Nerve Conduction and Antioxidant Levels in Experimentally Diabetic Rats: Effects of Streptozotocin Dose and Diabetes Duration

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Oxidative stress supposedly plays a role in the pathogenesis of diabetic neuropathy. We have studied whether a variation in the streptozotocin (STZ) dose or diabetes duration affects the outcome of measurements of oxidative damage in relation to nerve conduction. In experiment 1, we induced diabetes in rats using 40 or 60 mg/kg STZ intravenously and assessed sciatic nerve conduction velocity. After 18 weeks, we measured plasma malondialdehyde (MDA) and red blood cell (RBC) and nerve glutathione levels. We observed a dose-dependent effect of STZ on body weight, and to a lesser extent on nerve conduction, but not on RBC or nerve glutathione and plasma MDA. In experiment 2, we administered a fixed dose of STZ (40 mg/kg) and measured antioxidants and MDA in RBCs, plasma, and sciatic nerve after 2, 4, 8, and 18 weeks in diabetic and control rats. RBC glutathione decreased in diabetic animals initially, but did not differ from control values after week 4. Plasma total glutathione increased until week 8. The ratio of total to oxidized glutathione in the sciatic nerve from diabetic animals paralleled the decrease observed in RBCs, and subsequently increased compared with controls. Nerve catalase increased in diabetic animals. Endoneurial MDA remained unchanged, whereas plasma MDA increased and RBC superoxide dismutase (SOD) decreased in the diabetic group. We conclude that differences in antioxidant levels between STZ-diabetic and control rats depend on the duration of hyperglycemia. Furthermore, dose-related effects of STZ on nerve conduction are not reflected in endoneurial lipid peroxidation or glutathione.

ANTIOXIDANT TREATMENT has been shown to lead to a partial prevention of the development of nerve dysfunction in experimentally diabetic rats.[1-5] Chronic hyperglycemia causes increased production of reactive oxygen species (ROS) due to the autooxidation of monosaccharides, leading to the production of superoxide (O_2^-) and hydroxyl (·OH) radicals.[6] Consequently, the oxidation of cell structures of peripheral nerves may induce important functional and structural changes. Reduced antioxidant capacity has been reported in sciatic nerves from diabetic animals.[7,8]

The development of diabetic neuropathy is associated not only with metabolic changes in the nerve but also with reduced tissue oxygenation and nerve blood flow.[9,10] Therefore, changes in the antioxidant state of the erythrocyte and endothelium are of interest. The beneficial effects of antioxidant treatment on nerve blood flow[4,5] emphasize beneficial systemic effects leading finally to an improvement of nerve function. Previously, we have shown that changes in antioxidant capacity and lipid peroxidation after 18 weeks of experimental diabetes are systemic rather than endoneurial and can be reversed by insulin treatment.[11]

The changes in prooxidant and antioxidant factors that have been observed in diabetes are probably largely dependent on the duration and severity of hyperglycemia and the organs studied.[12] Therefore, the variety of experimental models complicates their interpretation. The first objective of this study was to investigate whether the dose of streptozotocin (STZ) administered to induce diabetes can influence glycemic control, nerve conduction, or antioxidant status. Secondly, we evaluated the influence of the duration of experimental hyperglycemia on the antioxidant capacity, both systemic and endoneurial. For this purpose, we studied red blood cell (RBC), plasma, and sciatic nerve antioxidants in rats with a different duration of STZ-induced diabetes.

MATERIALS AND METHODS

Animals

All experiments were approved by the University ethics committee for animal experiments. Male Wistar rats (initial weight, ~250 g) were housed on sawdust in macrolon cages (two rats per cage) and maintained on a 12-hour light-dark cycle. All animals received food and water ad libitum.

Experiment 1

Diabetes was induced by 40 or 60 mg/kg STZ (Serva, Heidelberg, Germany) intravenously. Each STZ-treated group initially consisted of 14 animals. Other animals (n = 12) served as nondiabetic controls. Diabetes was confirmed by measuring whole-blood glucose levels with a rapid glucose sensor system (Medisense; Amersfoort, The Netherlands). Blood glucose levels were measured 48 hours after STZ administration and at weeks 8, 12, and 16.

At week 0 (before STZ administration) and weeks 4, 8, 10, 12, 14, and 16, sensory and motor nerve conduction velocity (SNCV and MNCV) were measured in the left sciatic nerve as previously described.[13] Measurements were performed under general anesthesia (0.033 mL/100 g body weight Hypnorm containing 10 mg/mL fluanisone and 0.2 mg/mL fentanyl citrate; Janssen Pharmaceutica, Tilburg, The Netherlands). All measurements were performed by the same examiner. The body temperature of the animals was kept at a constant level of 36° to 37°C.

At week 18, all animals were decapitated and the blood was subsequently collected in heparinized tubes, separated into plasma and RBCs, and processed immediately. Both sciatic nerves were located, removed, desheathed, and stored in liquid nitrogen until homogenization and further processing. Nerve homogenization was performed on ice in 50-mmol/L sodium phosphate buffer (pH 7.5) on the same day.

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The following biochemical parameters were measured: whole-blood glycated hemoglobin (HbA_1), erythrocyte total glutathione and thiol (−SH), plasma malondialdehyde (MDA) and urea, and sciatic nerve total glutathione.

Experiment 2

Diabetes was induced in 32 rats by STZ 40 mg/kg intravenously. An age-matched control group was kept under identical circumstances. From the diabetic and control groups, eight animals were selected at random at weeks 2, 4, 8, and 18 (weeks after STZ injection). These animals were decapitated, and the blood and sciatic nerve were processed as described for experiment 1. Apart from whole-blood HbA_1 and plasma urea, the same biochemical measurements were performed as in experiment 1. Furthermore, we assessed plasma and sciatic nerve superoxide dismutase (SOD) and catalase, erythrocyte SOD, plasma total glutathione, and sciatic nerve MDA and oxidized glutathione (GSSG). Sciatic nerve glutathione was expressed as the total glutathione to GSSG ratio.

Biochemical Assay Procedures

MDA. The assay has been previously described by Aust[14] and is based on the concept that MDA, an end product of lipid peroxidation, forms a colored product with thiobarbituric acid measurable at 532 nm. We used MDA-bis (Merck, Darmstadt, Germany) to create a standard curve. The plasma was deproteinized with trichloroacetic acid (TCA) before MDA measurement. Sciatic nerve homogenate was not pretreated. Samples and standards were incubated with a solution containing 26 mmol/L thiobarbituric acid and 918 mmol/L TCA in 0.25N HCl for 20 minutes at 100°C. MDA concentrations were calculated from the extinction measured at 532 nm on a 96-well plate.[14]

SOD. No pretreatment of samples was performed. One hundred microliters of sample or standard was added to 800 µL of a solution containing 0.25 mmol/L hypoxanthine and 50 µmol/L cytochrome C; 100 µL xanthine oxidase solution (16 mU/mL) was added. At 25°C and pH 10. O_2^- is formed due to the oxidation of hypoxanthine. O_2^- is a reductant of cytochrome C. Reduced cytochrome C is measurable at 550 nm. If SOD is present, O_2^- is rapidly transformed into hydrogen peroxide (H_2O_2), and the reduction of cytochrome C is slowed. Thus, the increase in the reduced cytochrome C concentration measured at 0 and 3 minutes is inversely correlated with the sample SOD activity.[15]

Catalase. No pretreatment of the samples was performed. Ten microliters of sample (or standard) was added to 990 µL of 57 mmol/L H_2O_2 solution at 25°C, pH 7.0. An oxygen electrode was used to measure oxygen production, which is directly correlated with the amount of active catalase present.[16]

Total glutathione. The erythrocyte lysate, sciatic nerve homogenate, and plasma were deproteinized in a TCA/EDTA solution. The supernatant was extracted with diethylether. One hundred microliters of the sample or standard was added to 900 µL solution containing 0.5 mmol/L 5,5-dithiobis(2-nitrobenzoic acid)3-carboxy-4-nitrophenyl disulfide (DTNB) and 200 mg/L NADPH. Ten microliters of 20 U/mL glutathione reductase solution was added. At 37°C, DTNB is reduced by reduced glutathione (GSH) and measurable at 412 nm. As GSSG is reduced in the presence of glutathione reductase and NADPH, the rate of reduction of DTNB is directly correlated with the GSH equivalent concentration. The change in absorption was measured permanently for 5 minutes, and the overall slope was representative of the amount of glutathione present.[17]

Erythrocyte total thiol. Erythrocyte lysate was deproteinized with a solution of 0.04 mol/L sulfuric acid and 0.3 mol/L tungstic acid. In a 96-well microtiter plate, 50 µL deproteinized test sample or reference sample was added to a solution containing 59 µmol/L DTNB. The level of reduced DTNB was measured at 412 nm and was directly correlated with the sample −SH concentration.[18]

GSSG. After deproteinization with 30% $HClO_4$, *N*-ethyl-morpholin (NEM) was added to obtain alkylation of GSH. NEM was alkylated with 3 mol/L potassium phosphate; 15% $HClO_4$ was added. One hundred microliters of supernatant was added to 300 µL of 1.0 mmol/L DTNB solution. 300 µL of 0.4 mol/L potassium phosphate buffer, and 300 µL solution containing 0.48 mmol/L NADPH and 3.0 U/mL glutathione reductase. DTNB reduction was measured at 412 nm. The speed of reduction of DTNB was directly correlated with the amount of GSSG present. GSSG concentrations were calculated using standard solutions treated identically.[19]

Urea. Plasma urea was assessed using a standard laboratory urease assay.[20]

Protein. Protein concentrations in sciatic nerve were measured according to the method of Bradford.[21]

Hemoglobin. Hemoglobin concentrations in whole blood were assessed using hemiglobin-cyanide.[22]

HbA_1. HbA_1 levels were measured using a commercial kit (Sigma Diagnostics, St Louis, MO).

Statistics

In experiment 1, ANOVA for repeated measurements was used to evaluate weight and nerve conduction velocity curves. Biochemical data were compared using ANOVA with a post hoc Bonferroni test. In experiment 2, differences between diabetic and control animals were calculated per group using an unpaired Student's *t* test. A P value less than .05 was considered significant.

RESULTS

Experiment 1 (STZ dose-effect relation)

As previously observed,[2,11] MNCV and SNCV increased approximately 40% as a consequence of maturation in the nondiabetic controls. Body weight, SNCV, and MNCV were significantly reduced in all diabetic animals ($P < .001$). Within the diabetic groups, a STZ dose-dependent effect was observed for weight ($P < .001$). In the 60-mg/kg STZ group, five animals died shortly after STZ injection. For nerve conduction, a similar significant dose-dependent difference between the diabetic groups was found for SNCV ($P < .01$) and MNCV ($P < .05$) (Fig 1). Blood glucose and HbA_1 were largely increased in diabetic animals ($P < .001$), and there was a trend for increased glucose ($P = .073$) and HbA_1 ($P = .084$) in the 60-mg/kg STZ group (Table 1). To exclude the possibility of uremic neuropathy, we measured plasma urea levels. Within the diabetic groups, urea levels were similar, although they were elevated compared with the controls ($P < .001$), probably reflecting the catabolic state of the diabetic animals. Plasma MDA levels were higher in diabetic animals ($P < .05$), but no effect of STZ dose on MDA was observed. Erythrocyte −SH and sciatic nerve and erythrocyte total glutathione levels were similar in diabetic and control animals.

Experiment 2 (oxidative stress and diabetes duration)

Increased systemic lipid peroxidation in the diabetic animals was shown by an increase in plasma MDA at weeks 4, 8, and 18 ($P < .001$). This increase was associated with elevated plasma total glutathione concentrations at weeks 2, 4, and 8 but not week 18 ($P < .05$) and attenuated plasma catalase levels, which reached significance at weeks 8 and 18 ($P < .05$). Plasma SOD remained unchanged except for a decrease in STZ-treated animals at week 2 ($P < .001$). In erythrocytes, initially attenu-

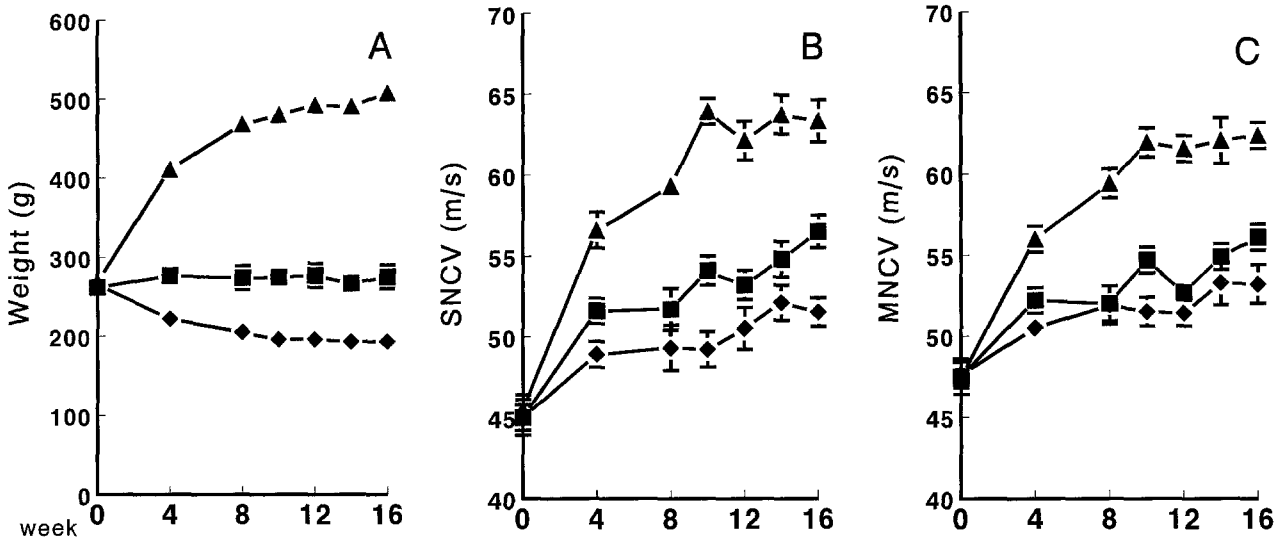

Fig 1. Experiment 1: weight (A), SNCV (B), and MNCV (C) in control (▲) rats and rats treated with 40 (■) and 60 (◆) mg/kg STZ. Statistical analysis (ANOVA for repeated measurements): weight, MNCV, and SNCV are reduced in both diabetic groups *v* controls ($P < .001$); significant differences between diabetic groups were observed for weight ($P < .001$), MNCV ($P < .05$), and SNCV ($P < .01$). Values are the mean ± SEM.

ated levels of −SH were observed in the diabetic group (week 2, $P < .001$; week 4, $P < .01$). A similar decrease was found in RBC total glutathione (week 2, $P < .01$; week 4, $P < .05$). Erythrocyte SOD was decreased in diabetic animals (week 2 and week 8, $P < .05$) (Fig 2 and Table 2).

Sciatic nerve MDA levels were not influenced by diabetes. A significantly elevated total glutathione to GSSG ratio ($P < .05$) was found in diabetic animals at weeks 8 and 18. Nerve catalase activity was elevated at most instances in diabetic animals (week 2 and week 18, $P < .05$; week 8, $P < .01$). Nerve SOD activity was elevated in diabetic animals at week 2 only ($P < .05$) (Fig 3).

Table 1. Experiment 1: Plasma, RBC, and Sciatic Nerve Biochemical Data After 18 Weeks of Diabetes

Parameter	Diabetics: STZ 40 mg/kg (n = 14)	Diabetics: STZ 60 mg/kg (n = 9)	Controls (n = 12)
Mean blood glucose (mmol/L)	24.4 ± 0.9	27.1 ± 1.2	8.7 ± 0.3*
Mean blood HbA_1 (%)	7.7 ± 0.2	8.3 ± 0.2	4.5 ± 0.1*
Plasma urea (μmol/L)	8.9 ± 1.1	8.8 ± 0.6	5.3 ± 0.2†
Plasma MDA (μmol/L)	1.46 ± 0.20	1.45 ± 0.16	0.83 ± 0.20‡
RBC total glutathione (μmol/g Hb)	8.77 ± 0.57	7.33 ± 0.51	8.13 ± 0.58
RBC-SH (μmol/g Hb)	13.2 ± 0.8	11.6 ± 0.7	13.3 ± 0.4
Nerve total glutathione (μmol/g protein)	20.4 ± 1.3	18.9 ± 0.7	23.7 ± 1.5

NOTE. Data are the group mean ± SEM. Rats received 40 or 60 mg/kg STZ to induce diabetes.

*Diabetic > control, F (ANOVA) < .001, no significant differences between diabetic groups.

†Diabetic > control, F (ANOVA) < .01, no significant differences between diabetic groups.

‡Diabetic > control, F (ANOVA) < .05, no significant differences between diabetic groups.

DISCUSSION

There is accumulating evidence that increased oxidative stress plays a role in the pathogenesis of nerve dysfunction in diabetes, since antioxidant treatment has been shown to protect against the development of experimental diabetic neuropathy.[2-5,23-27] The present data show that increased lipid peroxidation products, measured as MDA, are found in plasma but not in sciatic nerve from diabetic rats, independently of the STZ dose or duration of diabetes. The data from experiment 1 demonstrate that the highest dose of STZ is associated with increased mortality, weight loss, attenuated nerve conduction velocity, and some deterioration of glycemic control, but without further affecting MDA levels. Therefore, either STZ toxicity or decreased metabolic control, but not further enhancement of ROS-mediated toxicity, may explain the observed differences in body weight and nerve function.

Plasma MDA reflects a major part of the oxidation of lipid membranes from organs and cell structures in which lipid peroxidation occurs. Peroxidation of other products such as deoxy sugars, nucleosides, or benzoate may also form colored products with thiobarbituric acid, measurable at the same wavelength; therefore, the assay described is also often referred to as measurement of thiobarbituric acid–reactive substances.[28] Increased plasma MDA levels in diabetic animals indicate that more lipid peroxidation occurs either as a consequence of hyperglycemia or hyperlipidemia, which is also generally found in STZ-diabetic rats.[29] In both cases, they indicate an increased availability of ROS, ie, enhanced oxidative stress. Elevated MDA levels have been reported in the liver, brain, and kidney from diabetic animals.[30,31] Furthermore, other studies have shown an increase in MDA concentrations in RBC hemolysates from diabetic animals[31,32] or patients.[33-35] One study has demonstrated increased sciatic nerve MDA levels in rats after 3 weeks of diabetes,[36] but this was contradicted by others.[7,37]

Since nerve MDA levels were not elevated in diabetic

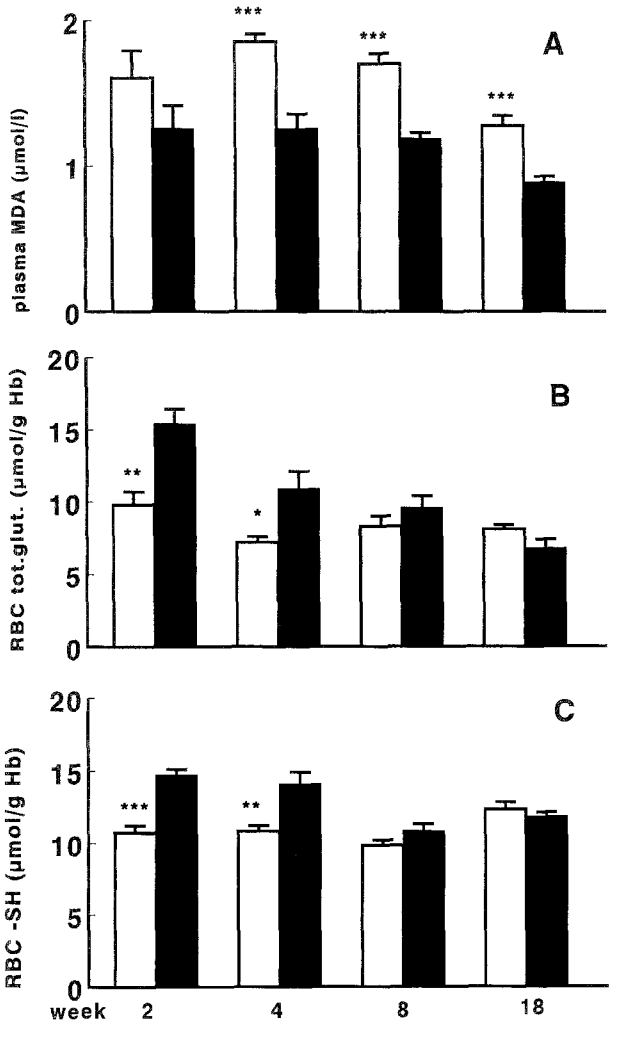

Fig 2. Experiment 2: plasma MDA (A) and RBC total glutathione (B) and total −SH (C) concentrations in diabetic (□) and control (■) rats after 2, 4, 8, and 18 weeks of hyperglycemia. Diabetics were compared with controls by Student's *t* test (*$P < .05$, **$P < .01$; ***$P < .001$). A significant age-dependent decrease in RBC total glutathione and −SH was observed in the controls by ANOVA with a post hoc Bonferroni test (F, ANOVA < .0001). Values are the mean ± SEM.

animals in this study, the present data suggest that the local endoneurial toxicity of ROS is less important than systemic oxidative stress. Sciatic nerve conjugated dienes, another parameter to evaluate lipid peroxidation, have been shown to be increased by experimental diabetes with a duration between 1 and 12 months.[7] However, in a second study from the same group,[38] no effects of hyperglycemia on conjugated dienes were observed, unless STZ-diabetes was combined with α-tocopherol deficiency. However, this effect disappeared with a longer duration of diabetes.[38] Previously, we observed an important increase in sciatic nerve MDA but no nerve conduction deficits in nondiabetic α-tocopherol–deficient rats.[37]

Besides lipid peroxidation, we studied both systemic and endoneurial endogenous ROS scavengers in experimental diabetes. In some cases, we observed changes in the antioxidant status of the control group. Age-related variations in antioxidant capacity have been described before, and it has been demonstrated that with maturation, hepatic glutathione output decreases.[39]

The present data confirm previous observations[11,37] of elevated sciatic nerve catalase activity in STZ-diabetic rats. Increased concentrations of this enzyme have also been reported in the heart and pancreas from diabetic animals.[40,41] Since comparable changes occurred during food restriction, it has been suggested that besides the hyperglycemia, the catabolic state of the diabetic animal contributes to this increase.[42] Possibly, increased local H_2O_2 production induces the enzyme activity, as increased mRNA production has been demonstrated in cell cultures incubated with H_2O_2.[43]

GSH plays a pivotal role in the protection against ROS in peripheral tissue. Alterations in GSH/GSSG balance may be the consequence of both increased polyol pathway flux and decreased glucose-6-phosphate dehydrogenase activity in diabetes.[44] The present data demonstrate that after 18 weeks of diabetes, there is an increase in the total to oxidized glutathione ratio, which is the consequence of both a small increase in total glutathione and attenuated GSSG levels. Decreased GSH concentrations have been reported in the sciatic nerve after 1 month of STZ-induced diabetes,[5,38] but this decrease disappeared with a longer duration of diabetes.[38] This suggests enhanced endoneurial synthesis or molecular reduction of glutathione as an adaptation to the increase of ROS.

Total and reduced glutathione in RBCs were significantly decreased in the first 4 weeks of diabetes only. Together with the

Table 2. Experiment 2: Weight, Blood Glucose, and Plasma and RBC Antioxidant Levels

Parameter	Week 2		Week 4		Week 8		Week 18	
	D	C	D	C	D	C	D	C
Weight (g)	298 ± 13*	355 ± 7	334 ± 14*	438 ± 12	320 ± 18*	445 ± 10	323 ± 15*	495 ± 16
Blood glucose (mmol/L)	30.0 ± 0.9*	5.8 ± 0.2	31.0 ± 1.0*	5.3 ± 0.1	29.1 ± 1.4*	5.4 ± 0.2	32.3 ± 0.9*	6.1 ± 0 3
Plasma total glutathione (µmol/L)	18.2 ± 1.8†	11.1 ± 2.3	12.3 ± 2.2†	6.2 ± 0.7	12.3 ± 1.8†	7.8 ± 0.6	7.3 ± 1.0	6.0 ± 0.3
Plasma SOD (mg/L)	4.51 ± 0.22*	6.05 ± 0.26	5.86 ± 0.11	5.73 ± 0.20	5.86 ± 0.19	6.08 ± 0.26	5.85 ± 0.19	6.05 ± 0.18
Plasma catalase (mg/L)	1.49 ± 0.13	2.51 ± 0.55	1.45 ± 0.15	1.58 ± 0.11	1.51 ± 0.14†	1.94 ± 0.07	1 38 ± 0.14†	2.06 ± 0.20
RBC SOD (mg/g Hb)	1.10 ± 0.04†	1.25 ± 0.04	1.23 ± 0.04	1.27 ± 0.04	1.11 ± 0.04†	1.24 ± 0.04	1 21 ± 0.05	1.32 ± 0 04

NOTE. Values are the mean ± SEM. All groups consisted of 8 animals. Significant differences between diabetic and control animals were calculated by Student's *t* test.

Abbreviations: D, STZ-diabetic rats after 2, 4, 8, and 18 weeks of hyperglycemia; C, age-matched controls.

*$P < .001$.

†$P < .05$.

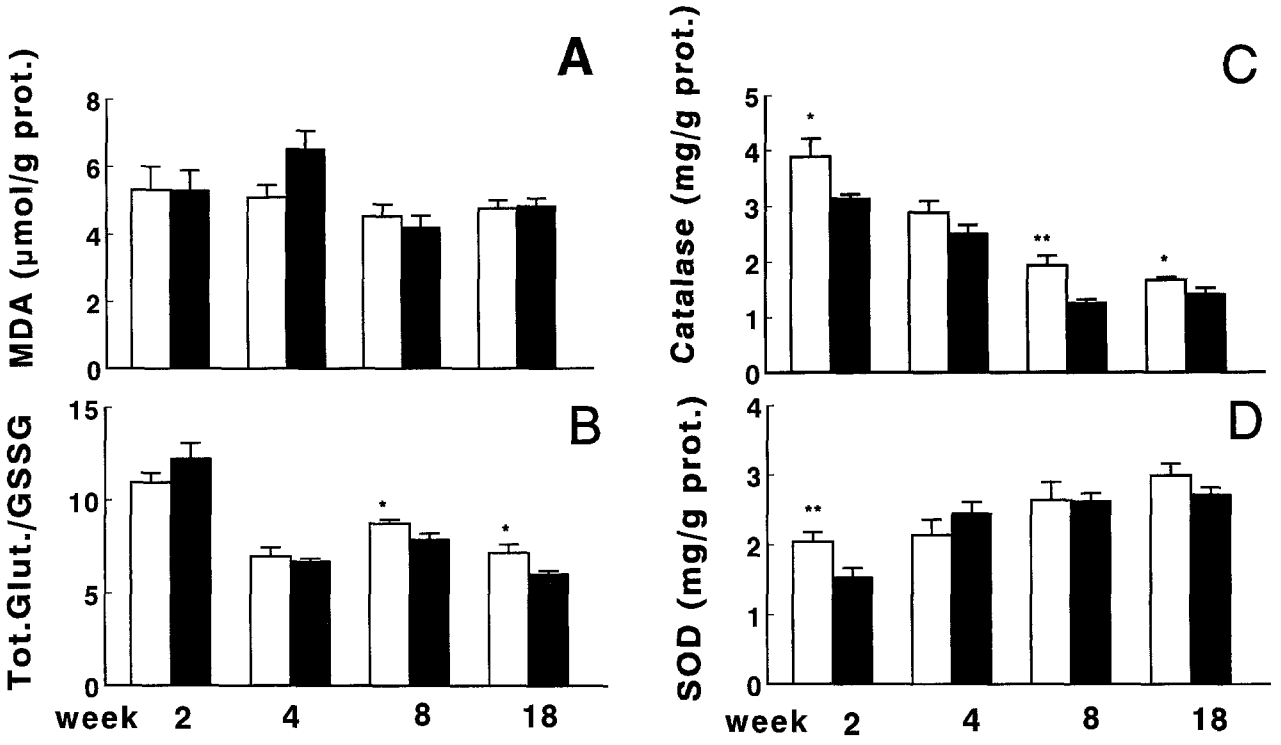

Fig 3. Experiment 2: sciatic nerve MDA (A), total glutathione to GSSG ratio (B), catalase (C), and SOD (D) in diabetic (□) and control (■) rats after 2, 4, 8, and 18 weeks of hyperglycemia. Diabetics were compared with controls by Student's *t* test (*$P < .05$, **$P < .01$). A significant age-dependent decline in the nerve total glutathione to GSSG ratio and catalase, as well as an increase in SOD, were observed in the controls by ANOVA with a post hoc Bonferroni test (F, ANOVA < .0001). Values are the mean ± SEM.

observed increase in plasma glutathione levels, this suggests a leakage of glutathione from the erythrocyte as a consequence of increased oxidation and the inability to maintain glutathione in its reduced form. Increased GSSG release from erythrocytes of diabetic patients has indeed been demonstrated.[44] The decreased erythrocyte SOD activity, leading to elevated superoxide levels, could be responsible for an increased consumption of glutathione.

In conclusion, increased oxidative stress in STZ-diabetic rats leads to an increase in plasma lipid peroxidation degradation products, but has no influence on endoneurial MDA concentrations. Furthermore, the observed sciatic nerve catalase activity and total versus oxidized glutathione balance probably reflect local adaptation to increased oxidative stress. The systemic effects of STZ-diabetes observed herein were a persistent reduction in plasma catalase and erythrocyte SOD activity, as well as a temporary decrease in total and reduced glutathione levels in erythrocytes. The dose-dependent effect of STZ on nerve conduction and body weight was not reflected in systemic lipid peroxidation or erythrocyte or nerve glutathione. Thus, to evaluate the effect of diabetes on nerve function or antioxidant capacity, the duration of hyperglycemia, the degree of maturation of the animal, and the dose of STZ must be taken into consideration. Further studies should focus on the effects of hyperglycemia and enhanced oxidative stress on local and systemic endogenous ROS scavenger synthesis, as our data suggest that diabetes is associated with protective antioxidant adaptive mechanisms.

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