

# Effect of Antioxidant Treatment of Streptozotocin-Induced Diabetic Rats on Endoneurial Blood Flow, Motor Nerve Conduction Velocity, and Vascular Reactivity of Epineurial Arterioles of the Sciatic Nerve

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We have shown that diabetes-induced reduction in endoneurial blood flow (EBF) and impaired endothelium-dependent vascular relaxation precede slowing of motor nerve conduction velocity (MNCV) and decreased sciatic nerve  $\text{Na}^+/\text{K}^+$  ATPase activity. Furthermore, vascular dysfunction was accompanied by an accumulation of superoxide in arterioles that provide circulation to the sciatic nerve. In the present study, we examined the effect that treatment of streptozotocin-induced diabetic rats with antioxidants has on vascular and neural function. Diabetic rats were treated with 0.5%  $\alpha$ -lipoic acid as a diet supplement or with hydroxyethyl starch deferoxamine (HES-DFO) by weekly intravenous injections at a dose of 75 mg/kg. The treatments significantly improved diabetes-induced decrease in EBF, acetylcholine-mediated vascular relaxation in arterioles that provide circulation to the region of the sciatic nerve, and MNCV. The treatments also reduced the production of superoxide by the aorta and superoxide and peroxynitrite by arterioles that provide circulation to the region of the sciatic nerve. Treating diabetic rats with  $\alpha$ -lipoic acid prevented the diabetes-induced increase in thiobarbituric acid-reactive substances in serum and significantly improved lens glutathione levels. In contrast, treating diabetic rats with HES-DFO did not prevent diabetes-induced changes of either of these markers of oxidative stress. Diabetes-induced increase in sciatic nerve conjugated diene levels was not improved by treatment with either  $\alpha$ -lipoic acid or HES-DFO. Treating diabetic rats with  $\alpha$ -lipoic acid but not HES-DFO partially improved sciatic nerve  $\text{Na}^+/\text{K}^+$  ATPase activity and *myo*-inositol content. The increase in sciatic nerve sorbitol levels in diabetic rats was unchanged by either treatment. These studies suggest that diabetes-induced oxidative stress and the generation of superoxide may be partially responsible for the development of diabetic

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Oxidative stress has been defined as a disturbance in the balance between the production of reactive oxygen species—oxygen-free radicals, i.e., hydroxyl radical ( $\text{OH}^\bullet$ ), superoxide anion ( $\text{O}_2^{\bullet-}$ ), and  $\text{H}_2\text{O}_2$ —and antioxidant defenses, which may lead to tissue injury (1). Oxidative stress and the damage that it causes have been implicated in a wide variety of natural and pathological processes, including aging, cancer, diabetes, atherosclerosis, neurological degeneration, schizophrenia, and autoimmune disorders, such as arthritis (2). Oxidative stress can be derived from a variety of sources and includes events such as the production of reactive oxygen species by mitochondrial oxidative phosphorylation, ionizing radiation exposure, and metabolism of exogenous compounds (2). In addition to these sources of oxidative stress, a decrease in the activity of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, and catalase, may contribute to oxidative stress in some disease states (3).

There is substantial evidence that oxidative stress occurs during the course of diabetes and is believed to be a contributing factor in the development of endothelial dysfunction and vascular disease (4–9). Studies using several types of diabetic rodent models have demonstrated impaired endothelium-dependent relaxation in both conduit and resistance arteries (4). Various factors have been proposed to contribute to this defect, including increased release of an endothelium-derived constricting factor, increased protein kinase C activity, inhibition of  $\text{Na}^+/\text{K}^+$  ATPase activity, a deficiency of substrate or cofactors for nitric oxide synthase, increased quenching of nitric oxide by advanced glycosylation end products, decreased nitric oxide release and decreased nitric oxide availability caused by increased quenching by superoxide, and subsequent formation of peroxynitrite (4). Studies showing that treatment with antioxidants prevents diabetes and hyperglycemia-induced impairment of endothelium-dependent relaxation suggest that oxidative stress is a major factor in the development of diabetic vascular

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EBF, endoneurial blood flow; EDHF, endothelium-derived hyperpolarizing factor; GSH, glutathione; HES-DFO, hydroxyethyl starch deferoxamine; MNCV, motor nerve conduction velocity; PSS, physiological saline solution; RLU, relative light unit; TBARS, thiobarbituric acid-reactive substance.

disease (4,10–23). In addition, 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 (13,15,22,24–26). Previously, we showed 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 levels in these vessels (27,28). These changes preceded the slowing of motor nerve conduction velocity (MNCV) and the reduction in  $\text{Na}^+/\text{K}^+$  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.

In the present study, we sought to determine the role of diabetes-induced oxidative stress on vascular function in arterioles that provide circulation to the region of the sciatic nerve as well as the relationship to EBF and nerve activity, as determined by measuring nerve conduction velocity. In these studies, we demonstrate that treating streptozotocin-induced diabetic rats with  $\alpha$ -lipoic acid or hydroxyethyl starch deferoxamine (HES-DFO) prevents the impairment of vascular function and the accumulation of superoxide and peroxynitrite induced by diabetes in arterioles that provide circulation to the region of the sciatic nerve and it also prevents the reduction in EBF and the slowing of MNCV.

## RESEARCH DESIGN AND METHODS

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

### Methods

**Animals.** Male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) 8–9 weeks old were used for these studies. The animals were housed in a certified animal care facility, and food (meal form, Harlan Teklad #7001, Madison, WI) and water were provided ad libitum. All institutional and National Institutes of Health 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 mol/l 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, Milpitas, CA). Rats with a blood glucose level  $\geq 300$  mg/dl (16.7 mmol/l) were considered to be diabetic. All studies were conducted ~3–4 weeks after the verification of diabetes. Rats receiving  $\alpha$ -lipoic acid were fed a diet supplemented with 0.5%  $\alpha$ -lipoic acid. On the basis of the rats' average daily consumption of diet, the daily dose of  $\alpha$ -lipoic acid was  $\sim 350$  mg  $\cdot$  kg  $\text{rat}^{-1} \cdot \text{day}^{-1}$ . This dose and method of treatment have been shown to prevent endothelial and neurogenic defects in the corpus cavernosum of diabetic rats (15). Diets were prepared by thoroughly mixing 0.5% of  $\alpha$ -lipoic acid in the meal diet. Control rats fed a diet containing 0.5%  $\alpha$ -lipoic acid or treated with HES-DFO gained weight similarly to control rats fed a nonsupplemented diet. Other evaluations, including nerve blood flow, markers for oxidative stress, MNCV, sciatic nerve  $\text{Na}^+/\text{K}^+$  ATPase activity, and vascular reactivity (data not shown), showed no differences between the nontreated and treated control groups. Rats receiving HES-DFO treatment were given weekly intravenous injections of HES-DFO (tail vein) at a dose of 5 ml/kg (26 mmol/l in saline). At this dose, the rats received 75 mg  $\cdot$  kg deferoxamine $^{-1} \cdot$  week $^{-1}$ . The HES-DFO used in these studies was provided by Dr. Bo Hedlund (Biomedical Frontiers, Minneapolis, MN). The rationale for the HES-DFO treatment protocol was arrived at after consultation with Drs. Hedlund and Norman Cameron. Cameron and Cotter (29) have shown that HES-DFO treatment (twice weekly at a dose of 34 mg/kg deferoxamine) reversed the diabetes-induced decrease in EBF and MNCV. In our study, we decided to keep the weekly dose of deferoxamine about the same but reduce the number of injections to one per week. For these experiments, several studies were conducted in which control and diabetic rats were treated with weekly injections of hydroxyethyl

starch at the same concentration as in the HES-DFO solution. Analysis of these experiments revealed that hydroxyethyl starch treatment alone had no effect on any of the evaluations made in these studies (data not shown). All studies using HES-DFO treatments were conducted 3–5 days after the final injection.

On the day of the experiment, rats were anesthetized with Nembutal administered intraperitoneally (50 mg/kg; Abbott Laboratories, North Chicago, IL). After the determination of MNCV and EBF, 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 (27,30) 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 killed by exsanguination, and body temperature was lowered with topical ice. Samples of the left sciatic nerve were then taken for determination of  $\text{Na}^+/\text{K}^+$  ATPase activity, sorbitol and *myo*-inositol content, and conjugated diene level. Conjugated diene level was also determined in liver samples. The lens was collected for determination of glutathione (GSH) levels. Levels of thiobarbituric acid-reactive substances (TBARSs) in the serum and liver and the lactate-to-pyruvate ratio in the aorta were also determined. A segment of the aorta was used to determine superoxide level using lucigenin. Serum samples were also taken for determination of free fatty acids and triglycerides. Samples for blood glucose measurements were taken on the day of the experiment.

**MNCV.** MNCV was determined as previously described using a noninvasive procedure in the sciatic-posterior tibial conducting system in a temperature-controlled environment (27,31). The left sciatic nerve was stimulated first at the sciatic notch and then at the Achilles tendon. Stimulation consisted of single 0.2-ms supramaximal (8 V) pulses through a bipolar electrode (Grass S44 Stimulator; Grass Medical Instruments, Quincy, MA). The evoked potentials were recorded from the interosseous muscle with a unipolar platinum electrode and displayed on a digital storage oscilloscope (model 54600A; Hewlett-Packard, Rolling Meadows, IL). MNCV was calculated by subtracting the distal from the proximal latency (measured in milliseconds) from the stimulus artifact of the take-off of the evoked potential, and the difference was divided into the distance between the two stimulating electrodes (measured in millimeters using a Vernier caliper). The MNCV was reported in meters per second.

**EBF.** Immediately after determination of MNCV, sciatic nerve endoneurial nutritive blood flow was determined as described by Cameron et al. (32,33). The trachea was intubated for mechanical ventilation and a carotid cannula inserted to monitor mean arterial blood pressure. Core temperature was monitored using a rectal probe and temperature regulated between 36 and 37°C using a heating pad and radiant heat. The right sciatic nerve was carefully exposed by a small surgical incision and the surrounding skin sutured to a plastic ring. The isolated area was filled with mineral oil at 37°C to a depth of 1 cm to minimize diffusion of hydrogen gas from the nerve. The rats were then mechanically ventilated. A glass-insulated platinum microelectrode (tip = 2  $\mu\text{m}$ ) was inserted into the sciatic nerve, proximal to the trifurcation, and polarized at 0.25 V with respect to a reference electrode inserted subcutaneously into the flank of the rat. Once the recording had stabilized, the inspired air was modified to contain 10% hydrogen gas, and this gas flow continued until the hydrogen current recorded by the electrode had stabilized, indicating equilibrium of the inspired air with arterial blood. The hydrogen gas supply was then discontinued and the hydrogen clearance curve recorded until a baseline was achieved. The hydrogen clearance data were fitted by computer to a mono- or biexponential curve using commercial software (Prism; GraphPad, San Diego, CA); vascular conductance (milliliter per minute per 100 g per millimeter Hg) was determined by dividing nutritive blood flow by the average mean arterial blood pressure (34); and nutritive blood flow (milliliter per minute per 100 g) was calculated using the equation described by Young in 1980 (35). Two recordings were made for each rat at different locations along the nerve, and the final blood flow value was averaged.

**Vascular reactivity.** 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 (27,28). The vessels used for these studies were generally oriented longitudinally in relation to the sciatic nerve; however, radially oriented vessels were also used on occasion. No differences were observed in acetylcholine-induced vasodilation based on the orientation of the vessel to the sciatic nerve. The arterioles used in this study should be regarded as epineurial rather than perineurial vessels. To isolate these vessels, the common iliac was exposed, and the branch points of the internal pudendal and superior gluteal arteries were identified. The vessels were then clamped, and tissue containing these vessels and the branches at the internal pudendal and superior gluteal arteries were dissected en bloc. The block of tissue was

immediately submerged in a cooled (4°C), oxygenated (20% O<sub>2</sub>, 5% CO<sub>2</sub>, and 75% N<sub>2</sub>) Krebs-Henseleit physiological saline solution (PSS) of the following composition (in millimoles per liter): NaCl 118, KCl 4.7, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 20, Na<sub>2</sub>EDTA 0.026, and glucose 5.5. Branches of the superior gluteal and internal pudendal arteries (50- to 150- $\mu$ m 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, Cornelia, GA). The pipettes were attached to a single pressure reservoir (initially set at 0 mmHg) 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 closed-circuit television camera (WV-BL200; Panasonic, Secaucus, NJ), a video monitor (Panasonic), and a video caliper (VIA-100K; Boeckeler Instruments, Tucson, AZ). The organ chamber was connected to a rotary pump (Masterflex; Cole Parmer Instrument, 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 mmHg. At this pressure, we found that KCl gave the maximal constrictor response. Therefore, all of the studies were conducted at 40 mmHg. Internal vessel diameter (resolution of 2  $\mu$ m) was measured by manually adjusting the video micrometer. After a 30-min equilibration, KCl was added to the bath to test vessel viability. Vessels failing to constrict >30% were discarded. After they were washed with PSS, vessels were incubated for 30 min in PSS and then constricted with U46619 (10<sup>-8</sup> to 10<sup>-7</sup> mol/l) (Cayman Chemical, Ann Arbor, MI) to 30-50% of passive diameter. There was no significant difference in the amount of U46619 required to induce constriction in control and diabetic vessels. Cumulative concentration-response relationships were evaluated for acetylcholine (10<sup>-8</sup> to 10<sup>-4</sup> mol/l) using vessels from control and diabetic rats. At the end of each acetylcholine dose response determination, sodium nitroprusside (10<sup>-4</sup> mol/l) was added to determine maximal vasodilation. In a separate set of experiments, the same arterioles were isolated to determine superoxide and peroxynitrite levels using hydroethidine and 3-nitrotyrosine immunoreactivity, respectively, as previously described (35).

**Detection of superoxide and peroxynitrite.** Hydroethidine (Molecular Probes, Eugene, OR), an oxidative fluorescent dye, was used to evaluate in situ levels of superoxide (O<sub>2</sub><sup>-</sup>) as described previously (28,35). Hydroethidine is permeable to cells, and in the presence of O<sub>2</sub><sup>-</sup> it is oxidized to fluorescent ethidium bromide, where it is trapped by intercalating with DNA. This method provides sensitive detection of O<sub>2</sub><sup>-</sup> in situ. Unfixed frozen ring segments were cut into 5  $\mu$ m-thick sections and placed on glass slides. Hydroethidine (2  $\times$  10<sup>-6</sup> mol/l) was topically applied to each tissue section and coverslipped. Slides were 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 rats and untreated and treated diabetic rats was processed and imaged in parallel. Laser settings were identical for acquisition of all images from control and diabetic specimens. It has been reported that desferrioxamine at high concentrations in vitro can react with superoxide to form a nitroxide free radical (36,37). To determine whether HES-DFO acutely reacts with superoxide produced by vessels, we preincubated epineurial vessels from a diabetic rat with 1 mmol/l, 100  $\mu$ mol/l, or 10  $\mu$ mol/l HES-DFO or hydroxyethyl starch alone for 30 min. Afterward, superoxide levels were determined as described previously.

Superoxide levels were also measured using the aorta by lucigenin-enhanced chemiluminescence, as described previously (35). Vessel segments were incubated in 0.5 ml phosphate-buffered saline containing lucigenin (5  $\mu$ mol/l); afterward, relative light units (RLUs) were measured using a Zylux FB12 luminometer. For these studies, chemiluminescence was measured for 5 min. Background activity was determined and subtracted, and RLUs were normalized to surface area.

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 (27). 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 (38). 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 (39,40). 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 the immunoreactivity of 3-nitrotyrosine—a stable biomarker of tissue peroxynitrite formation—using a commercial kit from Vector Laboratories (Burlingame, CA). Briefly, frozen tissue segments of arterioles were cut into 5- $\mu$ m sections and air dried. Sections were then incubated for 20 min with blocking

serum at room temperature; afterward, the blocking solution was removed, and the sections were blotted. Primary antiserum was added, and the sections were incubated for 30 min. Sections were then rinsed and incubated with Vectastain ABC-AP reagent for 30 min. After washing, the sections were incubated for 10 min in alkaline phosphatase substrate solution. Sections were then rinsed, fixed, counterstained, and mounted. Mounted tissue sections were visually analyzed for staining of 3-nitrotyrosine using a Zeiss light microscope equipped with a 35-mm camera.

**Sciatic nerve sorbitol and myo-inositol content.** The left sciatic nerve was removed, desheathed, and divided into three samples for determination of Na<sup>+</sup>/K<sup>+</sup> ATPase activity, conjugated diene levels, and sorbitol and myo-inositol content (28). Each nerve sample was weighed to standardize the data. For the determination of sorbitol and myo-inositol content, tissue samples were boiled for 10 min in water containing  $\alpha$ -methylmannopyranoside as an internal standard and deproteinized with 0.5 ml each of 0.19 mol/l Ba(OH)<sub>2</sub> and 0.19 mol/l ZnSO<sub>4</sub>. After centrifugation, the supernatant was collected, frozen, and lyophilized. Afterward, the samples were derivatized, and intracellular content of sorbitol and myo-inositol was determined by gas-liquid chromatography as previously described (27,31).

**Na<sup>+</sup>/K<sup>+</sup> ATPase activity.** Total and ouabain-inhibited Na<sup>+</sup>/K<sup>+</sup> ATPase activities were measured in crude homogenates of sciatic nerve (27,31). Sciatic nerves were homogenized in a glass homogenizer at 4°C in 1 ml of 0.2 mol/l sucrose and 0.02 mol/l Tris-HCl buffer, pH 7.5. The samples were then centrifuged at 100g for 10 min at 4°C. An aliquot of the supernatant (50  $\mu$ l) was added to two cuvettes containing 100 mmol/l NaCl, 10 mmol/l KCl, 2.5 mmol/l MgCl<sub>2</sub>, 2 mmol/l EGTA, 1 mmol/l Tris-ATP, 1 mmol/l 3-(cyclohexylammonium) phosphoenolpyruvate (Boehringer-Mannheim, Indianapolis, IN), 30 mmol/l imidazole-HCl buffer (pH 7.3), 0.15 mmol/l NADH, 50  $\mu$ g lactate dehydrogenase (Boehringer-Mannheim), and 30  $\mu$ g pyruvate kinase (Boehringer-Mannheim) with or without 1 mmol/l ouabain to inhibit the ouabain-sensitive Na<sup>+</sup>/K<sup>+</sup> ATPase fraction. After a 20-min stabilization period, the oxidation of NADH was recorded over a 30-min period. The activity was expressed as micromoles of ADP per gram of wet weight per hour. Each assay was conducted in triplicate.

**Additional biological parameters.** Lactate-to-pyruvate ratios for the aorta were determined using perchloric acid extracts and high-performance liquid chromatography as previously described (41). Lens glutathione, serum and liver TBARSs, and sciatic nerve and liver conjugated diene levels were determined as additional markers of oxidative stress. Lens GSH levels were determined according to Lou et al. (42). Lenses were weighed and homogenized in 1 ml of cold 10% trichloroacetic acid and centrifuged for 15 min at 1,000g. The supernatant (100  $\mu$ l) was mixed with 0.89 ml of 1.0 mol/l Tris, pH 8.2, and 0.02 mol/l EDTA. Afterward, 10  $\mu$ l dithionitrobenzene was added and change in absorbance measured at 412 nm. A GSH standard curve (100-500 ng) was performed for each assay. The data were recorded as micrograms per milligram of wet weight. TBARS levels in serum and liver were determined by the method of Mihara et al. (43) as modified by Siman and Eriksson (44). Briefly, 200  $\mu$ l serum was boiled in 0.75 ml phosphoric acid (0.19 mol/l), 0.25 ml thiobarbituric acid (0.42 mmol/l), and 0.3 ml water for 60 min. For liver, the biopsy was homogenized in distilled water and adjusted for weight to 200 mg/ml. We used 200  $\mu$ l of the homogenate for the assay. Afterward, the serum and liver 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 were reported as micrograms per milliliter of serum and nanograms per milligram of wet weight for liver. Sciatic nerve and liver conjugated diene levels were determined according to the method of Recknagel and Ghoshal (45) and Low and Nickander (46). Briefly, a segment of the sciatic nerve or biopsy of liver 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  $\times$  10<sup>4</sup> mol/l was used to determine the amount of conjugated diene present. The data were reported as micromoles per milligram of wet weight. Serum free fatty acid and triglyceride levels were determined using commercial kits from Roche Diagnostics (Mannheim, Germany) and Sigma Chemical, respectively.

**Data analysis.** The results are presented as means  $\pm$  SE. Comparisons between the groups for MNCV, EBF, sciatic nerve Na<sup>+</sup>/K<sup>+</sup> ATPase activity, sciatic nerve sorbitol and myo-inositol content, serum and liver TBARSs, sciatic nerve and liver conjugated diene, serum free fatty acid and triglyceride, aorta lactate-to-pyruvate ratio, and lens GSH 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 (27,28). Whenever significant interactions were noted,

TABLE 1  
Change in body weight and blood glucose levels

Animal	Change in body weight (g)	Blood glucose mg/dl
Control ( <i>n</i> = 9)	119 ± 11	97 ± 3
Diabetic ( <i>n</i> = 8)	4 ± 8*	424 ± 26*
Diabetic + HES-DFO ( <i>n</i> = 8)	17 ± 11*	450 ± 22*
Diabetic + α-lipoic acid ( <i>n</i> = 11)	1 ± 11*	373 ± 15*

Data are means ± SE. \**P* < 0.05 vs. control.

specific treatment dose effects were analyzed using a Bonferroni adjustment. A *P* value of <0.05 was considered significant. All computations were performed using SAS version 6.12 for Windows.

RESULTS

**Body weight and plasma glucose levels.** Data in Table 1 show that streptozotocin-induced diabetic rats on average gained less weight than age-matched control rats over the 3- to 4-week experimental period of this study. At the time of experimentation, plasma glucose levels were increased three- to fourfold in diabetic rats compared with control rats. Treating diabetic rats with either HES-DFO or α-lipoic acid had no significant effect on weight gain over the 3- to 4-week experimental period (data not shown). Treating diabetic rats with HES-DFO did not significantly affect blood glucose levels compared with untreated diabetic rats (data not shown). The 15% decrease in blood glucose in diabetic rats treated with α-lipoic acid was not significant. α-Lipoic acid has been reported to increase glucose transport (47). This finding may explain the small decrease in blood glucose level in diabetic rats treated with α-lipoic acid. The blood glucose level of control rats treated with α-lipoic acid was 93 ± 6 mg/dl (*n* = 3), which was not significantly different than the blood glucose level in untreated controls.

**Sciatic nerve Na<sup>+</sup>/K<sup>+</sup> ATPase activity and sorbitol and myo-inositol content.** Data in Fig. 1 demonstrate that diabetes causes a significant decrease in sciatic nerve Na<sup>+</sup>/K<sup>+</sup> ATPase activity. Treating diabetic rats with HES-DFO did not prevent the decrease in Na<sup>+</sup>/K<sup>+</sup> ATPase activity. In contrast, treating diabetic rats with α-lipoic acid partially prevented the diabetes-induced decrease in sciatic nerve Na<sup>+</sup>/K<sup>+</sup> ATPase activity. The change in sciatic nerve Na<sup>+</sup>/K<sup>+</sup> ATPase activity for diabetic rats treated with α-lipoic acid was not significantly different from control rats, nor was it significantly different from untreated diabetic rats. Data in Fig. 2 demonstrate that diabetes causes a significant increase in the sorbitol content in the sciatic nerve, and this result was not statistically improved by treating diabetic rats with either HES-DFO or α-lipoic acid. Data in Fig. 2 also demonstrate that diabetes causes a significant decrease in the myo-inositol content in the sciatic nerve. Treating diabetic rats with HES-DFO did not prevent the decrease in sciatic nerve myo-inositol content. In contrast, treating diabetic rats with α-lipoic acid partially but significantly improved the myo-inositol content in the sciatic nerve compared with untreated diabetic rats. However, treatment of diabetic rats with α-lipoic acid did not totally prevent the diabetes-induced decrease in the myo-inositol content of

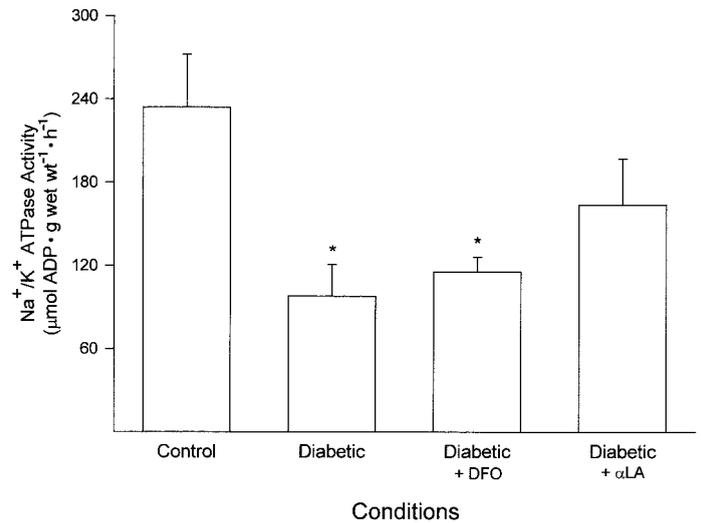


FIG. 1. Sciatic nerve Na<sup>+</sup>/K<sup>+</sup> ATPase activity. For these studies, control rats, diabetic rats, and diabetic rats treated with either HES-DFO (DFO) or α-lipoic acid (αLA) were used to determine sciatic nerve Na<sup>+</sup>/K<sup>+</sup> ATPase activity, as described in RESEARCH DESIGN AND METHODS. The duration of diabetes and treatments for these studies was 3–4 weeks. Data are presented as means ± SE for a minimum of eight separate samples. Sciatic nerve Na<sup>+</sup>/K<sup>+</sup> ATPase activity in control rats treated with HES-DFO or α-lipoic acid was 296 ± 10 (*n* = 3) and 260 ± 44 (*n* = 3) μmol ADP · g wet wt<sup>-1</sup> · h<sup>-1</sup>, respectively. \*A significant difference compared with control rats.

the sciatic nerve, which remained significantly reduced compared with control rats.

**Serum triglyceride and free fatty acid levels.** Data in Fig. 3 demonstrate that diabetes causes a significant increase in serum triglyceride and free fatty acid levels. Serum triglyceride levels were reduced in rats treated with either HES-DFO or α-lipoic acid compared with untreated diabetic rats. However, serum triglyceride levels appeared to still be elevated compared with control rats, although this difference was not significantly different because of the large standard error. Treating diabetic rats with either HES-DFO or α-lipoic acid did not prevent the increase in

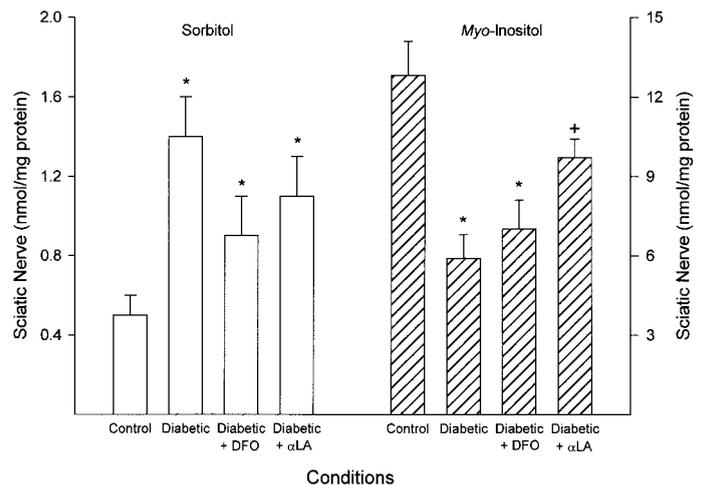


FIG. 2. Sciatic nerve sorbitol and myo-inositol content. Samples of the sciatic nerve from the animals described in Fig. 1 were used to determine the sorbitol and myo-inositol content, as described in RESEARCH DESIGN AND METHODS. Data are presented as means ± SE for a minimum of eight separate samples. \*A significant difference compared with control rats; +a significant difference compared with untreated diabetic rats.

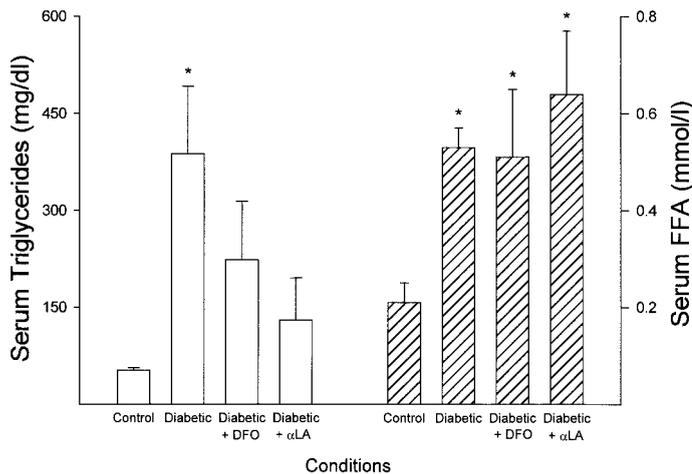


FIG. 3. Serum triglyceride and free fatty acid (FFA) levels. Serum collected from animals described in Fig. 1 was used to determine the triglyceride and free fatty acid levels, as described in RESEARCH DESIGN AND METHODS. Data are presented as means  $\pm$  SE for a minimum of eight separate samples. \*A significant difference compared with control.

serum free fatty acid levels. We also determined lactate-to-pyruvate ratios in the liver and found no significant differences between control and diabetic treated or untreated animals (data not shown).

**Evaluation of oxidative stress.** To assess the effect of diabetes and its treatment with either HES-DFO or  $\alpha$ -lipoic acid on oxidative stress, we examined three markers of oxidative stress in several different tissues. By doing so, we hoped to get a more complete understanding of the oxidative stress status of the rats used in these studies. Data in Fig. 4 demonstrate that diabetes causes an increase in TBARSs in both the liver and serum. Treating diabetic rats with HES-DFO did not significantly reduce the increase in liver or serum TBARS level caused by diabetes. However, treating diabetic rats with  $\alpha$ -lipoic acid did prevent the diabetes-induced increase in serum TBARS level and significantly decreased liver TBARS level com-

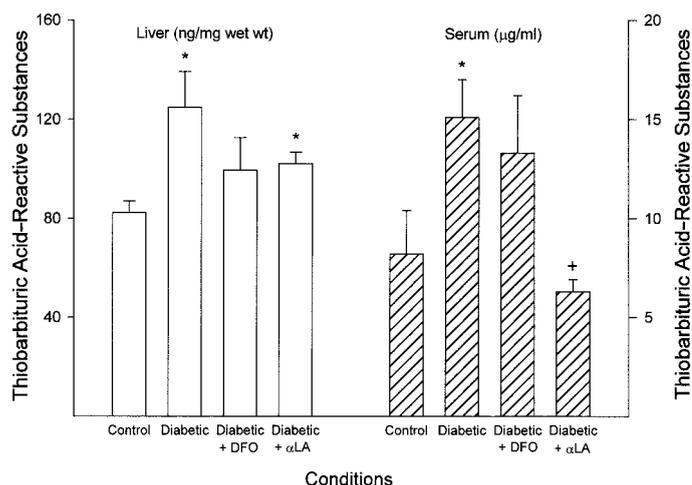


FIG. 4. Liver and serum TBARSs. Liver and serum samples were collected from the animals described in Fig. 1 and used to determine TBARS level as described in RESEARCH DESIGN AND METHODS. Serum TBARSs in control rats treated with HES-DFO or  $\alpha$ -lipoic acid were  $4.8 \pm 0.6$  ( $n = 3$ ) and  $5.6 \pm 1.2$  ( $n = 3$ )  $\mu$ g/ml, respectively. Data are presented as means  $\pm$  SE for a minimum of eight separate samples. \*, A significant difference compared with control rats; +, a significant difference compared with untreated diabetic rats.

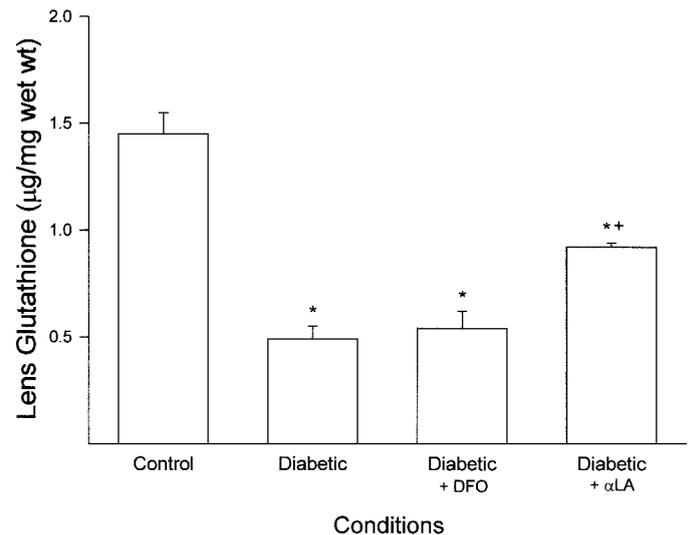


FIG. 5. Lens GSH level. The lens was collected from the animals described in Fig. 1 and used to determine GSH level as described in RESEARCH DESIGN AND METHODS. Data are presented as means  $\pm$  SE for a minimum of eight separate samples. Lens GSH level in control rats treated with HES-DFO or  $\alpha$ -lipoic acid was  $1.4 \pm 0.2$  ( $n = 3$ ) and  $1.6 \pm 0.3$  ( $n = 3$ )  $\mu$ g/mg wet wt, respectively. \*, A significant difference compared with control; +, a significant difference compared with untreated diabetic rats.

pared with untreated diabetic rats. Lens GSH level was significantly decreased in streptozotocin-induced diabetic rats compared with control rats (Fig. 5). Treating diabetic rats with HES-DFO did not significantly improve the decrease in lens GSH level compared with untreated diabetic rats. However, treating diabetic rats with  $\alpha$ -lipoic acid did significantly improve lens GSH level by  $\sim 50\%$ . Conjugated diene content of the liver was not significantly changed by diabetes in these studies (Fig. 6). However, conjugated diene level in the sciatic nerve was significantly increased by diabetes, and the increase was not significantly altered by treating diabetic rats with either HES-DFO or  $\alpha$ -lipoic acid.

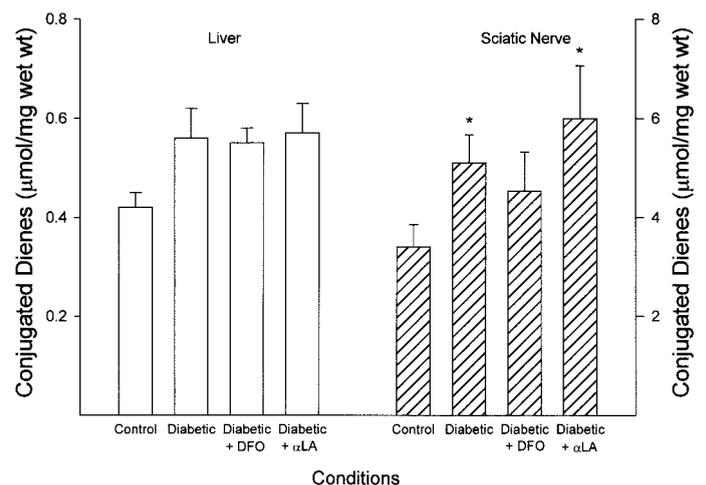


FIG. 6. Liver and sciatic nerve conjugated diene level. Samples of the liver and sciatic nerve were collected from the animals described in Fig. 1 and used to determine conjugated diene level as described in RESEARCH DESIGN AND METHODS. Data are presented as means  $\pm$  SE for a minimum of eight separate samples. Sciatic nerve conjugated diene level in control rats treated with HES-DFO or  $\alpha$ -lipoic acid was  $2.7 \pm 0.4$  ( $n = 3$ ) and  $3.1 \pm 1.5$  ( $n = 3$ )  $\mu$ mol/mg wet wt, respectively. \*, A significant difference compared with control rats.

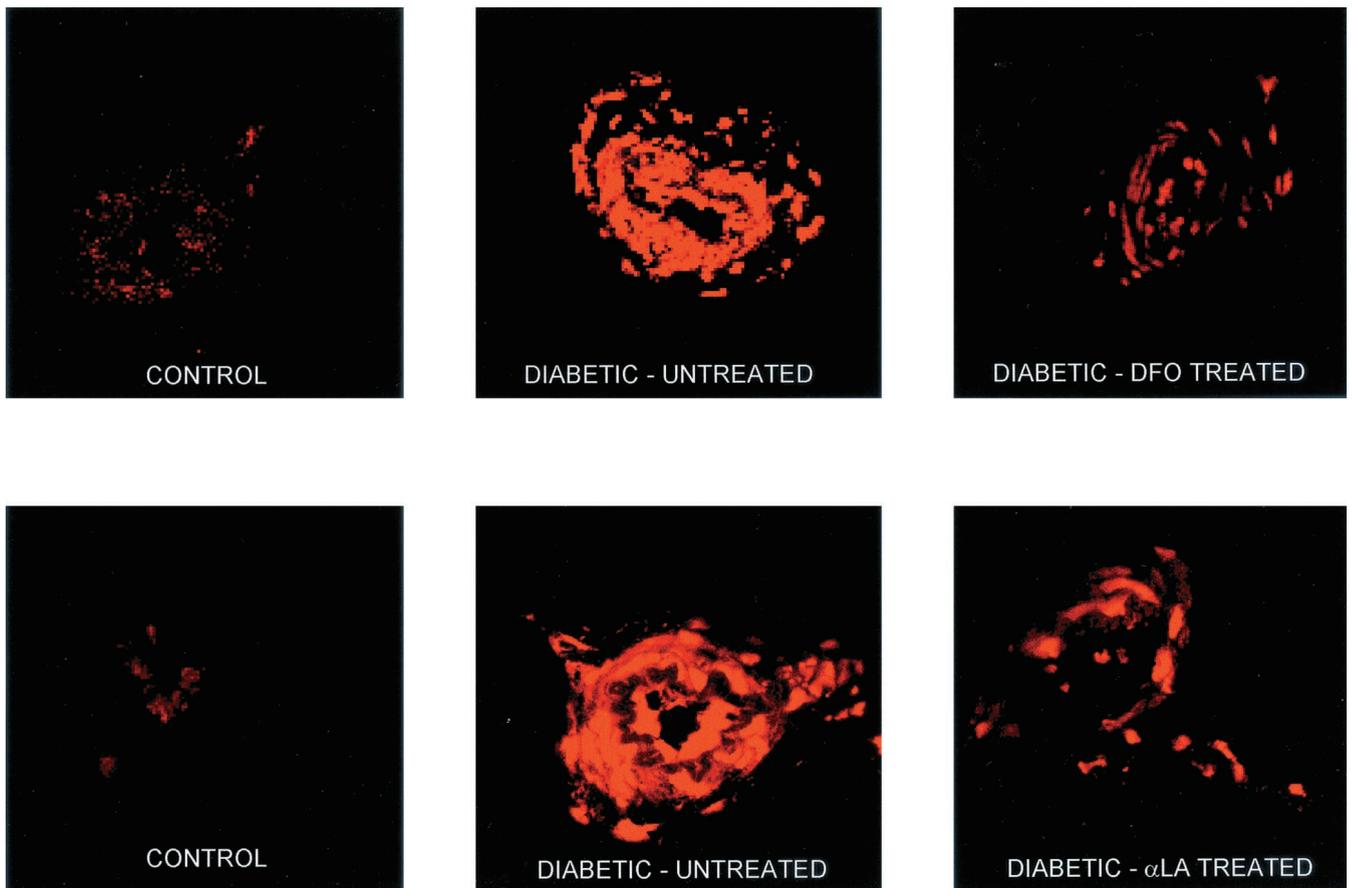


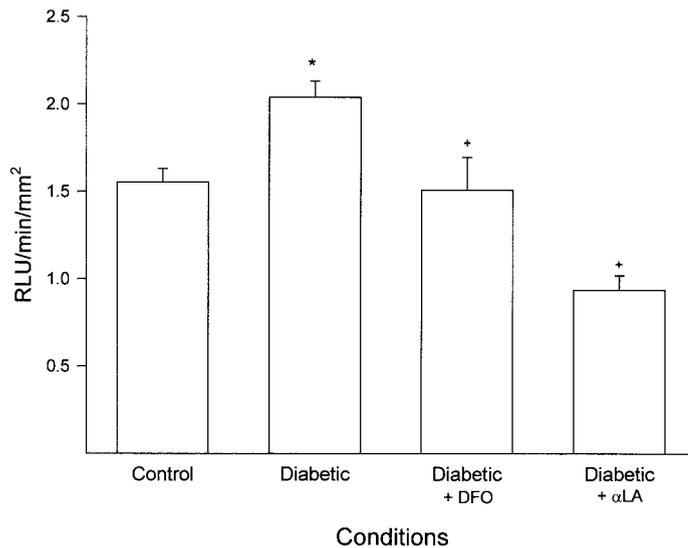
FIG. 7. Detection of superoxide level in arterioles from control rats, diabetic rats, and diabetic rats treated with either HES-DFO (DFO) or  $\alpha$ -lipoic acid ( $\alpha$ LA). The duration of diabetes and treatments for these studies was 3–4 weeks. Fluorescent photomicrographs of confocal microscopic 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 as described in RESEARCH DESIGN AND METHODS. Recordings of fluorescence were taken at identical laser and photomultiplier settings for both control rats 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.

We previously reported that diabetes causes an increase in superoxide level in arterioles that provide circulation to the region of the sciatic nerve (28). The increase in superoxide level was observed in endothelial cells as well as in the smooth muscle and adventitial cells. In these studies, we sought to determine whether treating diabetic rats with either HES-DFO or  $\alpha$ -lipoic acid prevented the increase in vascular superoxide level. Data in Fig. 7 demonstrate that treating streptozotocin-induced diabetic rats with either HES-DFO or  $\alpha$ -lipoic acid markedly decreased the diabetes-induced increase in the level of superoxide in these arterioles (as measured by hydroethidine fluorescence) compared with paired analysis of untreated diabetic rats. Similar results were obtained in two additional studies using control rats, untreated diabetic rats, and diabetic rats treated with HES-DFO or  $\alpha$ -lipoic acid. Preincubating epineurial vessels from a diabetic rat with 10  $\mu$ mol/l, 100  $\mu$ mol/l, 1 mmol/l HES-DFO, or hydroxyethyl starch alone did not quench the diabetes-induced increase in superoxide level (data not shown). We also measured the superoxide level in the aorta by lucigenin-enhanced chemiluminescence. Data in Fig. 8 demonstrate that the superoxide level is increased in the aorta of diabetic rats compared with control rats and could be prevented by treating diabetic rats with either HES-DFO or  $\alpha$ -lipoic acid.

Since superoxide can react with nitric oxide to form peroxynitrite, we analyzed arterioles that provide circulation to the region of the sciatic nerve for 3-nitrotyrosine immunoreactivity. Data in Fig. 9 visually demonstrate that diabetes induces the formation of 3-nitrotyrosine, a marker for peroxynitrite, in presumably endothelial cells and the adventitia of these arterioles. Treating diabetic rats with either HES-DFO or  $\alpha$ -lipoic acid prevented the formation of 3-nitrotyrosine, as indicated by the lack of staining in these arterioles.

**EBF and MNCV.** As previously reported, diabetes causes a reduction in EBF and a slowing of MNCV in the sciatic nerve conducting system (27,28). Data in Fig. 10 demonstrate that treating diabetic rats with either HES-DFO or  $\alpha$ -lipoic acid prevents the decrease in EBF compared with untreated diabetic rats. Likewise, data in Fig. 11 demonstrate that treating diabetic rats with either HES-DFO or  $\alpha$ -lipoic acid prevents the slowing in MNCV.

**Arteriolar vascular reactivity.** Stimulated changes in vascular diameter of arterioles that provide circulation to the region of the sciatic nerve were measured *in vitro* by application of acetylcholine (endothelium-dependent), as previously described (27,28). Baseline diameter of vessels from control and diabetic rats (untreated or treated) was similar, and the vessels were constricted to a similar degree with U46619 (10–100 nmol/l). As previously re-



**FIG. 8.** Determination of superoxide in the aorta using lucigenin-enhanced chemiluminescence. Samples of the aorta were collected from the animals described in Fig. 1 and used to determine superoxide level using lucigenin-enhanced chemiluminescence. Data are presented as mean RLU  $\pm$  SE for a minimum of eight separate samples. \*A significant difference compared with control; +a significant difference compared with untreated diabetic rats.

ported and as demonstrated in Fig. 12, diabetes causes a significant decrease in acetylcholine-mediated vascular relaxation in arterioles that provide circulation to the region of the sciatic nerve. Treating diabetic rats with either HES-DFO or  $\alpha$ -lipoic acid significantly prevents the diabetes-induced impairment in acetylcholine-mediated vascular relaxation in these vessels. In contrast, maximal vasodilation induced by sodium nitroprusside (endothelium-independent) in these vessels was not significantly affected by diabetes or treatment of diabetic rats with HES-DFO or  $\alpha$ -lipoic acid (data not shown).

## DISCUSSION

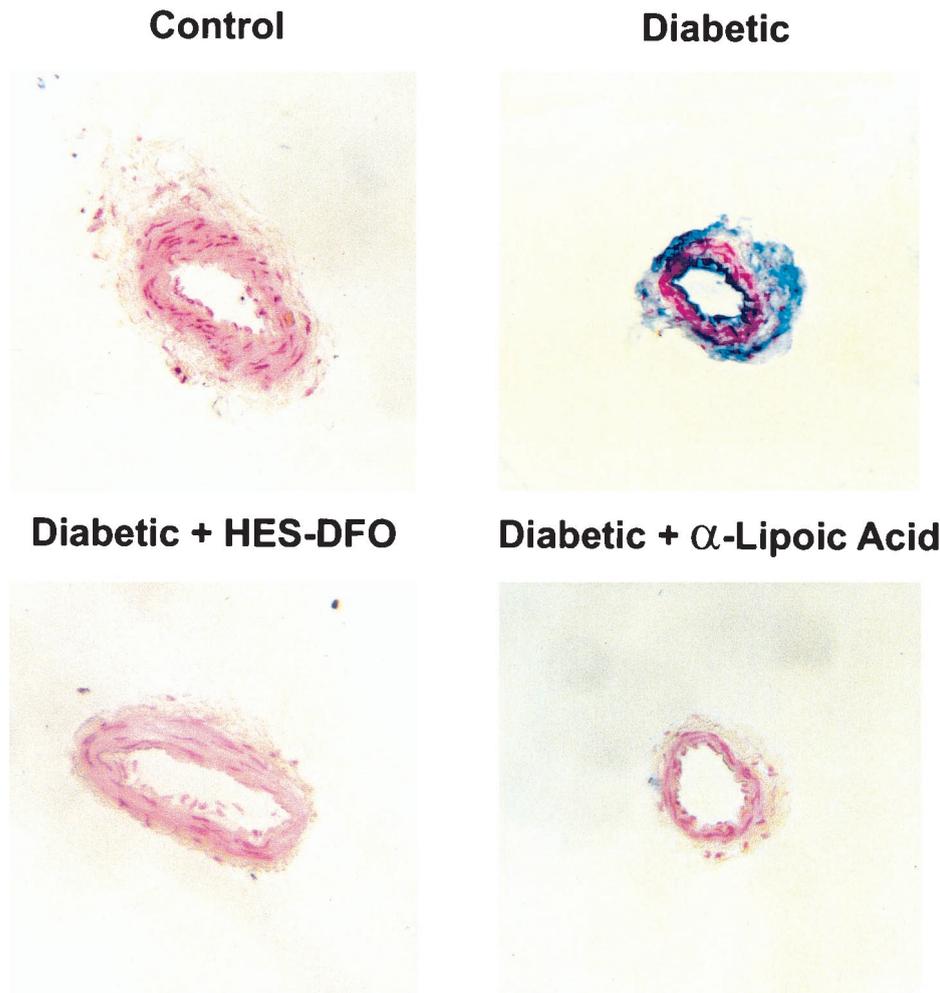
Previously, we demonstrated that diabetes-induced decrease in EBF and impairment of acetylcholine-induced vasodilation of arterioles that provide circulation to the region of the sciatic nerve precede slowing of motor nerve conduction and decrease in  $\text{Na}^+/\text{K}^+$  ATPase activity in the sciatic nerve (28). In addition, we showed that the generation of superoxide in the vasculature that provides circulation to the region of the sciatic nerve accompanies the diabetes-induced impairment in vasodilation (28). In these studies, we demonstrated that treating streptozotocin-induced diabetic rats with HES-DFO or  $\alpha$ -lipoic acid partially or totally prevents the diabetes-induced production of superoxide in the aorta and epineurial vessels, the slowing of MNCV, the reduction in EBF, and the impairment of acetylcholine-mediated vascular relaxation in arterioles that provide circulation to the region of the sciatic nerve.

$\alpha$ -Lipoic acid is a naturally occurring free-radical scavenger and transition metal chelator (15). It also is a cofactor for mitochondrial pyruvate dehydrogenase, it activates glucose uptake, and it has been termed a metabolic antioxidant (47). Cameron et al. (13) have shown that treatment of diabetic rats with  $\alpha$ -lipoic acid improved both motor and sensory nerve conduction deficits as well as

EBF. In addition, Keegan et al. (15) demonstrated that treating diabetic rats with  $\alpha$ -lipoic acid improved endothelium-dependent vascular relaxation of corpus cavernosum smooth muscle. We have obtained similar results in this study, and in addition, we report that treatment of streptozotocin-induced diabetic rats with  $\alpha$ -lipoic acid partially prevents the production of superoxide and peroxynitrite and significantly improves endothelium-dependent vascular relaxation in arterioles that provide circulation to the region of the sciatic nerve. In a study conducted by Stevens et al. (47), treatment of diabetic rats with  $\alpha$ -lipoic acid was shown to improve digital sensory but not sciatic-tibial MNCV, and it corrected endoneurial nutritive but not composite nerve blood flow. The reason for the differences in the latter study and those conducted by Cameron and colleagues and our laboratory is unknown. However, one possible explanation could be the difference in the delivery and dose of  $\alpha$ -lipoic acid. In the study by Stevens et al. (47), rats were treated with  $\alpha$ -lipoic acid by intraperitoneally injecting  $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ . In the studies by Cameron and colleagues and in our studies, however, rats were treated with a dietary supplement of  $\alpha$ -lipoic acid at a dose of  $\sim 300\text{--}350 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  (15). In addition to the studies conducted with diabetic animal models, treatment of diabetic humans with  $\alpha$ -lipoic acid reportedly improved microcirculation in patients with peripheral neuropathy as well as autonomic neuropathy and other symptoms of diabetic polyneuropathy (18,48).

In our studies, we found that treating diabetic rats with  $\alpha$ -lipoic acid generally improved markers of oxidative stress, including liver and serum TBARSs and lens GSH levels. In contrast, treating diabetic rats with HES-DFO had little effect on the diabetes-induced changes in these markers of oxidative stress. This finding could be attributable to the experimental protocol.  $\alpha$ -Lipoic acid was supplied in the diet; thus, the rats received a continuous dose. By way of comparison, diabetic rats treated with HES-DFO received a weekly injection. It could be that the efficacy of the weekly HES-DFO treatment protocol was not as effective as the daily  $\alpha$ -lipoic acid treatment in preventing the increase in liver and serum TBARSs or the decrease in lens GSH level. It is also possible that efficacy of HES-DFO may be tissue-specific. HES-DFO may work primarily in vascular tissue, where we have shown that it prevents the diabetes-induced increase in superoxide level. Additional studies will be required to answer these questions.

Our studies have demonstrated that treating diabetic rats with  $\alpha$ -lipoic acid reduces the accumulation of superoxide in arterioles that provide circulation to the region of the sciatic nerve. Presumably, this is caused by the free-radical scavenger capabilities of this antioxidant (15). This effect may be one explanation for the beneficial effects of  $\alpha$ -lipoic acid on vascular and nerve function. Reducing the level of superoxide may lead to a reduction in the formation of peroxynitrite and thus a reduction in the quenching of nitric oxide, thereby improving vascular function and reducing nerve ischemia. The formation of peroxynitrite is an early event in cardiovascular disorders, and it has been shown to inactivate antioxidant enzymes, thereby contributing further to oxidative stress (36,49). Our previous studies have shown that acetylcholine-induced generation



**FIG. 9.** Detection of peroxynitrite in arterioles from control rats, diabetic rats, and diabetic rats treated with either HES-DFO (DFO) or  $\alpha$ -lipoic acid ( $\alpha$ LA). Arterioles from control rats, untreated diabetic rats, and diabetic rats treated with either HES-DFO (DFO) or  $\alpha$ -lipoic acid ( $\alpha$ LA) were collected and treated for determination of 3-nitrotyrosine immunostaining as described in RESEARCH DESIGN AND METHODS. Shown is a representative sample of one set of animals. This experiment was repeated four separate times with similar results.

of nitric oxide and endothelium-derived hyperpolarizing factor (EDHF) mediate acetylcholine-induced vasodilation in arterioles that provide circulation to the region of the sciatic nerve (27). Therefore, any reduction in nitric oxide availability could alter vascular relaxation. In this regard, our studies indicate both that the production of peroxynitrite is increased by diabetes in arterioles that provide circulation to the region of the sciatic nerve and that it is prevented by treating diabetic rats with either HES-DFO or  $\alpha$ -lipoic acid. Therefore, quenching of nitric oxide may be partially responsible for impairment of endothelium-dependent vascular relaxation in arterioles from diabetic rats. However, we cannot rule out the possibility that  $\alpha$ -lipoic acid may also be affecting the production of EDHF. It is unknown whether the generation of reactive oxygen species alters the production of EDHF by the endothelium. Besides functioning as a scavenger of reactive oxygen species,  $\alpha$ -lipoic acid has also been reported to act as a chelator of transition metals (15). Furthermore, studies have shown that  $\alpha$ -lipoic acid supplementation reduces oxidative protein damage in the streptozotocin-induced diabetic rat (17). It has been suggested that increased oxidative protein damage may contribute to the development of diabetic complications (17). Further stud-

ies will be necessary to determine which of the oxidative stress mechanisms inhibited by  $\alpha$ -lipoic acid is responsible for diabetes-induced vascular and neural defects.

Our studies have demonstrated that diabetes causes the accumulation of superoxide in epineurial vessels in the endothelium, smooth muscle, and adventitia (28). In contrast, we have shown that peroxynitrite accumulates primarily in the endothelium and adventitia of epineurial vessels from diabetic rats. One possible reason for this difference is that the smooth muscle does not generally produce large amounts of nitric oxide compared with endothelial cells and the cells associated with the adventitia. Therefore, superoxide produced by smooth muscle cells may not have a sufficient amount of nitric oxide with which to react; thus, no detectable amount of peroxynitrite is formed.

Autoxidation and glycation reactions of glucose and metabolites, catalyzed by transition metal ions, are an important source of free radicals in diabetes (11,29). These free radicals contribute to vascular and neural disease in diabetes and can be prevented by free-radical scavengers. However, large concentrations of scavengers are generally required to prevent these complications. An alternative approach to scavenging for reducing the accumulation of

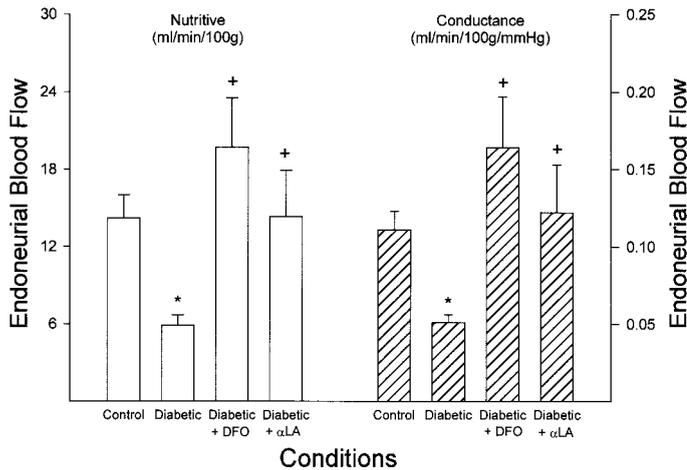


FIG. 10. Determination of EBF. EBF reported as nutritive or conductance was determined for the same rats described in Table 1 and Fig. 1. EBF was determined as described in RESEARCH DESIGN AND METHODS. Data are presented as means  $\pm$  SE for a minimum of eight separate samples. Nutritive EBF in control rats treated with HES-DFO or  $\alpha$ -lipoic acid was  $15.5 \pm 5.6$  ( $n = 3$ ) and  $15.3 \pm 2.8$  ( $n = 3$ )  $\text{ml} \cdot \text{min}^{-1} \cdot 100 \text{g}^{-1}$ , respectively. \*A significant difference compared with control rats; +a significant difference compared with untreated diabetic rats.

free radicals is to prevent their formation. In these studies, we treated diabetic rats with HES-DFO and obtained results similar to those we had obtained with diabetic rats treated with  $\alpha$ -lipoic acid. Hydroxyethyl starch-conjugated deferoxamine is a transition metal chelator, and in this form it provides a volume-active colloid that retains its antioxidant properties and significantly reduces the toxicity of free deferoxamine (50). Moreover, the half-life of the antioxidant activity of this conjugate is significantly increased (12,50). Pieper and Siebeneich (12) have shown that relaxation to acetylcholine in aortic rings from diabetic rats is impaired, and that long-term treatment with HES-DFO completely prevented this dysfunction. In addition,

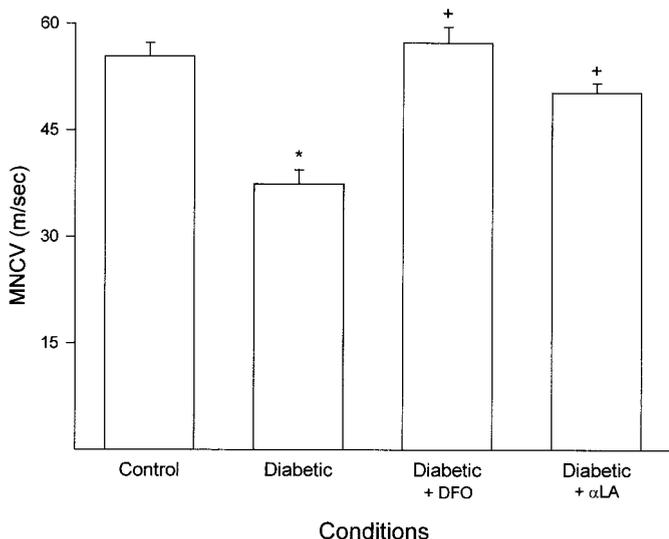


FIG. 11. Determination of MNCV. MNCV was determined for the same rats described in Table 1 and Fig. 1. MNCV was determined as described in RESEARCH DESIGN AND METHODS. Data are presented as means  $\pm$  SE for a minimum of eight separate samples. MNCV in control rats treated with HES-DFO or  $\alpha$ -lipoic acid was  $54.9 \pm 3.0$  ( $n = 3$ ) and  $51.3 \pm 7.2$  ( $n = 3$ ) m/s, respectively. \*A significant difference compared with control rats; +a significant difference compared with untreated diabetic rats.

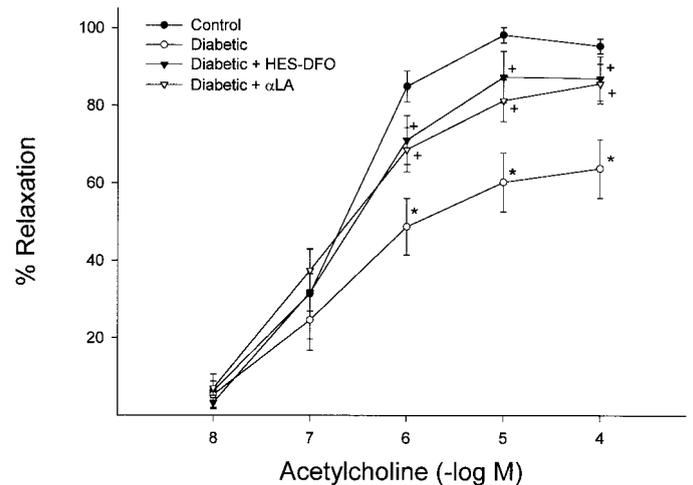


FIG. 12. Determination of the effect of treatment with HES-DFO or  $\alpha$ -lipoic acid ( $\alpha$ LA) on acetylcholine-mediated vascular relaxation in arterioles that provide circulation to the region of the sciatic nerve. Pressurized arterioles were constricted with U46619 (30–50%), and incremental doses of acetylcholine were added to the bathing solution while the steady-state vessel diameter was recorded. The number of experimental animals used in these studies was the same as that noted in Table 1. \*Denotes that the response to acetylcholine was significantly attenuated in the diabetic rat; +denotes that the response to acetylcholine was significantly different compared with the untreated diabetic rats.

tion, Cameron and Cotter (11,29) have demonstrated that treating diabetic rats with the transition metal chelators deferoxamine and trientine significantly improved slowing of MNCV and EBF. These studies as well as our own suggest that increased free-radical activity caused by transition metal-catalyzed reactions contributes to the early deficits in diabetes-induced nerve and vascular function (22). One possible explanation for the effects of HES-DFO is the prevention of the metal-catalyzed formation of hydroxyl radicals ( $\cdot\text{OH}$ ) (51). Studies by Pieper and colleagues (12,52) support a role for  $\cdot\text{OH}$  involvement in diabetes-induced endothelial dysfunction. Another possible explanation is derived from a theory proposed by Qian and Eaton (53). They found that nonenzymatic glycation of proteins yields products that bind metals such as copper and iron. They proposed that glycated proteins of the endothelial basement membrane bind transition metals and form a metal-rich layer that prevents normal endothelial-dependent vascular relaxation via metal-catalyzed destruction of nitric oxide. Chelating drugs, such as HES-DFO, may remove these metal deposits, restore endothelium-dependent vascular relaxation, and presumably prevent or reverse ischemia-induced dysfunction of peripheral nerves (53). As stated above, HES-DFO is primarily thought to be a metal chelator. Therefore, it is difficult to explain the effects of this drug in preventing superoxide generation in our studies. It is possible that HES-DFO may have other effects (in addition to its metal chelating properties) that are responsible for preventing superoxide generation. It could be promoting the production of natural antioxidants in rats. This possibility seems unlikely, however, because lens GSH levels are not restored in diabetic rats treated with HES-DFO, and liver and serum TBARS levels remain elevated. It is possible that the antioxidant properties of HES-DFO may be limited to vascular tissue, since markers of oxidative stress related

to diabetes in other tissues remained altered. We do not know whether HES-DFO increases superoxide dismutase activity in the diabetic rat or whether HES-DFO affects the formation of reactive oxygen species proximal to hydroxyl radical formation. In this regard, autoxidation reactions of molecules (like glucose) catalyzed by free transition metals promote the formation of oxygen-free radicals (11,54). It is possible that the formation of oxygen free radicals by autoxidation may further promote the formation of superoxide in vivo. Another possible explanation for the effect of HES-DFO on the generation of superoxide by epineurial vessels from diabetic rats is its nonspecific quenching. In vitro studies using high concentrations of deferoxamine have demonstrated that it can react with superoxide to form a nitroxide free radical (36,37). However, this reactivity is reduced when deferoxamine is conjugated to starch (55). In our studies, it is unlikely that high-enough concentrations of deferoxamine are achieved in vivo to quench superoxide in epineurial vessels and aorta from diabetic rats. Furthermore, our studies were conducted 72 h after the last injection of HES-DFO. At this time, HES-DFO in circulation cannot be detected. In addition, we demonstrated that acute exposure of epineurial vessels from diabetic rats to HES-DFO or hydroxyethyl starch alone did not reduce the detection of superoxide in these vessel segments. Therefore, we conclude that nonspecific quenching of superoxide by HES-DFO is not responsible for the decrease in superoxide in vascular tissue from diabetic rats treated with HES-DFO. At this time, we do not have a good understanding of the possible interactions between pathways involved in the formation of oxygen free radicals in vivo. Additional studies will clearly be necessary to determine the role and mechanism of transition metals in the formation of oxygen free radicals in diabetes and the potential benefit realized by treatment with transition metal chelators.

Our studies suggest that the production of superoxide and possibly peroxynitrite may be mediating factors in diabetes-induced vascular dysfunction in arterioles that provide circulation to the region of the sciatic nerve. It has been shown that sources of superoxide production in the vasculature include the mitochondria, xanthine oxidase, nitric oxide synthases, NAD(P)H-dependent oxidoreductases, cyclooxygenase, and lipoxygenase (56–58). Recent studies have suggested that the NAD(P)H oxidase system accounts for the majority of superoxide generation in the vessel wall and that NAD(P)H oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats (59–61). Hink et al. (62) have demonstrated that diabetes of 2 weeks' duration causes superoxide formation in the aorta, leading to a decrease in the bioavailability of vascular nitric oxide. In addition, Hink et al. (62) suggest that the overexpression of nitric oxide synthase III and/or increased activity of NAD(P)H oxidase is responsible for the production of superoxide in the diabetic aorta via a protein kinase C-sensitive mechanism. Therefore, in addition to increased autoxidation of glucose and increased activity of the mitochondrial electron transport chain, increased activity of NAD(P)H oxidase or specific isoforms of nitric oxide synthase need to be considered when determining the source for increased production of superoxide in vascular tissue in diabetes.

We do not know the effect that transition metal chelators such as HES-DFO or more general antioxidants like  $\alpha$ -lipoic acid may have on these pathways. Studies are now underway to determine the mechanism and possible source(s) of superoxide production in arterioles that provide circulation to the region of the sciatic nerve after the induction of diabetes.

In summary, these studies have demonstrated that treating streptozotocin-induced diabetic rats with two different antioxidants—HES-DFO or  $\alpha$ -lipoic acid—that are mechanistically different with regard to preventing the production of reactive oxygen species effectively prevents the production of superoxide and peroxynitrite in arterioles that provide circulation to the region of the sciatic nerve and also prevents the vascular and neural deficits associated with diabetes.

#### ACKNOWLEDGMENTS

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