# Oxygen Free Radical Effects in Sciatic Nerve in Experimental Diabetes

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**We previously reported the presence of endoneurial hypoxia, ischemia, impairment of the blood-nerve barrier, and reduction of norepinephrine and 6-ketoprostaglandin $F_{1\alpha}$ in chronic streptozocin-induced diabetic neuropathy (SDN) and interpreted these findings as suggesting the involvement of oxygen free radicals (OFRs) but did not directly measure indices of OFR activity. In this study, we report on sciatic nerve conjugated dienes, hydroperoxides, norepinephrine, and malondialdehyde in SDN at 1, 4, and 12 mo in male Sprague-Dawley rats. Severe hyperglycemia was present throughout in diabetic rats. Conjugated dienes were consistently increased at all time points, hydroperoxides were consistently reduced, and malondialdehyde was not significantly different in diabetes compared with controls. These findings are consistent with increased OFR activity in experimental diabetes. It is necessary to monitor several indices of OFR activity in a metabolically active tissue such as the peripheral nerve. *Diabetes* 40:873–77, 1991**

In chronic streptozocin (STZ)-induced diabetic neuropathy (SDN), nerve blood flow is reduced, endoneurial hypoxia is present (1), and reducing equivalents are increased (2). Should hypoxia result in the conversion of xanthine dehydrogenase to xanthine oxidase as occurs in other tissues, then the availability of substrate, enzyme, and cofactor for the xanthine-xanthine oxidase reaction (3) may result in the generation of reduced oxygen species, e.g., oxygen free radicals (OFRs; 4). Additional evidence for the OFR effect includes impairment of the blood-nerve barrier (5,6), reduced nerve norepinephrine (NE; 4), a reduction in the important scavenging enzyme superoxide dismutase (SOD), which is reduced in some diabetic tissues (7), and a reduction in in vivo biosynthesis of 6-ketoprostaglandin $F_{1\alpha}$, the stable metabolite of prostacyclin (4). We suggested that the latter changes are due to the inhibition of prostacyclin synthetase by lipid hydroperoxides (8,9). Further support for OFR activity derives from our ischemia-reperfusion experiments. We found that reperfusion after ischemia to the rat sciatic nerve resulted in reduced reflow and an accentuation of blood-nerve barrier impairment with reperfusion (10). In this study, we determined lipid hydroperoxides, conjugated dienes, malondialdehyde (MDA), and NE in the sciatic-tibial nerve of SDN at 1, 4, and 12 mo.

## RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing ~250 g were used throughout. Diabetes was induced by the injection of 65 mg/kg i.p. STZ.

Lipid extract for conjugated dienes and hydroperoxides was adapted from the method of Folch et al. (11) for peripheral nerve. The nerve was homogenized in 1 ml ice-cold 0.9% NaCl. Two 0.4-ml aliquots of homogenate were transferred to 4 ml chloroform/methanol (2:1 vol/vol). This mixture was vortexed for 1 min then centrifuged at 2500 × *g*. The lower layer was removed for evaporation. The upper layer was washed with chloroform/methanol/$H_2O$ (86:14:1 vol/vol) and centrifuged. The lower layer was combined with the first lower layer and evaporated under $N_2$. The lipids were then dissolved in 0.5 ml chloroform/methanol (2:1 vol/vol) and stored at −80°C until analysis.

Conjugated dienes were determined as an increase in absorbance at 233 nm with the method of Recknagel and Ghoshal (12) adapted for peripheral nerve. An aliquot of lipid extract was evaporated and redissolved in 1 ml cyclohexane. Absorbance spectra were determined in quartz cuvettes (1-cm light path) with extraction blanks used as references. An extinction coefficient of $2.52 \times 10^4$/M was used.

We measured lipid hydroperoxides with the iodometric method of Buege and Aust (13). The lipid extract was dried

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TABLE 1
Duration of diabetes, initial and final weights, blood glucose, and $HbA_{1c}$ of control and diabetic rats

| Time point | Duration of diabetes* | Initial weight | Final weight | Glucose (mM) | $HbA_{1c}$ |
|---|---|---|---|---|---|
| 1 mo | | | | | |
| Control | 28.3 ± 0.2 | 250 ± 2.6 | 341 ± 5 | 5.9 ± 0.2 | 3.7 ± 0.2 |
| Diabetic | 28.4 ± 0.2 | 252 ± 1.5 | 155 ± 10† | 23.6 ± 1.1† | 12.9 ± 0.3† |
| 4 mo | | | | | |
| Control | 120.8 ± 0.5 | 310 ± 2.5 | 484 ± 7 | 6.2 ± 0.2 | 4.8 ± 0.2 |
| Diabetic | 121.0 ± 0.6 | 311 ± 3.1 | 210 ± 18† | 29.2 ± 0.9† | 12.8 ± 0.5† |
| 12 mo | | | | | |
| Control | 352.3 ± 7.5 | 234 ± 5.1 | 547 ± 22 | 5.8 ± 0.3 | 6.8 ± 1.1 |
| Diabetic | 336.7 ± 10.3 | 239 ± 7.4 | 214 ± 16† | 20.2 ± 0.9† | 18.3 ± 0.9† |

*Streptozocin induced or sham injection.
†$P < 0.001$ vs. control.

under $N_2$ and mixed with 1 ml glacial (17.4 M/L) acetic acid/chloroform (3:2 vol/vol) and then 50 μl 1.2 g/ml potassium iodide. The tube was capped and kept in the dark for 5 min. Next, 3 ml of 0.5% cadmium acetate was added, mixed, and centrifuged at 2500 rpm for 10 min. The top layer was read at absorbance 353 nm ($Abs_{353}$). Lipid hydroperoxides were expressed as $Abs_{353}$ per gram wet weight or milligram protein and compared to a standard curve (cumene hydroperoxide).

MDA was adapted for the nerve from the method of Therasse and Lemonnier (14). Nerve was homogenized in 0.25 ml 0.1 M phosphate buffer, pH 6.7, and centrifuged. The pellet was resuspended in 0.2 ml buffer. Both supernatant and pellet were assayed for MDA. Thiobarbituric acid dissolved in phosphate buffer, pH 6.7, containing 0.02% 2,6-di-*tert*-butyl-*p*-methyl-phenol as antioxidant was added (0.8 ml) to the supernatant and the pellet, heated at 80°C for 15 min to form the adduct, then extracted with ethyl acetate, and the emulsion was broken down with diethyl ether. The mixture was centrifuged and the organic layer evaporated under $N_2$. The residue was dissolved in mobile phase, consisting of 0.1% ethanolamine/acetonitrile (2:1 vol/vol). MDA was detected with high-performance liquid chromatography (HPLC), reversed phase, at a flow rate of 0.4 ml/min on a 2 × 150-mm Phenomenex Ultramex (Rancho Palos Verdes, CA) octyldecylsilane C-18 column, and the absorbance was monitored at 539 nm. A standard curve was generated with pure malonaldehyde bis(dimethyl acetal) run under identical conditions.

We used the method of Crapo et al. (15) with modifications including reducing assay volumes for use in a 1-ml cuvette and increasing xanthine concentration twofold. Sciatic nerve was homogenized in phosphate buffer, pH 7.8, and centrifuged for 15 min at 14,600 × *g*. The supernatant was recovered, and spectrophotometric determination was determined at 550 nm as percent inhibition of ferricytochrome c by SOD and calculated from a standard curve of percent inhibition against SOD.

Tissue NE was determined with HPLC with electrochemical detection methodology (4). Briefly, the tissue was homogenized in 0.1 M perchloric acid containing 0.1 mM ascorbic acid and 35 pg/μl dihydroxybenzylamine (the internal standard) and then centrifuged. The supernatant was then added to alumina to adsorb the catecholamines and washed three times with distilled water. The catecholamines were then eluted with acetic acid and injected into the HPLC column. Electrochemical detection was performed on a BAS LC-4B detector (Bioanalytical Systems, West Lafayette, IN) set at 0.7 V. The mobile phase consisted of 0.1 M phosphate buffer, pH 3, containing 1 mM octyl sulfate, 0.1 mM EDTA, and 4% methanol at a flow rate of 0.45 ml/min on a 2 × 150-mm Phenomenex Ultremex octyldecylsilane C-18 column. Recent improvements consist of the use of microbore columns, a thinner membrane, and dual versus the previously used single electrode.

Serum glucose was determined with a glucose oxidase method (2). Measurements were done in duplicate at 450 nm on an LKB spectrophotometer (Ultraspec II). $HbA_{1c}$ was assayed by affinity column (1,16).

Statistical comparison of control versus diabetic nerves was done with the unpaired *t* test. Analysis of variance was used with Duncan's multiple comparison analysis for multiple comparisons. Regression analysis was used for evaluation of age effects. Significance was accepted at $P < 0.05$.

## RESULTS

The weight and diabetic characteristics are shown in Table 1. Control rats were well matched against diabetic groups in weight at onset. The onset weights of 4-mo control and diabetic rats were significantly heavier than the 1- and 12-mo groups ($P < 0.001$). Diabetic rats lost weight and remained lighter than control rats throughout the duration of the study. Diabetic rats were markedly hyperglycemic and had increased $HbA_{1c}$ by 1 mo. Hyperglycemia was maintained throughout the study. Rats were anesthetized with 50 mg/kg pentobarbital sodium before nerve harvest.

In control nerves, there was a progressive nonsignificant increase with age in conjugated dienes and a significant increase in hydroperoxides in data expressed on a per milligram of protein basis (Table 2). There was no statistical

TABLE 2
Regression of control rat sciatic nerve $HbA_{1c}$, conjugated dienes, hydroperoxides, and malondialdehyde (MDA) with age in days

| Variable | df | Slope | Intercept | *r* | Slope (*P*) |
|---|---|---|---|---|---|
| $HbA_{1c}$ | 19 | +0.009 | 3.163 | 0.75 | 0 |
| Conjugated dienes | 18 | +0.024 | 26.025 | 0.36 | 0.124 |
| Hydroperoxides | 22 | +0.015 | 4.146 | 0.62 | 0.002 |
| MDA | 23 | −0.174 | 94.000 | 0.74 | 0 |

TABLE 3
Conjugated dienes, lipid hydroperoxides, malondialdehyde (MDA), and norepinephrine (NE) in control and diabetic rat sciatic nerve

| Time point | Conjugated dienes (nmol/mg protein) | Hydroperoxides (nmol/mg protein) | MDA (pM/mg wet wt) | NE (ng/mg wet wt) |
|---|---|---|---|---|
| 1 mo | | | | |
| Control | 26.6 ± 0.8 | 5.8 ± 1.0 | 91.1 ± 8.2 | 0.29 ± 0.02 |
| Diabetic | 36.8 ± 2.1* | 3.0 ± 0.7† | 104.7 ± 20.4 | 0.23 ± 0.01† |
| 4 mo | | | | |
| Control | 32.3 ± 5.1 | 5.9 ± 0.7 | 50.4 ± 7.8 | 0.31 ± 0.02 |
| Diabetic | 39.7 ± 4.1 | 3.5 ± 1.2 | 49.8 ± 9.8 | 0.24 ± 0.02† |
| 12 mo | | | | |
| Control | 35.4 ± 1.9 | 10.6 ± 1.2 | 25.1 ± 4.7 | 0.29 ± 0.02 |
| Diabetic | 41.5 ± 2.3 | 5.9 ± 1.0‡ | 26.2 ± 2.2 | 0.28 ± 0.03 |

*$P < 0.001$, †$P < 0.05$, ‡$P < 0.01$, vs. control.

difference in data expressed against wet weight, suggesting that the increase was due to a progressive lower protein–wet weight ratio with aging. Indeed protein per milliliter regressed significantly against wet weight per milliliter ($y = 0.109–0.002x$, $r = 0.60$, $P = 0.003$, where $x$ is age in mo). MDA progressively decreased with age ($P < 0.001$, analysis of variance with Duncan's multiple comparison test). NE was not significantly different with increasing age (Table 3).

Compared with age-matched control rats, diabetic rats had sciatic nerve conjugated dienes that were consistently increased (Fig. 1). These alterations were present by 1 mo and were maintained at 4 and 12 mo of diabetes. MDA was not significantly altered for the duration of the study (Table 3; Fig. 3). Hydroperoxides (Table 3; Fig. 2) and NE (Table 3; Fig. 4) were consistently reduced, and the changes were similar for all time points, reaching statistical significance for some but not all time points. Concentrations were expressed in units of both wet weight and protein for conjugated dienes and hydroperoxides, because the diabetic state may potentially affect nerve-protein concentration. The alterations at different time points were of similar degree with whichever method was used.

To evaluate the role of nerve SOD, we evaluated Cu-Zn-SOD in control rats, severely hyperglycemic STZ-induced diabetic rats (duration 1 mo), and moderately hyperglycemic insulin-treated STZ-induced diabetic rats. The untreated diabetic rat sciatic nerve had reduced SOD that was correctable with insulin treatment.

## DISCUSSION

The findings of this study of an increase in sciatic nerve conjugated dienes associated with a reduction in tissue NE (at 1 mo) are consistent with chronic and increased OFR activity in STZ-induced diabetes. These abnormalities developed by 1 mo, and the increase in conjugated dienes persisted throughout the study period.

Both metabolic and microvascular abnormalities are probably responsible for the abnormalities of chronic progressive experimental diabetic neuropathy. At 1 mo, slowing of motor nerve conduction velocity is present (17), but abnormalities of nerve prostacyclin are absent (4). By 4 mo, electrophysiological abnormalities are well established (1,18), endoneurial hypoxia is present, and nerve prostacyclin is reduced by 43% (4). By 12 mo, the microvascular abnormalities are present, but nerve *myo*-inositol may no longer be reduced (19).

The pattern of OFR indices consisting of an increase in conjugated dienes and a decrease in hydroperoxides and NE with little change in MDA requires explanation. The detection of lipid peroxidation in vitro is straightforward. However, the study of in vivo peroxidation poses many problems, because the peroxidation products may be metabolized or removed from the tissue of interest (13,20). In this study, the divergent results reflect the complex metabolic alterations of hydroperoxides and diabetes. In such a situation, multiple indices are preferable to a single index. Lipid peroxidation begins with hydrogen abstraction from a polyunsaturated

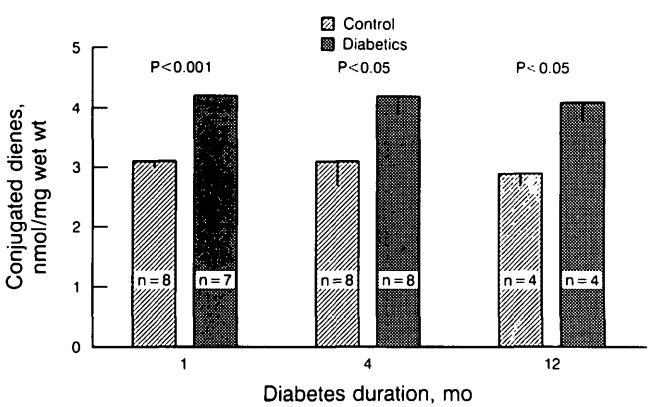


FIG. 1. Sciatic nerve conjugated dienes at 1, 4, and 12 mo of diabetes.

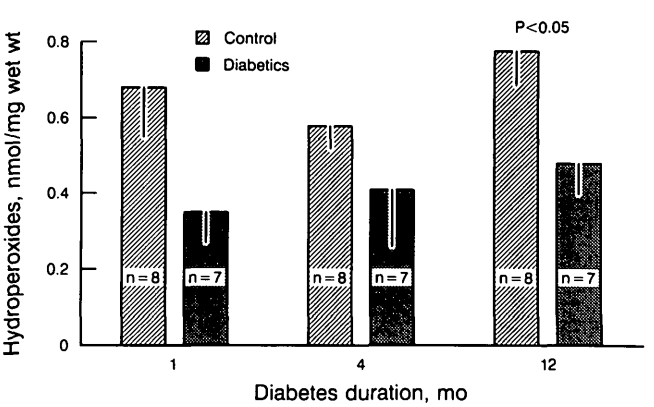


FIG. 2. Sciatic nerve hydroperoxides at 1, 4, and 12 mo of diabetes.

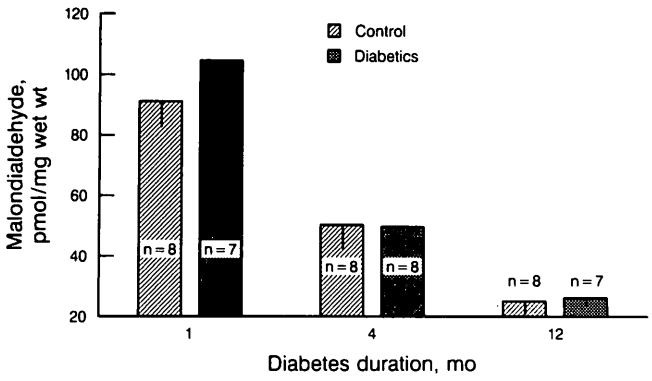


**FIG. 3. Sciatic nerve malondialdehyde at 1, 4, and 12 mo of diabetes.**

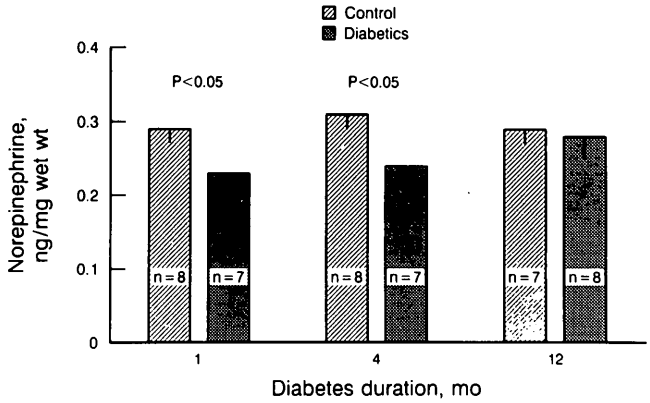


**FIG. 4. Sciatic nerve norepinephrine at 1, 4, and 12 mo of diabetes.**

fatty acid forming a lipid radical, the rearrangement of which results in conjugated dienes. The lipid peroxyradical results from subsequent interaction with molecular oxygen, and hydrogen abstraction from an adjacent lipid results in lipid hydroperoxide. MDA is formed as a degradation product (13). Conjugated dienes were most likely increased in this study because the diene structure, once formed, is better retained. In contrast, hydroperoxides and MDA may reflect metabolic and diffusion factors unrelated to OFR. The reduced hydroperoxides could reflect the increase in glutathione peroxidase found in some diabetic tissues (21). The main detoxifying systems for lipid peroxides are glutathione peroxidase and glutathione reductase, especially the former (22), with reactions of the NADPH regeneration system as the rate-determining step of peroxide metabolism. However, the activity of this enzyme in nerve is not known.

MDA is formed distal to conjugated dienes and hydroperoxides, and the lack of an increase may reflect the alterations of hydroperoxides. MDA has been suggested to be an unsatisfactory indicator of lipid peroxidation in vivo (13,23) because MDA is readily metabolized in vivo and in tissue suspensions (24), partly by a mitochondrial aldehyde oxidase (25). It also reacts with tissue components to form cross-linked lipofuscin pigments, decreasing intracellular composition. Furthermore, positive thiobarbituric acid reactions may be generated by other carbonyl compounds (26), sialic acid (27), and aldehydes, e.g., 2,4-alkadienals and 2-alkenals (28). Certain derivatives of glucose, sucrose, sialic acid, and Cu inhibit MDA reaction (29) or interfere with thiobarbituric acid estimation (30).

Increasing age is associated with a small progressive increase in hydroperoxides per milligram protein and reduction in MDA. This pattern suggests the possible involvement of OFRs with increasing age. OFRs have been suggested to affect aging in two ways. First, OFRs have been suggested to attack polyunsaturated fatty acid proteins and nucleic acids, causing a general "wear and tear" effect on biopolymer molecules. OFRs have also been suggested to influence the rate of development of specific chronic diseases. Aging has been reported to increase the rate of lipid peroxidation in the brain, heart, and liver of rats (31,32). Cytoplasmic SOD progressively declines with age in the liver but not in the brain in rats and mice (33).

SOD is significantly reduced in the peripheral nerve after 1 mo of diabetes, and this reduction appears to be correctable even with only moderate diabetes control, suggesting that scavenging-enzyme alterations and oxygen radical generation in the nerve are related to the diabetic state (Table 4). Beyond the peripheral nerve, there is considerable evidence that oxygen radical activity is produced in excess in the diabetic state (34–36). There are alterations in the antioxidant-enzyme systems that can be correctable with insulin (35,36). The changes are probably not due to STZ, because similar changes are seen with alloxan administration and in the spontaneously diabetic BB Wistar rat (34,35). The degree and type of alterations may vary considerably among tissues in these experimental animals and in humans (34).

Considerable evidence exists to suggest a role of OFRs in microvascular atherogenesis in experimental and human diabetes, although the focus has hitherto not been directed to the peripheral nerve. MDA increased 126% in STZ-induced diabetic rats (37). MDA is also increased in human diabetes (38,39). Of particular note is that lipid peroxide levels are highest in diabetic patients with angiopathy and are relatively normal in patients with well-controlled diabetes (39).

At the tissue level, peroxidized lipids are better substrate for phospholipase $A_2$ (40), and atheroma has a high level of

TABLE 4
Effect of diabetes on superoxide dismutase (SOD)

| Group | *n* | Final weight | Glucose (mM) | $HbA_{1c}$ | Cu-Zn-SOD (ng/mg wet wt) |
|---|---|---|---|---|---|
| Control | 8 | 343 ± 9 | 5.7 ± 0.2 | 4.2 ± 0.1 | 47.6 ± 2.8 |
| Diabetic given insulin | 8 | 301 ± 5* | 17.1 ± 1.3† | 9.5 ± 0.2 | 42.1 ± 2.4 |
| Diabetic | 8 | 203 ± 10† | 24.9 ± 1.2† | 16.7 ± 0.5 | 36.3 ± 2.3‡ |

Values are means ± SE.
*$P < 0.05$, †$P < 0.001$, ‡$P < 0.01$, vs. control.

lipid peroxide (41). Proposed as the primary defense against the superoxide radical (42), SOD is reduced in some experimental diabetic tissue, including liver, kidney, spleen, heart, testis, pancreas, skeletal muscle erythrocytes (7,38,43,44), and now in the peripheral nerve. Erythrocyte Cu-Zn-SOD is reduced in patients with non-insulin-dependent diabetes (38), and SOD is reduced in various organs of rats and humans with diabetes of sufficient severity (44). The reduction is consistently present in recent diabetes but usually absent in chronic diabetes, suggesting the development of compensatory mechanisms in the chronic state (35,36).

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