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Ischemia–Reperfusion Injury Causes Oxidative Stress and Apoptosis of Schwann Cell in Acute and Chronic Experimental Diabetic Neuropathy

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ABSTRACT

Mild ischemia–reperfusion (IR) injury to diabetic peripheral nerve is known to cause severe ischemic fiber degeneration. Little information is available on its effects on Schwann cell (SC). In this study, we evaluated oxidative stress and apoptosis of SC following mild IR, using immunohistochemistry in streptozotocin (STZ)-induced diabetic rats. Twenty-six rats were divided into four groups according to the duration of diabetes: 1-month STZ-induced diabetic group (*n* = 7) and age-matched control group (*n* = 7); 4-month STZ-induced diabetic group (*n* = 6) and age-matched control group (*n* = 6). Using our established IR model of 3 h of ischemia followed by 7 days of reperfusion, sciatic and tibial nerves were harvested and labeled with 8-hydroxy-deoxyguanosine (8-OHdG; oxidative stress marker), caspase-3 (apoptotic executor), and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) activity (apoptotic indicator). Marked positive staining with 8-OHdG, caspase-3, and TUNEL were found in diabetic ischemic nerves (right side) following IR in both 1-month and 4-month groups. Only mild positive staining or no staining was seen in the nonischemic side (left side) of diabetic and age-matched control groups. Co-labeling with S-100 confirmed that the cells labeled with 8-OHdG, caspase3, and TUNEL were SC. SC was susceptible to oxidative injury and apoptosis in experimental diabetic neuropathy when subjected to mild IR injury. *Antioxid. Redox Signal.* 7, 1513–1520.

INTRODUCTION

Neuropathy is a common complication of diabetes, affecting >50% of diabetic patients after 15 years of hyperglycemia (8). The precise pathogenesis of diabetic neuropathy is unknown. However, a number of putative pathophysiologic mechanisms exist. Hyperglycemia is reported to result in polyol pathway overactivity (10), increased advanced glycation end products (2), nerve hypoxia/ischemia (17), deficiency of γ-linolenic acid (4), increase in protein kinase C, especially the β-isoform (5), and growth factor(s) deficiency (11). These pathways all converge in producing oxidative stress (18, 31). Nerve ischemia and oxidative stress may be key factors involved (18, 26). Apoptosis can be induced in the nervous system by oxidative stress through activation of the caspase pathway (37). Recently, studies have shown that apoptosis is involved in the pathogenesis of diabetic sensory neuropathy associated with dysfunction of mitochondria and activation of the caspase pathway (29, 31, 33, 35).

Ischemia–reperfusion (IR) injury insufficient to cause fiber degeneration of normal nerves will readily cause ischemic fiber degeneration (IFD) of diabetic peripheral nerves (25, 36). Demyelination occurs in diabetic neuropathy. Although this could be secondary to axonal atrophy, an alternative or additional mechanism is a Schwannopathy. In the pres-

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ent study, we test the hypothesis that IR has a second target, the Schwann cell (SC), which is subject to oxidative injury and apoptosis.

For evaluation of oxidative stress and apoptosis, we undertook immunohistochemical staining of 8-hydroxydeoxyguanosine (8-OHdG), caspase-3, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) positivity; for confirmation of SC, double-staining with S-100 was applied.

RESEARCH DESIGN AND METHODS

Animals

Male Sprague–Dawley rats weighing 250 ± 5 g at the beginning of the study were used. They were divided into four groups according to the duration of diabetes: 1-month streptozotocin (STZ)-diabetic group (1moD, $n = 7$) and age-matched control group (1moC, $n = 7$); 4-month STZ-diabetic group (4moD, $n = 6$) and age-matched control group (4moC, $n = 6$), with 3 h of ischemia and 7 days of reperfusion. The STZ-diabetic groups were produced by intraperitoneal injection of STZ in 0.05 ml/L citrate buffer at pH 4.5 (65 mg/mL; dose 1.32 ml/kg). The control groups were injected with citrate buffer alone. They did not receive insulin treatment. The rats were accepted as diabetic when their fasting blood glucose exceeded 16.7 mmol/L 3 days after injection of STZ and remained >16.7 mmol/L throughout the study. The experimental protocol was approved by the Mayo Clinic Institutional Animal Care and Use Committee and conformed to NIH guidelines for the use of animals in research.

IR model

Ischemia was produced by ligating the abdominal aorta, the right iliac artery, the right femoral artery, and all identifiable collateral vessels supplying the right sciatic–tibial nerve with 6-0 silk sutures, as previously described (21) in our lab. After 3 h of ischemia, while maintaining the right hind limb at 35°C, the ties were released using a slipknot technique for ready release and rapid reperfusion. The limb temperature was monitored with an intramuscular thermistor probe and was maintained at 35°C during ischemia by using an infrared lamp. Tibial and sciatic nerves were harvested 1 week after ischemic surgery for immunohistochemistry evaluation. The left side served as the nonischemic control side.

Immunohistochemistry

The rat received intracardiac perfusion with 2% paraformaldehyde for 30 min at a rate of 20–22 ml/min. The entire lengths of sciatic–tibial nerves were harvested. For cryostat sectioning (10 μm), the proximal sciatic and midtibial nerves were postfixed in 2% paraformaldehyde for 24 h, immersed in 30% sucrose for 24 h, covered with ornithine carbamyltransferase compound, frozen with liquid nitrogen, and then stored at −80°C.

Sections were stained with the following antibodies: goat anti-8-OHdG and rabbit anti-active caspase-3 polyclonal antibodies (Chemicon International, Inc., Temecula, CA, U.S.A.) and TUNEL (TdT-FragEL DNA Fragmentation Detection Kit; Oncogene Research Products, Boston, MA, U.S.A.) as previously described (31). Filtered 3,3′-diaminobenzidine (DAB) was used as the chromogen. Negative controls were produced by omission of the primary antibody in every protocol.

Immediately following the DAB step, the above sections were blocked with 10% normal donkey serum and incubated with S-100 [rabbit anti-S-100 polyclonal antibody (Chemicon International, Inc.) and goat anti-S-100 polyconal antibody (Santa Cruz Biotechnology, Inc., CA, U.S.A.)] for 1 hour at 37°C. Sections were then incubated with donkey anti-rabbit IgG-fluorescein-5-isothiocyanate (FITC) or donkey anti-goat IgG-cy3 (Chemicon International, Inc.) and then coverslipped using slow-fade media.

Evaluation

Sections were semiquantitatively graded by two independent observers. In brief, cells were considered positive if clear, cell-like staining was visible. The percentage of positive cells was graded as follows: grades 0 to 4 represent ≤5%, 6–15%, 16–25%, 26–35%, and >35% positive cells, respectively.

Statistics

Data are expressed as means ± SE. To examine grading for 8-OHdG, caspase-3, and TUNEL, Mann–Whitney U tests were used for nonparametric analysis. To compare the difference between right side (ischemic side) and left side (nonischemic side), Wilcoxon signed rank tests were used for nonparametric paired analysis. The correlation between 8-OHdG, caspase-3, and TUNEL was performed using Spearman rank correlation.

RESULTS

8-OHdG

For the ischemic (right) limb, excessive positive staining for 8-OHdG was seen in both the 1-month (Fig. 1) and 4-month (Fig. 2) diabetic groups, and these alterations were consistently greater than those seen in 1-month and 4-month control groups when subjected to IR (Figs. 1 and 2). The grades of 8-OHdG were increased significantly to 3.7 ± 0.3 (tibial nerve, diabetic versus control, $p < 0.05$) and 2.6 ± 0.7 (sciatic nerve, diabetic versus control, $p < 0.01$) for 1-month diabetic groups, compared with 1.5 ± 0.5 (tibial nerve) and 0.0 ± 0.0 (sciatic nerve) for 1-month control groups. Similarly, a significant increase in 8-OHdG was found for the 4-month diabetic group when compared with the 4-month control group (tibial nerve, diabetic 3.7 ± 0.3 versus control 0.3 ± 0.2, $p < 0.01$; sciatic nerve, diabetic versus 3.3 ± 0.7 versus control 0.3 ± 0.2, $p < 0.05$) (Fig. 3). There were significant differences between right side (ischemic side) and left side (nonischemic side) in 1-month diabetic group and tibial nerve of 4-month diabetic groups, but no significant difference was found in all control groups and 4-month sciatic nerve ($p = 0.0625$).

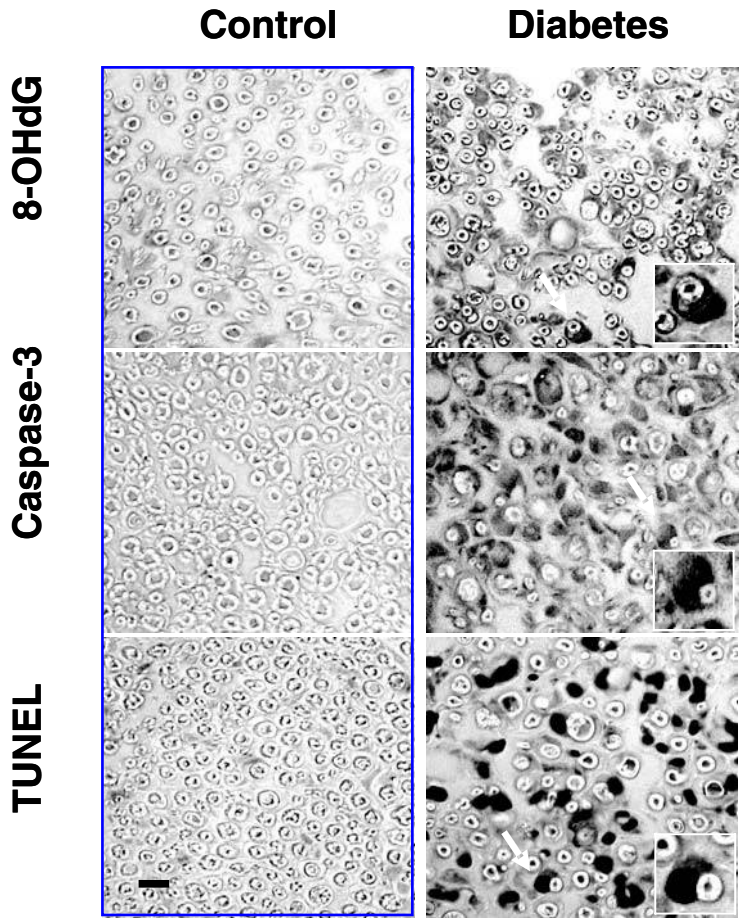

FIG. 1. Microscopic findings of immunoreactivity of 8-OHdG, caspase-3, and TUNEL in 1-month diabetic and age-matched control groups (right tibial nerve only). After 3 h of ischemia and 7 days of reperfusion, extensive positive cellular labeling of 8-OHdG, caspase-3, and TUNEL was seen in right tibial nerve of diabetic groups (arrows and white-framed pictures in the right corner represent positive cell). Only mild positivity or negative staining was shown in control groups for right tibial and sciatic nerves. Bar = 10 μm.

Caspase-3

A marked increase in the number of caspase-3-positive cells was seen for both the 1-month (Fig. 1) and 4-month (Fig. 2) diabetic groups. In contrast, few positive staining cells were found in the age-matched control group. The grades of caspase-3 were higher for both sciatic and tibial nerves in 1-month (tibial, diabetic 3.8 ± 0.2 versus control 0.8 ± 0.6, $p < 0.05$; sciatic, diabetic 2.7 ± 0.6 versus control 0.1 ± 0.1, $p < 0.01$) and 4-month diabetic groups (tibial, diabetic 3.7 ± 0.3 versus control 0.5 ± 0.2, $p < 0.01$; sciatic, diabetic 3.3 ± 0.7 versus control 0.2 ± 0.2, $p < 0.05$) than in age-matched control groups subjected to IR injury. There was no significant difference between the 1-month and 4-month groups (Fig. 4). The grade of caspase-3 was very low (equal to or very close to 0.0 ± 0.0) for left side nerves (nonischemic side) in all control and diabetic rats, except for left tibial nerve in 4-month diabetic rats (grade was 0.7 ± 0.2). There were significant differences found between right side (ischemic side) and left side (nonischemic side) in 1-month diabetic group and tibial nerve of 4-month diabetic group ($p < 0.05$) (Fig. 4).

TUNEL

The number of TUNEL-positive cells was increased dramatically in 1-month and 4-month diabetic nerves compared with that of age-matched control groups when subjected to IR injury (Figs. 1 and 2). The grade of TUNEL was increased in 1-month (tibial, diabetic 3.5 ± 0.5 versus control 1.00 ± 0.5, $p < 0.05$; sciatic, diabetic 2.7 ± 0.6 versus control 0.1 ± 0.1, $p < 0.01$) and 4-month diabetic groups (tibial, diabetic 3.8 ± 0.2 versus control 0.2 ± 0.2, $p < 0.01$; and sciatic, diabetic 3.5 ± 0.5 versus control 0.5 ± 0.2, $p < 0.01$) (Fig. 5).

For the left (nonischemic side), there was a consistent trend where TUNEL grade of both tibial and sciatic nerves in 4-month diabetic rats was higher than that of 1-month diabetic rats and 4-month control. Significant differences were shown in all diabetic groups between right side (ischemic side) and left side (nonischemic side) (Fig. 5).

A majority of immunolabeled cells were identified by double staining with S-100 as SCs (Fig. 6). Based on double staining of 8-OHdG-, caspase-3-, and TUNEL-positive cells with S-100, we concluded that the majority of cells undergoing oxidative stress and apoptosis were the SC.

Correlation

The correlations between 8-OHdG, caspase-3, and TUNEL in diabetic groups were performed by Spearman correlation. 8-OHdG and caspase-3 significantly correlated with TUNEL in both 1-month and 4-month sciatic nerves (*1-month:* 8-OHdG: $r = 0.9661$, $p = 0.0028$; caspase-3: $r = 0.9555$, $p = 0.0028$; *4-month:* 8-OHdG: $r = 1$, $p = 0.0004$;

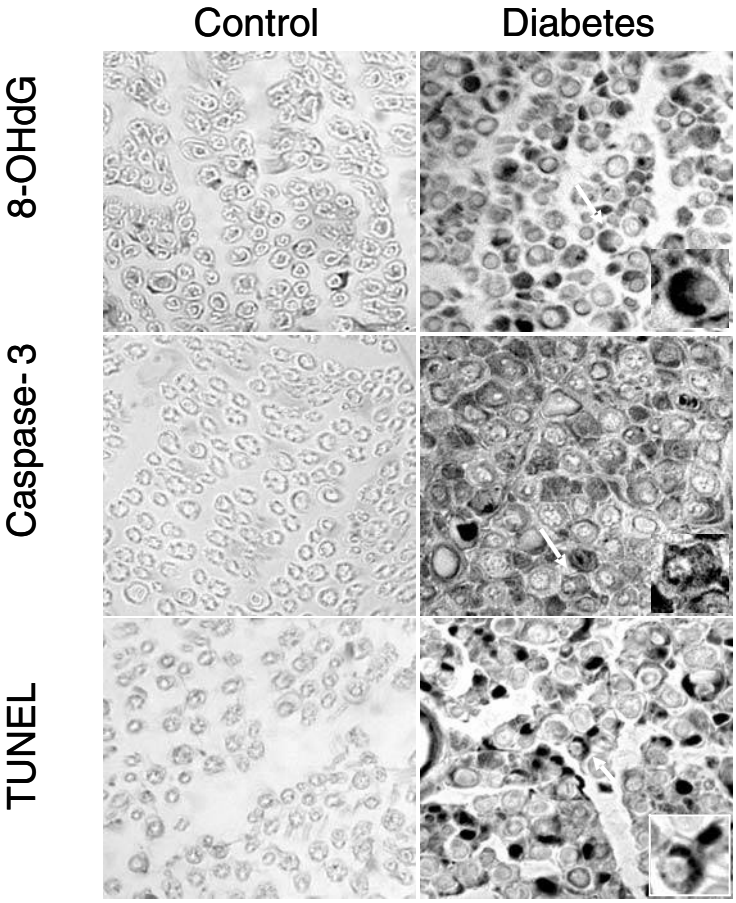

FIG. 2. Microscopic findings of immunoreactivity of 8-OHdG, caspase-3, and TUNEL in 4-month diabetic and age-matched control groups (right tibial nerve only). After 3 h of ischemia and 7 days of reperfusion, markedly positive labeling of 8-OHdG, caspase-3, and TUNEL was seen in right tibial nerve of 4-month diabetic groups (arrows and white-framed pictures in the right corner represent positive cell). The staining was diffuse in 4-month diabetic when compared with control or 1-month diabetic groups. Cells in control groups were either negative or demonstrated only mild imunoreactivity. Bar = 10 μm.

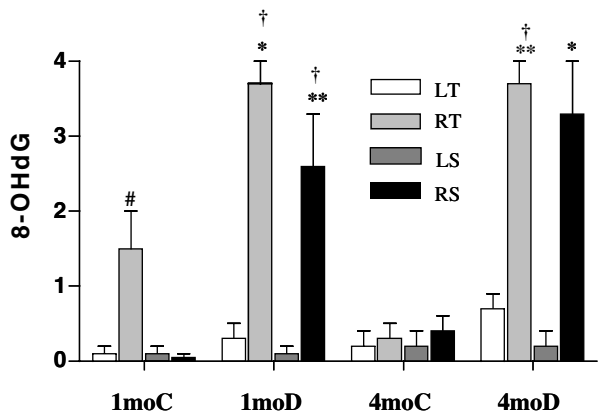

FIG. 3. Grades of immunohistochemical staining with 8-OHdG of left and right tibial and sciatic nerves in 1-month and 4-month diabetic and age-matched control groups. All right tibial and sciatic nerves of diabetic groups (1moD and 4moD) exhibited higher grades than those of control groups (1moC and 4moC). All grades of left-sided nerves (nonischemic nerve) were very low. There were significant differences between right-sided nerves (ischemic nerve) and left-sided nerves (nonischemic nerve) in 1-month diabetic and tibial nerve of 4-month diabetic groups. There was a significant increase for right tibial nerve in 1-month control group when compared with 4-month control group. For this and subsequent figures, all data are expressed as means ± SE. *$p < 0.05$, **$p < 0.01$, control versus diabetic groups; †$p < 0.05$, ‡$p < 0.01$, left side versus right side; #$p < 0.05$, 1-month versus 4-month groups. 1moC, 1-month control group; 1moD, 1-month diabetic group; 4moC, 4-month control group; 4moD, 4-month diabetic group; LT, left tibial nerve; RT, right tibial nerve; LS, left sciatic nerve; RS, right sciatic nerve.

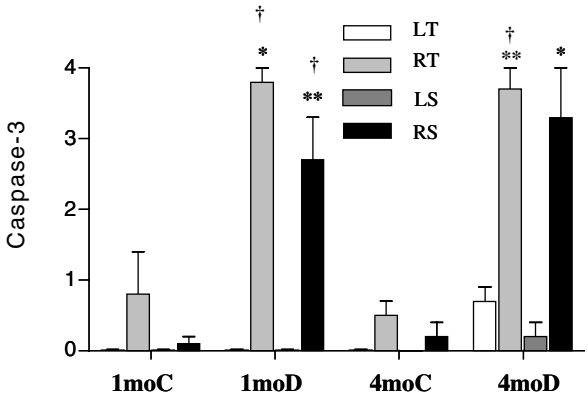

FIG. 4. Grades of immunohistochemical staining with caspase-3 of left and right tibial and sciatic nerves in 1-month and 4-month diabetic groups and age-matched control groups. The grades of caspase-3 in all right diabetic nerves (tibial and sciatic nerves, 1moD and 4moD) were higher than those of age-matched control groups (1moC and 4moC). The grades of caspase-3 in left-sided nerves were close to 0, except left tibial nerve in 4-month diabetic rats (increased at 0.7). Significant differences were shown between right-sided nerves (ischemic side) and left-sided nerves (nonischemic side) in 1-month diabetic group and tibial nerve of 4-month diabetic group. There was no significant difference between 1-month and 4-month groups. For symbols and abbreviations, see Fig. 3 legend.

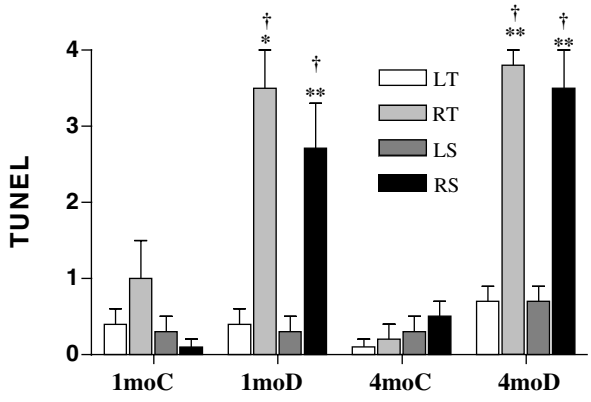

FIG. 5. Grades of TUNEL activity of left and right tibial and sciatic nerves in 1-month and 4-month diabetic and age-matched control groups. All right diabetic nerves had higher TUNEL positivity than right age-matched control nerves. The grades in control groups did not exceed 1.0. Significant differences were found between right-sided nerves (ischemic side) and left-sided nerves (nonischemic side) in all diabetic groups. No significant difference was found between 1-month and 4-month groups. For symbols and abbreviations, see Fig. 3 legend.

caspase-3: $r = 1$, $p = 0.0004$). There was no significant correlation in the diabetic tibial nerves.

DISCUSSION

For the first time, we have demonstrated that SC is susceptible to apoptosis induced by oxidative stress secondary to IR injury in experimental diabetic rats. Oxidative injury and apoptosis of SC occurred in diabetic rats with short-duration (1 month, acute) and long-duration (4 months, chronic) diabetes, affecting both tibial nerve (severe ischemia) and sciatic nerve (moderate ischemia).

The alterations in nerve conduction, blood flow, and biochemical abnormalities in experimental diabetes closely mimic those of human diabetic neuropathy, but the severity of microvascular disease is much milder in experimental diabetes. In certain types of focal diabetic neuropathy in humans, such as radiculoplexus neuropathy and radiculopathies, there is severe ischemia associated with prominent inflammatory infiltration (9). Combining experimental diabetes with IR, as we have done in this study, results in a similar pattern of focal IFD with prominent inflammatory changes.

Peripheral nerve is physiologically unique in its vasoregulation and response to ischemia. It has poor autoregulation so that flow directly reflects pressure (16), and there is a dramatic reduction in endoneurial oxygenation in response to a small change in blood volume (34), rendering it particularly vulnerable to ischemia injury. Additionally, it undergoes an enhanced and prolonged vasoconstriction in response to topical application of endothelin-1 in diabetes (38).

The susceptibility of diabetic peripheral nerve subjected to a reduction in blood flow by embolization (12) or IR has been well demonstrated in our previous studies (21, 32). Although the pathogenesis of IFD is still not fully understood, oxidative stress is likely to be important (23). Additionally, treatment with the potent antioxidant α-lipoic acid is neuroprotective against IR (21) and prevents the blood flow deficits in experimental diabetes (22).

The sensitivity of SC to IR injury in diabetic peripheral nerve is due in part to reduced free radical defenses in peripheral nerve compared with brain and liver, a problem that is accentuated in the diabetic state (19). Reduced glutathione (GSH) and GSH-containing enzyme scavengers are only 10% that of brain in the normal rodent sciatic nerve (28). Further reduction of cuprozinc superoxide dismutase occurs in sciatic nerve of experimental diabetic neuropathy (EDN) (15). An elevated lipid peroxidation production (malondialdehyde and 4-hydroxyalkenals) and decreased GSH and taurine were shown in diabetic rats, even at very early time points (22, 27). These conditions render peripheral nerve, and especially SC (with its higher metabolic rate), vulnerable to IR injury. Although these studies have focused on oxidative stress and antioxidant defenses, there has been no attempt to separately study SC. Clearly, such a study is needed in the future.

Recent studies have focused on the molecular details of oxidative injury and apoptosis. A sustained high level of glucose increases mitochondrial-generated reactive oxygen species (24), inducing mitochondrial dysfunction with release of proapoptotic factor and leading to the activation of caspase-3, finally resulting in apoptosis or cell death in diabetic complications (3, 30, 33). Our IR model has the advantage of permitting us to separately study ischemia and reperfusion by ligating (aorta and collaterals on one side) and then releasing ligatures of blood vessels supplying sciatic and

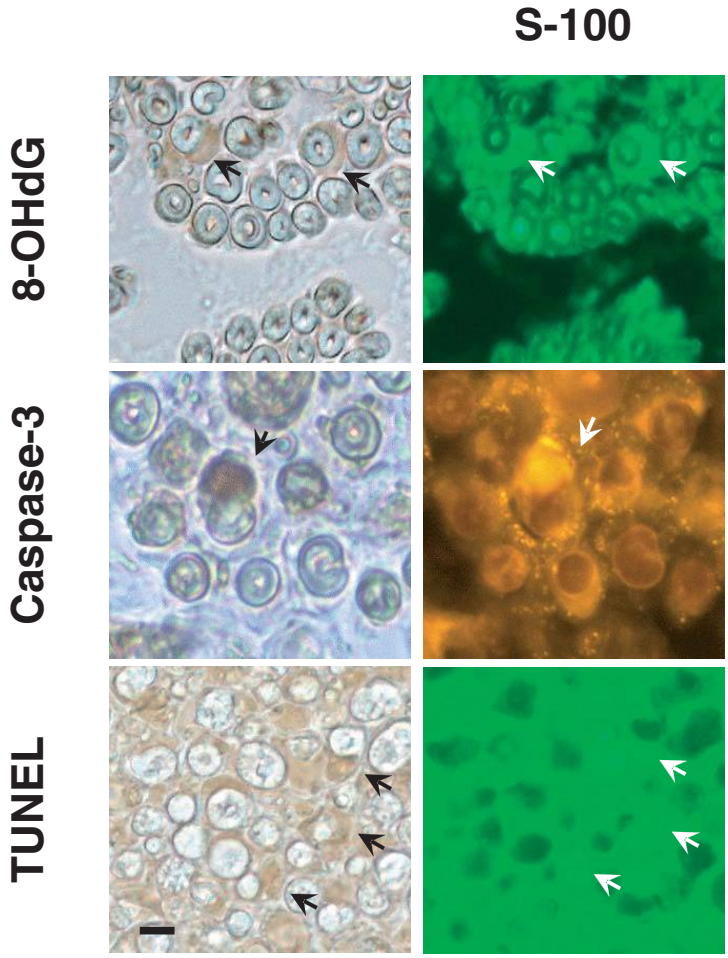

FIG. 6. Fluorescence double labeling of S-100 with 8-OHdG, caspase-3, and TUNEL. Double labeling of 8-OHdG, caspase-3, and TUNEL (brown) and S-100 (FITC: green for 8-OHdG and TUNEL; Cy3: red for caspase-3) (arrows: positive staining) is shown. Cells positive for 8-OHdG, caspase-3, and TUNEL co-labeled with S-100, confirming that the cell undergoing oxidative stress and apoptosis after IR injury is SC. Bar = 10 μm.

tibial nerves (32). We also have the scenario where we have three levels of ischemia: greatest in ipsilateral tibial nerve, moderate in ipsilateral sciatic nerve, and minimal in contralateral sciatic–tibial nerve. We selected immunohistochemical labeling so that particular cells can be identified. We selected conditions of an ischemic duration of 3 h at 35°C because this duration causes only minimal IFD in normal nerves, which requires 5 h to consistently cause IFD (20, 21, 32). A reperfusion duration of 7 days provides optimal conditions for the detection of oxidative injury and IFD (20, 21, 32). Immunolabeling with 8-OHdG, caspase-3, and TUNEL has the advantage of identifying the number and cells affected by oxidative injury and commitment to the apoptotic pathway (31). Using this approach, our results indicate that, on average, ~35% of SC (a score of 3 indicates 26–35% of cells are labeled) are undergoing oxidative injury in tibial nerve and ~20% in the sciatic nerve in both 1-month and 4-month STZ-diabetic rats. Co-labeling with S-100 confirms that the cells positive for 8-OHdG, caspase-3, and TUNEL are indeed SC, indicating that oxidative stress and apoptosis of SC occur in diabetic peripheral neuropathy. The minimal positive staining found in the left-side nerves for control and diabetic rats suggests that oxidative stress and apoptosis in this model were caused mainly by IR injury. Although there is no statistical significance, there is a consistent trend that grades of all three markers (8-OHdG, caspase-3, and TUNEL) for 4-month diabetic left tibial nerve are higher than those of 1-month diabetic rats, implicating that 4-month diabetic nerve is more susceptible to oxidative stress than 1-month diabetic nerve. In our previous work, IFD was found in 1-month and 4-month diabetic right tibial and sciatic nerves when subjected to IR injury. The same process (*i.e.,* oxidative stress) resulting from IR is responsible for two different results (necrosis and apoptosis) to two different targets (axon and SC). It causes necrosis (IFD) of the axon, but apoptosis of SC in diabetic nerves subjected to moderate ischemia (sciatic nerve).

Apoptosis has been involved in various diabetic complications, including retinopathy (1), encephalopathy (14), and neuropathy (29). The report of activation of caspase-3 (with negative TUNEL) in long-term STZ-induced diabetic dorsal root ganglion sensory neurons without resultant cell loss has appeared (6). This finding is at variance with other reports of both caspase-3 and TUNEL positivity, with confirmatory chromatin clumping and ganglion neuron loss (13, 29). The discrepancy likely reflects severity of oxidative injury to the neurons. Apoptosis, with caspase-3 expression and TUNEL positivity, unequivocally demonstrates apoptosis to the SC in our model of EDN, subjected to the additional insult of mild IR. Diabetic SC is excessively vulnerable to oxidative dam-

age and apoptosis. The significant correlation between oxidative stress (8-OHdG), apoptotic executioner (caspase-3), and apoptosis (TUNEL) in all diabetic sciatic nerve supported our hypothesis that diabetic SC, as a second target, was susceptible to apoptosis induced by IR oxidative damage. The lack of significant correlation in diabetic tibial nerves was due to negative immunolabeling for markers of apoptosis by too severe ischemia, resulting in necrotic loss of SC.

The importance of recognizing SC as a separate target has significant implications. SC is needed to maintain myelin. SC injury may result in an inability to maintain myelin, and demyelination is a feature of human diabetic neuropathy. SC is also essential in fiber regeneration so that abnormal SC might result in impaired regeneration and symptoms of neuropathy in human diabetic peripheral nerve (7).

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ABBREVIATIONS

DAB, 3,3′-diaminobenzidine; EDN, experimental diabetic neuropathy; FITC, fluorescein-5-isothiocyanate; GSH, glutathione; IFD, ischemic fiber degeneration; IR, ischemia–reperfusion; 8-OHdG, 8-hydroxydeoxyguanosine; SC, Schwann cell; STZ, streptozotocin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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