Role for nitrosative stress in diabetic neuropathy: evidence from studies with a peroxynitrite decomposition catalyst

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SPECIFIC AIMS

Diabetic distal symmetric sensorimotor polyneuropathy affects up to 60–70% of patients with diabetes mellitus in the U.S. The best-studied mechanisms of diabetic neuropathy include increased aldose reductase (AR) activity, nonenzymatic glycation/glycooxidation, activation of protein kinase C, and impaired neurotrophic support. These mechanisms contribute to enhanced oxidative stress resulting from increased production of reactive oxygen species (ROS) and insufficient up- or down-regulation of antioxidative defense.

Increased formation of the potent oxidant peroxynitrite (the product of superoxide anion radical reaction with nitric oxide) has been documented in experimental and clinical diabetic neuropathy. Peroxynitrite causes nitration and nitrosylation of biomolecules including proteins, lipids, and DNA. Nitrosative stress has been implicated in DNA single strand breakage, followed by poly(ADP-ribose) polymerase (PARP) activation, in pathological conditions associated with oxidative stress. The pathogenetic role of reactive nitrogen species in diabetic neuropathy remains unexplored. This study was designed to evaluate the role for nitrosative stress in early diabetic neuropathy in two animal models of Type 1 diabetes mellitus [streptozotocin (STZ)-diabetic mice and diabetic NOD mice], using the potent peroxynitrite decomposition catalyst FP15.

PRINCIPAL FINDINGS

1. FP15 corrects physiological abnormalities characteristic for peripheral diabetic neuropathy in two models of Type 1 diabetes mellitus

Experiments were performed in STZ-diabetic mice and NOD-diabetic mice. In experiment 1, control and STZ-diabetic mice were treated with or without FP15 (5 mg kg^{-1} d^{-1}, in drinking water) for 1 wk after initial 8 wk without treatment. Sciatic nerve motor and hind-limb digital sensory nerve conduction velocities (MNCV and SNCV) were measured at the beginning of the study (prior to induction of diabetes), at 8 wk time point (prior to FP15 treatment) and at the end of the study. After completion of nerve functional studies, the mice were killed and both sciatic nerves removed for biochemical [phosphocreatine (PCr), creatine (Cr), glucose, sorbitol, fructose] and immunohistochemical [nitrotyrosine (NT), poly(ADP-ribose), and PARP-1] measurements. MNCV and SNCV were reduced in diabetic mice 8 wk after induction of diabetes. They remained reduced in the untreated group 9 wk after induction of diabetes, whereas 1 wk FP15 treatment normalized MNCV and essentially normalized SNCV (**Fig. 1*A***). FP15 treatment did not affect either MNCV or SNCV in control mice. In experiment 2, diabetic NOD mice were treated with either 1 or 3 mg kg^{-1} d^{-1} FP15 in drinking water for 1 wk. FP15 dose-dependently reversed increased tail-flick latency (a sign of hypoalgesia), and the effect of the higher dose was significant as early as 3 days after the beginning of treatment (Fig. 1*B*). FP15 treatment did not affect weight gain or blood glucose concentrations in either model of Type 1 diabetes. Body weights were similarly reduced and blood glucose concentrations similarly increased in untreated and FP15-treated diabetic animals compared with corresponding nondiabetic controls.

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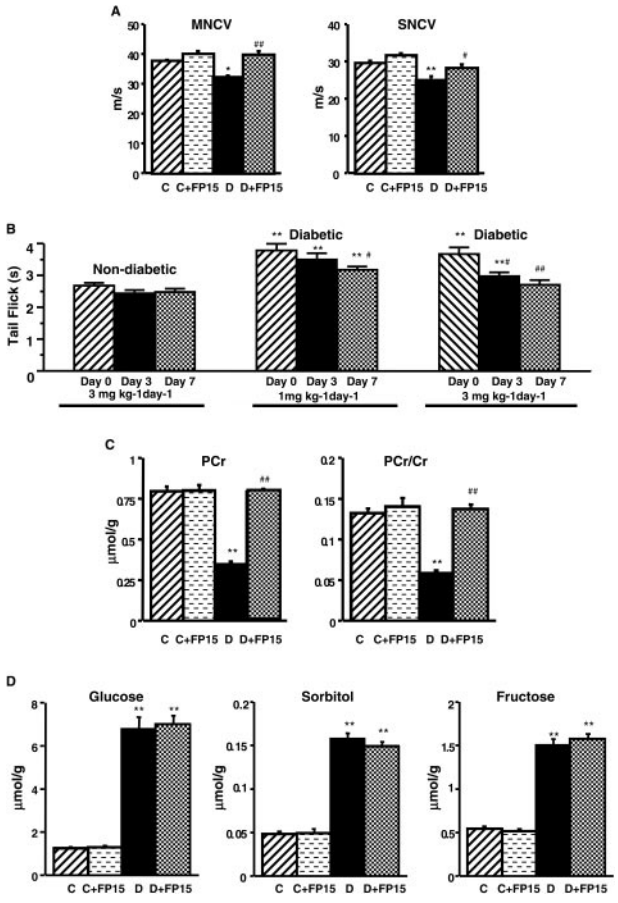

Figure 1. *A)* Final sciatic motor nerve conduction velocities and hind-limb digital sensory nerve conduction velocities in control and STZ-diabetic mice treated with or without FP15. $n = 12$–14/group. C = control; D = diabetic; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. untreated diabetic group. *B)* Tail flick latencies in nondiabetic and diabetic NOD mice treated FP15. $n = 5$/group. $^{**}P < 0.01$ vs. nondiabetic group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. corresponding values in NOD diabetic mice before treatments. *C)* Phosphocreatine (PCr) concentrations and phosphocreatine/creatine (PCr/Cr) ratios in the sciatic nerve of control and STZ-diabetic mice treated with or without FP15. $n = 5$–6/group. $^{**}P < 0.01$ vs. control group; $^{\#\#}P < 0.01$ vs. untreated diabetic group. *D)* Glucose and sorbitol pathway intermediate concentrations in control and STZ-diabetic mice treated with or without FP15. $n = 6$/group. $^{**}P < 0.01$ vs. control group; $^{\#\#}P < 0.01$ vs. untreated diabetic group.

2. FP15 treatment corrects energy failure, but does not interfere with accumulation of glucose or sorbitol pathway intermediates in the peripheral nerve in streptozotocin-diabetic mice

PCr concentrations and PCr/Cr ratios were decreased in peripheral nerve of streptozotocin-diabetic mice compared with nondiabetic controls (Fig. 1*C*). Diabetes-associated energy failure was corrected by FP15 treatment. Nerve glucose, sorbitol, and fructose concentrations were elevated in diabetic mice compared with nondiabetic controls, and neither glucose nor sorbitol pathway intermediate concentrations were affected by FP15 treatment (Fig. 1*D*).

3. FP15 treatment counteracts nitrosative stress and PARP activation without affecting PARP-1 abundance in peripheral nerve of STZ-diabetic mice

Nitrotyrosine (NT) and poly(ADP-ribose) immunoreactivities were increased in the peripheral nerve of STZ-diabetic mice, and diabetes-associated increase in both variables was essentially corrected by FP15 treatment (**Fig. 2*A, B***, respectively). PARP-1 abundance in the peripheral nerve was not affected by either diabetes or FP15 treatment (Fig. 2*C*).

CONCLUSIONS AND SIGNIFICANCE

Nitrosative stress (peroxynitrite-induced injury) is an important factor in numerous pathological conditions associated with oxidative stress. Our results provide the first evidence for the important role of peroxynitrite in the pathogenesis of diabetic neuropathy in two models of Type 1 (insulin-dependent) diabetes mellitus. Short term treatment with a peroxynitrite decomposition catalyst corrected motor and sensory nerve conduction deficits as well as hypoalgesia characteristic for diabetic neuropathy. FP15 appeared remarkably effective at such low doses as 1, 3, and 5 mg kg^{-1} d^{-1}. Thus, the effective doses for the peroxynitrite decomposition catalyst are, at least, 100-fold lower than the effective doses for conventional, even the most potent (e.g., α-lipoic acid) antioxidants. These findings suggest that peroxynitrite is the most important oxidant involved in the pathogenesis of diabetic neuropathy.

Another important finding in the present study is the demonstration of the major role of nitrosative stress in PARP activation in the diabetic peripheral nerve. PARP activation is responsible for diabetes-associated down-regulation (or, like in the peripheral nerve, insufficient up-regulation) of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, and activation of several major pathways involved in the pathogenesis of diabetic complications. PARP-1 is involved in transcriptional regulation via direct binding or poly(ADP-ribosyl)ation of transcription factors (NF-κB, activator protein-1, STAT-1, p53, and others). Our recent findings indicate that PARP activation is a fundamental mechanism in the pathogenesis of diabetic neuropathy, and that motor and sensory nerve conduction deficits do not develop in diabetic PARP-deficient (PARP−/−) mice. Further studies are needed to identify pathways triggered by nitrosative stress and subsequent PARP activation in the pathogenesis of diabetic complications.

Our findings provide further support to previous observations of our group and others indicating that from a variety of metabolic parameters nerve energy state correlates best with nerve conduction. Nerve PCr/Cr ratio, the best marker of peripheral nerve energy state, was compromised in streptozotocin-diabetic mice, and corrected by short-term FP15 treatment. Thus, nitrosative stress is a major contributor to energy failure in experimental diabetic neuropathy. The latter is consistent with the key role

for peroxynitrite and PARP activation in ATP or PCr depletion and resulting energy failure in other pathological conditions associated with oxidative stress.

Our results contribute to a better understanding of the relationship between increased AR activity and oxidative stress/PARP activation in the pathogenesis of diabetic complications, and in particular, diabetic neuropathy. Numerous findings obtained with AR inhibitors as well as AR-overexpressing mice indicate that increased AR activity is one of the most important mechanisms contributing to such complex phenomenon as oxidative stress in tissue sites for diabetic complications. Recently, one group has hypothesized that oxidative stress and PARP activation precede and account for diabetes-associated increase in the sorbitol pathway activity. In our study, FP15 counteracted nitrosative stress and PARP activation, but did not affect glucose or sorbitol pathway intermediate accumulation in diabetic peripheral nerve. The latter is consistent with downstream localization of oxidative-nitrosative stress and PARP activation consequent to increased AR activity in the pathogenesis of diabetic complications. Oxidative-nitrosative stress generated by increased AR activity, nonenzymatic glycation/glycooxidation, activation of protein kinase C, mitochondrial respiratory chain, extramitochondrial xanthine oxidase, and NAD(P)H oxidase affects downstream signaling by mitogen-activated protein kinases, PARP, and other mechanisms, which in turn causes up-regulation of inducible nitric oxide synthase, cyclooxygenase-2, endothelin-1, inflammatory genes (secondary contributors to diabetes-associated oxidative damage) (**Fig. 3**). The role of these secondary mechanisms remains to be explored.

Our results provide the first evidence of the pathogenetic role of reactive nitrogen species in peripheral diabetic neuropathy in two experimental models of Type 1 insulin-dependent diabetes. The findings justify development of pharmacological agents counteracting peroxynitrite formation and promoting peroxynitrite decomposition and their further study in animal and cell culture models of diabetic neuropathy and other chronic complications of diabetes.

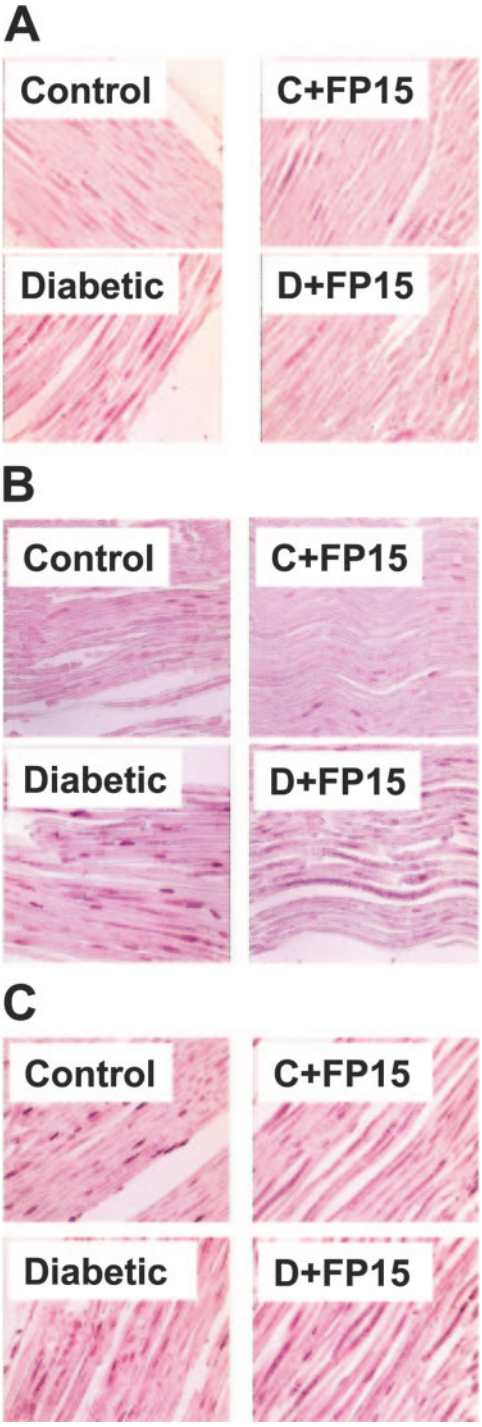

Figure 2. Representative microphotographs of immunohistochemical staining of nitrotyrosine (*A*), poly(ADP-ribose) (*B*) and poly(ADP-ribose) polymerase-1 (*C*) in the sciatic nerve of control and streptozotocin-diabetic mice treated with or without FP15. $n = 5–6$/group. ×400.

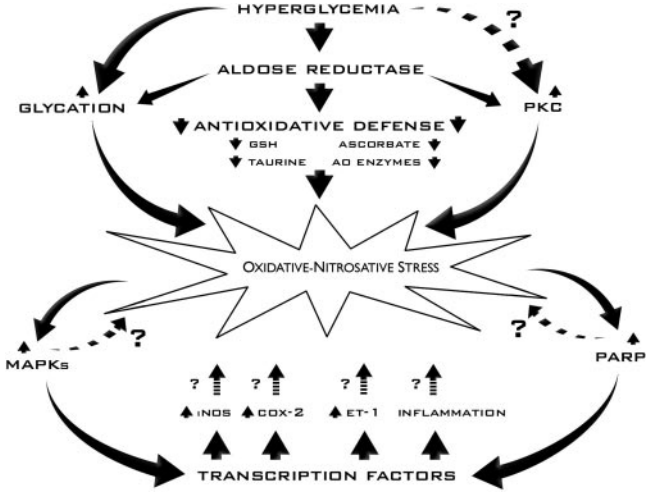

Figure 3. Interactions among oxidative-nitrosative stress and other mechanisms implicated in the pathogenesis of peripheral diabetic neuropathy.