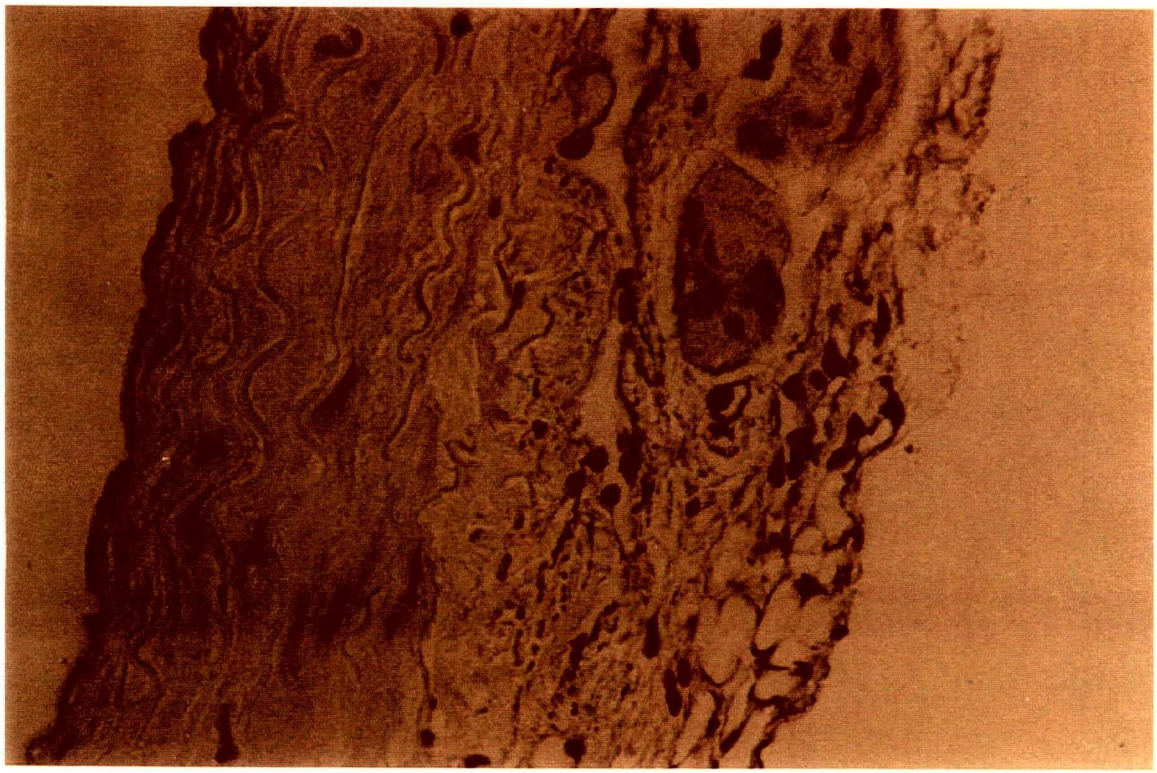


Figure 11

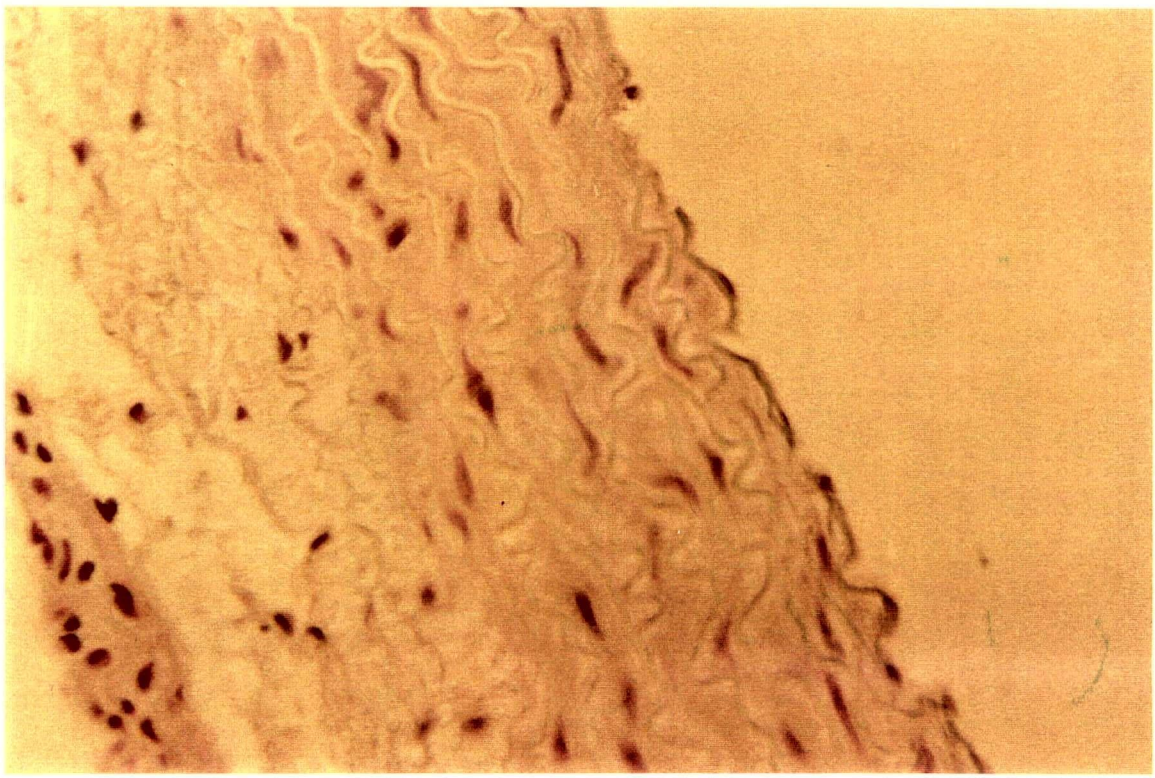
Immunostaining of endothelium-intact control (**A,B**) and 12-14 week STZ-diabetic (**C,D**) superior mesenteric arteries and mouse brain (**E**), with the polyclonal anti-nNOS antibody.

Scale bar: 100μm

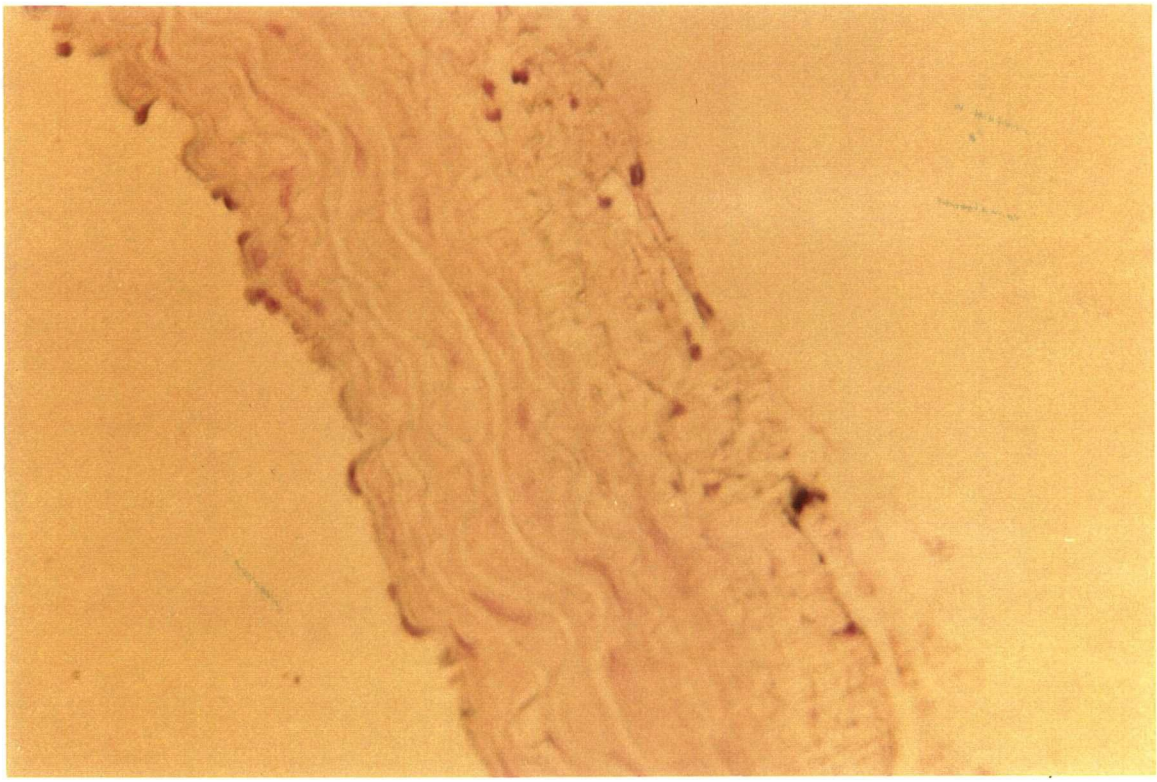
A



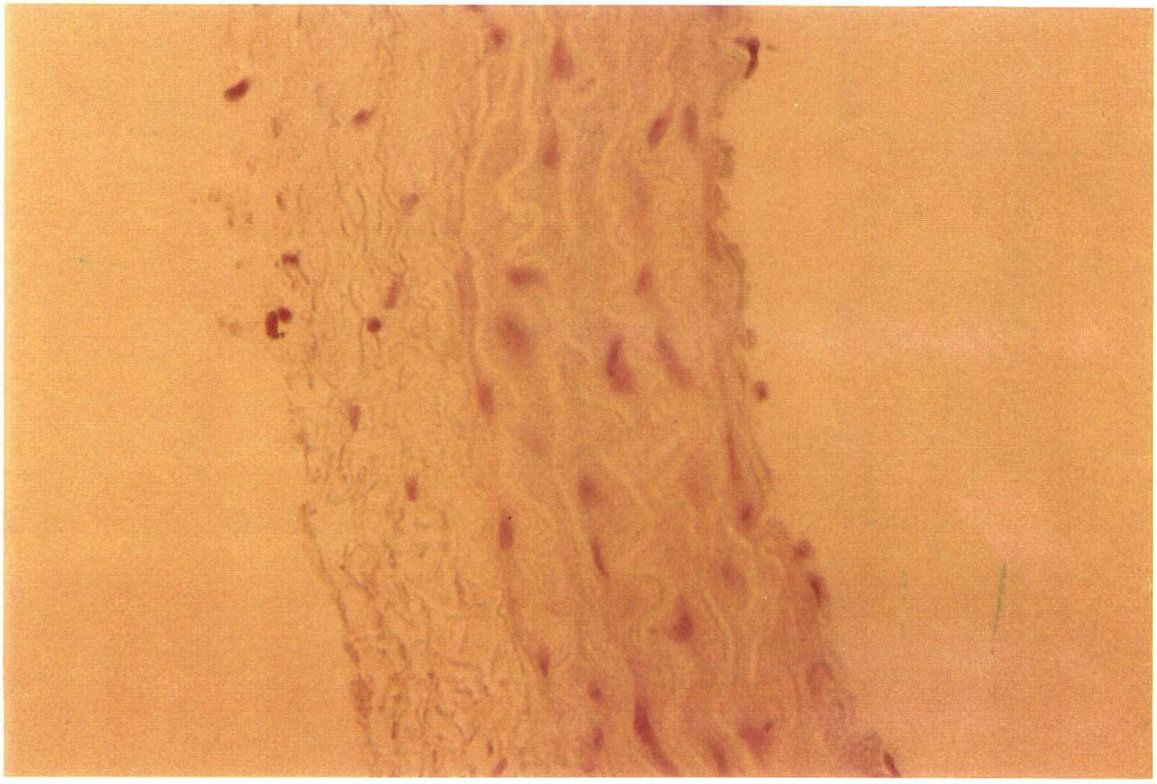
B



C



D



E

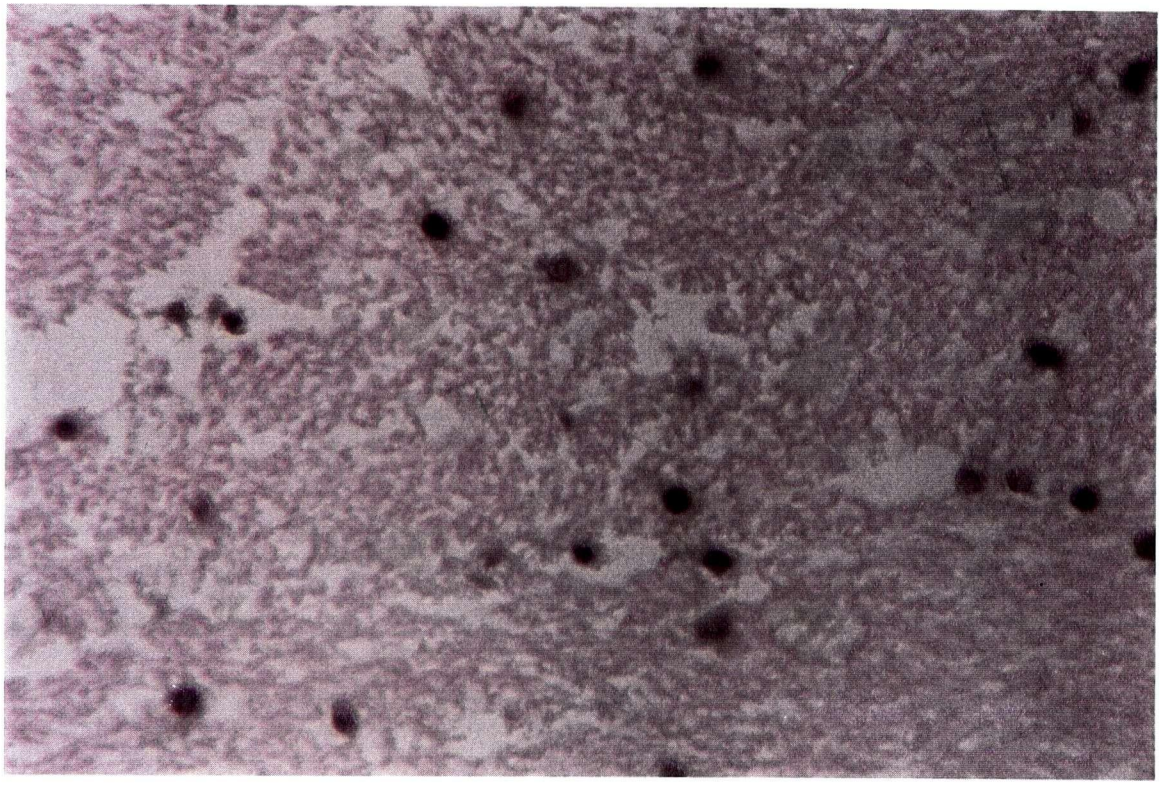
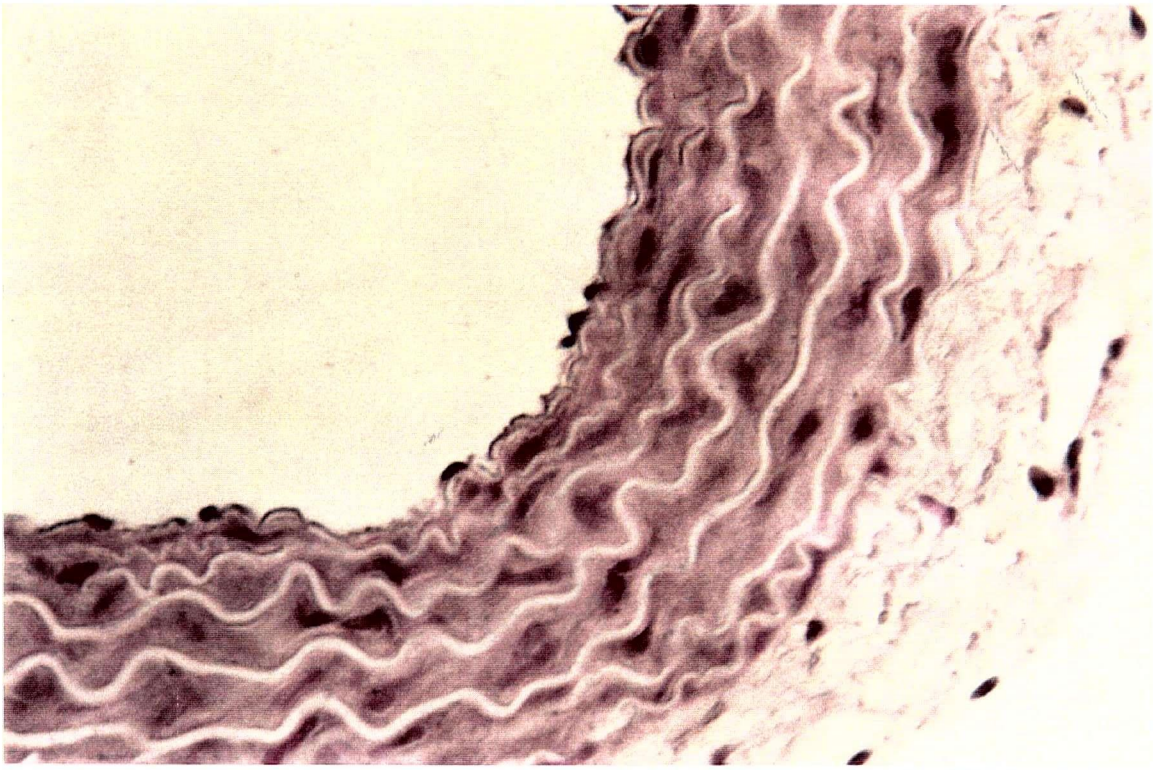


Figure 12

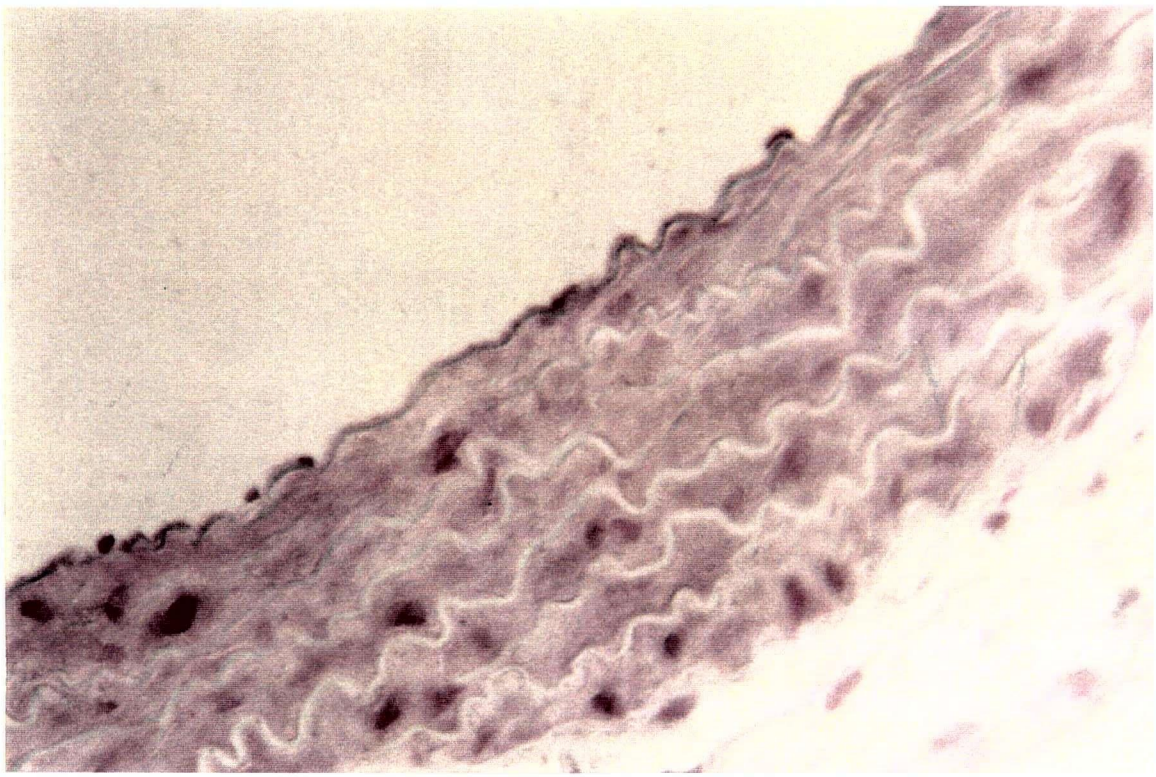
Immunostaining of endothelium-intact control (**A-C**) and 12-14 week STZ-diabetic (**D-G**) superior mesenteric arteries with the polyclonal anti-iNOS antibody.

Scale bar: 100μm

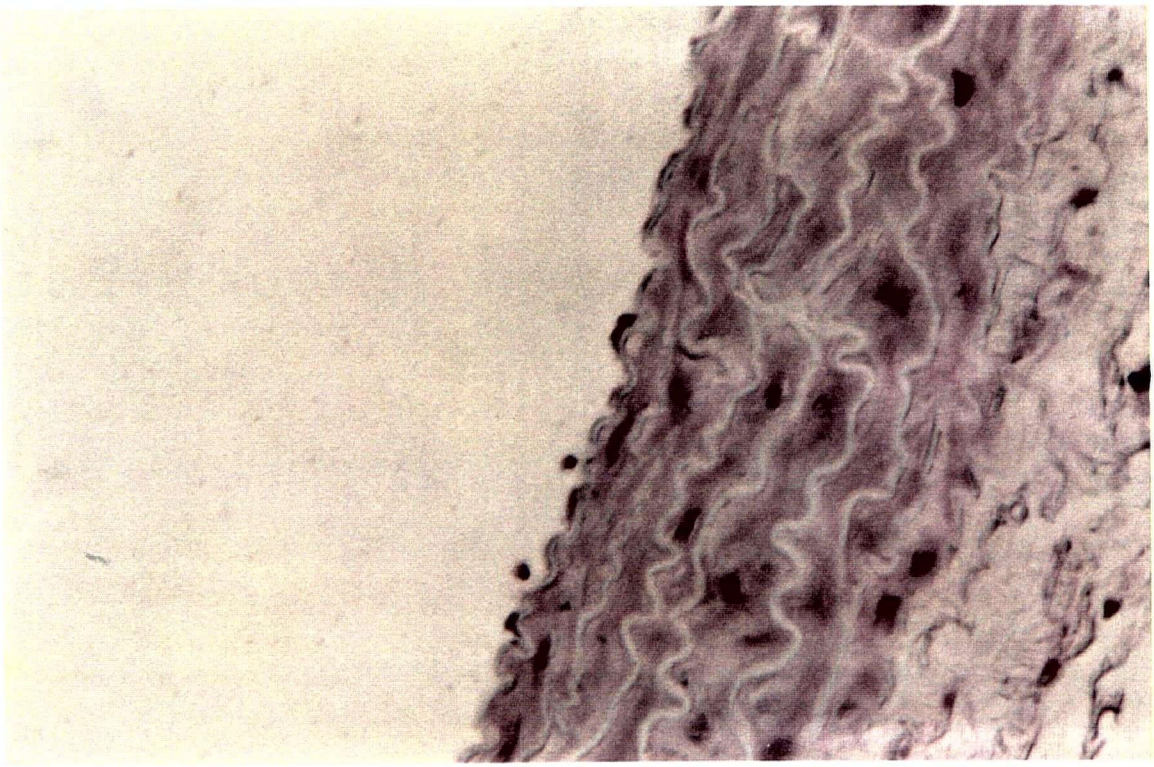
A



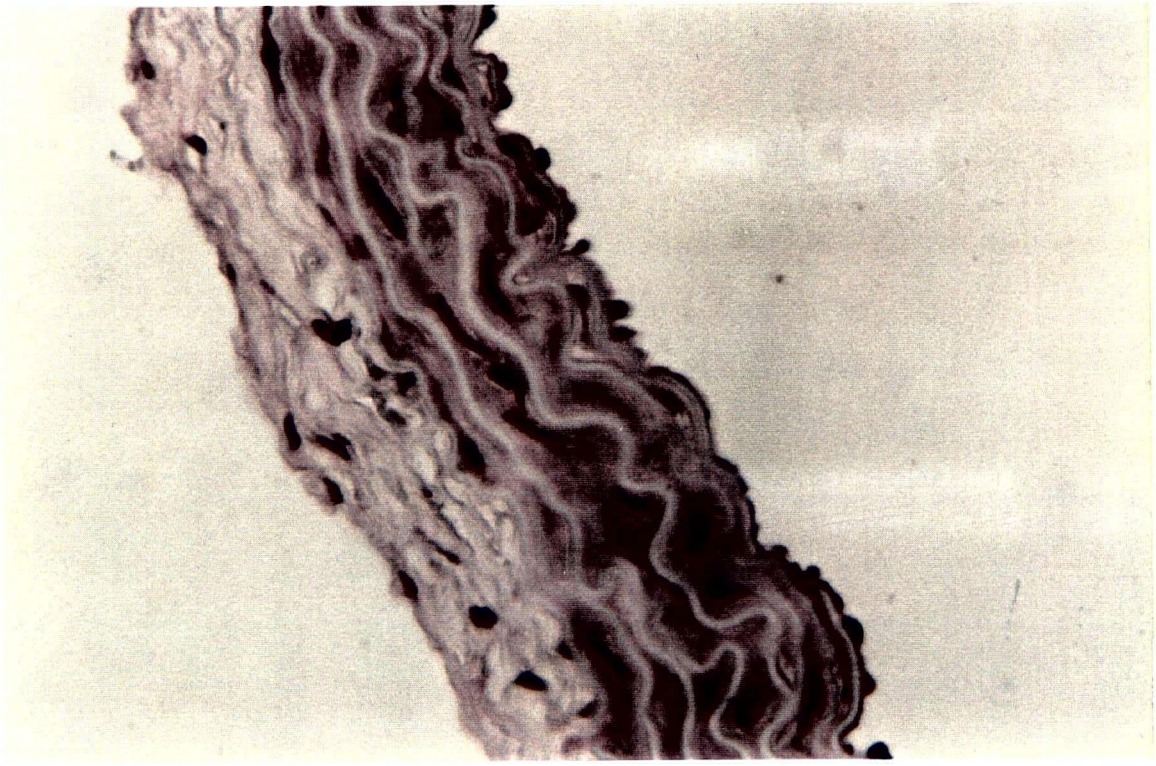
B



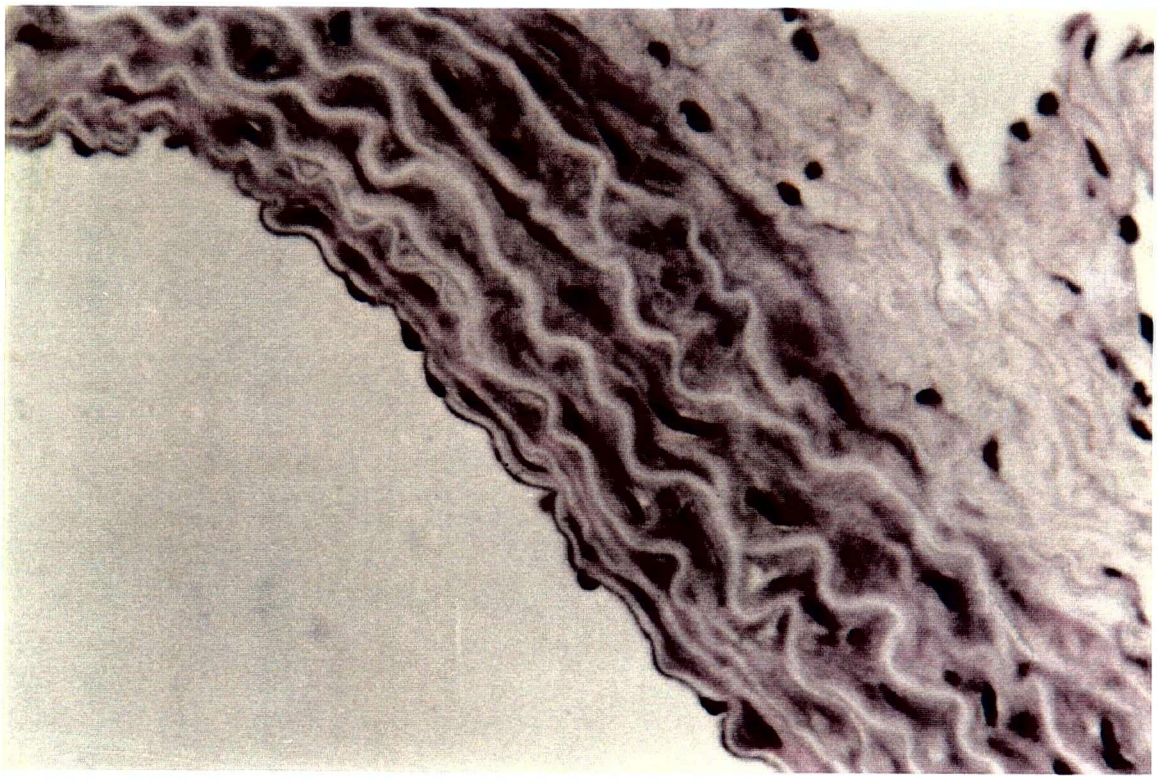
C



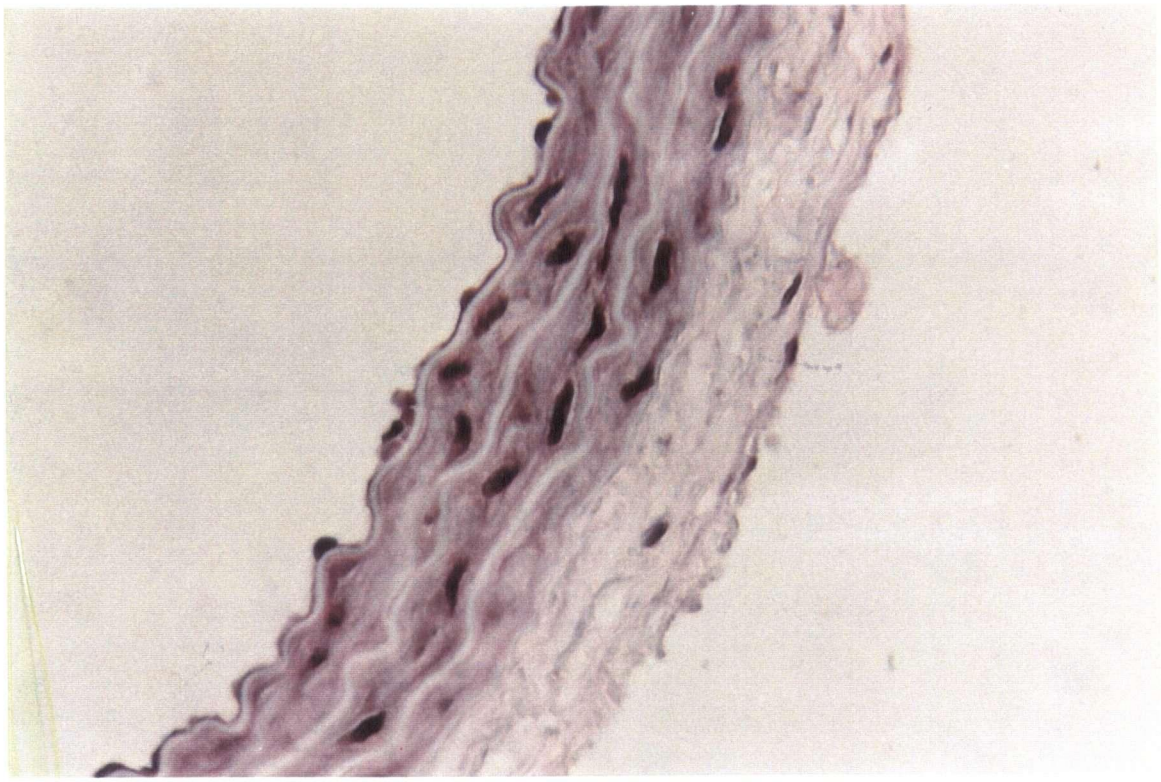
D



E



F



G

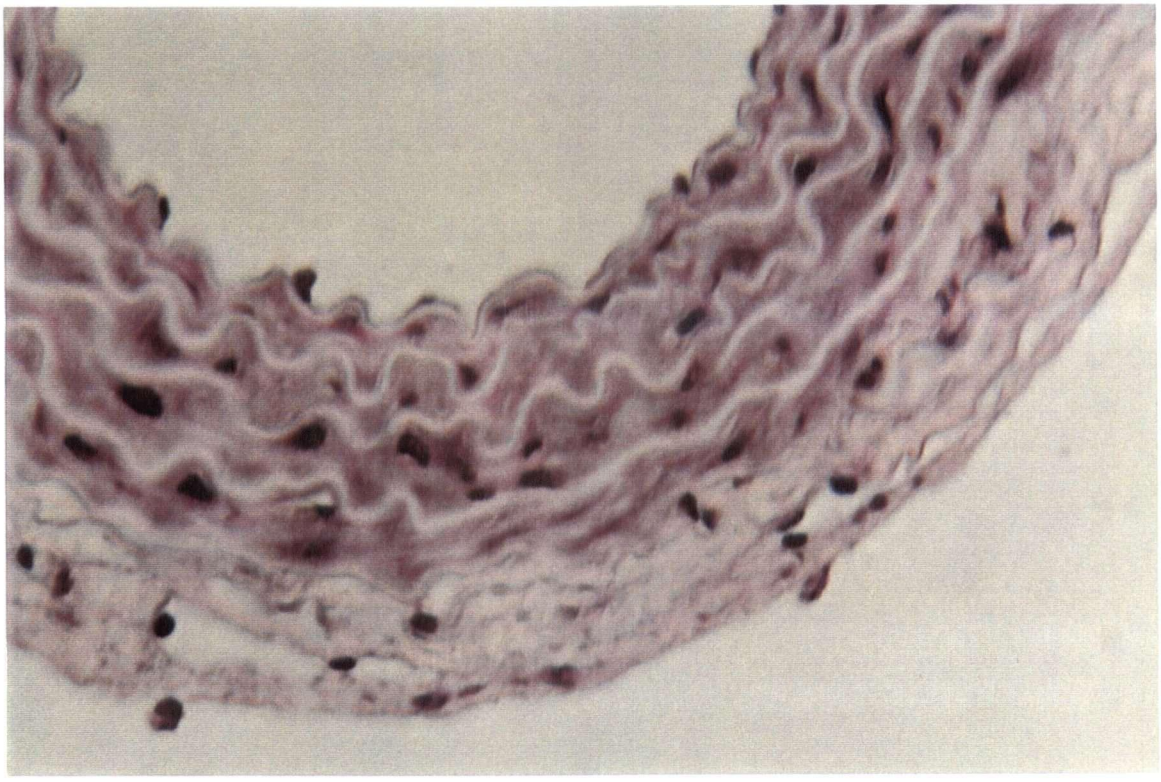
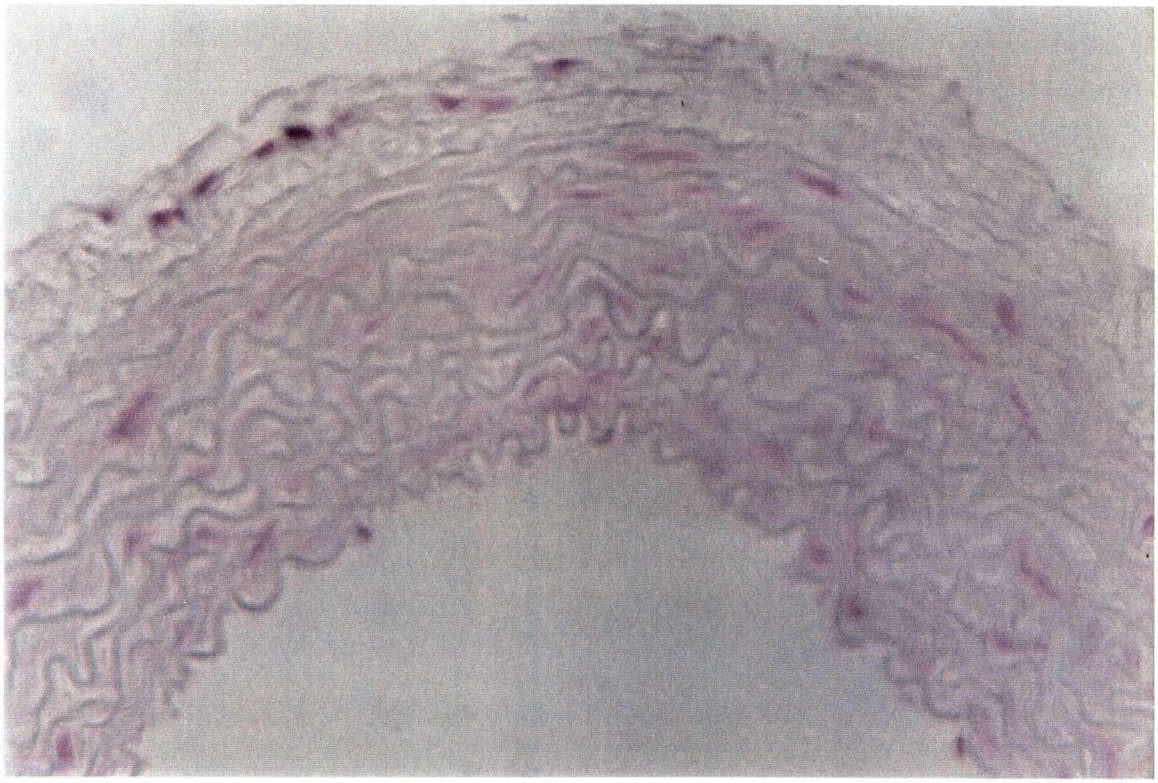


Figure 13

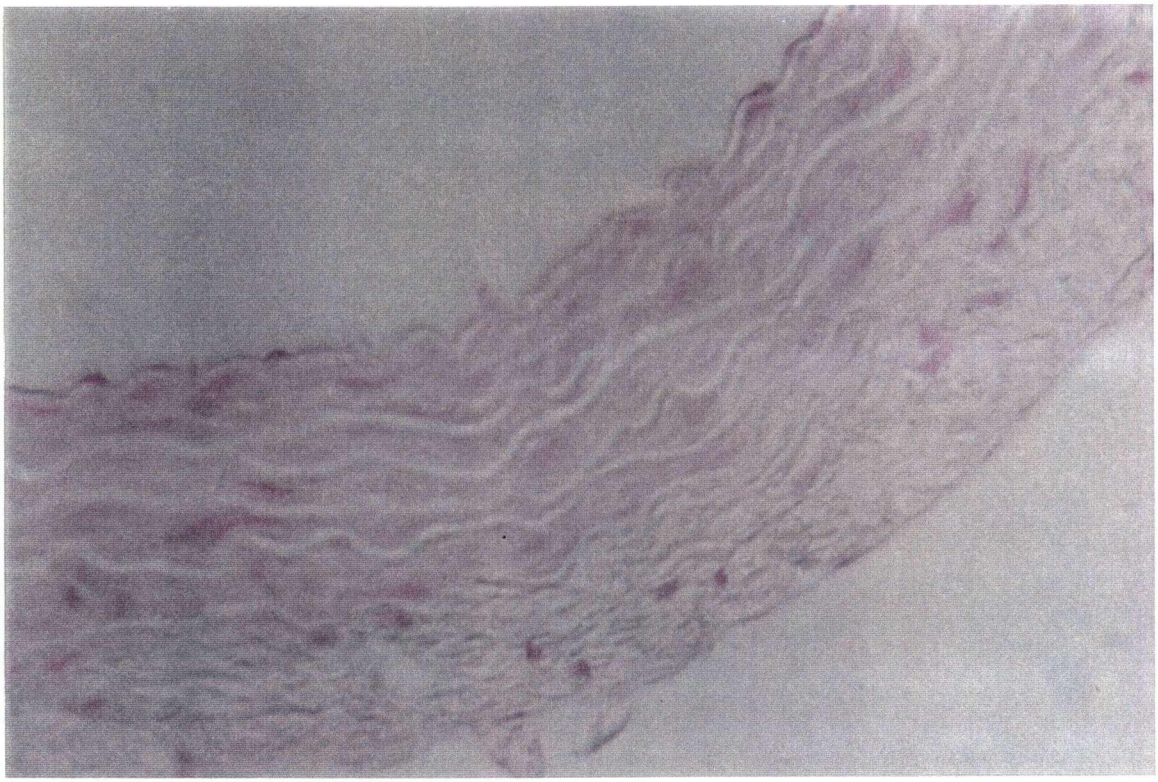
Immunostaining of endothelium-intact control (**A**) and 12-14 week STZ-diabetic (**B**) superior mesenteric arteries and rat spleen (**C**) with the monoclonal anti-macrophage (clone ED2) antibody.

Scale bar: 100μm

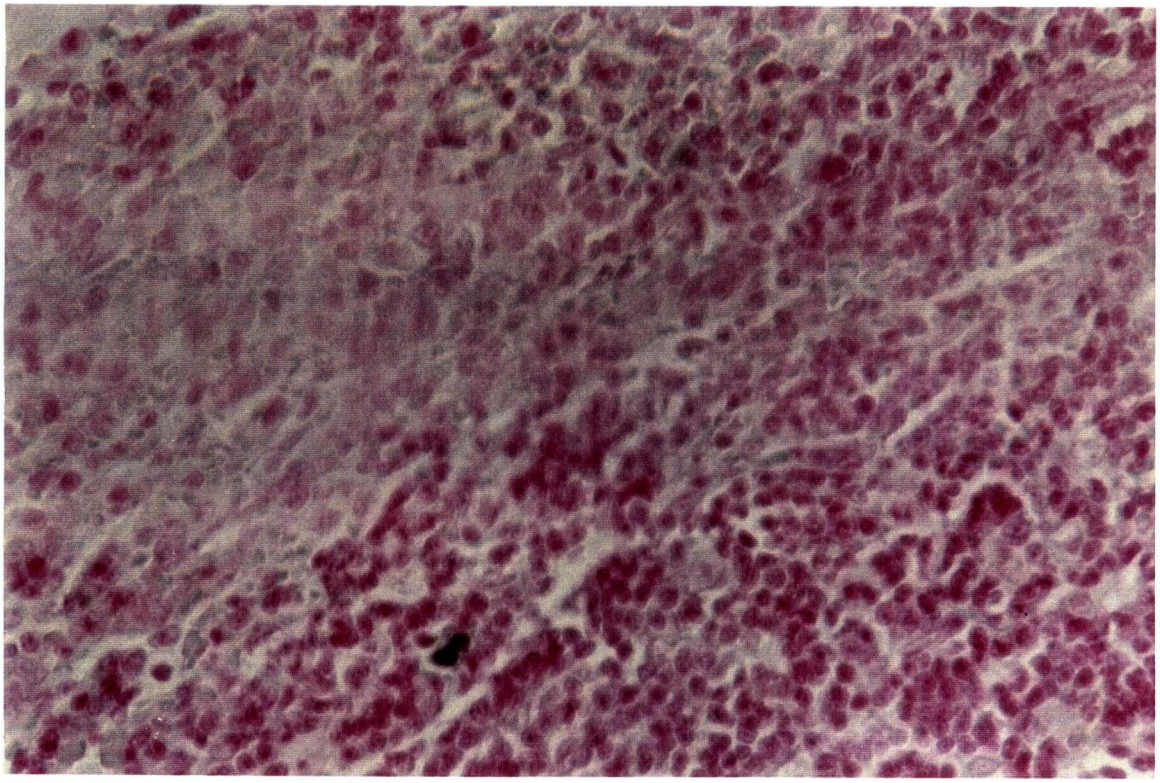
A



B



C



H. Quantitative measurement of cytosolic NOS activity at different time points following STZ-injection

To investigate NOS activity in control and STZ-diabetic superior mesenteric arteries at 2-3 weeks, 6-8 weeks, and 12-14 weeks following STZ-injection, the citrulline assay for quantitative analysis of NOS activity was performed. Cytosolic calcium-dependent (nNOS) and independent (iNOS) activity was determined.

No significant calcium-dependent (nNOS) activity was observed in either control or diabetic arteries at any time point following STZ-injection (Fig.14, Table 8). Similarly, no significant calcium-independent activity was detected in control mesenteric arteries at any time point (Fig.15, Table 8). Furthermore, no calcium-independent activity was observed in STZ-diabetic arteries at 2-3 weeks or 6-8 weeks following STZ-injection (Fig.15, Table 8). However, a significant and marked elevation in calcium-independent (iNOS) activity was detected in superior mesenteric arteries from 12-14 week STZ-diabetic rats (Fig.15, Table 8).

Table 8

Cytosolic NOS specific activity of control and STZ-diabetic superior mesenteric arteries 2-3 weeks, 6-8 weeks, and 12-14 weeks following STZ-injection

Animals	Time-point	Calcium-dependent activity (pmol/min/mg)	Calcium-independent (pmol/min/mg)
Control, N=6	2-3 weeks	0.39±0.37	0.92±0.39
Control, N=5	6-8 weeks	2.17±0.27	1.47±0.87
Control, N=7	12-14 weeks	0.24±0.39	1.43±0.39
Diabetic, N=6	2-3 weeks	1.70±0.94	0.66±0.40
Diabetic, N=6	6-8 weeks	0.24±0.23	2.31±0.63
Diabetic, N=7	12-14 weeks	0.25±0.46	18.06±4.11*

* $P<0.05$ as compared to background levels and corresponding control levels

All values are mean ± s.e.m.

Figure 14

Calcium-dependent NOS specific activity of the cytosolic fraction of control (solid bars) and STZ-diabetic (open bars) 2-3 weeks, 6-8 weeks, and 12-14 weeks following STZ-injection. Each bar represents the mean±s.e.m.

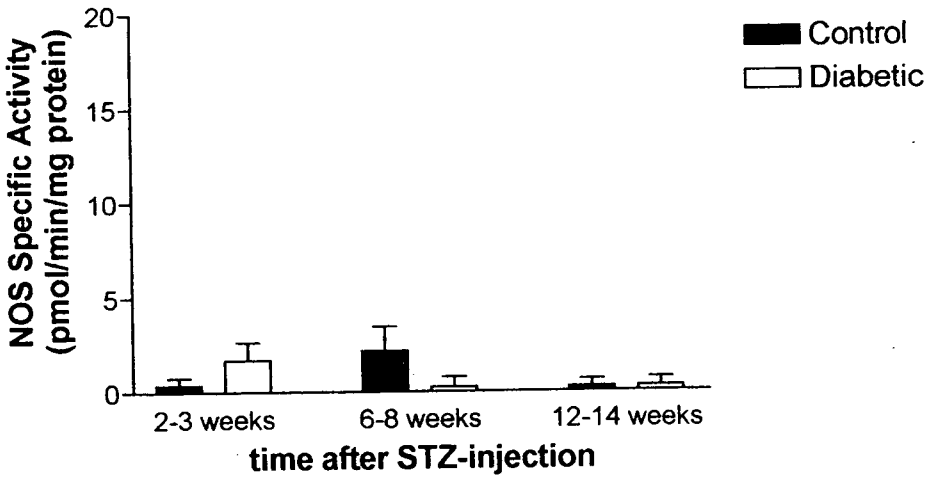
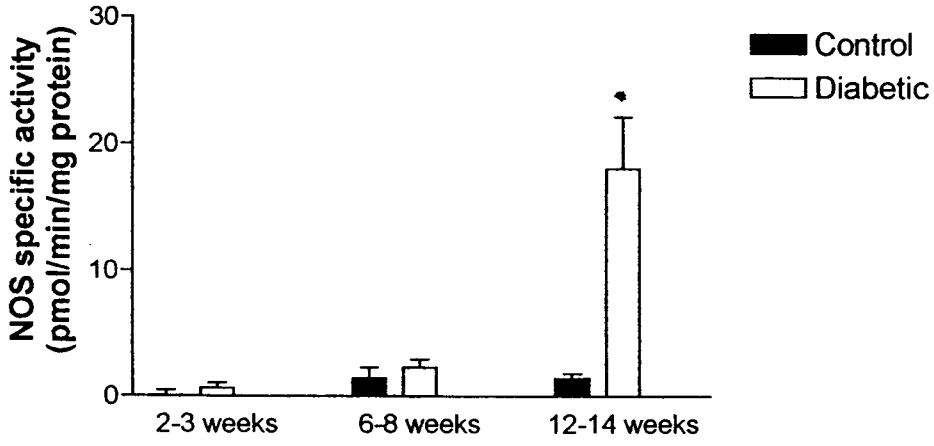

NOS Specific Activity
(pmol/min/mg protein)
20
15
10
5
0
2-3 weeks
6-8 weeks
12-14 weeks
time after STZ-injection
Control
Diabetic

Figure 15

Calcium-independent NOS specific activity of the cytosolic fraction of control (solid bars) and STZ-diabetic (open bars) 2-3 weeks, 6-8 weeks, and 12-14 weeks following STZ-injection. Each bar represents the mean±s.e.m.

* $P<0.05$ compared to control


NOS specific activity
(pmol/min/mg protein)
30
20
10
0
2-3 weeks
6-8 weeks
12-14 weeks
Control
Diabetic

Discussion

The results of the present investigation provide evidence for the novel finding that iNOS is functionally expressed in VSM of superior mesenteric arteries from 12-14 week STZ-diabetic rats, but not their age and gender matched controls. Furthermore, induction of iNOS does not occur in diabetic arteries until a time point after 6-8 weeks following STZ-injection. Induction of iNOS in STZ-diabetic arteries and the elevated NO levels that result may be implicated in the cardiovascular dysfunction associated with chronic diabetes mellitus.

In the present study, endothelium-intact mesenteric arteries from 12-14 week STZ-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 NA compared to responses of age and gender-matched control rats. No difference in maximum responses was observed between control and 2-3 week STZ-diabetic arteries. These data are consistent with previous reports from our laboratory, which have found increased maximum responses of 3 month diabetic arteries to NA but not KCl, with little or no change in NA pD_2 value (reviewed in Subramanian and MacLeod, 1999). Neither endothelial-denudation nor preincubation with NOS inhibitors produced any significant effect on maximum responses in control or diabetic arteries suggesting that the enhanced maximum responsiveness to NA in long-term diabetic arteries is independent of the endothelial-cell layer and of NO.

Endothelial denudation or non-selective inhibition of NOS with LNMMA or L-NIO resulted in a leftward shift of the concentration-response curves to NA in both control and diabetic arteries, suggesting that NO release in these arteries normally

limits NA sensitivity. However the magnitude of the shift produced by L-NIO in endothelium-intact arteries was significantly greater in 12-14 week STZ-diabetic arteries. The LNMMA or L-NIO-induced increase in sensitivity to NA in control arteries is likely due primarily to inhibition of eNOS from the endothelial cell layer, since NOS inhibition produced no further effect on NA sensitivity in these vessels following endothelial denudation. In contrast, the presence of a leftward shift in the NA concentration-response curve with LNMMA or L-NIO in endothelium-denuded 12-14 week STZ-diabetic arteries suggests the presence of NOS activity in diabetic VSM.

Following preincubation with LNMMA, the NA sensitivity of endothelium-denuded 12-14 week STZ-diabetic arteries was significantly greater than that of endothelium-intact (treated) diabetic arteries. However, there was no difference in NA pD_2 values between treated endothelium-intact and treated endothelium-denuded 12-14 week STZ-diabetic arteries with L-NIO. This maybe due to differences between the two inhibitors (Figure 16). Both LNMMA and L-NIO act to inhibit NOS activity by competing with L-arginine for the active site on the NOS enzyme (Kerwin et al, 1995). However, L-NIO may diffuse through the endothelial cell layer to the VSM more efficiently than LNMMA and may also compete with L-arginine for uptake into the cell at its transporter, the cationic y+ transporter (Kerwin et al, 1995).

It is unlikely that the observed increase in NA sensitivity produced by L-NIO is caused by a non-specific effect of the compound, since the stereospecificity of the interaction of L-NIO with NOS and/or competition with L-arginine for intracellular transport was confirmed by reversal of the leftward shift of the concentration-response curve with L-arginine but not D-arginine.

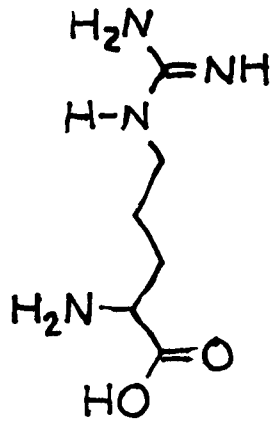
Figure 16

Chemical structures of L-Arginine (**A**), LNMMA (**B**)

and L-NIO (**C**)

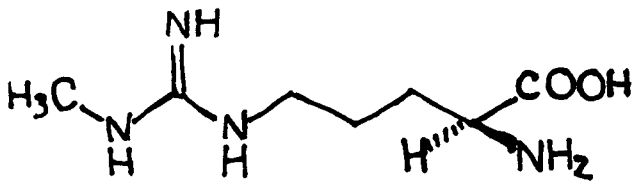
A

L-arginine



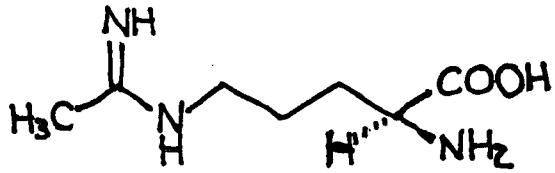
B

LNMMA



C

L-NIO



Results from the present study do not provide evidence for a significant difference in eNOS activity or expression between control and diabetic arteries at any time point following STZ-injection. Endothelial-denudation produced increases in pD2 values of similar magnitude in control and diabetic arteries. Furthermore, immunohistochemical analysis revealed a positive signal for eNOS in the endothelial cell layer in both control and diabetic arteries. These observations are consistent with previous findings from our laboratory, that there is no significant difference in the maximum ACh-induced relaxation or in the ACh-induced increase in cGMP levels between control and STZ-diabetic arteries (Harris and MacLeod, 1988). However, numerous studies from other laboratories have reported impaired endothelium-dependent relaxation in isolated arterial rings from STZ-diabetic rats. Explanations for reported impairments of endothelium-dependent vasodilation include enhanced cGMP metabolism, reduced activity of cGMP-dependent protein kinase, decreased availability of L-arginine, and increased inactivation of NO by free radicals (Kamata et al, 1989; Hattori et al, 1991; Taylor et al, 1992; Rodriguez-Manas et al, 1998). Further controversy in this area comes from reports from other laboratories that endothelial-derived NO is enhanced in diabetic arteries (Bhardwaj and Moore, 1988; Langenstroer and Pieper, 1992). The reasons for these discrepancies remain unclear at present.

Although endothelial-derived NO (from eNOS) may be of primary importance under normal conditions, both nNOS and iNOS may be expressed in VSM under certain conditions. The expression of nNOS in VSM has been reported in uterine arteries during pregnancy (Garvey et al, 1994) and in carotid arteries from

spontaneously hypertensive rats (Boulanger et al, 1998). However, in the present study, incubation of endothelium-denuded control and 12-14 week STZ-diabetic arteries with 100μM 7-NINA had no effect on NA 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, although a positive signal was present in the positive control (mouse brain). Furthermore, quantitative measurement of NOS activity of the cytosolic fraction (containing nNOS and iNOS) revealed no significant calcium-dependent activity in 2-3 week, 6-8 week, or 12-14 week control or STZ-diabetic superior mesenteric arteries. 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 including exposure to inflammatory cytokines and endothelial injury (Schulz and Triggle, 1994; Hanson et al, 1994; Gonzalez-Fernandez et al, 1998). The results of the present study provide substantial evidence that iNOS is the subtype that is expressed in VSM from STZ-diabetic rats.

Aminoguanidine (1mM) has been used as a selective inhibitor of iNOS in arterial preparations (Tilton et al, 1993; Joly et al, 1994). In the present study, preincubation of endothelium-denuded 12-14 week STZ-diabetic arteries with 1mM aminoguanidine produced an increase in sensitivity to NA similar to that seen with L-NIO or LNMMA, suggesting the presence of NOS activity in VSM of 12-14 week STZ-diabetic arteries. However, the results of the present study also indicate that at the concentration used (1mM), aminoguanidine is not selective for iNOS. Preincubation

of endothelium-intact control arteries with aminoguanidine (1mM) produced a significant increase in NA sensitivity, suggesting that aminoguanidine also inhibits eNOS at this concentration.

EIT has been reported to be 40-50 fold more selective for iNOS than for nNOS or eNOS (Nakane et al., 1995). In arterial ring preparations, EIT has been reported to inhibit iNOS at concentrations ranging from 2μM-30μM (Southan et al, 1995). EIT (5μM) produced no effect on NA responses in our experimental conditions. However, EIT (10μM) mimicked the increase in NA sensitivity seen with L-NIO in endothelium-denuded 12-14 week STZ-diabetic mesenteric arteries. In the present investigation, EIT (10μM) had no effect on NA responses in endothelium-intact control arteries, providing evidence that EIT is selective for iNOS over eNOS at this concentration in mesenteric arteries. L-arginine but not D-arginine reversed the leftward shift in the concentration-response curve to NA, confirming that EIT acts as a stereoselective competitive inhibitor of NOS. EIT had no effect on NA responses in endothelium-denuded control arteries from rats of any age, suggesting that iNOS activity is not present in VSM from control rats. Similarly, EIT had no effect on responses to NA of endothelium-denuded 2-3 week or 6-8 week STZ-diabetic mesenteric arteries, suggesting that iNOS is not induced in STZ-diabetic arteries until after 6-8 weeks following STZ-injection.

Immunohistochemical analysis demonstrated a strong positive signal for iNOS expression in mesenteric arteries from 12-14 week STZ-diabetic but not control rats. Immunostaining for iNOS protein was observed in the intimal, medial, and adventitial layers of diabetic arteries.

Further evidence for the functional expression of iNOS in 12-14 week STZ-diabetic arteries was obtained from quantitative measurement of cytosolic NOS activity. Levels of calcium-independent (iNOS) activity in 12-14 week STZ-diabetic arteries were significantly increased above both background and levels in control arteries. No significant calcium-independent NOS activity was observed in control arteries at any time point. Similarly, no significant calcium-independent activity was detected in the cytosolic fraction of 2-3 week or 6-8 week STZ-diabetic arteries, further suggesting that iNOS is not functionally expressed in diabetic VSM until after 6-8 weeks following STZ-injection.

The possibility that the presence of iNOS in the 12-14 week STZ-diabetic arteries was due to its induction *in vitro* seems unlikely. All experiments were conducted in the presence of dexamethasone, at a concentration (0.1μM) that has been reported to inhibit iNOS induction *in vitro* (Knowles et al., 1990b). 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. Finally, it is unlikely that iNOS was induced in 12-14 week STZ-diabetic arteries as a result of experimental procedures, since no evidence for the presence of iNOS in control arteries or 2-3 week or 6-8 week STZ-diabetic arteries, treated in the exact same manner, was obtained in the present study.

Immunohistochemical results from the present study suggest that iNOS is present throughout all layers of superior mesenteric arteries from 12-14 week STZ-diabetic rats. iNOS appears to be induced in the tunica intima in endothelial cells. It is

assumed that iNOS is expressed in VSM cells in the tunica media. However, the specific cell types that express iNOS in the tunica media and tunica adventitia cannot be stated with certainty from results of the present study since no double antibody immunohistochemical analysis for the co-detection of iNOS with specific cell types was performed. Macrophage infiltration has been recently identified as a source of iNOS expression in smooth muscle preparations under certain conditions (Zheng et al., 1997). However, it is not likely that the iNOS detected in VSM of 12-14 week STZ-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. Therefore, iNOS induction in 12-14 week STZ-diabetic arteries is likely due to pathophysiological changes that occur with the chronic diabetic state.

The factors contributing to iNOS induction in diabetic arteries remain unclear. However, AGE-mediated alterations in cytokine production, increased oxidative stress and/or enhanced PKC activation could all be implicated in the process of iNOS induction in long-term diabetes (DeVera et al., 1996, Paul et al., 1997; Hecker et al, 1999).

As described in the Introduction, the formation of AGEs with chronic hyperglycemia may result in AGEs binding to their receptor, RAGE, resulting in the generation of ROS and alterations in gene expression (Brownlee, 2000). AGEs have been shown to upregulate various cytokines that are involved in iNOS gene transcription, including TGF-β, IL-1, and NF-κB (Vlassara et al, 1988). Preliminary data from our laboratory indicate that levels of glycosylated hemoglobin, a measure of AGE formation, increase in STZ-diabetic rats with time. Increased AGE formation and

the enhanced cytokine production that may result over time may help to explain why iNOS is not expressed in STZ-diabetic rats until after 8 weeks following induction of diabetes.

Increased oxidative stress has also been associated with chronic diabetes and may result in the activation of factors involved in iNOS induction, including NF-κB (Guigliano et al, 1996; Li and Karen, 1999). In addition, long-term diabetes may be associated with enhanced activation of specific PKC isoforms, which may also potentiate cytokine-induced iNOS expression in VSM. The individual PKC isoforms that are elevated in different diabetic tissues remains to be elucidated.

The mechanism by which iNOS is induced in diabetic VSM also remains unclear. As described in the Introduction, the promoter region of the iNOS gene in mouse, rat, and human contains several putative binding elements, including response elements to transcription factors that may be elevated in long-term diabetes, including NF-κB and IL-1β (DeVera et al, 1996). Induction of iNOS by individual cytokines varies depending on the tissue, which may be due to variable expression of cytokine receptors or cell-specific differences in iNOS gene transcription factors (Kolyada et al, 1996). Although the underlying mechanism remains unclear, activation of PKC has been reported to potentiate iNOS induction in VSM, depending on the isoform of PKC and the individual blood vessel (Hecker et al, 1999; Paul et al, 1997). The individual isoforms of PKC capable of potentiation (or attenuation) of iNOS induction in different tissues and cell types also remains to be determined.

Once induced, iNOS synthesizes a prolonged and increased release of NO as compared to 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 Bredt, 1992). Since there has been considerable recent evidence that there is an increase in the generation of oxygen-derived free radicals in diabetes, increased NO production in diabetic arteries has potential for considerable damage.

In other conditions in which the expression of iNOS in all three arterial layers has been demonstrated, the overproduction of NO and subsequent peroxynitrite formation have been reported to contribute to vascular damage and to atherosclerosis (Luoma and Yla-Herttuala, 1999; Gutterman, 1999).

Peroxynitrite itself has been reported to have vasoconstrictor properties (Elliot et al, 1998). Furthermore, oxidation of LDL and other plasma lipids by peroxynitrite results in the production of various potentially detrimental substances, including lipid peroxides, modified aldehydes, and lipids with vasoconstrictor properties (Darley-Usmar and White, 1997). Peroxynitrite has been shown to react with plasma lipids and lipoproteins (including LDL) to produce vasoconstrictor isoprostanes (Moore et al, 1995). The mechanism of isoprostane action remains unclear although it has been suggested that isoprostanes may be involved in the alteration of cyclo-oxygenase-dependent signal transduction pathways (Moore et al, 1995). Increased plasma levels and urinary excretion of isoprostanes have been reported in type I and type II diabetic patients (Gopaul et al, 1995; Davi et al, 1999). The vasoconstrictor properties of peroxynitrite and isoprostane *in vivo* remain to be determined. However, one potential implication of iNOS induction in human diabetic arteries may be an increase in blood pressure and/or peripheral resistance in response to the vasoconstrictor actions of peroxynitrite and/or isoprostanes.

In humans, iNOS has been detected in VSM cells and megakaryocytes from

atherosclerotic lesions (Butterly et al, 1996). Oxidation of LDL by peroxynitrite is thought to be involved in the initiation of macrophage infiltration and inflammatory response in the process of atherosclerotic lesion formation in humans (Graham et al, 1993). It is not fully understood why experimental STZ-diabetic rats do not appear to develop atherosclerosis. However, rats have higher levels of high density lipoproteins (HDL) than LDL (Bennani-Kabchi et al, 2000). Therefore, unlike human arteries, levels of oxidized LDL in rat arteries may not be sufficient to stimulate atherogenesis. In humans, peroxynitrite-mediated nitration of tyrosine residues results in a stable, highly immunogenic adduct, which also has been implicated in an inflammatory response and the initiation of atherogenesis (Darley-Usmar and White, 1997). Therefore, if iNOS is expressed in VSM in humans with diabetes, there is significant potential for an increase in the formation of atherosclerotic lesions.

Abnormal production of NO may also contribute to endothelial dysfunction due to an imbalance in the release of other endothelium-derived factors, including endothelin-1 (ET-1) (Kiff et al, 1991). ET-1 is a potent vasoconstrictor whose production has been reported to be both increased (Takeda et al, 1991) and decreased (Wu and Tang, 1998) in experimental diabetes. 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., 1999). Results from the present study would predict a decrease in ET-1 release from diabetic mesenteric arteries, since induction of iNOS would result in overproduction of NO.

The consequences of iNOS induction in diabetic VSM need not be entirely 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 of this study, that the sensitivity of diabetic arteries to NA is not significantly different from control in the absence of L-NIO, but is enhanced in it's presence. The induction of iNOS in VSM of STZ-diabetic arteries may also help to explain why these animals have generally not been reported to be hypertensive. Induction of iNOS could help to compensate for the decreased release of or responsiveness to endothelial-derived NO that has been commonly reported in diabetic arteries. Although the overproduction of NO by iNOS may contribute to atherogenesis as a result of peroxynitrite production, NO itself acts to prevent atherosclerosis by inhibition of platelet aggregation and VSM proliferation (Moncada and Higgs, 1995; Yates et al, 1992). Whether induction of iNOS in diabetic VSM results in detrimental or protective effects remains unclear but may be dependent on levels of oxygen derived free radicals in the local environment of NO production.

Summary and Conclusions

A. Summary

1. STZ-diabetic rats exhibited characteristics associated with the diabetic state including hyperglycemia, hypoinsulinemia, decreased body weight gain, polyuria, and osmotic diarrhea.

2. Maximum responsiveness to NA but not sensitivity was significantly greater in 12-14 week STZ-diabetic superior mesenteric arteries as compared to control regardless of the status of the endothelium or the absence or presence of NOS inhibitors. No difference in NA responses were observed between 2-3 week or 6-8 week STZ-diabetic arteries and their age and gender-matched controls.

3. Endothelial-denudation or preincubation of endothelium-intact control arteries with L-NIO or LNMMA produced an increase in NA sensitivity, suggesting that NO derived from the endothelial cell layer normally limits NA sensitivity. Following endothelial-denudation, no further change in NA sensitivity was observed in control superior mesenteric arteries following preincubation with LNIO, LNMMA, aminoguanidine, EIT, or 7-NINA, suggesting that NOS activity is not present in VSM from control rats.

4. Endothelial-denudation or incubation of 12-14 week STZ-diabetic arteries with L-NIO or LNMMA produced an increase in NA sensitivity. However, in superior mesenteric arteries from 12-14 week STZ-diabetic rats, LNMMA or L-NIO

produced a further increase in NA sensitivity following endothelial denudation, suggesting the presence of NOS activity in VSM in diabetic arteries.

5. L-NIO produced a greater increase in NA sensitivity than LNMMA in endothelium-intact 12-14 week STZ-diabetic arteries. There was no observed difference between the two inhibitors in NA responses in endothelium-denuded arteries, suggesting that L-NIO may be more efficiently transported through the endothelial cell layer.

6. 7-NINA had no significant effect on NA responses in endothelium-denuded 12-14 week STZ-diabetic arteries, suggesting that nNOS is not the subtype of NOS present in diabetic VSM. Furthermore, no positive signal for nNOS protein was obtained in either control or 12-14 week STZ-diabetic arteries, suggesting the lack of nNOS expression in these arteries.

7. EIT (10μM) produced a significant increase in NA sensitivity in endothelium-denuded 12-14 week but not 2-3 week or 6-8 week STZ-diabetic mesenteric arteries, suggesting that iNOS is functionally expressed in diabetic VSM, but not until after 8 weeks following STZ injection.

8. Immunohistochemical analysis indicated eNOS expression in the endothelial cell monolayer of both control and 12-14 week STZ-diabetic superior mesenteric arteries.

9. Immunohistochemical staining for iNOS protein indicated extensive iNOS expression in 12-14 week STZ-diabetic arteries but not control. The presence of iNOS protein in diabetic VSM was not due to macrophage infiltration, since immunostaining for macrophages produced no positive signal.

10. Quantitative measurement of cytosolic NOS activity revealed no significant calcium-dependent NOS (nNOS) activity in control, 2-3 week, 6-8 week, or 12-14 week STZ-diabetic rats.

11. No calcium-independent NOS (iNOS) activity was obtained in control, 2-3 week, or 6-8 week STZ-diabetic arteries. However, there was a marked elevation in calcium-independent NOS activity in 12-14 week STZ-diabetic rats, providing further evidence for the functional expression of iNOS in 12-14 week STZ-diabetic arteries.

B. Conclusion and Future experiments

Conclusion

The results of the present study demonstrate the novel finding that iNOS is functionally expressed in VSM from rats with chronic STZ-induced diabetes, at a time when vasoconstrictor responsiveness is also enhanced. Induction of iNOS in diabetic VSM may play a role in the cardiovascular complications associated with diabetes mellitus.

Future research

Further investigation will be necessary to determine whether iNOS is expressed in chronic STZ-diabetic animals in other tissue types and/or VSM from different arteries. Immunohistochemical detection for the presence of iNOS in other arteries and arterial beds of chronic STZ-diabetic rats should be performed. In addition, investigation of iNOS activity in arteries from other species that are susceptible to atherosclerosis, such as rabbits or guinea pigs with STZ-induced diabetes, may also be of interest to investigate the effects of iNOS induction on atherogenesis.

The relationship between the duration of hyperglycemia and the induction of iNOS, as well as the factors associated with long-term STZ-diabetes responsible for iNOS induction in VSM are also areas for further research. The PKC isoforms that are activated with chronic diabetes in different arteries, and that are responsible for enhancement of iNOS induction in individual arteries remains to be determined. The time course of AGE formation and/or elevations in cytokine production with chronic diabetes also remains to be investigated.

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