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Original Research

# Direct assessment and diminished production of morphine stimulated NO by diabetic endothelium from saphenous vein

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**KEY WORDS** nitric oxide; diabetes mellitus; myocardial revascularizations; opioid receptors; saphenous vein

## ABSTRACT

**AIM**: to directly measure in real time basal and stimulated levels of NO released from human saphenous vein endothelium and to quantify the expression of the $\mu$ opiate receptor, which has been linked with NO release. **METHODS**: Saphenous vein segments from patients with type 2 diabetes ($n$ = 12) and patients without diabetes ($n$ = 8) were obtained. The release of NO was measured directly from the endothelium using a NO-specific amperometric probe. $N^{\Omega}$-nitro-*L*-arginine methyl ester (*L*-NAME, 0.1 mmol/L), a NO synthase (NOS) inhibitor, or morphine (1 $\mu$mol/L), a stimulant, was administered and the measurements were repeated. Values were reported relative to the mean initial measurement of NO release from diabetic endothelium, which was defined as the relative zero level of NO release. A RT-PCR was then performed on the endothelium to measure $\mu$ opiate receptor expression. **RESULTS**: Diabetic patients ($n$ = 12) showed a relative and significantly diminished basal level of released NO, (0.049 ± 0.012) nmol/L, compared with non-diabetic patients ($n$ = 8), (0.42 ± 0.12) nmol/L ($P$ < 0.05). Application of *L*-NAME to non-stimulated tissues resulted in no change in NO release from the diabetic group and a decrease in NO release of (0.21 ± 0.09) nmol/L from the non-diabetic group ($P$ < 0.05). Morphine stimulation of the diabetic endothelium resulted in a lower peak and shorter duration of NO release compared to the non-diabetic tissue, (21 ± 6) nmol/L *vs* (38 ± 4) nmol/L and (7.3 ± 1.4) min *vs* (12.2 ± 2.2) min, respectively ($P$ < 0.01). Lastly, evaluation of the $\mu$ opiate receptor expression was found to be diminished in the diabetics by 59.1 %. **CONCLUSION**: Maturity-onset diabetes attenuates both the constitutive basal and morphine stimulated NO release from human saphenous vein endothelium. In this study, after NOS inhibition, the actual basal NO release in diabetes was negligible. One explanation for the impaired capacity of diabetic endothelium to release NO was the diminished $\mu$ opiate receptor that was seen in diabetic endothelium.

## INTRODUCTION

Maturity-onset diabetes mellitus (type 2) is the most common form of diabetes, affecting approximately 10 % to 20 % of the United States population older than 45 years of age, or more than 100 million people worldwide. The early development of cardiovascular complications, including accelerated atherosclerosis and microangiopathy, which occurs with this disease, is responsible for a high morbidity and mortality[1].

Recently, the importance of a functionally intact endothelium for preventing vascular dysfunction has been realized and with that the importance of endothelium-derived nitric oxide (NO). NO inhibits the adhesion and aggregation of platelets and the release of their contained growth factors, the chemotaxis and activation of mononuclear leukocytes, the expression of leukocyte adhesion molecules by activated vascular endothelium, and the migration and proliferation of smooth muscle cells. It decreases endothelial permeability for

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macromolecules and lipoproteins[2]. In addition, NO is essential for the maintenance of basal vascular tone and its regulation in response to various physiologic and pathophysiological stimuli. Therefore it has been surmised that impaired activity of NO diminishes the resistance of the vascular wall to disease and disrupts vascular homeostasis.

Previous reports had examined NO activity in diabetic endothelium, but contradicting results have emerged[3,4]. All of these reports have used indirect methods in an attempt to determine NO, and no conclusive direct evidence currently exists on the levels of NO release in diabetic endothelium. To our knowledge, the present report described the first attempt to directly measure in real time the basal and stimulated release of NO from diabetic endothelium. Additionally, it is the first reported investigation of endothelial $\mu$ opiate receptor expression, which has been linked with NO release, in diabetes mellitus.

## MATERIALS AND METHODS

**Subjects** The study group included 12 patients with long standing maturity-onset diabetes mellitus (type 2) and 8 non-diabetic patients as controls, all of who underwent coronary artery bypass grafting at University Hospital and Medical Center. The two groups were closely matched with respect to age and gender [(68 ± 5) a and 4:8 (female : male) *vs* (67 ± 5) a and 3:5 (female : male), respectively]. The average duration of diabetes was 9.5 a (range 5 to 21 a). All diabetic patients required daily insulin injections for adequate glycemic control at the time of the study, despite previous and initial management with only diet and/or oral hypoglycemic medications. Their insulin requirements varied, but exceeded 10 U regular Insulin and 10 U of NPH Insulin twice a day in every case.

From a coronary disease standpoint the patients were also closely matched. The left ventricular ejection fraction was (38 ± 5) % in the diabetic group *vs* (39 ± 7) % in the control group. Diabetics required 3.8 ± 2.1 grafts *vs* 3.4 ± 1.8 for controls.

The study was approved by the Committee on Research Involving Human Subjects of the State University of New York at Stony Brook.

**Tissue preparation and NO measurement** At the time of operation, remaining harvested saphenous vein segments from each patient, normally regarded as pathogenic waste, were placed in a balanced electrolyte solution (plasmalyte and 5000 U heparin per 500 mL), and transported on ice (4 ℃) to the laboratory. The vessels were cut transversely into 5.0 mm rings, then cut open longitudinally, and placed in 2.0 mL test tubes. A NO-selective amperometric microprobe with a detection limit of 1 pmol/L (World Precision Instruments, Sarasota, FL) was used to measure the levels of NO released from the endothelium. The redox current was detected by a current-voltage converter circuit and continuously recorded by the DUO 18 software (World Precision Instruments). The tip diameter of the probe (200 $\mu$m) permitted the use of a micromanipulator (Zeiss-Eppendorff, Erlangen, FRG) attached to the stage of an inverted microscope to position the sensor 10 $\mu$m above the cell surface (Nikon Diaphot, Melville, NY). The system was calibrated daily through the liberation of a known quantity of NO, by adding potassium nitrite to a solution of potassium iodide (World Precision Instruments). Baseline levels of NO release were determined in PBS. Baseline levels of NO were determined in the presence of superoxide dismutase (SOD; 200 kU/L, Sigma, Inc, St Louis, MO) and 0.1 mol/L $N^{\Omega}$-nitro-*L*-arginine methyl ester (*L*-NAME), a NOS inhibitor. The probe was allowed to equilibrate for 24 h in the incubation medium, free of tissue, before being transferred to the vials containing the tissue. Manipulation/handling of the tissue was performed only with glass instruments.

Levels of endothelial NO release were measured at constitutive basal conditions, after the application of SOD and *L*-NAME, and the administration of 1 $\mu$mol/L of morphine sulfate. Each experiment was repeated three times on new tissue segments. Results were expressed relative to the basal constitutive level of NO release of diabetic endothelium at time zero. The NO mean values were graphed to represent the actual release phenomenon. All NO measurements were performed in a blinded fashion without knowledge of the patient data. At the end of the 3-year study period, the data were examined after code identification.

**Isolation of total RNA** Tissue endothelium, gently scraped of tissue samples, was homogenized in Tri-Reagent (Molecular Research Center, INC, Cincinnati, OH) using a polytron homogenizer. The homogenates were stored at room temperature for 5 min to allow complete dissociation of nucleoprotein. 1-Bromo-3-chloropropane (BCP) 0.1 mL per 1 mL of Tri-Reagent was added to the homogenates. The samples were vortexed vigorously for 15 s and then stored at room

temperature for 7 min. After centrifugation of the samples for 15 min at 12 000 × $g$, the aqueous phase was transferred to a fresh tube. RNA was ethanol precipitated, washed with 75 % ethanol, air-dried and resuspended in water. RNA was analyzed on a 1 % agarose gel and purity was determined spectrophotometrically.

**Reverse transcription-polymerase chain reaction (RT-PCR) of total RNA** First strand cDNS synthesis was performed using random hexamers (GIBCO, BRL, Gaithesburg, MD). Three μg of total RNA isolated from vascular tissues was denatured at 95 ℃ and reverse transcribed at 42 ℃ for 1 h using Superscript Ⅱ Rnase H-RT (GIBCO BRL, MD). Seven μL of the RT product were added to the PCR mixture containing specific primers for the μ opioid receptor gene and Taq DNA polymerase (GIBCO, BRL, MD). In order to demonstrate that the PCR was in the linear phase of amplification, various cycles were performed (15, 20, 25, 30, 35, and 40 cycles, respectively). PCR reactions were denatured at 95 ℃ for 5 min followed by 30 cycles at 95 ℃ for 1 min, 57 ℃ for 1 min, and 72 ℃ for 1 min, and then an extension step cycle at 72 ℃ for 10 min. PCR products were analyzed on a 2 % agarose gel (Sigma) and visualized by ethidium bromide staining. The only μ opioid receptor-specific primers used in the PCR reactions that yielded a specific PCR product amplified a 441 bp fragment starting at map position 896 (Primer M1 – 5′GGTACTGGGAAAACCTGCTGAAGATCTGTG3′), and at map position 1336 (primer M4 – 5′GGTCTCTAGTGTTCTGACGAATTCGAGTGG3′). This segmenu of the gene encodes the third extracellular loop of the receptor that is important for μ opioid receptor agonist selectivity. The bands corresponding to the expected size fragment were excised, purified with the StrataPrep purification kit (Stratagene), ligated into the PCR-Script Amp SK (+) vector (Stratagene) and transformed into XL10-Gold kan competent cells (Stratagene). Primers specific for the internal control gene (G3PDH) were used to amplify a 451 bp fragment. Five μL of the RT products was used in the PCR reaction followed by 25 cycles at 95 ℃ for 1 min, 60 ℃ for 1 min, and 72 ℃ for 1 min, and then an extension step cycle at 72 ℃ for 10 min.

Band density readings were determined by Gel Pro Density Analysis (Media Cybemetrics, Inc, MD). Pharmacological agents were purchased from Sigma (St Louis, MO).

**Sequencing of the cloned PCR Products** Purified plasmid DNA was sequenced with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems-Perkin-Elmer) and the ABI Prisms 310 Genetic Analyzer and ABI 377 DNA sequencer.

**Statistical analysis** Data were expressed as $\bar{x} \pm s$. Differences in the measured levels of NO between the two study groups, diabetics and non-diabetics, were evaluated by a two-tailed Student's $t$ test. Differences in NO levels within each group after experimental manipulation were evaluated with a paired two-tailed Student's $t$ test. For time-sensitive experiments, an analysis of variance for repeated measures and Tukey test were used for measurements between the groups. $P$-value of less than 0.05 was considered significant. Data acquisition was by the computer-interfaced DUO-18 software (World Precision Instruments). The experimental values were then transferred to Sigma Plot and Sigma Stat (Jandel, Corte Madera, CA) for graphic representation and evaluation.

## RESULTS

**Basal levels of NO release** Baseline basal measurements of the endothelium were made in each group. The diabetic patients ($n$ = 12) were found to have a significant difference in relative basal levels of NO release, (0.049 ± 0.012) nmol/L, compared to the non-diabetics ($n$ = 8), (0.42 ± 0.12) nmol/L [($P < 0.05$) analysis of variance for repeated measures] (Fig 1). Subsequently, to determine the actual basal level of NO release, the endothelial cells were treated with $L$-NAME (0.1 mmol/L), a NOS inhibitor. The diabetic tissue was found to have no significant difference in its level of release [($P \geqslant 0.05$) analysis of variance of repeated measures before *vs* after $L$-NAME] and the non-diabetic tissue was found to have a significant decrease of (0.21 ± 0.09) nmol/L [($P < 0.05$) analysis of variance of repeated measures before *vs* after $L$-NAME] (Fig 1). This signified that the actual basal level of release in diabetic endothelium was zero. Superoxide dismutase (SOD) (200 kU/L) was then applied to the incubation medium to eliminate any extracelluar NO scavengers, which could alter the measured level from the real level of endothelial NO release. No significant change was seen in the diabetic group ($P > 0.05$) analysis of variance of repeated measures before *vs* after SOD (Fig 2), whereas

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in control vein pieces ($n = 8$) the NO level increased, indicating NO was produced and a degree of NO scavenging was taking place ($P < 0.05$) analysis of variance of repeated measures before *vs* after SOD (Fig 2).

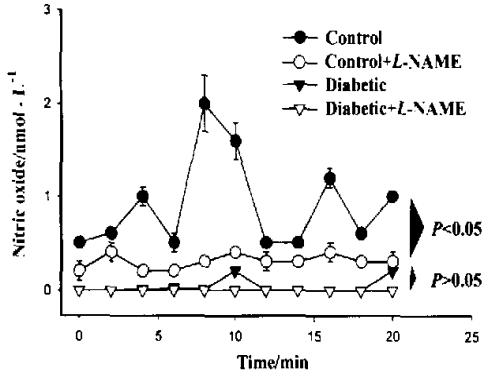


**Fig 1. Basal amperometric nitric oxide measurement of human saphenous vein segments from diabetic patients ($n = 12$) or non-diabetic endothelium ($n = 8$). $\bar{x} \pm s$. $P < 0.05$ *vs* control. $P > 0.05$ *vs* diabetic.**

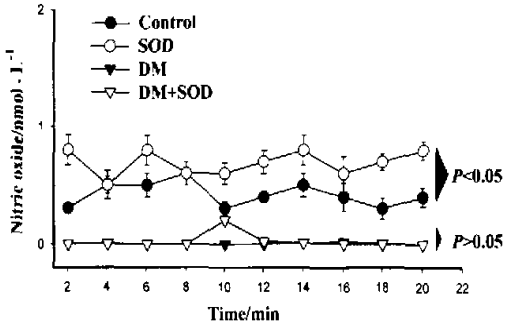


**Fig 2. To eliminate the possibility of free oxygen radicals interfering with the amperometric measurements, superoxide dismutase (SOD) 200 kU/L was added to the diabetic ($n = 12$) and control ($n = 8$) saphenous vein rings. $P < 0.05$ *vs* control. $P > 0.05$ *vs* DM.**

**Stimulated levels of NO release** A known NO stimulant, morphine sulfate, was used to evaluate the capacity of the endothelium to release NO. We have previously shown that morphine stimulated NO release from the saphenous vein endothelium in a dose-dependent manner[2,5]. In the diabetic vessels, the duration of NO release in response to morphine was significantly diminished, (7.3 ± 1.3) min *vs* (12.2 ± 2.3) min [$P < 0.05$; diabetes mellitus (DM) *vs* non DM], as was the peak level of morphine induced release, (38.4 ± 4.7) nmol/L *vs* (21.2 ± 6.1) nmol/L ($P < 0.05$; DM *vs* non-DM) in comparison to the non-diabetic vessels (Fig 3).

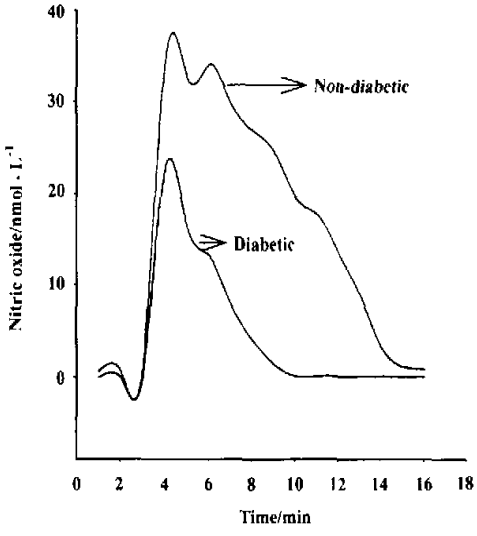


**Fig 3. Spline curve represents controls (non-diabetic) after morphine 1 μmol/L. The measurements were obtained on-line at 1 s intervals. The mean is depicted. The *s* was ≤ 10.0 %. Spline curve from the diabetic vessels shows a significantly diminished response to morphine; analysis of variance of repeated measures and a Tukey test at time points from 4 min to 10 min reveal significant difference ($P < 0.05$).**

**Mu opiate receptor expression** To determine if μ opiate receptors are expressed in surgical specimens of human saphenous vein endothelia, we examined the tissue with specific primers designed to amplify a fragment of the coding region of the μ opiate receptor (Fig 4). Only one pair of μ-specific primers (see methods) amplified a transcript of the expected size for the μ receptor (441 bp) was amplified in RNA isolated from saphenous vein endothelia (Fig 4, lane 2). Sequence analysis of the PCR product demonstrates that this fragment exhibits 100 % sequence identity with the human brain μ opiate receptor. Furthermore, tissues denuded of their endothelial layer were found not to express these transcripts[6]. The other primer pairs that spanned the coding region of the μ receptor did not yield specific PCR products (data not shown). In the saphenous vein endothelia obtained from the severe diabetic group μ transcript expression was diminished compared to its normal level of expression (−59.1 %; Fig 4, Lane 3 compared to Lane 2). The G3PDH

internal control demonstrated that equal sample amounts were loaded in each lane (Fig 4, Lane 1).

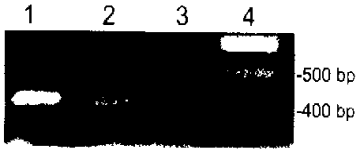


**Fig 4. Sample Gel 1 out of 5: Identification of μ opioid receptor mRNA transcript. Total RNA isolated from these tissues was reverse transcribed and PCR amplified and run on agarose gels. Lane 1: G3PDH housekeeping gene (expected transcript size is 451 bp). Lane 2: μ opioid receptor transcript in healthy saphenous vein endothelia (expected size 441 bp). Lane 3: Diminished receptor transcript expression in severe diabetic saphenous vein endothelia. Lane 4: 100 bp DNA markers.**

## DISCUSSION

Dysfunction of multiple aspects of the endothelium has been identified in diabetes mellitus (DM). Initially, studies in animals were performed which found both conduit and resistance arteries of chemically induced diabetes to have attenuated endothelium-dependent relaxation[7,8]. To date, numerous studies have confirmed that endothelium-dependent relaxation is impaired in DM. The question that remains to be answered pertains to whether this phenomenon is caused by altered NO activity.

Nitric oxide release from the endothelium has been shown to have an important role in vascular homeostasis and may play a pivotal role in regulation of vascular disease. In 1980, a sentinel observation was made by Furchgott and Zowadzi[9]: an intact endothelial lining was required for acetylcholine-induced relaxation of an *in vitro* aortic preparation, with this phenomenon being mediated by a labile, secreted substance that they called endothelium derived relaxation factor (EDRF). Since this time, the identification of EDRF as NO or a related nitroso-containing compound has led to the appreciation that this chemically simple compound plays multiple critical roles in vascular biology and pathophysiology.

Several indirect methods have been used to attempt to ascertain whether altered NO activity exists in DM. Guanylate cyclase inhibitors have been used to inhibit nitric oxide synthase (NOS), whilst hemoglobin and methylene blue were used to inhibit NO reactivity[10,11]. These substances increase the pre-contracted tone of normal arteries through the elimination of NO effect. Through this methodology indirect information was obtained regarding the contribution of NO to basal tone and agonist-induced relaxation in these studies. The assumption that endothelial-dependent relaxation to certain vasodilators, such as acetylcholine, correlated with decreased basal NO activity of diabetic arteries under both *in vitro* and *in vivo* conditions was made.

NO trapping agents were used to indirectly assess the basal NO activity in the rat aorta[12]. These scavengers bind and eliminate NO after its release without altering NOS activity. Analysis of tension in rings pre-contracted with phenylephrine revealed that the application of NO trapping agents produced an additional increment in tension that was greater in the control than the diabetic rings. These findings were thought to suggest that the basal NO activity in diabetics is less than that in non-diabetics.

Lastly, information regarding potential deficits in NO synthesis has been indirectly derived from measurements of cGMP in vascular tissue[13]. A decrease in acetylcholine-stimulated cGMP production is observed in multiple diabetic animal models. No apparent intrinsic change in either guanylate cyclase or phosphodiesterase activity of the vascular smooth muscle was found to account for defective cGMP production in these blood vessels. Therefore the conclusion that decreased basal NO bioactivity in experimental diabetes occurs has been suggested.

Unfortunately, all of these investigations in both animal models and human studies make the assumption that endothelium-dependent relaxation in both control and diabetic blood vessels is exclusively mediated via NO to reach their conclusions. The present study is the first to employ real time direct measurements of NO release with an amperometric probe in order to conclusively provide information on NO levels. In this regard, we definitively demonstrate diabetic endothelium to have a diminished basal NO release in comparison to non-diabetic controls. Through the application of a NO synthase inhibitor, further information on the actual basal level of NO release was obtained. Diabetic endothelium showed no decrease in its basal release level. We believe this signifies that the actual level of release is negligible. By exposing the tissues to superoxide dismutase we confirmed that the depressed levels of released NO seen were not the result of extracellular NO scavengers. Unfortunately we can not exclude the possibility of intracellular scavengers. Lastly, we

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evaluated the capacity of diabetic endothelium to produce NO when stimulated by the agonist, morphine sulfate. Although not the main physical receptor for release of NO, the $\mu$ opiate receptor has unique anti-inflammatory characteristics. The bulk of the work pertaining to NO release has been performed by stimulating cholinergic receptors. The results obtained were consistent with the premise that the previous findings support[14]. NO metabolism is impaired in diabetes mellitus. A lower peak level and shorter duration of stimulated release, as well as a decreased expression of $\mu$ opiate receptors were seen in diabetic compared to non-diabetic tissue.

In conclusion, we surmise that basal NO levels promote the health of the endothelium by limiting its immune and vascular activating potential. Thus, in diabetic individuals, the decrease in the capacity for this vital action leads to both enhanced vascular and immune activity, as noted by increased platelet-derived plaque formation for example[15]. The fact that stimulated NO levels are significantly diminished but still exists denotes the progressive nature of the vascular pathology associated with diabetes. The remaining capacity for stimulated NO release may help down-regulate the vascular and immune tissues. However, since it is not continuous and diminished in the diabetic, its effect is probably only partial, allowing for a progressive decrease in the ability to down-regulate these tissues over the long term. Certainly, this may not be the only explanation for the vascular abnormalities found in diabetes mellitus given the complexity of the pathological processes, however we do believe that this impaired NO metabolism plays a significant role.

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