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Preventing Superoxide Formation in Epineurial Arterioles of the Sciatic Nerve from Diabetic Rats Restores Endothelium-dependent Vasodilation

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We have previously reported that in streptozotocin-induced diabetic rats that increased formation of superoxide and peroxynitrite is associated with impairment in vascular relaxation in epineurial arterioles of the sciatic nerve. In this study we demonstrate that pretreating epineurial arterioles from diabetic rats *in vitro* with α-lipoic acid, dihydrolipoic acid, tempol or arginine restores acetylcholine-mediated vascular relaxation to near the reactivity observed in vessels from control rats. Suggesting that increased oxidative stress and reduction in nitric oxide availability is partially responsible for the impairment in endothelium-dependent vasodilation observed in epineurial arterioles from diabetic rats. In contrast, pretreating epineurial arterioles from diabetic rats with aminoguanidine or allopurinol had no effect. Studies designed to investigate the source of superoxide formation provided results suggesting that complex I of the mitochondrial electron transport chain and NAD(P)H oxidase are responsible for the increase in superoxide formation observed with epineurial arterioles from the sciatic nerve. Pretreating epineurial arterioles from diabetic rats with the protein kinase C inhibitor bisindolymaleimide I (GF 109203X) improved acetylcholine-mediated vascular relaxation but did not prevent the increase in superoxide formation suggesting that activation of protein kinase C by oxidative stress is downstream of superoxide formation. These studies imply that increased superoxide formation via the mitochondrial electron transport chain and perhaps NAD(P)H oxidase is partially responsible for reduced vascular reactivity observed in epineurial arterioles of the sciatic nerve from diabetic rats.

Keywords: Oxidative stress; Diabetes; Vascular reactivity superoxide; Protein kinase C

INTRODUCTION

Diabetic neuropathy is a multifactorial problem with a unique etiology. It has been described by some investigators to be a disease of the vasculature leading to nerve ischemia and altered nerve function.[1–8] Other investigators have proposed that diabetic neuropathy is caused by metabolic defects associated with an increased flux of glucose through the aldose reductase pathway and depletion of intracellular myo-inositol levels leading to a defect in Na^+/K^+ ATPase activity and an alteration of signal transduction pathways in the nerve.[9–11] Our studies suggest that vascular dysfunction of epineurial vessels and reduction in endoneurial blood flow (EBF) is responsible for the early stages of diabetic neuropathy.[12] In addition, we have shown that oxidative stress, likely due to the generation of superoxide and perhaps peroxynitrite, causes vascular dysfunction in epineurial arterioles and accompanies the reduction in EBF. These abnormalities precede the slowing of motor nerve conduction velocity (MNCV).[13,14] Recently, Brownlee and colleagues have linked several of the molecular mechanisms thought to contribute to diabetic complications to the overproduction of superoxide by the mitochondrial electron-transport chain.[15,16]

In order to further examine the role of superoxide formation in the impairment of

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endothelium-dependent vascular relaxation in epineurial arterioles of the sciatic nerve from diabetic rats, we investigated the source of superoxide formation in these vessels and the effect of treatment with antioxidants, arginine, allopurinol, aminoguanidine and a protein kinase C inhibitor *in vitro* on the diabetes-induced decrease in acetylcholine-mediated vascular relaxation. Studies employing rotenone and diphenylene iodonium suggest that the mitochondria and perhaps NAD(P)H oxidase are sources for diabetes-induced superoxide production by epineurial arterioles of the sciatic nerve. In addition, the activation of protein kinase C may contribute to the diabetes-induced impairment of vascular relaxation mediated by increased superoxide formation.

MATERIALS AND METHODS

Materials

Unless stated otherwise all chemicals used in these studies were obtained from Sigma Chemical Co. (St Louis, MO).

Animals

Male Sprague–Dawley (Harlan Sprague Dawley, Indianapolis, IN) 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 (meal form), Madison, WI) 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 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 anesthetized 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). Rats having blood glucose level of 300 mg dl^{-1} (16.7 mM) or greater were considered to be diabetic. The duration of diabetes for the rats used in these studies was 3–4 weeks. Previously, we had demonstrated that after a period of 2-weeks of diabetes endothelium-dependent vascular relaxation in epineurial arterioles of the sciatic nerve is maximally impaired.[17] On the day of experimentation blood glucose level for control and diabetic rats was 102 ± 21 and 412 ± 45 mg/dl, respectively.

Vascular Reactivity

Rats were anesthetized with Nembutal i.p. (50 mg/kg, i.p., Abbott Laboratories, North Chicago, IL) and the abdominal aorta was isolated and occluded 1–2 cm above the branch of the common iliac artery. Distal to the occlusion a solution containing India ink with 2% gelatin was injected to facilitate the identification of the superior gluteal and internal pudendal arteries, which arise from the common iliac artery. The rat was then sacrificed by exsanguination, and body temperature lowered with topical ice.[17] Videomicroscopy was used to investigate *in vitro* vasodilatory responsiveness of arterioles supplying the region of the sciatic nerve (branches of the superior gluteal and internal pudendal arteries) as previously described.[17] To isolate these vessels the common iliac was exposed and the branch points of the internal pudendal and superior gluteal arteries identified. The vessels were then clamped, and tissue containing these vessels and its branches dissected *en bloc*. The block of tissue was immediately submerged in a cooled (4°C), oxygenated (20% O_2, 5% CO_2 and 75% N_2) Krebs Henseleit physiological saline solution (PSS) of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 20, Na_2EDTA 0.026, and 5.5 glucose. Branches of the superior gluteal and internal pudendal arteries (50–150 μm internal diameter and 2 mm in length) were carefully dissected and trimmed of fat and connective tissue. Both ends of the isolated vessel segment were cannulated with glass micropipettes filled with PSS (4°C), and secured with 10–0 nylon Ethilon monofilament sutures (Ethicon, Inc., Cornelia, GA). The pipettes were attached to a single pressure reservoir (initially set at 0 mm Hg) under condition of no flow. The organ chamber containing the cannulated vessels was then transferred to the stage of an inverted microscope (CK2, Olympus, Lake Success, NY). Attached to the microscope were a CCTV camera (WV-BL200, Panasonic, Secaucus, NJ), video monitor (Panasonic), and a video caliper (VIA-100 K, Boeckeler Instruments, Inc., Tucson, AZ). The organ chamber was connected to a rotary pump (Masterflex, Cole Parmer Instrument Co., Vernon Hills, IL), which continuously circulated 37°C oxygenated PSS at 30 ml/min. The pressure within the vessel was then slowly increased to 40 mm Hg. At this pressure we found that KCl gave the maximal constrictor response. Therefore, all the studies were conducted at 40 mm Hg. Internal vessel diameter (resolution of 2 μm) was measured by manually adjusting the video micrometer. After 30 min equilibration, KCl was added to the bath to test vessel viability. Vessels, which failed to constrict more than 30%, were discarded. After washing with PSS, vessels were incubated for 30 min in PSS and then constricted with U46619 (10^{-8}–10^{-7} M) (Cayman Chemical, Ann Arbor, MI) to 30–50% of passive diameter. Cumulative concentration-response relationships were evaluated for acetylcholine (10^{-8}–10^{-4} M) using vessels from control and

diabetic rats. At the end of each acetylcholine dose response determination sodium nitroprusside (10^{-4} M) was added to determine maximal vasodilation. Prior to examining vascular reactivity responsiveness to acetylcholine vessels from diabetic rats were pre-incubated for 1 h in the organ chamber in the presence of α-lipoic acid (10^{-3} M), L-arginine (10^{-3} M), aminoguanidine (3×10^{-4} M), bisindolymaleimide I (GF 109203X; 40×10^{-6} M), dihydrolipoic acid (10^{-3} M), tempol (10^{-3} M), allopurinol (3×10^{-4} M), rotenone (5×10^{-6} M), diphenylene iodonium (DPI; 10^{-4} M), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 10^{-6} M), thenoyltrifluoroacetone (TTFA; 10×10^{-6} M) or vehicle. Vessels from non-diabetic, control rats were also pre-incubated for 1 h in vehicle prior to examining vascular reactivity responsiveness to acetylcholine.

Detection of Superoxide

Hydroethidine (Molecular Probes Inc., Eugene, OR), an oxidative fluorescent dye, was used to evaluate *in situ* levels of superoxide (O_2^-) as described previously.[12–14] Hydroethidine is permeable to cells and in the presence of O_2^- is oxidized to fluorescent ethidium bromide, where it is trapped by intercalating with DNA. This method provides sensitive detection of O_2^- *in situ*. Unfixed frozen sections of epineurial arterioles of the sciatic nerve from control and diabetic rats were cut into 5-μm-thick sections and placed on glass slides. These ring segments were then incubated for 1 h at 37°C in saline containing the same compounds and concentration presented above. Afterwards, hydroethidine (2×10^{-6} M) was topically applied to each tissue section and cover slipped. Slides were then incubated in a light protected humidified chamber at 37°C for 30 min. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Fluorescence was detected with a 585-nm long pass filter. Tissue from control and diabetic rats were processed and imaged in parallel. Laser settings were identical for acquisition of all images from control and diabetic specimens.

Data Analysis

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.[12–14] 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.

RESULTS

Vascular Reactivity

Data in Fig. 1 demonstrate the effect of acute treatment of epineurial arterioles from diabetic rats with antioxidants on acetylcholine-mediated vasodilation. Acetylcholine-mediated vasodilation in epineurial arterioles from diabetic rats is significantly decreased compared to arterioles from control animals (Fig. 1). Treating epineurial arterioles of the sciatic nerve *in vitro* with 1 mM α-lipoic acid, 1 mM tempol or 1 mM dihydrolipoic acid for 1 h significantly improved the diabetes-induced decrease in acetylcholine-mediated vasodilation compared to non-treated diabetic arterioles incubated for 1 h with vehicle alone. Treating epineurial arterioles from control or diabetic rats with vehicle (DMSO) for 1 h had no effect on acetylcholine-mediated vasodilation (data not shown). Following treatment of epineurial arterioles from diabetic rats with α-lipoic acid, tempol or dihydrolipoic acid, acetylcholine-mediated relaxation was similar to acetylcholine-mediated relaxation observed in control animals. Treating epineurial arterioles of the sciatic nerve from diabetic rats with 0.3 mM allopurinol, to inhibit xanthine oxidase, for 1 h did not improve the diabetes-induced decrease in acetylcholine-mediated vasodilation compared to

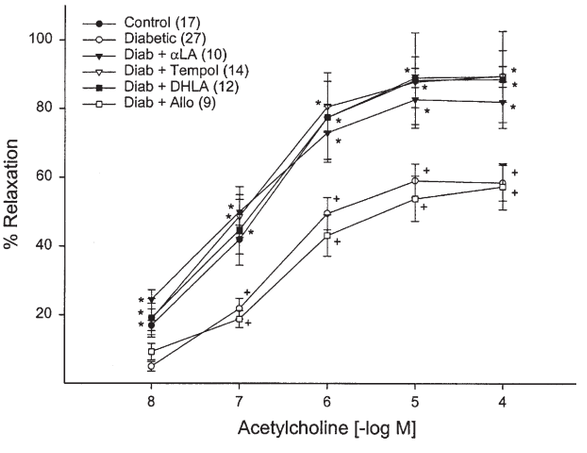

FIGURE 1 The effect of α-lipoic acid, tempol, dihydrolipoic acid or allopurinol on acetylcholine-mediated vascular relaxation of epineurial arterioles of the sciatic nerve from diabetic rats. Epineurial arterioles were collected from either control or diabetic rats. Pressurized arterioles from diabetic rats were preincubated with 1 mM α-lipoic acid (αLA), 1 mM tempol, 1 mM dihydrolipoic acid (DHLA), 0.3 mM allopurinol (Allo) or vehicle for 1 h and afterwards constricted with U46619 (30–50%). Control represents acetylcholine-mediated vasodilation in normal rats preincubated with vehicle alone. Incremental doses of acetylcholine were then added to the bathing solution while recording steady state vessel diameter. The number of experimental observations for each condition is shown in parenthesis. The + denotes that the response to acetylcholine was significantly attenuated ($p < 0.05$) compared to arterioles from control rats. The * denotes that the response to acetylcholine was significantly improved ($p < 0.05$) compared to arterioles from diabetic rats.

acetylcholine-induced vasodilation in control animals.

To further examine the possible mechanisms linking increased oxidative stress and impaired vasodilation in epineurial arterioles, vessels from diabetic rats were preincubated for 1 h with 40 μm GF 109203X, 1 mM L-arginine, 0.3 mM aminoguanidine or vehicle. Afterwards, acetylcholine-mediated vasodilation was examined. Data in Fig. 2 demonstrate that preincubating epineurial arterioles from diabetic rats with the protein kinase C inhibitor, GF 109203X, or L-arginine for 1 h significantly improved acetylcholine-mediated vasodilation compared to non-treated diabetic epineurial arterioles incubated for 1 h with vehicle alone. In contrast, preincubating epineurial arterioles with aminoguanidine for 1 h, to inhibit inducible nitric oxide synthase activity, did not significantly improve acetylcholine-mediated vasodilation compared to non-treated diabetic epineurial arterioles.

Superoxide Formation

Data in Fig. 3 illustrate the effect of these compounds on superoxide production by epineurial arterioles of the sciatic nerve. The size of the arterioles used for these studies was 100–150 μm internal diameter. Data in Fig. 3 (top and middle) demonstrate that superoxide formation is minimal in arterioles from control rats, whereas superoxide formation is increased in arterioles from diabetic rats. The increase in superoxide in epineurial arterioles from diabetic rats was observed in endothelial cells as well as in the smooth muscle and adventitial cells. Preincubating epineurial arterioles from diabetic rats *in vitro* with 1 mM α-Iipoic acid, 1 mM dihydrolipoic acid and to lesser extent 1 mM tempol for 1 h decreased superoxide formation compared to non-treated diabetic rats (Fig. 3, top). The decrease in superoxide formation was observed throughout the vessels. In contrast, preincubating epineurial arterioles from diabetic rats for 1 h with 0.3 mM allopurinol did not decrease superoxide formation (Fig. 3, top). Data in Fig. 3 (middle) demonstrate that preincubating epineurial arterioles of the sciatic nerve from diabetic rats with 40 μM GF 109203X, 1 mM L-arginine or 0.3 mM aminoguanidine did not decrease superoxide formation compared to non-treated diabetic rats.

Experiments were conducted to localize the site(s) of superoxide production by epineurial arterioles of the sciatic nerve from diabetic rats. Data in Fig. 3 (bottom) demonstrate that preincubating epineurial vessels with 100 μM DPI or 5 μM rotenone for 1 h decreased superoxide production by epineurial arterioles compared to non-treated diabetic rats. The decrease in superoxide formation was observed throughout the vessels. In contrast, preincubating epineurial arterioles with 1 μM CCCP or 10 μM TTFA for 1 h had little to no effect on decreasing superoxide production.

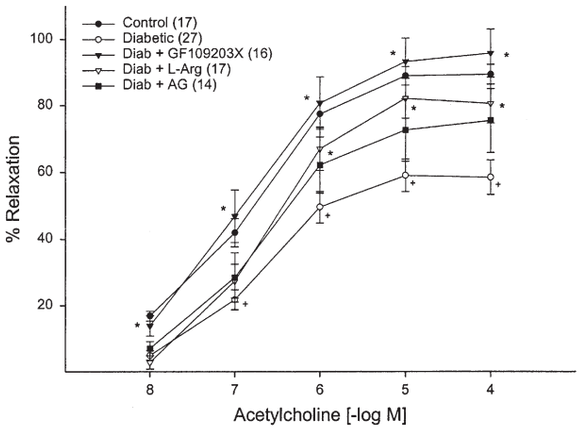

FIGURE 2 The effect of GF109203X, L-arginine, or aminoguanidine on acetylcholine-mediated vascular relaxation of epineurial arterioles of the sciatic nerve from diabetic rats. Epineurial arterioles were collected from either control or diabetic rats. Pressurized arterioles from diabetic rats were preincubated with 40 μM GF109203X, 1 mM L-arginine (L-Arg), 0.3 mM aminoguanidine (AG), or vehicle for 1 h and afterwards constricted with U46619 (30–50%). Control represents acetylcholine-mediated vasodilation in normal rats preincubated with vehicle alone. Incremental doses of acetylcholine were then added to the bathing solution while recording steady state vessel diameter. The number of experimental observations for each condition is shown in parenthesis. The + denotes that the response to acetylcholine was significantly attenuated ($p < 0.05$) compared to arterioles from control rats. The * denotes that the response to acetylcholine was significantly improved ($p < 0.05$) compared to arterioles from diabetic rats.

DISCUSSION

We have previously demonstrated that reducing superoxide formation and oxidative stress in diabetic rats by treatment with several different types of antioxidants improves vasodilation by acetylcholine in epineurial arterioles of the sciatic nerve.[13,14] In these studies we investigated the source of superoxide formation in epineurial arterioles of the sciatic nerve from diabetic rats and whether treatment of these vessels with antioxidants *in vitro* can improve acetylcholine-mediated vascular relaxation.

The recent studies clearly demonstrated that antioxidants are capable of preventing superoxide formation and reversing diabetes-induced vascular impairment *in vitro*. Dihydrolipoic acid and to a lesser extent α-lipoic acid were effective in decreasing superoxide formation and restoring acetylcholine-mediated vasodilation to arterioles from diabetic rats. α-Lipoic acid is capable of scavenging hydroxyl radicals, hypochlorous acid and singlet oxygen, but not superoxide or peroxyl radicals[18,19] α-Lipoic acid

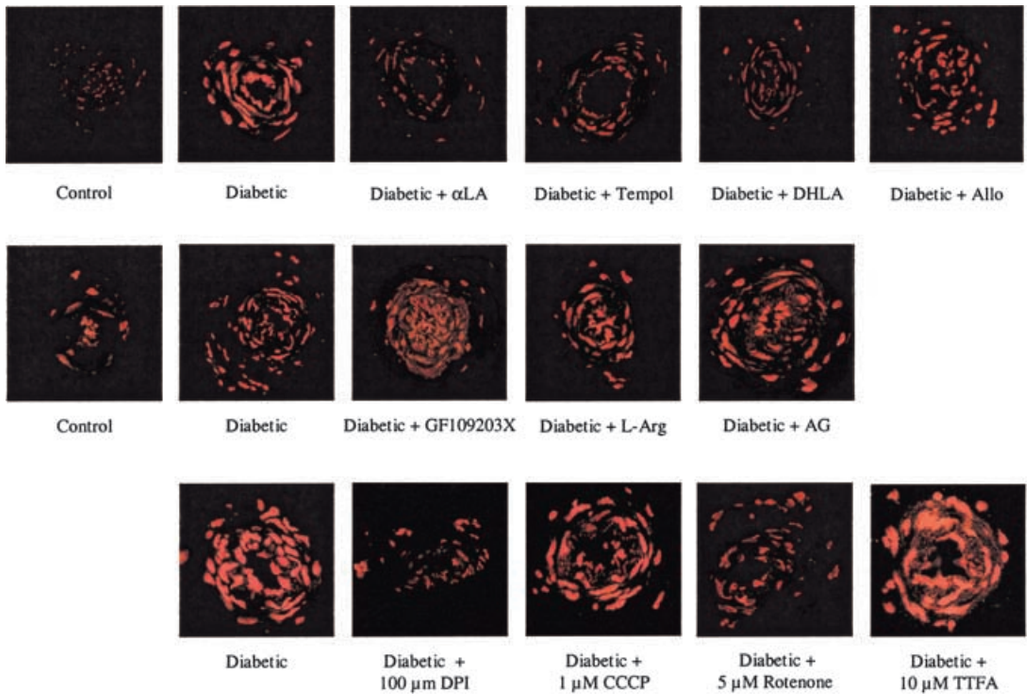

FIGURE 3 Top: The effect of α-lipoic acid, tempol, dihydrolipoic acid or allopurinol on superoxide formation by epineurial arterioles of the sciatic nerve from diabetic rats. For this experiment, epineurial arterioles of the sciatic nerve from diabetic rats were pretreated with or without, 1 mM α-lipoic acid (αLA), 1 mM tempol, 1 mM dihydrolipoic acid (DHLA), 0.3 mM allopurinol (Allo) or vehicle (diabetic) for 1 h. Middle: The effect of GF109203X, L-arginine, or aminoguanidine on superoxide formation by epineurial arterioles of the sciatic nerve from diabetic rats (middle). For this experiment, epineurial arterioles of the sciatic nerve from diabetic rats were pretreated with or without 40 μM GF109203X, 1 mM L-arginine (L-Arg), 0.3 mM aminoguanidine (AG), or vehicle (diabetic) for 1 h. Control in the top and middle sections represents epineurial arterioles from normal rats preincubated with vehicle alone. Bottom: The effect of diphenylene iodonium (DPI), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), rotenone, thenoyltrifluoroacetone (TTFA) or vehicle on superoxide formation by epineurial arterioles of the sciatic nerve from diabetic rats. Epineurial arterioles of the sciatic nerve from diabetic rats were pretreated with 100 çM DPI, 1 μM CCCP, 5 μM rotenone, or 10 μM TTFA or vehicle (diabetic) for 1 h. Afterwards, arterioles were labeled with the hydroethidine. Shown are fluorescent photomicrographs of confocal microscopic sections of epineurial arterioles of the sciatic nerve from one representative experiment that was repeated at least three times, which showed similar results. Recordings of fluorescence were taken at identical laser and photomultiplier settings for each experiment.

is also effective at chelating transition metals. In contrast, in its reduced form as dihydrolipoic acid, it is a good scavenger of superoxide and prevents initiation of lipid peroxidation.[18,19] *In vivo* α-lipoic acid can be converted into dihydrolipoic acid.[18] In addition, both α-lipoic acid and dihydrolipoic acid can regenerate other cellular antioxidants including dehydroascorbate, ubiquinol, oxidized glutathione, and indirectly, the tocopherols.[18] The combination of these properties are likely responsible for the effectiveness of α-lipoic acid and dihydrolipoic acid in decreasing superoxide formation. Tempol, a superoxide dismutase mimetic, also reversed the diabetes-induced impairment of acetylcholine-mediated vasodilation and increased superoxide formation in epineurial arterioles.[20] This is in agreement with other studies, which demonstrated that tempol or M40403, another superoxide dismutase mimetic, restores diabetes-induced endothelial dysfunction.[14,20,21] The decrease in superoxide formation by α-lipoic acid, dihydrolipoic acid or tempol and the reversal of the diabetes-induced impairment in vasodilation, suggests that the increased formation of superoxide and perhaps scavenging of nitric oxide is responsible for the reduced vascular response to acetylcholine in epineurial arterioles from diabetic rats. This is supported by our previous studies demonstrating the formation of peroxynitrite by epineurial arterioles of the sciatic nerve from diabetic rats and prevention with treatment by antioxidants *in vivo*.[13,14] This is further supported by studies demonstrating that pretreatment with L-arginine *in vitro* improves acetylcholine-mediated vasodilation in epineurial arterioles from diabetic rats without decreasing the formation of superoxide by these vessels. Acute pretreatment with L-arginine of aortic rings from diabetic rats as well as L-arginine treatment of diabetic animal models and humans has led to the suggestion that reduced availability of nitric oxide during periods of hyperglycemia may by responsible for impaired vascular relaxation.[22–26] This may be due to a limitation in arginine as a substrate for nitric oxide synthase in diabetes or an increase in scavenging of nitric oxide by superoxide.[13,14,27] Our studies would support the latter conclusion.

We have demonstrated increased superoxide and peroxynitrite formation in epineurial arterioles of diabetic rats and impairment in endothelium-dependent vascular relaxation that is prevented by antioxidant treatment.[13,14] Moreover, these studies demonstrate prevention of superoxide formation *in vitro* also improves endothelium-dependent vascular relaxation in epineurial arterioles of diabetic rats.

Like many chronic diseases, diabetes is widely believed to involve increased oxidative stress.[28,29] In diabetes an increase in oxidative stress arises due to a compromise in the natural antioxidant mechanisms and an increase in oxygen free radical production.[13,14,29] In these studies we investigated the possible sources of superoxide formation in epineurial arterioles of diabetic rats. Increased formation of superoxide by epineurial arterioles of diabetic rats was attenuated by preincubation with rotenone but not CCCP or TTFA. Rotenone is an inhibitor of complex 1 of the mitochondrial electron transport chain, TTFA is an inhibitor of complex II and CCCP is an uncoupler of oxidative phosphorylation.[15] We are unsure why CCCP was less effective than rotenone in reducing superoxide formation by epineurial arterioles of the sciatic nerve of diabetic rats. It is possible that CCCP could not penetrate the vascular wall under the incubation conditions. Nonetheless, this study implicates complex 1 of the mitochondrial electron transport chain in the production of superoxide by epineurial arterioles of the sciatic nerve of the diabetic rat. In our studies increased formation of superoxide by epineurial arterioles from diabetic rats was also partially decreased by diphenylene iodonium (DPI). DPI has been used for many years as a NAD(P)H oxidase inhibitor.[30] Therefore, our studies would suggest that NAD(P)H oxidase may also be a source for the production of superoxide by epineurial arterioles of the diabetic rat. However, Li and Trush have reported in studies with monocytes that DPI at concentrations that inhibit NAD(P)H oxidase diminished the production of superoxide by mitochondrial respiration.[31] They found that DPI was as potent as rotenone in inhibiting the production of superoxide by the mitochondria, likely by complex 1. If the studies by Li and Trush are correct, we can not unequivocally state that NAD(P)H oxidase is a source of superoxide formation by epineurial arterioles of the sciatic nerve.

Using cultured endothelial cells Brownlee and colleagues demonstrated that increased mitochondria-derived reactive oxygen species might be responsible for hyperglycemia-induced activation of protein kinase C, formation of advanced glycation end products, sorbitol accumulation and activation of NF-κB.[15] Thereby, linking three of the four pathways thought to be involved in hyperglycemia-induced diabetic complications.[16] Our studies also demonstrate that the activation of protein kinase C may be involved in impairment of vascular relaxation in epineurial arterioles of the sciatic nerve from diabetic rats. Preincubating epineurial arterioles with GF109203X, a specific protein kinase C inhibitor, reversed the impairment in acetylcholine-mediated vasodilation caused by diabetes without affecting superoxide production by these vessels. Thereby, suggesting that activation of protein kinase C is downstream of superoxide formation in epineurial arterioles of the sciatic nerve. Inoguchi *et al.* have demonstrated that activation of protein kinase C is responsible for activation of NAD(P)H oxidase, another possible source of reactive oxygen species generation.[32] However, in our studies, unlike those by Inoguchi, which were conducted with cultured vascular cells exposed to 400 mg/dl glucose for 72 h, preincubation with GF109203X alone did not prevent the formation of superoxide.[32] Our results conducted with intact vessels from diabetic rats *in vitro* suggests that activation of NAD(P)H oxidase by protein kinase C is not directly responsible for increased formation of superoxide, but rather protein kinase C activation is downstream of superoxide formation and may be responsible in part for regulation of vascular reactivity. These studies support the studies of Brownlee and colleagues and others indicating that activation of protein kinase C via reactive oxygen species are partially responsible for diabetes related vascular dysfunction.[15,33,34]

Other potential sources for superoxide formation are increased activity of nitric oxide synthase and xanthine oxidase.[35,36] Our studies demonstrated that neither aminoguanidine nor allopurinol were capable of preventing superoxide formation or impairment of acetylcholine-mediated vascular relaxation by epineurial arterioles of the sciatic nerve *in vitro*. One of the reported effects of aminoguanidine is inhibition of inducible nitric oxide synthase (iNOS) and allopurinol is an inhibitor of xanthine oxidase.[35,36] Unpublished studies from our laboratory have shown that the expression of endothelial nitric oxide synthase (eNOS) or iNOS in endoneurial arterioles of the sciatic nerve is unchanged by diabetes. Combined these studies suggest that it is unlikely that increased activity of iNOS contributes to the increased formation of superoxide by endoneurial arterioles of the sciatic nerve from diabetic rats. Desco *et al.* demonstrated that aortic rings from diabetic rabbits produced superoxide in the presence of xanthine, which was completely inhibited by allopurinol, thereby decreasing oxidative stress.[37] These authors also demonstrated that xanthine oxidase is increased in plasma and liver of diabetic rats.[37] Xanthine oxidase is shed by

the liver into the plasma and is bound to vascular endothelial cells. Besides allopurinol, treating aortic rings from diabetic rabbits with heparin, which releases xanthine oxidase from the endothelial cell surface, inhibits the diabetes-induced formation of superoxide.[37] It is unknown to what extent xanthine oxidase may bind to the endothelium of resistance vessels. Because of the smaller surface area of the lumen of these vessels compared to the aorta the contribution by xanthine oxidase to superoxide formation by resistance vessels may be minimal.

In summary, these studies demonstrate that increased superoxide formation by endoneurial arterioles of the sciatic nerve from diabetic rats is responsible for decreasing endothelium-dependent vascular relaxation perhaps by decreasing nitric oxide availability. The likely source of increased superoxide production by epineurial arterioles from diabetic rats is complex 1 of the mitochondrial oxidative phosphorylation pathway and perhaps NAD(P)H oxidase activity.

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