Protective Effects of Cyclooxygenase-2 Gene Inactivation Against Peripheral Nerve Dysfunction and Intraepidermal Nerve Fiber Loss in Experimental Diabetes

Aaron P. Kellogg,[1] Tim D. Wiggin,[2] Dennis D. Larkin,[1] John M. Hayes,[2] Martin J. Stevens,[3] and Rodica Pop-Busui[1]

OBJECTIVE—Activation of the cyclooxygenase (COX) pathway with secondary neurovascular deficits are implicated in the pathogenesis of experimental diabetic peripheral neuropathy (DPN). The aim of this study was to explore the interrelationships between hyperglycemia, activation of the COX-2 pathway, and oxidative stress and inflammation in mediating peripheral nerve dysfunction and whether COX-2 gene inactivation attenuates nerve fiber loss in long-term experimental diabetes.

RESEARCH DESIGN AND METHODS—Motor and sensory digital nerve conduction velocities, sciatic nerve indexes of oxidative stress, prostaglandin content, markers of inflammation, and intraepidermal nerve fiber (IENF) density were measured after 6 months in control and diabetic COX-2–deficient (COX-$2^{-/-}$) and littermate wild-type (COX-$2^{+/+}$) mice. The effects of a selective COX-2 inhibitor, celecoxib, on these markers were also investigated in diabetic rats.

RESULTS—Under normal conditions, there were no differences in blood glucose, peripheral nerve electrophysiology, markers of oxidative stress, inflammation, and IENF density between COX-$2^{+/+}$ and COX-$2^{-/-}$ mice. After 6 months, diabetic COX-$2^{+/+}$ mice experienced significant deterioration in nerve conduction velocities and IENF density and developed important signs of increased oxidative stress and inflammation compared with nondiabetic mice. Diabetic COX-$2^{-/-}$ mice were protected against functional and biochemical deficits of experimental DPN and against nerve fiber loss. In diabetic rats, selective COX-2 inhibition replicated this protection.

CONCLUSIONS—These data suggest that selective COX-2 inhibition may be useful for preventing or delaying DPN. ***Diabetes*** **56:2997–3005, 2007**

From the [1]Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan; the [2]Department of Neurology, University of Michigan, Ann Arbor, Michigan; and the [3]University of Birmingham, Birmingham, U.K.

Address correspondence and reprint requests to Rodica Pop-Busui, MD, PhD, University of Michigan, Department of Internal Medicine, 5570D MSRB II, 1150 W. Medical Center Dr., Ann Arbor, MI 48109. E-mail: rpbusui@umich.edu.

Received for publication 31 May 2007 and accepted in revised form 22 August 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 24 August 2007. DOI: 10.2337/db07-0740.

COX, cyclooxygenase; DPN, diabetic peripheral neuropathy; DRG, dorsal root ganglia; GSH, glutathione; IENF, intraepidermal nerve fiber; MDA, malondialdehyde plus 4-hydroxyalkenals; MNCV, motor nerve conduction velocity; NF, nuclear factor; PG, prostaglandin; ROS, reactive oxygen species; SNCV, sensory nerve conduction velocity; STZ, streptozotocin; TNF, tumor necrosis factor; TXB, thromboxane B_2.

Diabetic peripheral neuropathy (DPN) commonly complicates diabetes and is the leading cause of nontraumatic lower-limb amputations in the U.S. (available at www.diabetes.org), with major impact on quality of life and disability (1). In addition, the consequences of diabetic nerve dysfunction often influence the final outcome of the disease. Significant correlations have been also reported between the presence of DPN and increased cardiovascular and overall mortality risks, further raising the economic burden for diabetes care (2–4). Although there is conclusive evidence demonstrating that intensive diabetes management reduces the incidence and progression of DPN (4,5), most clinical trials that have prospectively evaluated various classes of pharmacological agents for treating DPN have failed to show therapeutic benefit. One of the reasons is the complexity of mechanisms involved in its pathogenesis. Several contributing factors such as increased production of reactive oxygen species (ROS) by the mitochondrial respiratory chain, nonenzymatic glycation, increased glucose flux through the polyol pathway, protein kinase C activation, and neurovascular dysfunction have all been proposed (6–10).

We have previously demonstrated a link between hyperglycemia and cyclooxygenase (COX)-2 activation in the pathogenesis of experimental DPN (11). We postulated that upregulation of COX-2, by inflammatory stimuli (12, 13), protein kinase C activation (14), and/or oxidative stress (15), contributes to an altered prostaglandin (PG) profile in diabetes that favors vasoconstriction and additional ROS generation, further exacerbating oxidative stress (16). We have also shown that short-term selective chemical COX-2 inhibition in rats and COX-2 gene inactivation in mice prevented diabetes-induced functional and biochemical peripheral nerve deficits (11,16).

The aims of this study were to further explore the links between hyperglycemia, activation of the COX-2 pathway, and increased oxidative stress and inflammation in mediating peripheral nerve dysfunction and whether the protective effects of COX-2 gene inactivation against diabetes-induced nerve functional deficits will attenuate epidermal nerve fiber loss in long-term experimental diabetes. The effects of a selective COX-2 inhibitor, celecoxib (Pfizer), on nerve electrophysiology, markers of oxidative stress, PG production, and inflammatory cytokines were also investigated in diabetic rats.

TABLE 1
Initial and final body weights and blood glucose concentrations in various experimental groups: effects of STZ-induced diabetes and COX-2 gene inactivation or treatment on body weight and blood glucose

Experimental group ($n \geq 8$)	Body weight (g)		Blood glucose (mmol/l)	
	Start	End	Start	End
Mice				
Nondiabetic COX-$2^{+/+}$	18.3 ± 0.9	33.5 ± 0.8	4.9 ± 1.1	5.6 ± 1.5
Diabetic COX-$2^{+/+}$	17.2 ± 0.5	26 ± 0.5*	5.3 ± 1.2	16 ± 3.4*
Nondiabetic COX-$2^{-/-}$	15.4 ± 0.6	34 ± 0.5	5.3 ± 1.4	5.3 ± 1.3
Diabetic COX-$2^{-/-}$	16.7 ± 0.8	25.1 ± 1*	4.8 ± 1.1	16.7 ± 2.9*
Rats				
Nondiabetic	258 ± 4	743 ± 12	4.9 ± 1.4	5.1 ± 1.7
Diabetic	260 ± 3	493 ± 8*	5.1 ± 1.3	16.5 ± 2.9*
Nondiabetic (celecoxib)	259 ± 4	729 ± 14	5.1 ± 1.1	4.8 ± 1.4
Diabetic (celecoxib)	261 ± 4	519 ± 13*	5.2 ± 1.6	17.3 ± 3.1*

Data are means ± SE. *$P < 0.05$ vs. nondiabetic. COX-$2^{+/+}$, wild-type mice; COX-$2^{-/-}$, knockout mice.

RESEARCH DESIGN AND METHODS

Chemicals. Unless otherwise stated, all chemicals were of reagent-grade quality and were purchased from Fisher Scientific (Hanover Park, IL) and Sigma (St. Louis, MO). Kits for measurements of malondialdehyde plus 4-hydroxyalkenals (MDA) and glutathione (GSH) were purchased from Oxis International (Portland, OR). PG and cytokine enzyme-linked immunosorbent assay kits were purchased from Cayman Chemical (Ann Arbor, MI).

Animal models. The experiments were performed in accordance with regulations specified by the National Institutes of Health's *Principles of Laboratory Animal Care, 1985 Revised Version* and the institutional animal care and use committees at the University of Toledo and the University of Michigan.

Mouse experiments. Mice heterozygous for disruption of the COX-2 gene ($Ptgs2^{tm1Jed}$) on a C57B6:129S7 strain were purchased from The Jackson Laboratories (Bar Harbor, ME) and subsequently bred at our institutions. COX-$2^{-/-}$ and littermate COX-$2^{+/+}$ male mice were rendered diabetic by streptozotocin (STZ) as previously described (16). Genotype and phenotype were confirmed through PCR and Western blot as previously published (16). At least eight animals per experimental group were maintained for 6 months for end point measurements.

Rat experiments. Male Wistar rats (200–250 g) were purchased from Charles River (Wilmington, MA). After fasting overnight, rats were rendered diabetic by STZ as previously described (11). Experimental groups comprised of nondiabetic and diabetic rats (eight or more per group) were randomly assigned to either no treatment or treatment with the selective COX-2 inhibitor (IC50 4.8 nmol/l) celecoxib (50 mg · kg^{-1} · day^{-1} in drinking water) and maintained for up to 6 months.

After all electrophysiological measurements were obtained, animals were killed by CO_2 inhalation followed by cervical dislocation. Sciatic nerves and dorsal root ganglia (DRG) were removed, snap frozen in liquid nitrogen, and stored at −80°C for end point measurements.

Sciatic motor nerve and digital sensory nerve conduction velocity. Motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV) were assessed as previously described (16,17). Briefly, animals were anesthetized with 3:1 ketamine:xylene (80 mg/kg i.p.). Hindlimb skin temperature was monitored using a thermistor and was maintained at ~36°C by radiant heat. Sciatic MNCV was recorded by stimulating proximally at the sciatic notch and distally at the ankle. Digital SNCV was measured by stimulating at the second toe and recording at the medial malleolus. All readings are an average of 10 recordings.

Measurements of nerve MDA and GSH. These measurements were performed independently using commercially available kits as previously described (16,17). Briefly, mouse and rat sciatic nerve tissue was homogenized in 125 μl of 0.1 mol/l sodium phosphate buffer, pH 6.5. A total of 5 mmol/l butylated-hydroxy toluene was added to each sample to prevent further lipid peroxidation. A total of 20 μl homogenate at ~15% weight to buffer were used for spectophotometric measurements of MDA performed in accordance with the manufacturer's instructions. GSH was measured by using a ~15% wt/vol homogenate using 5% metaphosphoric acid, which prevents enzymatic GSH regeneration, as the homogenizing buffer. GSH was assessed spectophotometrically in accordance with manufacturer's instructions.

PG and inflammatory cytokines. Thromboxane B_2 (TXB_2), PGE_2, 6-keto-PGF_{1a} (stable metabolite of prostacycline), and tumor necrosis factor (TNF)-α were measured by enzyme-linked immunosorbent assay in the sciatic nerve using commercially available kits.

Western blot analysis. Nerve tissue from treated and untreated nondiabetic and diabetic rats were analyzed as previously described (11). Rabbit anti–COX-1 and –COX-2 antibodies (1:100) (Santa Cruz, CA) or mouse anti–glyceraldehyde 3-phosphate dehydrogenase (1:5,000) (Chemicon, Temecula, CA) were used to visualize the selected proteins.

Immunohistochemistry. COX-2 immunoreactivity in sciatic nerves and DRG neurons was assessed by immunofluorescent histochemistry. After in vivo fixation with 4% paraformaldehyde, sciatic nerves and DRG were frozen in imbedding compound (OCT). Sections were stained with COX-2 (1:100) (rabbit; Santa Cruz) and S-1001:50 (goat; Santa Cruz) overnight at 4°C. Slides were stained with fluorescent secondary antibodies (donkey anti–goat 488 and donkey anti–rabbit 594; Invitrogen, Carlsbad, CA). Coverslips were mounted with Prolong Gold mounting media, which contains the nuclear stain DAPI.

Intraepidermal nerve fiber density. Intraepidermal nerve fiber (IENF) density was examined in hindpaw footpads using previously reported techniques (18). Briefly, footpads were collected from the plantar surface of the hindpaws before animal perfusion, fixed in Zamboni's fixative (2% paraformaldehyde, 0.2% picric acid, and 0.1 mol/l sodium phosphate buffer) for 14 h, and cryoprotected through a series of sucrose gradients and embedded in OCT. Free-floating 30-μm footpad sections were stained with anti–PGP 9.5 antibody (a panaxonal marker) (19). Analysis of IENF density was performed using an Olympus FluoView 500 laser-scanning confocal microscope. Fluorescein isothiocyanate fluorescence was excited with a 488-nm blue argon laser, and emission was measured through a 505- to 525-nm barrier filter. Samples were scanned on an Olympus IX-71 inverted microscope using a ×20 objective. Individual IENFs were counted once they crossed the basement membrane so that fibers that branched after crossing the membrane were counted as one fiber. Data are shown as number of fibers per linear millimeter.

Biomarker cluster analysis. Hierarchical clustering was performed on a subset of data evaluating biomarkers of oxidative stress and inflammation using Gene Pattern (available at www.broad.mit.edu/cancer/software/genepattern), specifically adapted with the support of the National Center for Integrative Biomedical Informatics (available at https://portal.ncibi.org/portal). Clusters were assessed by pairwise complete linkage analysis, comparing each column to the others to determine the closest matches. The silhouette width index, a measure of the variability within a cluster versus the variability between clusters, was used to evaluate the separation between defined sample groups (20).

Statistical analysis. Data are expressed as means ± SE. Differences among experimental groups were determined by ANOVA using SPSS and PRISM 3 software, and the significance of between-group differences was assessed by Tukey multiple-range test. Significance was defined as $P \leq 0.05$. If the variances for the variables were found to differ significantly, a logarithmic transformation was performed that corrected the unequal variances. All analyses were then performed on the transformed data.

RESULTS

In nondiabetic animals, COX-2 gene inactivation/COX-2 selective inhibition did not affect body weight or blood

glucose levels compared with wild-type mice/untreated rats at baseline or thereafter (Table 1).

COX-2 gene inactivation/COX-2 selective inhibition protects against diabetes-induced electrophysiology deficits. There was no significant difference in MNCV and SNCV between nondiabetic COX-$2^{+/+}$ and COX-$2^{-/-}$ mice at any time point. Consistent with our previous short-term observations (16), 6 months of STZ-induced diabetes significantly reduced MNCV and SNCV in diabetic COX-$2^{+/+}$ mice compared with nondiabetic mice ($P < 0.01$), whereas diabetic COX-$2^{-/-}$ mice were completely protected against diabetes-induced MNCV and SNCV slowing ($P = 0.9$ and $P = 0.9$ vs. COX-$2^{-/-}$ nondiabetic group; $P < 0.01$ vs. COX-$2^{+/+}$ diabetic group) (Fig. 1*A*).

As expected, in untreated rats diabetes induced a significant slowing in both MNCV and SNCV compared with nondiabetic rats, and the deficits in both variables progressed significantly with 3- and 6-month durations of STZ-induced diabetes ($P < 0.001$ for both vs. nondiabetic rats) (Fig. 1*B* and *C*). Celecoxib treatment prevented both MNCV and SNCV slowing in diabetic rats at each time point without affecting either variable in nondiabetic rats ($P = 1.0$ and 0.977, respectively, vs. nondiabetic rats).

Oxidative stress, PG imbalance, and inflammation are prevented by COX-2 gene inactivation/COX-2 selective inhibition. Oxidative stress was assessed by measurements of MDA and GSH. Under nondiabetic conditions, COX-2 gene inactivation/COX-2 selective inhibition had no effect on sciatic nerve MDA or GSH levels. MDA was significantly increased after 6 months of STZ-induced diabetes by 2.2-fold in COX-$2^{+/+}$ mice ($P < 0.05$ vs. nondiabetic mice) and by 3.1-fold in untreated diabetic rats ($P < 0.01$ vs. nondiabetic rats) (Fig. 2*A* and *C*). In contrast, diabetic COX-$2^{-/-}$ mice and celecoxib-treated diabetic rats exhibited similar MDA levels to nondiabetic COX-$2^{-/-}$ mice ($P = 0.8$) and rats ($P = 1.0$). GSH was significantly reduced in the sciatic nerves of diabetic COX-$2^{+/+}$ mice ($P < 0.01$ vs. nondiabetic COX-$2^{+/+}$ mice) and untreated diabetic rats ($P < 0.05$ vs. nondiabetic rats). COX-$2^{-/-}$ diabetic mice preserved similar nerve GSH levels with nondiabetic mice (Fig. 2*B* and *D*), and in diabetic rats celecoxib prevented nerve GSH depletion.

Measurements of various PGs were performed in peripheral nerves to determine trends of PG changes induced by diabetes and the effects of interventions. Under nondiabetic conditions, the production of TXB_2 in the peripheral nerve was not different between COX-$2^{-/-}$ and COX-$2^{+/+}$ mice at any time point. However, nondiabetic COX-$2^{-/-}$ mice had significantly higher nerve 6-keto-PGF_{1a} levels compared with nondiabetic COX-$2^{+/+}$ mice ($P < 0.01$). Sciatic nerve TXB_2 was significantly increased and 6-keto-PGF_{1a} was significantly decreased after 6 months of diabetes in COX-$2^{+/+}$ mice ($P < 0.001$ for both vs. COX-$2^{+/+}$ nondiabetic mice), whereas diabetic COX-$2^{-/-}$ mice were completely protected against this imbalance in PG synthesis ($P = 0.8$ and 0.7, respectively, vs. nondiabetic COX-$2^{-/-}$ mice) (Table 2). Likewise in rats, diabetes induced a significant increase in nerve PGE_2 and TXB_2 and a significant decrease in 6-keto-PGF_{1a} levels at all time points ($P < 0.01$ vs. nondiabetic rats). Celecoxib protected diabetic rats against the increase in the vasoconstrictor and the decrease in the vasodilator PG levels (Table 2).

To further examine the link between COX-2 metabolites and inflammation in the peripheral nerve, we measured levels of nuclear factor (NF)-κB–derived cytokines. Sciatic nerve TNF-α levels were not different in nondiabetic COX-$2^{-/-}$ and COX-$2^{+/+}$ mice or any nondiabetic rats. Diabetes induced a significant increase in nerve TNF-α levels in COX-$2^{+/+}$ mice ($P < 0.05$ vs. nondiabetic mice) and rat nerve TNF-α in untreated rats ($P < 0.01$ vs. nondiabetic rats), whereas diabetic COX-$2^{-/-}$ mice and

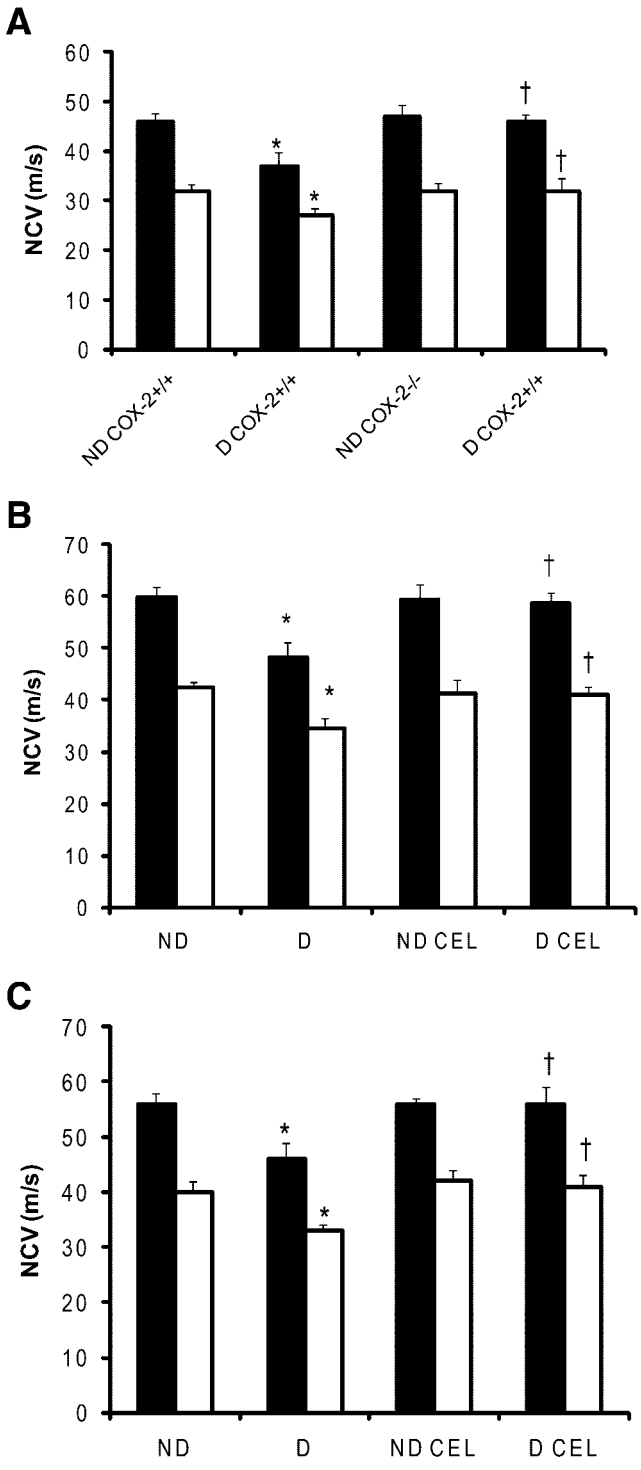

FIG. 1. Effects of STZ-induced diabetes and COX-2 gene inactivation or treatment on nerve conduction. MNCV (■) and SNCV (□) were measured in mice after 6 months (*A*) and rats after 3 and 6 months (*B* and *C*) as described in RESEARCH DESIGN AND METHODS. Data are expressed as means ± SE. **P* < 0.01 vs. nondiabetic (ND); †*P* < 0.01 vs. other diabetic (D). CEL, celecoxib; COX-$2^{+/+}$, wild-type mice; COX-$2^{-/-}$, knockout mice.

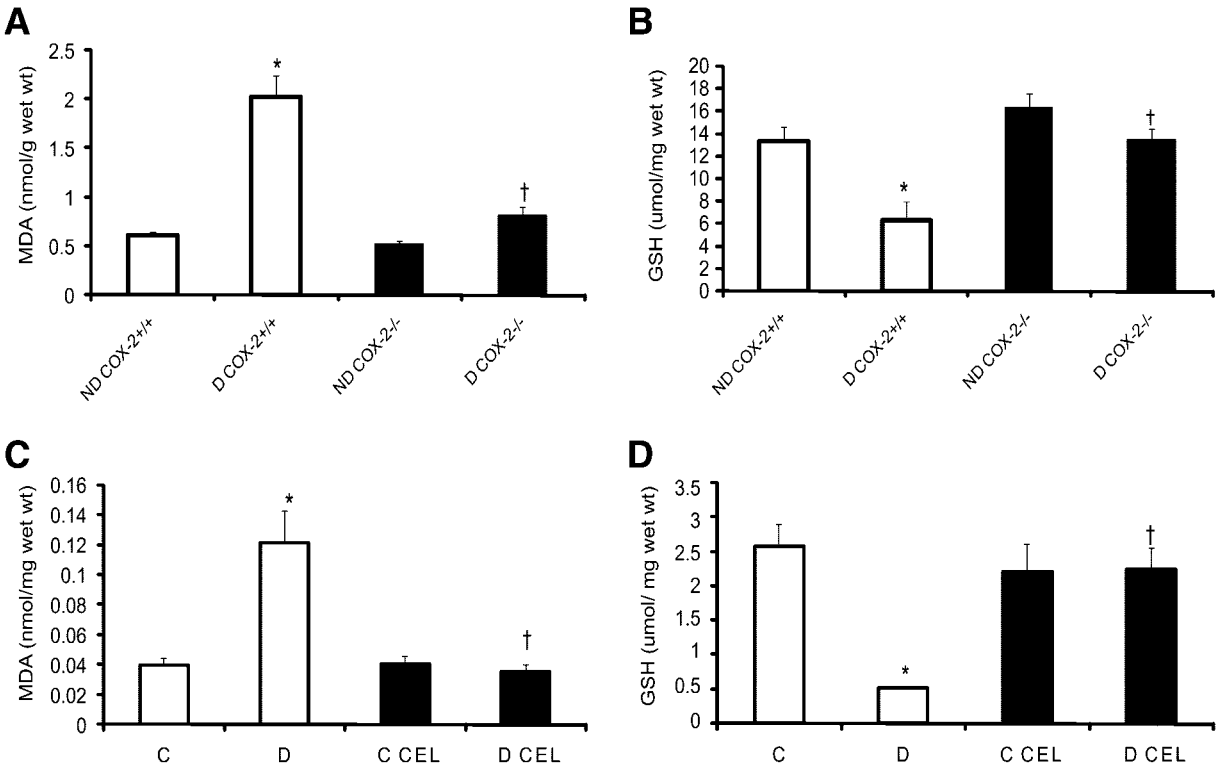

FIG. 2. Effects of STZ-induced diabetes and COX-2 gene inactivation or treatment on biomarkers of oxidative stress. MDA and GSH were measured in mice (*A* and *B*) and rats (*C* and *D*) after 6 months as described in RESEARCH DESIGN AND METHODS. Data are expressed as means ± SE. *$P < 0.01$ vs. nondiabetic (ND); †$P < 0.01$ vs. other diabetic (D). CEL, celecoxib. COX-$2^{+/+}$, wild-type mice; COX-$2^{-/-}$, knockout mice.

celecoxib-treated diabetic rats were protected against the increase in TNF-α (Fig. 3*A* and *B*).

To evaluate the clustering pattern in the change of several biomarkers of oxidative stress and inflammation induced by diabetes and/or intervention, we used methods commonly used for high throughput gene expression analysis (21), specifically adapted for our analysis as described in RESEARCH DESIGN AND METHODS. As shown in Fig. 4, cluster analysis of these biomarkers identified specific diabetes signatures and shows that COX-$2^{-/-}$ diabetic mice and celecoxib-treated diabetic rats cluster with respective nondiabetic animals for markers of oxidative stress, PG production, and inflammation (silhouette width index = 0.85 and 0.83, respectively) (Fig. 4).

Diabetes upregulates COX-2 expression in the peripheral nerve and DRG. Western blot analysis of COX-2 protein expression demonstrates that COX-2 was undetectable in the sciatic nerve of nondiabetic rats, regardless of treatment group. However, COX-2 protein expression was increased in the sciatic nerve of all diabetic rats, and this increase was not affected by treatment with celecoxib. In contrast, diabetes had no effect of COX-1 protein expression (Fig. 5*A*).

Immunofluorescence of mouse sciatic nerves and DRG demonstrates a diffusely increased COX-2 immunoreactivity within the axons and DRG of COX-$2^{+/+}$ diabetic mice compared with COX-$2^{+/+}$ nondiabetic mice, as confirmed by labeling serial adjacent sections with antibody to S-100

TABLE 2
Sciatic nerve PGs were measured in mice after 6 months and in rats after 3 and 6 months as described in RESEARCH DESIGN AND METHODS: effects of STZ-induced diabetes and COX-2 gene inactivation or treatment on PG production

Experimental group ($n \geq 8$)	TXB_2 (pg/mg wet wt)	6-keto-PGF_{1a} (pg/mg wet wt)	PGE_2 (pg/mg wet wt)
Mice			
Nondiabetic COX-$2^{+/+}$	10 ± 1	29 ± 2	
Diabetic COX-$2^{+/+}$	44 ± 4*	15 ± 2*	
Nondiabetic COX-$2^{-/-}$	11 ± 2	38 ± 2	
Diabetic COX-$2^{-/-}$	10 ± 2†	36 ± 2†	
Rats (3/6 months)			
Nondiabetic	13 ± 3/15 ± 2	20 ± 4/22 ± 1	6 ± 1/9 ± 1
Diabetic	27 ± 4*/37 ± 2*	5 ± 1*/6 ± 1*	25 ± 5*/35 ± 3*
Nondiabetic (celecoxib)	13 ± 3/9 ± 1	21 ± 3/25 ± 1	6 ± 1/7 ± 1
Diabetic (celecoxib)	10 ± 3†/11 ± 1†	18 ± 5†/24 ± 1†	8 ± 2†/6 ± 1†

Data are means ± SE. *$P < 0.01$ vs. nondiabetic; †$P < 0.01$ vs. other diabetic. COX-$2^{+/+}$, wild-type mice; COX-$2^{-/-}$, knockout mice.

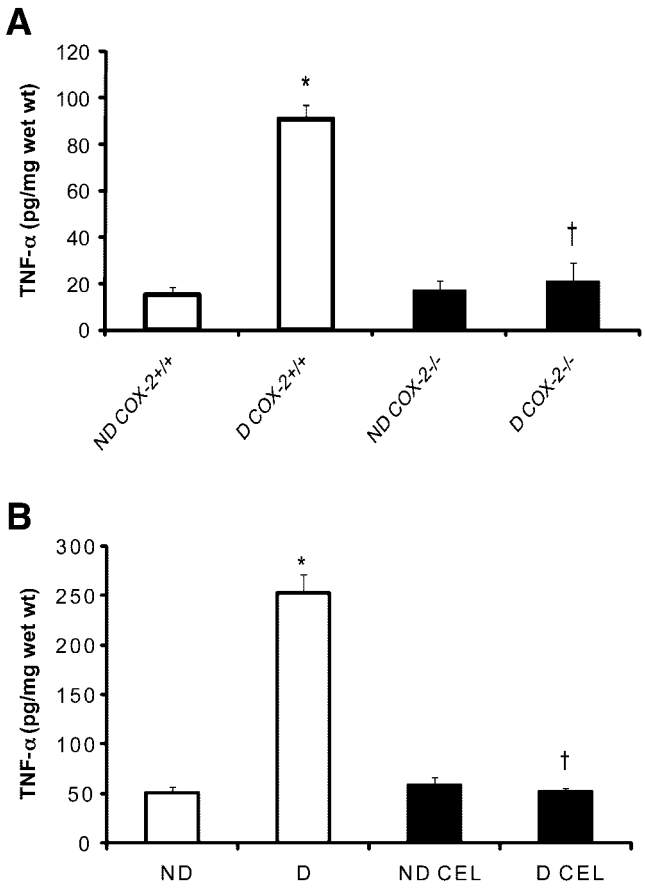

FIG. 3. Effects of STZ-induced diabetes and gene inactivation or treatment on inflammation biomarkers. TNF-α was measured in mice (*A*) and rats (*B*) after 6 months as described in RESEARCH DESIGN AND METHODS. Data are expressed as means ± SE. **P* < 0.01 vs. nondiabetic (ND); †*P* < 0.01 vs. other diabetic (D). CEL, celecoxib; COX-2$^{+/+}$, wild-type mice; COX-2$^{-/-}$, knockout mice.

and DAPI. No significant COX-2 immunofluorescence was found in the axons or DRG of COX-2$^{-/-}$ diabetic mice (Fig. 5*B* and *C*).

COX-2 gene inactivation attenuates diabetes-induced nerve fiber loss. COX-2 gene inactivation had no effect on IENF density in nondiabetic mice. After 6 months of diabetes, IENF density was significantly decreased in COX-2$^{+/+}$ mice compared with respective nondiabetic mice ($P < 0.05$). In contrast, diabetic COX-2$^{-/-}$ mice were completed protected against diabetes-induced nerve fiber loss ($P = 1.0$ vs. nondiabetic COX-2$^{-/-}$ mice) (Fig. 6).

DISCUSSION

The data reported herein confirm the critical role played by COX-2 pathway activation in mediating peripheral nerve dysfunction in experimental diabetes. Furthermore, we demonstrate that the long-term preventive effects of COX-2 gene inactivation on nerve electrophysiology and specific biomarkers of peripheral nerve function parallel the beneficial effects reported in short-term experiments (16). In addition, we also show that COX-2 gene inactivation protects against diabetes-induced IENF loss. Long-term treatment with the selective COX-2 inhibitor, celecoxib, replicates these effects in diabetic rats.

Several mechanisms may explain these beneficial effects of COX-2 inhibition on experimental DPN. As discussed below, our data suggest that modulation of peripheral nerve oxidative stress and inflammation appear to be important mechanisms explaining the neurovascular protection associated with COX-2 pathway inhibition in diabetes. Vascular mechanisms associated with either PG imbalance and/or reduced nitric oxide availability (14) with effects on endoneurial perfusion are also involved. For instance, our laboratory has previously reported that treatment with a selective COX-2 inhibitor prevented diabetes-induced endoneurial nerve blood flow deficits (11). In addition, interactions between COX-2 and the receptor for advanced glycation end products signaling pathway were recently described (22).

Consistent with our prior findings in short-term experiments (16) and with other groups (6,17), we found that GSH levels were decreased in the sciatic nerves of diabetic COX-2$^{+/+}$ mice and diabetic rats. However, diabetic COX-2$^{-/-}$ mice or celecoxib-treated diabetic rats were protected against GSH depletion, which suggests that in diabetes, COX-2 pathway metabolites act independently of, but synergistically with, hyperglycemia in inducing oxidative stress. This contribution is further underscored by the significant accumulation of lipid peroxidation products in the sciatic nerves of diabetic COX-2$^{+/+}$ mice and untreated diabetic rats, whereas diabetic COX-2$^{-/-}$ mice and celecoxib-treated diabetic rats were protected against this additional marker of oxidative injury.

Activation of the COX-2 pathway is an important consequence of oxidant- and inflammation-initiated metabolic response in various tissues. A chain of events linking ROS generation with NF-κB activation and subsequent COX-2 protein production is supported by multiple studies (14, 23,24). The increased lipid peroxidation associated with oxidative stress has been also shown to be an independent and potent inducer of COX-2 (25). However, our data indicate that COX-2 activation itself promotes oxidative stress in the diabetic peripheral nerves. This may be explained through vascular and nonvascular mechanisms induced by imbalanced PG generation and subsequent metabolic and inflammatory alterations in the peripheral diabetic neurovascular structures.

COX is the rate-limiting enzyme in PG synthesis and plays an essential role in neuroinflammation. Two isoforms of the enzyme, COX-1 and COX-2, have been isolated in mammalian cells. While COX-1 is ubiquitous, COX-2 operates as an inducible enzyme with low or undetectable levels under normal conditions but is rapidly upregulated in various disease states including diabetes.

Our laboratory (11) and other groups (26,27) have demonstrated that the COX-2 expression is elevated in the peripheral nerves and vascular tissues in various models of experimental diabetes, and this was further confirmed in the present study (Fig. 5*A*). We have now extended these studies to show that COX-2 upregulation subsequently induces a progressive PG imbalance due to increased levels of vasoconstrictor TXB_2 and proinflammatory PGE_2 and decreased prostacyclin in the peripheral nerves, which was maintained during long-term diabetic conditions in both animal models (Table 2). This imbalance, in turn, may contribute to the observed oxidative and neurovascular deficits. These findings are consistent with several studies (28–30) reporting a similar shift in the PG balance induced by diabetes. A COX-2–dependent imbalanced PG response favoring PGE_2 has been shown to promote oxidative stress and related neuronal damage in a model of Alzheimer's disease via PGE_2 receptor signaling

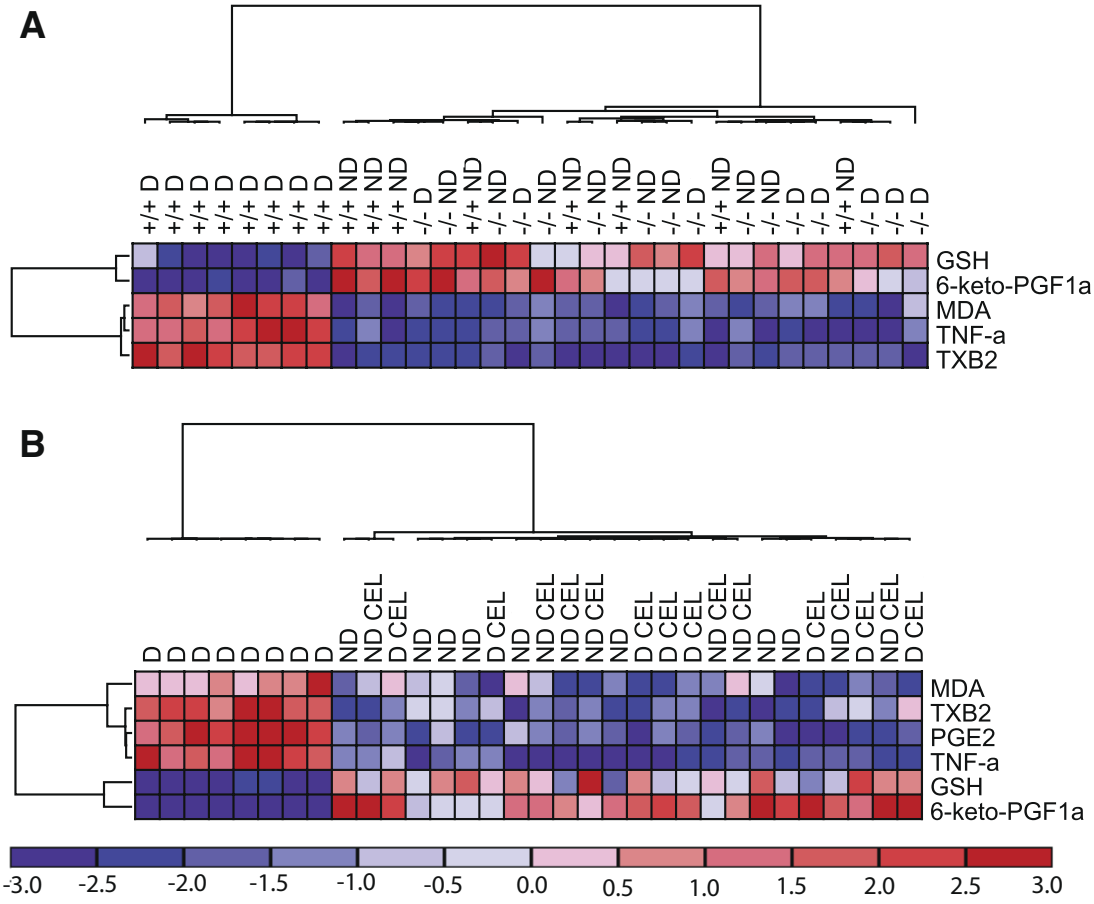

FIG. 4. Cluster analysis of the pattern of change of biomarkers of oxidative stress and inflammation induced by experimental diabetes and interventions. Clusters were generated using datasets for oxidative stress, PG production, and markers of inflammation obtained from mouse (*A*) or rat (*B*) sciatic nerve as described in RESEARCH DESIGN AND METHODS. Each row corresponds to a marker measured and each column represents an individual sample. Genotype (*A*) and treatment group (*B*) are listed above each corresponding column. Data were normalized to a mean of zero and an SD of 1. Measures above the mean are shaded in red, and those below the mean are shaded in blue. The color scale indicates the SD above or below the mean. CEL, celecoxib; COX-2$^{+/+}$, wild-type mice; COX-2$^{-/-}$, knockout mice; D, diabetic; ND, nondiabetic.

(31). In addition, COX-2 upregulation stimulates a peroxidase-dependent conversion of PGG_2 to PGH_2, resulting in an increased superoxide production and subsequent lipid peroxidation and protein nitrosylation (32). Although we have not directly measured ROS generation in this study, several reports (32,33) demonstrate a direct relationship between COX-2 and ROS production.

Our data indicate that additional markers of inflammation, such as the NF-κB–derived TNF-α (32), are elevated by diabetes in the peripheral nerves and that either COX-2 gene inactivation or pharmacological COX-2 blockade prevents this increase. Several molecular possibilities may explain different cellular effects involving the interaction between PGs, inflammation, and COX-2 in the peripheral nerves. For instance, COX-2 has an NF-κB binding site in its promoter region, and the NF-κB pathway directly modulates cellular inflammatory processes within the nervous system (32). It has been shown that while PGE_2 upregulates NF-κB activity, with subsequent increasing inflammatory response, other PGs such as PGD_2 and PGJ_2 suppress NF-κB activation and decrease inflammation (32). Therefore, in diabetes, COX-2–derived PGE_2, in concert with oxidative stress, generates a positive feedback loop resulting in NF-κB activation and subsequent inflammation. These data suggest that the relationship between oxidative stress, inflammation, and COX-2 activation in our diabetic models appear to be bi- rather than unidirectional, since COX-2 activation not only results from but also leads to increased oxidative stress and inflammation in the diabetic peripheral nerves.

Clustering analysis was applied to show a regulatory pattern linking PG, oxidative stress, and inflammatory markers as well as the presence of a specific diabetic phenotype signature (Fig. 4). The net cluster demarcation between diabetic COX-2$^{+/+}$ mice or untreated diabetic rats and their COX-2$^{-/-}$ and celecoxib-treated counterparts is an additional strong and original indicator of a similar nondiabetic phenotype induced by COX-2 inactivation/inhibition, supporting our hypothesis that increased COX-2 activity in diabetes mediates an unbalanced PG/ROS/inflammation interplay in peripheral nerves.

As reported previously (11) and confirmed in this communication (Fig. 5), the expression of COX-2, but not COX-1, is elevated during diabetes in the peripheral nerves and DRG. It has also been shown that in the nervous system, different PGs have the ability to antagonize one

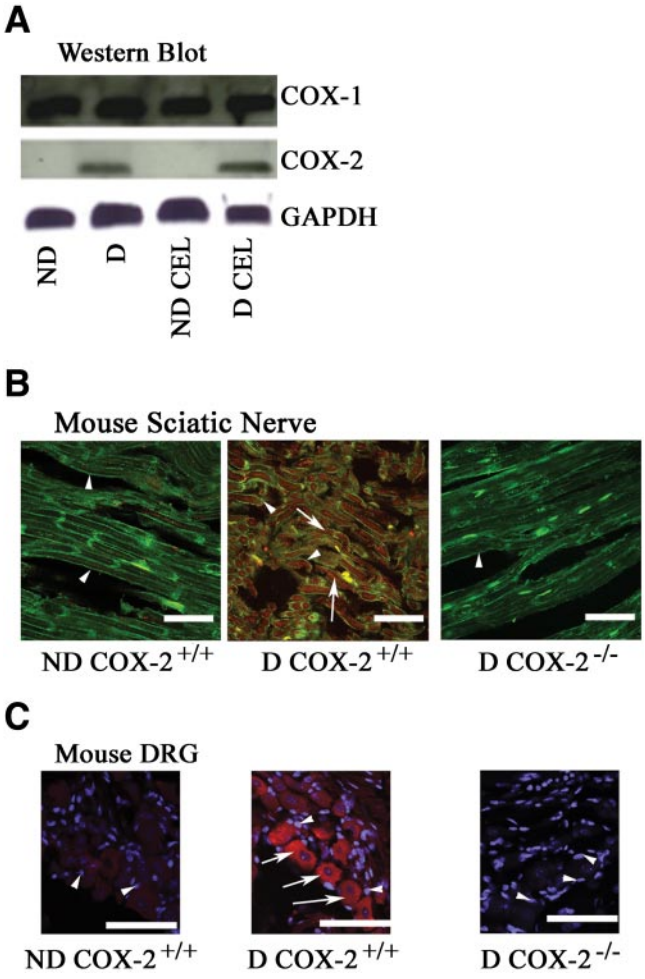

FIG. 5. Western blot analysis and immunoflorescence of COX-2 in sciatic nerves and DRGs. *A*: Western blot analysis was performed using equal amounts of protein obtained from fresh frozen rat sciatic nerve. Blots were immunostained using polyclonal antibodies against COX-1 and COX-2 as described in RESEARCH DESIGN AND METHODS. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. COX-2 immunofluorescence of mouse sciatic nerves (*B*) and DRG neurons (*C*) was performed as described in RESEARCH DESIGN AND METHODS. S-100 was used as a Schwann cell anatomical marker in the sciatic nerve and DAPI as a nuclear stain in DRG. *B* and *C*: Bar = 50 μm. *B*: Arrows indicate COX-2 staining in axons, and arrowheads indicate Schwann cells. *C*: Arrows indicate DRG, and arrowheads indicate nuclei. Images were captured with an oil immersion ×60 objective. S-100 (green), COX-2 (red), and DAPI (blue). All images were simultaneously enhanced to increase clarity. CEL, celecoxib; COX-$2^{+/+}$, wild-type mice; COX-$2^{-/-}$, knockout mice; D, diabetic; ND, nondiabetic. (Please see http://dx.doi.org/10.2337/db07-0740 for a high-quality digital representation of this figure.)

another and a PG can exert opposite effects in different cell types (32). This spectrum of responses, associated with antagonizing the actions of growth factors, has direct effects on neuron survival and nerve function (32). Studies in purified preparations of human COX-1 and COX-2 have also shown that even when both isoforms are present in the same intracellular compartment, downstream PG generation follows disease-specific paradigms and effective PG synthesis may proceed predominantly through one of the COX isoforms (31,34). Our observations in nondiabetic COX-$2^{-/-}$ mice, which had consistently significantly higher nerve 6-keto-PGF_{1a} levels compared with nondiabetic wild-type mice, support the hypothesis that formation of the vasodilator PG occurs mainly by a mechanism independent of the COX-2 pathway. Celecoxib treatment had no effect on COX-2 protein expression in nondiabetic or diabetic rats, suggesting that the inhibition of COX-2 activity is responsible for the beneficial effects observed with celecoxib (Fig. 5*A*).

In this report, we utilized animal models of type 1 diabetes, and, therefore, it is unclear as to whether inhibition of the COX-2 pathway will be as effective in treating DPN in type 2 diabetes. However, recently reported data (35,36) demonstrate that COX-2 is also upregulated in animal models of type 2 diabetes and that this upregulation is associated with similar inflammatory and metabolic changes to those reported herein and ultimately the development of microangiopathic complications. Therefore, COX-2 inhibition may also provide a potential therapeutic benefit for DPN in type 2 diabetes.

Finally, we have observed that our diabetic COX-$2^{+/+}$ mice have a marked decrease in IENF density compared with their nondiabetic littermates, whereas the diabetic COX-$2^{-/-}$ mice were protected against nerve fiber loss for 6 months of experimental diabetes. In animal models, nerve fiber density is often used to verify nerve fiber degeneration (37,38). Small-diameter nerve fibers can be affected early in peripheral neuropathy, and terminal sensory nerve endings might have degenerated despite normal sural morphometry (39,40). Therefore, routine NCV tests lack the sensitivity to detect small fiber impairment. On the contrary, footpad skin biopsy has proved to be a reliable tool to examine unmyelinated nerve fibers, as assessed by the quantification IENF density (38,41–43), and it is now accepted as a sensitive method in assessing the presence of DPN.

The data presented herein support the concept that COX-2 inhibition has beneficial effects in preventing diabetes-induced peripheral nerve dysfunction and could be used as a new potential therapeutic tool. A limitation of our study, however, was its preventive design, and so an interventional (or reversal) study could potentially yield different results.

Also, emerging data from several clinical trials (44–46) have recently questioned the cardiovascular safety of COX-2 selective inhibitors. One of the potential mechanisms involved includes a predominant decrease in PGI_2, but not TXB_2, production (44,47) and increased blood pressure. However, our data demonstrate the presence of specific PG and inflammatory imbalances in diabetes, which could potentially alter the cardiovascular risk association with COX-2 inhibition. Moreover, significant differences in cardiovascular outcomes were reported with various agents within class, which suggest rather a drug- and not a class-specific effect (44,45,48,49). Indeed, a recent trial specifically exploring cardiovascular risk measures in subjects with type 2 diabetes randomized to celecoxib, rofecoxib, or naproxen reported no increase in systolic blood pressure with celecoxib, while rofecoxib induced a significant increase in systolic blood pressure after 12 weeks (50). Nevertheless, an interesting possibility to be explored in future studies is a conditional tissue-specific COX-2 inhibition approach, which would promote the desired effects in target tissues without the unwanted consequences that may result from a general COX-2 inhibition.

Therefore, in summary, our data support an important role for increased flux through the COX-2 pathway in *1*) loss of sensory nerve function assessed by electrophysiological measurements, *2*) increased oxidative stress and inflammation in the peripheral nerves, and *3*) nerve fiber loss measured by IENF density in footpads. Selective

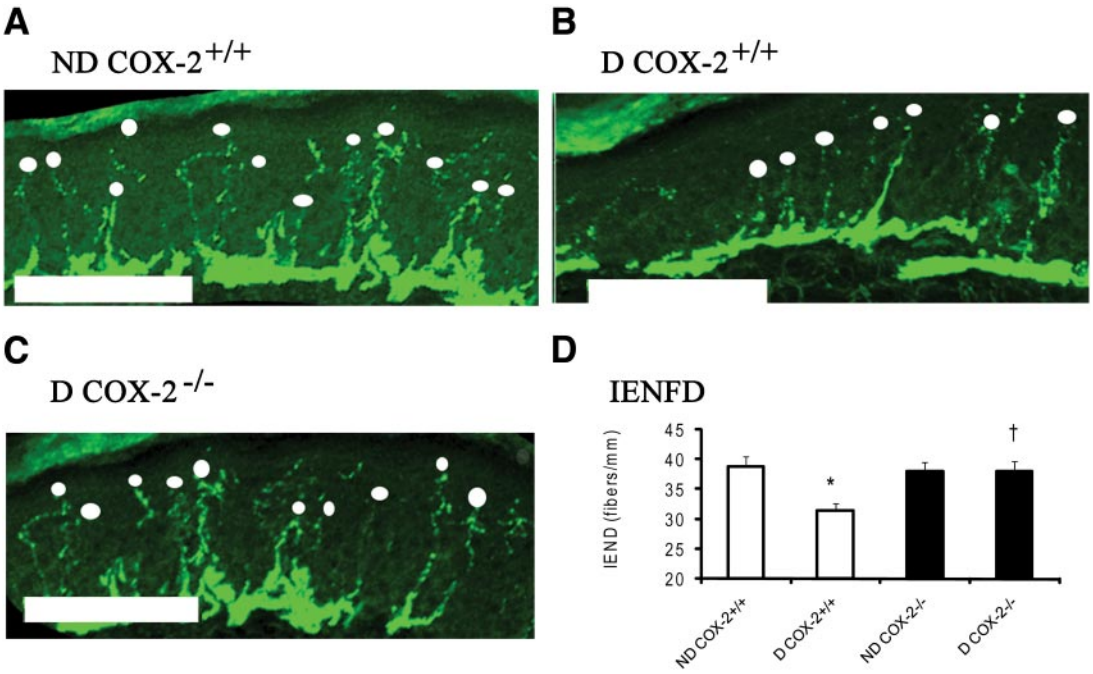

FIG. 6. Effects of STZ-induced diabetes and COX-2 gene inactivation on IENF density. IENF density was measured in nondiabetic (ND) and diabetic (D) COX-2$^{+/+}$ and COX-2$^{-/-}$ mice after 6 months as described in RESEARCH DESIGN AND METHODS. Fiber density is expressed as number of fibers per linear mm epidermis (*D*). Bar = 100 μm. ○, individual nerve fibers. Images were acquired with an oil immersion ×20 objective. Data are means ± SE. **P* < 0.01 vs. nondiabetic; †*P* < 0.01 vs. other diabetic. COX-2$^{+/+}$, wild-type mice; COX-2$^{-/-}$, knockout mice. (Please see http://dx.doi.org/10.2337/db07-0740 for a high-quality digital representation of this figure.)

COX-2 inhibition may therefore be useful for preventing or delaying DPN.

ACKNOWLEDGMENTS

This research is supported by National Institutes of Health Grant NINDS-NS047653-03 (to R.P.B). M.J.S. is supported by grant R01AT002146 and Eli Lilly. This work utilized the Morphology and Image Analysis Core of the Michigan Diabetes Research and Training Center funded by NIH5P60 DK20572 from the National Institute of Diabetes and Digestive and Kidney Diseases.

We thank Pfizer for the gift of the selective COX-2 inhibitor celecoxib and Dr. Kelli Sullivan of the University of Michigan for immunohistochemical advice.

REFERENCES

1. Vileikyte L, Leventhal H, Gonzalez JS, Peyrot M, Rubin RR, Ulbrecht JS, Garrow A, Waterman C, Cavanagh PR, Boulton AJ: Diabetic peripheral neuropathy and depressive symptoms: the association revisited. *Diabetes Care* 28:2378–2383, 2005
2. Gordois A, Scuffham P, Shearer A, Oglesby A, Tobian JA: The health care costs of diabetic peripheral neuropathy in the US. *Diabetes Care* 26:1790–1795, 2003
3. American Diabetes Association: Economic consequences of diabetes mellitus in the U.S. in 1997. *Diabetes Care* 21:296–309, 1998
4. The Diabetes Control and Complications Trial Research Group: The effect of intensive diabetes therapy on the development and progression of neuropathy. *Ann Intern Med* 122:561–568, 1995
5. The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329:977–986, 1993 [see comments]
6. Ho EC, Lam KS, Chen YS, Yip JC, Arvindakshan M, Yamagishi S, Yagihashi S, Oates PJ, Ellery CA, Chung SS, Chung SK: Aldose reductase-deficient mice are protected from delayed motor nerve conduction velocity, increased c-Jun NH_2-terminal kinase activation, depletion of reduced glutathione, increased superoxide accumulation, and DNA damage. *Diabetes* 55:1946–1953, 2006
7. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001
8. Yagihashi S, Yamagishi SI, Wada Ri R, Baba M, Hohman TC, Yabe-Nishimura C, Kokai Y: Neuropathy in diabetic mice overexpressing human aldose reductase and effects of aldose reductase inhibitor. *Brain* 124:2448–2458, 2001
9. Song Z, Fu DT, Chan YS, Leung S, Chung SS, Chung SK: Transgenic mice overexpressing aldose reductase in Schwann cells show more severe nerve conduction velocity deficit and oxidative stress under hyperglycemic stress. *Mol Cell Neurosci* 23:638–647, 2003
10. King GL, Ishii H, Koya D: Diabetic vascular dysfunctions: a model of excessive activation of protein kinase C. *Kidney Int Suppl* 60:S77–S85, 1997
11. Pop-Busui R, Marinescu V, Van Huysen C, Li F, Sullivan K, Greene DA, Larkin D, Stevens MJ: Dissection of metabolic, vascular, and nerve conduction interrelationships in experimental diabetic neuropathy by cyclooxygenase inhibition and acetyl-L-carnitine administration. *Diabetes* 51:2619–2628, 2002
12. Herschman HR, Reddy ST, Xie W: Function and regulation of prostaglandin synthase-2. *Adv Exp Med Biol* 407:61–66, 1997
13. Wu KK: Inducible cyclooxygenase and nitric oxide synthase. *Adv Pharmacol* 33:179–207, 1995
14. Cosentino F, Eto M, De Paolis P, van der Loo B, Bachschmid M, Ullrich V, Kouroedov A, Delli Gatti C, Joch H, Volpe M, Luscher TF: High glucose causes upregulation of cyclooxygenase-2 and alters prostanoid profile in human endothelial cells: role of protein kinase C and reactive oxygen species. *Circulation* 107:1017–1023, 2003
15. Feng L, Xia Y, Garcia GE, Hwang D, Wilson CB: Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-alpha, and lipopolysaccharide. *J Clin Invest* 95:1669–1675, 1995
16. Kellogg A, Pop-Busui R: Peripheral nerve dysfunction in experimental diabetes is mediated by cyclooxygenase-2 and oxidative stress. *Antioxid Redox Signal* 7:1521–1529, 2005
17. Stevens MJ, Obrosova I, Cao X, Van Huysen C, Greene DA: Effects of DL-α-lipoic acid on peripheral nerve conduction, blood flow, energy metabolism, and oxidative stress in experimental diabetic neuropathy. *Diabetes* 49:1006–1015, 2000
18. Cain DM, Wacnik PW, Turner M, Wendelschafer-Crabb G, Kennedy WR, Wilcox GL, Simone DA: Functional interactions between tumor and peripheral nerve: changes in excitability and morphology of primary

afferent fibers in a murine model of cancer pain. *J Neurosci* 21:9367–9376, 2001
19. Polydefkis M, Hauer P, Griffin JW, McArthur JC: Skin biopsy as a tool to assess distal small fiber innervation in diabetic neuropathy. *Diabetes Technol Ther* 3:23–28, 2001
20. Rousseeuw P: Silhouettes: a graphical aid to the interpretation and validation of cluster analysis. *J Comput Appl Math* 20:53–65, 1987
21. Welsh JB, Zarrinkar PP, Sapinoso LM, Kern SG, Behling CA, Monk BJ, Lockhart DJ, Burger RA, Hampton GM: Analysis of gene expression profiles in normal and neoplastic ovarian tissue samples identifies candidate molecular markers of epithelial ovarian cancer. *Proc Natl Acad Sci U S A* 98:1176–1181, 2001
22. Cipollone F, Iezzi A, Fazia M, Zucchelli M, Pini B, Cuccurullo C, De Cesare D, De Blasis G, Muraro R, Bei R, Chiarelli F, Schmidt AM, Cuccurullo F, Mezzetti A: The receptor RAGE as a progression factor amplifying arachidonate-dependent inflammatory and proteolytic response in human atherosclerotic plaques: role of glycemic control. *Circulation* 108:1070–1077, 2003
23. Adderley SR, Fitzgerald DJ: Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. *J Biol Chem* 274:5038–5046, 1999
24. Kiritoshi S, Nishikawa T, Sonoda K, Kukidome D, Senokuchi T, Matsuo T, Matsumura T, Tokunaga H, Brownlee M, Araki E: Reactive oxygen species from mitochondria induce cyclooxygenase-2 gene expression in human mesangial cells: potential role in diabetic nephropathy. *Diabetes* 52:2570–2577, 2003
25. Uchida K, Kumagai T: 4-hydroxy-2-nonenal as a COX-2 inducer. *Mol Aspects Med* 24:213–218, 2003
26. Bagi Z, Erdei N, Toth A, Li W, Hintze TH, Koller A, Kaley G: Type 2 diabetic mice have increased arteriolar tone and blood pressure: enhanced release of COX-2-derived constrictor prostaglandins. *Arterioscler Thromb Vasc Biol* 25:1610–1616, 2005
27. Guo Z, Su W, Allen S, Pang H, Daugherty A, Smart E, Gong MC: COX-2 up-regulation and vascular smooth muscle contractile hyperreactivity in spontaneous diabetic db/db mice. *Cardiovasc Res* 67:723–735, 2005
28. Baber SR, Champion HC, Bivalacqua TJ, Hyman AL, Kadowitz PJ: Role of cyclooxygenase-2 in the generation of vasoactive prostanoids in the rat pulmonary and systemic vascular beds. *Circulation* 108:896–901, 2003
29. Belton OA, Duffy A, Toomey S, Fitzgerald DJ: Cyclooxygenase isoforms and platelet vessel wall interactions in the apolipoprotein E knockout mouse model of atherosclerosis. *Circulation* 108:3017–3023, 2003
30. Quilley J, Chen YJ: Role of COX-2 in the enhanced vasoconstrictor effect of arachidonic acid in the diabetic rat kidney. *Hypertension* 42:837–843, 2003
31. Liang X, Wang Q, Hand T, Wu L, Breyer RM, Montine TJ, Andreasson K: Deletion of the prostaglandin E2 EP2 receptor reduces oxidative damage and amyloid burden in a model of Alzheimer's disease. *J Neurosci* 25:10180–10187, 2005
32. Consilvio C, Vincent AM, Feldman EL: Neuroinflammation, COX-2, and ALS: a dual role? *Exp Neurol* 187:1–10, 2004
33. Kaufmann WE, Worley PF, Pegg J, Bremer M, Isakson P: COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex. *Proc Natl Acad Sci U S A* 93:2317–2321, 1996
34. Kulmacz RJ, Wang LH: Comparison of hydroperoxide initiator requirements for the cyclooxygenase activities of prostaglandin H synthase-1 and -2. *J Biol Chem* 270:24019–24023, 1995
35. Yabuki A, Tahara T, Taniguchi K, Matsumoto M, Suzuki S: Neuronal nitric oxide synthase and cyclooxygenase-2 in diabetic nephropathy of type 2 diabetic OLETF rats. *Exp Anim* 55:17–25, 2006
36. Komers R, Zdychova J, Cahova M, Kazdova L, Lindsley JN, Anderson S: Renal cyclooxygenase-2 in obese Zucker (fatty) rats. *Kidney Int* 67:2151–2158, 2005
37. Yagihashi S, Kamijo M, Ido Y, Mirrlees DJ: Effects of long-term aldose reductase inhibition on development of experimental diabetic neuropathy: ultrastructural and morphometric studies of sural nerve in streptozocin-induced diabetic rats. *Diabetes* 39:690–696, 1990
38. Kennedy WR, Wendelschafer-Crabb G, Johnson T: Quantitation of epidermal nerves in diabetic neuropathy. *Neurology* 47:1042–1048, 1996
39. Hanson P, Schumacker P, Debugne T, Clerin M: Evaluation of somatic and autonomic small fibers neuropathy in diabetes. *Am J Phys Med Rehabil* 71:44–47, 1992
40. Lacomis D: Small-fiber neuropathy. *Muscle Nerve* 26:173–188, 2002
41. McArthur JC, Stocks EA, Hauer P, Cornblath DR, Griffin JW: Epidermal nerve fiber density: normative reference range and diagnostic efficiency. *Arch Neurol* 55:1513–1520, 1998
42. Herrmann DN, Griffin JW, Hauer P, Cornblath DR, McArthur JC: Epidermal nerve fiber density and sural nerve morphometry in peripheral neuropathies. *Neurology* 53:1634–1640, 1999
43. Periquet MI, Novak V, Collins MP, Nagaraja HN, Erdem S, Nash SM, Freimer ML, Sahenk Z, Kissel JT, Mendell JR: Painful sensory neuropathy: prospective evaluation using skin biopsy. *Neurology* 53:1641–1647, 1999
44. Mukherjee D, Nissen SE, Topol EJ: Risk of cardiovascular events associated with selective COX-2 inhibitors. *JAMA* 286:954–959, 2001
45. Bombardier C, Laine L, Reicin A, Shapiro D, Burgos-Vargas R, Davis B, Day R, Ferraz MB, Hawkey CJ, Hochberg MC, Kvien TK, Schnitzer TJ: Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis: VIGOR Study Group. *N Engl J Med* 343:1520–1528, 2000
46. Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgan K, Lines C, Riddell R, Morton D, Lanas A, Konstam MA, Baron JA: Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med* 352:1092–1102, 2005
47. Cheng Y, Austin SC, Rocca B, Koller BH, Coffman TM, Grosser T, Lawson JA, FitzGerald GA: Role of prostacyclin in the cardiovascular response to thromboxane A2. *Science* 296:539–541, 2002
48. Solomon SD, McMurray JJ, Pfeffer MA, Wittes J, Fowler R, Finn P, Anderson WF, Zauber A, Hawk E, Bertagnolli M: Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med* 352:1071–1080, 2005
49. Levesque LE, Brophy JM, Zhang B: The risk for myocardial infarction with cyclooxygenase-2 inhibitors: a population study of elderly adults. *Ann Intern Med* 142:481–489, 2005
50. Sowers JR, White WB, Pitt B, Whelton A, Simon LS, Winer N, Kivitz A, van Ingen H, Brabant T, Fort JG: The Effects of cyclooxygenase-2 inhibitors and nonsteroidal anti-inflammatory therapy on 24-hour blood pressure in patients with hypertension, osteoarthritis, and type 2 diabetes mellitus. *Arch Intern Med* 165:161–168, 2005