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Effect of M40403 treatment of diabetic rats on endoneurial blood flow, motor nerve conduction velocity and vascular function of epineurial arterioles of the sciatic nerve

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1 To further explore the effect of antioxidants in preventing diabetes-induced vascular and neural dysfunction we treated streptozotocin-induced diabetic rats daily with subcutaneous injections of 10 mg kg^{-1} of M40403 ($n=11$) and compared the results obtained from 17 control rats and 14 untreated diabetic rats. M40403 is a manganese(II) complex with a bis(cyclo-hexylpyridine)-substituted macrocyclic ligand that was designed to be a selective functional mimetic of superoxide dismutase. Thus, M40403 provides a useful tool to evaluate the roles of superoxide in disease states.

2 Treatment with M40403 significantly improved diabetes-induced decrease in endoneurial blood flow, acetylcholine-mediated vascular relaxation in arterioles that provide circulation to the region of the sciatic nerve, and motor nerve conduction velocity ($P<0.05$). M40403 treatment also reduced the appearance of superoxide in the aorta and epineurial vessels and peroxynitrite in epineurial vessels. Treating diabetic rats with M40403 reduced the diabetes-induced increase in thiobarbituric acid reactive substances in serum but did not prevent the decrease in lens glutathione level. Treating diabetic rats with M40403 did not improve sciatic nerve Na^+/K^+ ATPase activity or the sorbitol, fructose or myo-inositol content of the sciatic nerve.

3 These studies provide additional evidence that diabetes-induced oxidative stress and the generation of superoxide and perhaps peroxynitrite may be partially responsible for the development of diabetic vascular and neural complications.

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Keywords: Diabetes; vasodilation; diabetic neuropathy; acetylcholine; oxygen radicals; superoxide; peroxynitrite; endothelium

Abbreviations: DTNB, dithionitrobenzene; EBF, endoneurial blood flow; GSH, glutathione; H_2O_2, hydrogen peroxide; HES-DFO, hydroxyethyl starch deferoxamine; M40403, dichloro[(4a*R*, 13a*R*, 17a*R*,21a*R*)-1,2,3,4,4a,5,6,12,13,13a,14, 15,16,17,17a,18,19,20,21,21a - eicosahydro - 11,7 - nitrilo - 7H-dibenzo[1,4,7,10]tetraazacycloheptadecine-κN^5,κN^{13}, κN^{18},κN^{21},κN^{22}]manganese; MNCV, motor nerve conduction velocity; Na^+/K^+ ATPase, sodium/potassium ATPase; NF-κB, nuclear factor κB; $O_2^{\cdot-}$, superoxide anion; $OH^{\cdot}$, hydroxyl radical; PSS, Krebs Henseleit physiological saline solution; RLU, relative light units; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances

Introduction

Oxidative stress is an important component of diabetes and its complications (Pieper *et al*., 1993; Cameron & Cotter, 1995; 1999; Pieper & Siebeneich, 1997; 1998; Cameron *et al*., 1998; Keegan *et al*., 1999; Pieper, 2000; Obrosova *et al*., 2000; Cakatay *et al*., 2000; Haak *et al*., 2000; Andrew *et al*., 2000; Gocmen *et al*., 2000; Ishii *et al*., 1998; Ammar *et al*., 2000). Moreover, treatment of streptozotocin-induced diabetic rats with antioxidants has demonstrated that oxidative stress and vascular dysfunction may be a major factor in the development of diabetic neuropathy (Cameron *et al*., 1993; 1994; 1998; Karasu *et al*., 1995; Keegan *et al*., 1999; Cameron & Cotter, 1999). Previously, we have shown that acetylcholine-induced vasodilation by arterioles that provide circulation to the region of the sciatic nerve is impaired early in diabetes and is accompanied by a reduction of endoneurial blood flow (EBF) and an increase in superoxide in these vessels (Terata *et al*., 1999; Coppey *et al*., 2000). These changes preceded the slowing of motor nerve conduction velocity (MNCV) and the reduction in Na^+/K^+ ATPase activity in the sciatic nerve, suggesting that vascular dysfunction rather than hyperglycemia-induced metabolic abnormalities of the nerve is responsible for the nerve disorders associated with the early onset of diabetes. We have also demonstrated that treating streptozotocin-induced diabetic rats with α-lipoic acid or hydroxyethyl starch deferoxamine (HES-DFO) prevents the impairment of vascular function and accumulation of superoxide and peroxynitrite induced by diabetes in the arterioles that provide circulation to the region of the sciatic nerve as well

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as the reduction in endoneurial blood flow and slowing of motor nerve conduction velocity (Coppey *et al*., 2001).

In spite of these studies identification of the particular free radical(s) involved in diabetic complications has not been accomplished. This stems from the fact that selective antioxidants have not been available. In numerous disease states the use of native superoxide dismutase (SOD) enzymes both pre-clinically and clinically shed light on the importance of $O_2^{\cdot-}$ in disease and, thus, the therapeutic potential of exogenous SOD enzymes (Huber *et al*., 1980; Flohe, 1988; Uematsu *et al*., 1994). However, the native SOD enzyme has not been evaluated in animal models of diabetes. Thus, the role of superoxide in this condition is to date not defined. There are drawbacks or problematic issues associated with the use of the native enzymes as therapeutic agents (e.g., solution instability, immunogenicity of non-human enzymes, bell-shaped dose-response curves, high susceptibility to proteolytic digestion) and as pharmacological tools (e.g., they do not penetrate cells or cross the blood-brain barrier, limiting the dismutation of superoxide only to the extracellular space or compartments). To overcome the limitations associated with native enzyme therapy, we have developed a series of SOD mimetics that catalytically remove $O_2^{\cdot-}$. M40403 is a prototypic example of a stable, low molecular weight, manganese-containing, non-peptidic molecule possessing the function and catalytic rate of native SOD enzymes, but with the advantage of being a much smaller molecule (MW 483 *vs* MW 30,000 for the mimetic and native enzyme, respectively) (Salvemini *et al*., 1999). An important property of these SOD mimetics is that they catalytically remove superoxide at a high rate without interacting with other biologically important reactive species including nitric oxide, peroxynitrite, hydrogen peroxide, oxygen or hydroxyl radicals (Riley *et al*., 1996, 1997). This property is not shared by other classes of SOD mimetics or scavengers, including several metalloporphyrins such as tetrakis-(N-ethyl-2-pyridyl) porphyrin and tetrakis-(benzoic acid)porphyrin, that interact with other reactive species such as nitric oxide and peroxynitrite that clearly play important roles in inflammation (Patel & Day, 1999). The purpose of our study was to evaluate the role of superoxide by using M40403 in the development of diabetic vascular and neural complications.

Methods

Materials

Unless stated otherwise all chemicals used in these studies were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). M40403 was synthesized at MetaPhore Pharmaceuticals, Inc (St. Louis, MO, U.S.A.) as described previously (Salvemini *et al*., 1999).

Methods

Animals Male Sprague-Dawley (Harlan Sprague Dawley, Indianapolis, IN, U.S.A.) rats 8–9 weeks of age were used for these studies. The animals were housed in a certified animal care facility and food (Harlan Teklad, #7001, Madison, WI, U.S.A.) and water were provided *ad libitum*. All institutional and NIH guidelines for use of animals were followed. Diabetes was induced by intravenously injecting streptozotocin (60 mg kg^{-1} in 0.9% NaCl, adjusted to a pH 4.0 with 0.2 M sodium citrate). Control rats were injected with vehicle alone. The rats were anaesthetized with methoxyflurane before injection. Diabetes was verified 48 h later by evaluating blood glucose levels with the use of glucose-oxidase reagent strips (Lifescan Inc., Milpitas, CA, U.S.A.). Rats having blood glucose level of 300 mg dl^{-1} (16.7 mM) or greater were considered to be diabetic. At this time the diabetic rats were randomly divided into two groups one to receive M40403 and the other to receive vehicle. All studies were conducted approximately 3–4 weeks after the verification of diabetes. Rats treated with M40403 received a subcutaneous injection of 10 mg kg^{-1} daily. The M40403 was dissolved in sterile saline and control and non-treated diabetic rats received vehicle alone. Treatments with M40403 were started on the day hyperglycemia was verified.

Motor nerve conduction velocity MNCV was determined as previously described using a noninvasive procedure in the sciatic-posterior tibial conducting system in a temperature controlled environment (Terata *et al*., 1999; Yorek *et al*., 1993).

Endoneurial blood flow Immediately after determination of MNCV, sciatic nerve endoneurial nutritive blood flow was determined using the hydrogen clearance method as described by Cameron *et al*. (1991; 1997) and adapted by our laboratory (Terata *et al*., 1999; Coppey *et al*., 2000). The hydrogen clearance data was fitted by computer to a mono- or bi-exponential curve using commercial software (Prism, GraphPad, San Diego, CA, U.S.A.) and nutritive blood flow, (ml min^{-1} 100 g^{-1}), calculated using the equation described by Young (1980) and vascular conductance, (ml min^{-1} 100 g^{-1} mm Hg^{-1}) determined by dividing nutritive blood flow by the average mean arterial blood pressure.

Vascular reactivity Videomicroscopy was used to investigate *in vitro* vasodilatory responsiveness of epineurial arterioles supplying the region of the sciatic nerve (branches of the superior gluteal and internal pudendal arteries) to acetylcholine (10^{-4} and 10^{-8} mol l^{-1}) or sodium nitroprusside (10^{-4} mol l^{-1}) as previously described (Terata *et al*., 1999; Coppey *et al*., 2000).

Detection of superoxide and peroxynitrite Hydroethidine (Molecular Probes Inc., Eugene, OR, U.S.A.), an oxidative fluorescent dye, was used to evaluate *in situ* levels of superoxide ($O_2^{\cdot-}$) in epineurial vessels as described previously (Coppey *et al*., 2000; 2001). Hydroethidine is permeable to cells and in the presence of $O_2^{\cdot-}$ is oxidized to fluorescent ethidium bromide, where it is trapped by intercalating with DNA. This method provides sensitive detection of $O_2^{\cdot-}$ *in situ*. Superoxide levels were also measured in the aorta by lucigenin-enhanced chemiluminescence as described previously (Miller *et al*., 1998; Coppey *et al*., 2000; 2001).

One of two mechanisms by which acetylcholine mediates vascular relaxation in arterioles that provide circulation to the sciatic nerve is through the production of nitric oxide (Terata *et al*., 1999). The chemistry of nitric oxide is very complex, and several biochemical pathways other than nitric oxide production can influence nitric oxide action. For

example, superoxide anion can interact with nitric oxide to form peroxynitrite (Wattanapitayakul *et al*., 2000). This reaction reduces the efficacy of nitric oxide to act as a signal transduction agent. Peroxynitrite is a highly reactive intermediate known to nitrate protein tyrosine residues and cause cellular oxidative damage (Pryor & Squadrito, 1995; Beckman, 1996). To determine whether the diabetes-induced formation of superoxide by arterioles that provide circulation to the region of the sciatic nerve promotes the formation of peroxynitrite we measured 3-nitrotyrosine, a stable biomarker of tissue peroxynitrite formation, immunoreactivity using a commercial kit from Vector Laboratories (Burlingame, CA, U.S.A.) (Coppey *et al*., 2001)

Sciatic nerve Na^+/K^+ ATPase activity and sorbitol, fructose and myo-inositol content The left sciatic nerve was removed, desheathed, and divided into three samples for determination of Na^+/K^+ ATPase activity, conjugated diene levels (see below) and sorbitol, fructose and myo-inositol content as previously described (Coppey *et al*., 2001).

Additional biological parameters Lens glutathione (GSH), serum TBARS and sciatic nerve conjugated diene levels were determined as additional markers of oxidative stress. Lens glutathione levels were determined according to Lou *et al*. (1988). Lens were weighed and homogenized in 1 ml of cold 10% trichloroacetic acid and centrifuged for 15 min at $1000 \times g$. The supernatant (100 μl) was mixed with 0.89 ml of 1.0 M Tris, pH 8.2, and 0.02 M EDTA. Afterwards, 10 μl of dithionitrobenzene (DTNB) was added and change in absorbance measured at 412 nm. A glutathione standard curve (100–500 ng) was performed for each assay. The data were recorded as μg mg wet weight^{-1}. TBARS level in serum was determined by the method of Mihara *et al*. (1980) as modified by Siman & Eriksson (1997). Briefly, 200 μl of serum was boiled in 0.75 ml of phosphoric acid (0.19 M), 0.25 ml thiobarbituric acid (0.42 mM) and 0.3 ml water for 60 min. Afterwards, the samples were precipitated with methanol/NaOH and centrifuged for 5 min. The supernatant was measured fluorometrically at excitation wavelength 532 nm and emission wavelength 553 nm. Standards were prepared by the acid hydrolysis of 1,1,3,3-tetraethoxypropane. The data was reported as μg ml^{-1} serum. Sciatic nerve conjugated diene level were determined according to the method of Recknagel & Ghoshal (1996) and Low & Nickander (1991). Briefly, a segment of the sciatic nerve was extracted with chloroform and methanol. The lipid extract was evaporated and redissolved in 1 ml cyclohexane. Conjugated diene levels were determined by measuring the absorbance at 233 nm with extraction blanks used as references. An extinction coefficient of 2.52×10^4 M was used to determine the amount of conjugated diene present. The data was reported as μmol mg wet weight^{-1}. Serum free fatty acid and triglyceride levels were determined using commercial kits from Roche Diagnostics, Mannheim, Germany and Sigma Chemical Co. (St. Louis, MO, U.S.A.) respectively.

Data analysis The results are presented as mean ± s.e.mean. Comparisons between the groups for MNCV, EBF, sciatic nerve Na^+/K^+ ATPase activity, sciatic nerve sorbitol, fructose and myo-inositol content, serum TBARS, sciatic nerve conjugated diene, serum free fatty acid and triglyceride, aorta lactate/pyruvate ratio and lens glutathione levels were conducted using independent unpaired Student's *t*-tests. Dose response curves for acetylcholine-induced relaxation were compared using a two way repeated measures analysis of variance with autoregressive covariance structure using proc mixed program of SAS (Terata *et al*., 1999; Coppey *et al*., 2000). Whenever significant interactions were noted specific treatment-dose-effects were analysed using a Bonferroni adjustment. A P value of less 0.05 was considered significant. All computations were performed using SAS for Windows version 6.12.

Results

Body weight and plasma glucose levels

For these studies we used 17 control rats, 14 untreated diabetic rats and 11 diabetic rats treated with M40403. Data in Table 1 show that streptozotocin-induced diabetic rats treated with or without M40403 on average gained less weight than age-matched control rats over the 3–4 week experimental period of this study. At the time of experimentation plasma glucose levels were increased 3–4 fold in diabetic rats and diabetic rats treated with M40403 compared to control rats. Treating diabetic rats with M40403 had no significant effect on weight gain or blood glucose levels compared to non-treated diabetic rats.

Sciatic nerve Na^+/K^+ ATPase activity and sorbitol, fructose and myo-inositol content

Data in Table 2 demonstrate that diabetes causes a significant decrease in sciatic nerve Na^+/K^+ ATPase activity. Treating diabetic rats with M40403 did not prevent the diabetes-induced decrease in Na^+/K^+ ATPase activity compared to control rats. Data in Table 2 also demonstrate that diabetes causes a significant increase in the sorbitol and fructose content and a decrease in myo-inositol levels in the sciatic nerve and this was not improved by treating diabetic rats with M40403.

Serum triglyceride and free fatty acid levels

Data in Figure 1 demonstrate that diabetes causes a significant increase in serum triglyceride and free fatty acid levels ($P<0.05$). Treating diabetic rats with M40403 did not affect the diabetes-induced increase in serum free fatty acid levels. In contrast, serum triglyceride levels were significantly reduced in diabetic rats treated with M40403 compared to untreated diabetic rats ($P<0.05$). However, serum triglyceride levels in diabetic rats treated with M40403 remained significantly elevated compared to control rats ($P<0.05$).

Table 1 Change in body weight and blood glucose levels

Animal	*Change in body weight* (g)	*Blood glucose nmol l^{-1}*
Control ($n=17$)	123 ± 11	5.1 ± 0.2
Diabetic ($n=14$)	34 ± 10$^+$	22.6 ± 1.1$^+$
Diabetic + M40403 ($n=11$)	31 ± 10$^+$	24.9 ± 0.9$^+$

Data are means ± s.e.mean. $^+P<0.05$ vs control.

Table 2 Effect of treatment of streptozotocin-induced diabetic rats with M40403 on sciatic nerve Na^+/K^+ ATPase activity and sorbitol, fructose and myo-inositol levels

Animal	*Na^+/K^+ ATPase activity* (μmol ADP mg wet wt^{-1} h^{-1})	*Intracellular content* (nmol mg wet wt^{-1}) *Sorbitol*	*Fructose*	*myo-Inositol*
Control ($n=17$)	249.2±18.5	0.45±0.09	0.71±0.06	12.8±1.3
Diabetic ($n=14$)	93.4±18.3†	1.43±0.25†	3.63±0.33†	5.9±0.9†
Diabetic+M40403 ($n=11$)	146.2±20.5†	1.31±0.40†	3.32±0.37†	6.8±1.0†

Data are means±s.e.mean. †$P<0.05$ vs control.

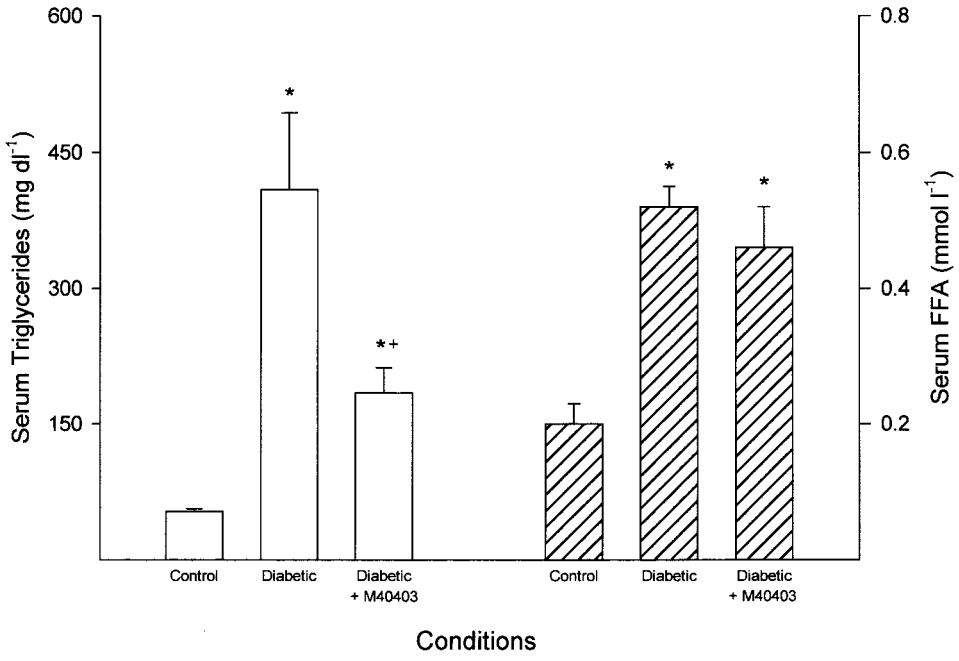

Figure 1 Serum triglyceride and free fatty acid levels. Data are presented as the mean±s.e.mean for 17 control rats, 14 untreated diabetic rats and 11 diabetic rats treated with M40403. The * denotes a significant difference compared to control, $P<0.05$. The+denotes a significant difference compared to untreated diabetic rats, $P<0.05$.

Evaluation of oxidative stress

Data in Figure 2 demonstrate that diabetes causes an significant increase in thiobarbituric acid reactive substances (TBARS) in serum ($P<0.05$). Treating diabetic rats with M40403 significantly reduced the increase in serum TBARS level caused by diabetes ($P<0.05$). Data in Figure 2 also demonstrate that conjugated diene level in the sciatic nerve was significantly increased ($P<0.05$) by diabetes and was reduced by about 40% when diabetic rats were treated with M40403. The level of conjugated dienes in the sciatic nerve of diabetic rats treated with M40403 remained significantly increased compared to control rats ($P<0.05$). Lens GSH level was significantly decreased in streptozotocin-induced diabetic rats compared to control rats (0.5±0.1 vs 1.5±0.1 μg mg wet wt^{-1}, respectively, $P<0.05$, $n=14$ and 17, respectively). Treating diabetic rats with M40403 did not improve the decrease in lens GSH level induced by diabetes (0.4±0.1 μg mg wet wt^{-1}, $P<0.05$ compared to control, $n=11$).

Data in Figure 3 demonstrate that treating streptozotocin-induced diabetic rats with M40403 markedly decreased the diabetes-induced increase in the level of superoxide in epineurial vessels as measured by hydroethidine fluorescence compared to paired analysis of untreated diabetic rats. Similar results were obtained in two additional studies using control, untreated diabetic rats and diabetic rats treated with M40403. We also measured the superoxide level in the aorta by lucigenin-enhanced chemiluminescence. These studies demonstrated that the superoxide level is significantly increased in the aorta of diabetic rats compared to control rats (3.1±0.3 vs 2.0±0.1 mean RLU min^{-1} mm^{-1}, respectively, $P<0.05$, $n=14$ and 17, respectively) and treating diabetic rats with M40403 prevented the diabetes-induced increase of superoxide (2.3±0.2 mean RLU min^{-1} mm^{-1} $P<0.05$ compared to untreated diabetic rat, $n=11$).

Data in Figure 4 visually demonstrate that diabetes induces the formation of 3-nitrotyrosine (as indicated by the blue staining), a marker for peroxynitrite, in presumably endothelial cells and the adventitia of these arterioles. Treating diabetic rats with M40403 prevented the formation of 3-nitrotyrosine as indicated by the lack of staining in these arterioles.

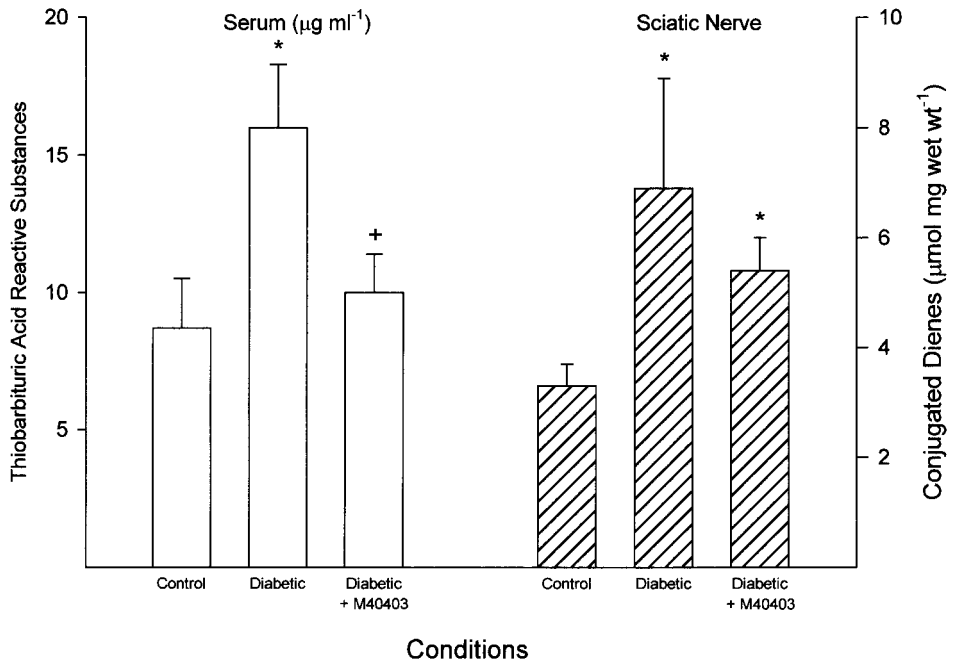

Figure 2 Serum thiobarbituric acid reactive substances and sciatic nerve conjugated diene level. Serum samples were collected and used to determine thiobarbituric acid reactive substances level (left side of figure). The sciatic nerve was also collected and a portion used to determine the conjugated diene level (right side of figure). Data are presented as the mean ± s.e.mean for 17 control rats, 14 untreated diabetic rats and 11 diabetic rats treated with M40403. The * denotes a significant difference compared to control, $P<0.05$. The + denotes a significant difference compared to untreated diabetic rats, $P<0.05$.

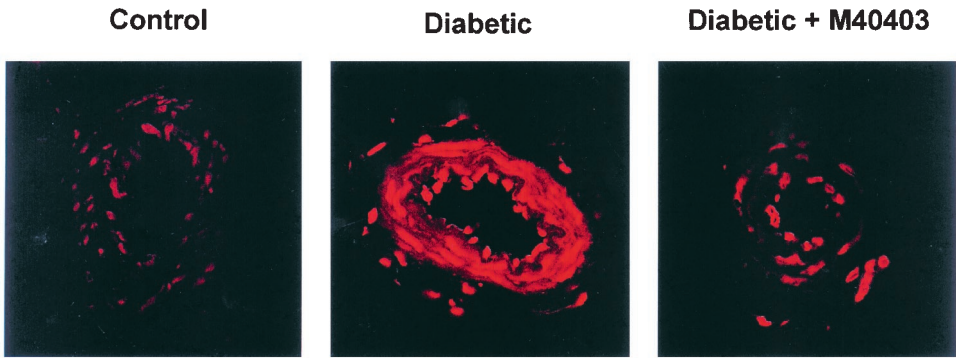

Figure 3 Detection of superoxide level in arterioles from control, diabetic rats and diabetic rats treated with M40403. The duration of diabetes and treatments for these studies was 3 weeks. Fluorescent photomicrographs of confocal microscopic sections of arterioles that provide circulation to the region of the sciatic nerve from the three individual groups of animals were examined on the same day. Arterioles were labeled with the oxidative dye hydroethidine. Recording of fluorescent were taken at identical laser and photomultiplier settings for both control and untreated and treated diabetic rats. Shown is a representative sample of one set of animals. This experiment was repeated three separate times on separate sets of animals on three different days with similar results.

Endoneurial blood flow and motor nerve conduction velocity

Data in Figure 5 demonstrate that treating diabetic rats with M40403 prevents the decrease in EBF compared to untreated diabetic rats. Likewise, data in Figure 6 demonstrate that treating diabetic rats with M40403 prevents the slowing in MNCV.

Arteriolar vascular reactivity

As demonstrated in Figure 7, diabetes causes a significant decrease ($P<0.05$) in acetylcholine (10^{-4} and 10^{-8} mol l^{-1}) mediated vascular relaxation in arterioles that provide circulation to the region of the sciatic nerve. Treating diabetic rats with M40403 significantly improves the diabetes-induced impairment in acetylcholine mediated vascular relaxation ($P<0.05$). In contrast, maximal vasodilation induced by sodium nitroprusside (10^{-4} M), endothelium-independent, in these vessels was not affected by diabetes or treatment of diabetic rats with M40403 (112.8 ± 8.5, 105.1 ± 8.8 and 113.9 ± 7.1 in control ($n=17$), untreated diabetic ($n=14$) and diabetic rats treated with M40403 ($n=11$), respectively. Baseline diameters of the vessels used in these studies was not different for control, untreated diabetic and diabetic rats treated with M40403 (129 ± 9, 111 ± 9 and 117 ± 7 μm, respectively).

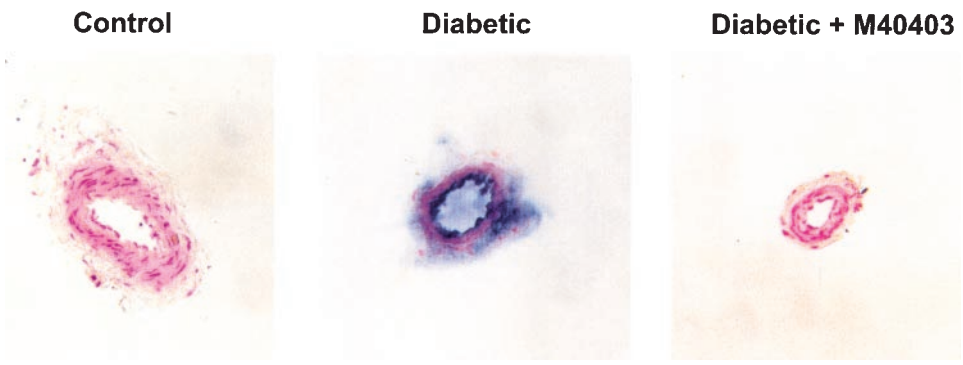

Figure 4 Detection of peroxynitrite in arterioles from control, diabetic rats, and diabetic rats treated with M40403. Arterioles from control, diabetic rats and diabetic rats treated with M40403 were collected and treated for determination of 3-nitrotyrosine immunostaining. Shown is a representative sample of one set of animals. This experiment was repeated three separate times with similar results.

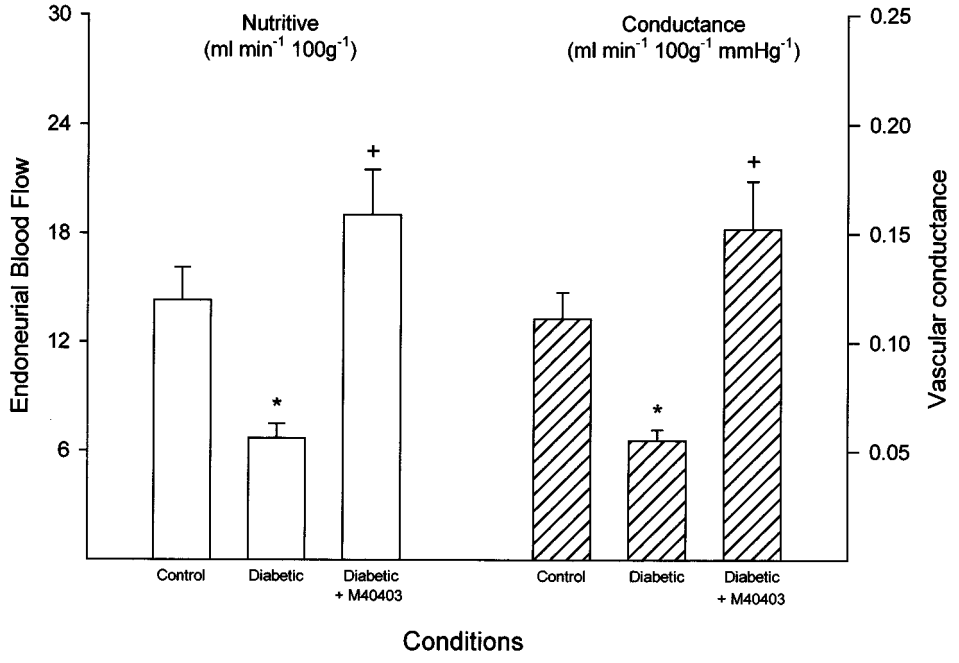

Figure 5 Determination of endoneurial blood flow. Endoneurial blood flow reported as nutritive flow (left) or conductance (right) was determined. Data are presented as the mean ± s.e.mean for 17 control rats, 14 untreated diabetic rats and 11 diabetic rats treated with M40403. The * denotes a significant difference compared to control, $P<0.05$. The + denotes a significant difference compared to untreated diabetic rats, $P<0.05$.

Discussion

M40403 has been described to be a nonpeptidyl mimetic of SOD (Salvemini *et al*., 1999; Salvemini & Riley, 2000). M40403 was derived from the macrocyclic ligand, 1,4,7,10,13-pentaazacyclopentadecane, containing the added bis(cyclohexylpyridine) substitution pattern (Salvemini *et al*., 1999). M40403 selectively catalyzes the dismutation of superoxide ($O_2^{\cdot -}$) with rates approaching that of the native Mn SOD (Salvemini & Riley, 2000). M40403 has a molecular weight of 484.4 and a catalytic rate $>2\times10^8$ M^{-1} s^{-1}, at the pH of $\sim$6.5 (Salvemini *et al*., 1999). It is thermodynamically stable and stable for up to 10 h in whole rat blood at 37°C (Salvemini *et al*., 1999; Salvemini & Riley, 2000). After intravenous injection into rats, M40403 distributes widely into the heart, lungs, brain, liver, and kidneys, while retaining its intact chemical identity (Salvemini *et al*., 1999; Salvemini & Riley, 2000). The compound is excreted intact in urine and feces with no detectable dissociation of the manganese (Salvemini & Riley, 2000). M40403 does not react with nitric oxide, hydrogen peroxide, or peroxynitrite (Salvemini & Riley, 2000). This unique selectivity of M40403 activity for superoxide in the presence of other reactive oxygen species makes it possible to dissect the role of superoxide in disease models such as diabetes in which other reactive oxygen species may be implicated.

Treating diabetic rats with M40403 we found that it inhibited the generation of superoxide by aorta and

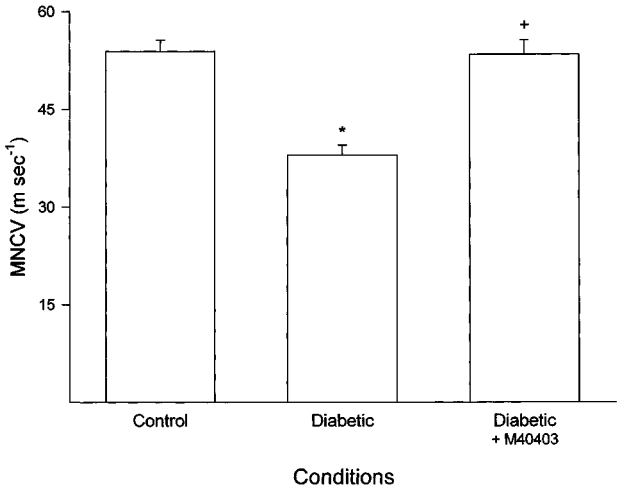

Figure 6 Determination of motor nerve conduction velocity. Data are presented as the mean ± s.e.mean for 17 control rats, 14 untreated diabetic rats and 11 diabetic rats treated with M40403. The * denotes a significant difference compared to control, $P<0.05$. The + denotes a significant difference compared to untreated diabetic rats, $P<0.05$.

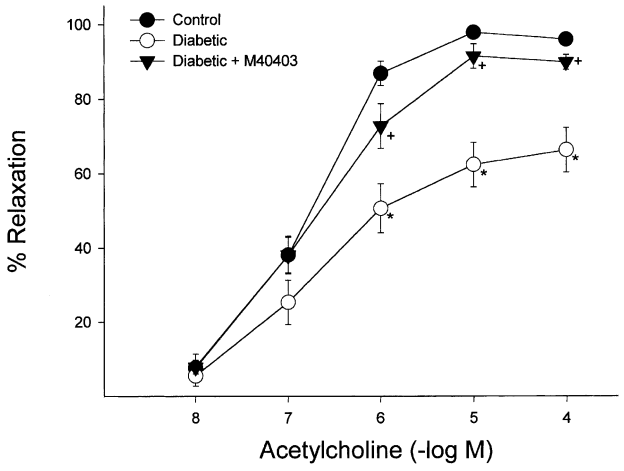

Figure 7 Determination of the effect of treatment with M40403 on acetylcholine-mediated vascular relaxation in arterioles that provide circulation to the region of the sciatic nerve. Pressurized arterioles (40 mm Hg) were constricted with U46619 (30–50%) and incremental doses of acetylcholine were added to the bathing solution while recording steady state vessel diameter. For control, diabetic and diabetic rats treated with M40403 the number of experimental observations was 17, 14 and 11, respectively. The * denotes that the response to acetylcholine was significantly attenuated in the diabetic rat, $P<0.05$. The + denotes that the response to acetylcholine was significantly different compared to the untreated diabetic rats, $P<0.05$.

epineurial vessels, the formation of peroxynitrite by epineurial vessels, the reduction in endoneurial blood flow, the slowing of MNCV and impairment of endothelium-dependent vasodilation of arterioles that provide circulation to the sciatic nerve. It also improved the diabetes induced changes in serum TBARS and sciatic nerve conjugated diene level, two additional markers of oxidative stress. In contrast, lens glutathione level remained reduced in diabetic rats treated with M40403. Treating diabetic rats with M40403 did not improve the metabolic related defects in the sciatic nerve such as the decrease in Na^+/K^+ ATPase activity and the reciprocal change in sorbitol and myo-inositol levels. This result raises the question whether these metabolic changes are a primary contributor to diabetes-induced vascular and neural dysfunction.

In a previous study we found that treating diabetic rats with α-lipoic acid prevented the generation of superoxide and peroxynitrite in epineurial vessels, reduced serum TBARS and improved lens GSH levels (Coppey *et al*., 2001). The level of conjugated dienes in the sciatic nerve remained increased in diabetic rats treated with α-lipoic acid. Both M40403 and α-lipoic acid treatment was found to prevent the decrease in endoneurial blood flow, slowing of motor nerve conduction velocity and impairment in acetylcholine-mediated vasodilation in arterioles that provide circulation to the region of the sciatic nerve. Therefore, treatment of diabetic rats with either M40403 or α-lipoic acid seem to have a similar efficacy in preventing oxidative stress and vascular and neural dysfunction in diabetic rats. This occurs even though the antioxidant properties of these two compounds are different. M40403 is a mimetic of superoxide dismutase whereas; α-lipoic acid is a naturally occurring free radical scavenger and transition metal chelator (Salvemini *et al*., 1999; Keegan *et al*., 1999).

Our studies have shown that treating diabetic rats with M40403 or α-lipoic acid improved vascular and neural function (Coppey *et al*., 2001). One common feature of these treatments in diabetic rats was the prevention of the generation of superoxide/peroxynitrite in vascular tissue. This suggests that the formation of superoxide may be a main factor in diabetes-induced vascular disease. This is supported by the present studies. Unlike the non-selective antioxidant properties of α-lipoic acid, M40403 is selective in preventing superoxide formation and does not directly influence the generation of other reactive oxygen species (Salvemini *et al*., 1999; Salvemini & Riley, 2000). Since treatment of diabetic rats with M40403 prevented vascular and neural dysfunction we conclude that the generation of superoxide and subsequent formation of peroxynitrite are likely responsible for the diabetes-induced vascular and neural defects associated with the early stages of diabetic neuropathy. Combined, these results suggest that diabetes-induced vascular dysfunction is caused by oxidative stress *via* the formation of superoxide and that vascular dysfunction rather than metabolic related defects of the sciatic nerve is responsible for the early neural defects associated with diabetic neuropathy.

In diabetes the production of superoxide anion radicals plays a role in impaired endothelium-dependent relaxation (Pieper *et al*., 1997). We have shown that in diabetes the accumulation of superoxide and peroxynitrite in epineurial vessels accompanies the reduction in EBF and impairment of endothelium-dependent vasodilation in these vessels and precedes the slowing of MNCV (Coppey *et al*., 2000). In addition, Mayhan (1997) has demonstrated that treating the basilar artery from diabetic rats with SOD improves nitric oxide synthase-dependent vasodilation. As further evidence that oxidative stress is a contributing factor in the development of diabetic neuropathy, antioxidant therapy has been shown to improve neural function in diabetic animal models (Cameron *et al*., 1993; 1994; 1998; Karasu *et al*., 1995; Keegan *et al*., 1999; Cameron & Cotter, 1999). In diabetes, one possible explanation for reactive oxygen species

mediated endothelial dysfunction comes from studies by Soriano *et al*. (2001). These studies have implicated the generation of reactive oxygen species with the activation of poly (ADP-ribose) polymerase in vascular tissue from diabetic mice and endothelial cells exposed to increased concentration of glucose. In the endothelium, the activation of poly (ADP-ribose) polymerase has been linked to vascular dysfunction, the activation of nuclear factor κB (NF-κB) and reduction in cellular ATP and pyridine nucleotide levels. In summary, these studies provide evidence that oxidative stress induced by diabetes contributes to vascular and neural dysfunction and that superoxide plays a significant role in these events.

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