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Peripheral Nerve Dysfunction in Experimental Diabetes Is Mediated by Cyclooxygenase-2 and Oxidative Stress

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ABSTRACT

Glucose-mediated oxidative stress and alterations in cyclooxygenase (COX) pathway activity with secondary deficits of endoneurial perfusion have been implicated in the pathogenesis of experimental diabetic neuropathy (EDN). We have previously reported that activation of the COX-2 pathway is an important mediator of neurochemical and neurovascular defects in EDN in a rat model. Considering that chemical COX inhibition may exert other pharmacological effects in addition to inhibition of COX activity, the aim of this study was to explore the role of COX-2 in experimental diabetic neuropathy, using a COX-2 knockout mouse model. Here we provide evidence that COX-2 inactivation had a protective effect against diabetes-induced motor and sensory nerve conduction slowing and impaired nerve antioxidative defense that were clearly manifest in the wild-type (COX-2$^{+/+}$) diabetic mice. These preliminary data support the role of the activation of the COX-2 pathway in mediating sensory and motor nerve conduction velocity deficits in EDN. These findings also suggest that the COX-2 pathway seems to be an important modulator of oxidative stress in EDN. *Antioxid. Redox Signal.* 7, 1521–1529.

INTRODUCTION

Diabetic peripheral neuropathy (DPN) affects most patients with diabetes and is the leading cause of nontraumatic lower limb amputations, accounting for ~85,000 amputations per year in the U.S. To date, besides a tight glycemic control, a viable treatment for human DPN is not available.

The contribution of hyperglycemia to the pathogenesis of microvascular complications in both type 1 (1, 2) and most recently type 2 diabetic subjects (3) is now beyond dispute. Although mounting evidence provides support for a microvascular basis of DPN (8, 10, 14), the pathophysiology of DPN is still quite poorly understood.

Increased glucose-induced oxidative stress (10, 24, 31), impaired protein kinase C (PKC) activity, redox imbalance secondary to enhanced aldose reductase (AR) activity, impaired nitric oxide synthesis (8, 12, 13, 15, 42), and endothelial dysfunction (19, 33) have all been identified as critical factors that precipitate the development of diabetic complications, including DPN.

Additional studies implicate alterations in cyclooxygenase (COX) activity with subsequent perturbations in prostaglandin (PG) metabolism (9, 12, 38, 41) in the pathogenesis of experimental DPN at a neurovascular level. Some pharmacological studies of experimental DPN in the streptozotocin (STZ)-induced diabetes (STZ-D) rat model attribute reduced nerve blood flow (NBF) (6, 7, 31) to reactive oxygen species (ROS)-induced vascular dysfunction (8, 14, 34). Conversely, endoneurial ischemia may precipitate ROS-related damage to the cellular elements of peripheral nerve tissue (31). This decrease in vascular perfusion can be corrected by a variety of different treatments, including AR inhibitors, essential fatty acids, PGs, and aminoguanidine (11, 12, 18, 28, 50).

We have recently reported (38) beneficial effects of cyclooxygenase-2 (COX-2)-selective inhibitors in preventing NBF and nerve conduction velocity (NCV) deficits in experimental DPN in a rat model.

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However, as chemical COX inhibition may exert other pharmacological effects in addition to strictly inhibition of COX enzymatic activity (30), the effects observed with these agents may not always reflect the physiological roles of the COX isoforms.

Recently, targeted gene disruption of COX-2 has been achieved in the mouse (20, 32). To this end, considering that the use of knockout mice offers a powerful tool, superior to strictly pharmacological studies, in the studies reported herein, we investigated the effects of partial and total COX-2 genetic inactivation on selected biochemical and neurofunctional deficits of experimental DPN in this murine model.

MATERIALS AND METHODS

Animal model

The experiments were performed in accordance with regulations specified by the National Institutes of Health *Principles of Laboratory Animal Care, 1985 Revised Version*, and the Institutional Animal Care and Use Committee at the Medical College of Ohio approved the procedures.

Mice heterozygous for disruption of the COX-2 gene ($Ptgs2^{tm1Jed}$) on a B6;129S7 strain were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.) and subsequently bred at our institution.

Homozygous and heterozygous ($COX\text{-}2^{-/-}$, $COX\text{-}2^{+/-}$)–deficient male mice and littermate, wild-type (+/+) male mice were rendered diabetic (STZ-D) by five consecutive intraperitoneal injections of 40 mg/kg STZ (Upjohn, Kalamazoo, MI, U.S.A.) in 0.2 ml of 10 m*M* citrate buffer, pH 4.5. Diabetes was defined as a nonfasting plasma glucose of ≥250 mg/dl in tail-vein blood (One Touch II, Lifescan, Inc., Milpitas, CA, U.S.A.) ~48 h after the last STZ injection. Animals were subsequently randomly assigned to the experimental groups listed in Table 1, and maintained for 6 weeks in individual air-filtered metabolic cages. The animals were fed standard chow with *ad libitum* access to water. After measurement of motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV), groups of 10–15 animals per condition were euthanized by CO_2 inhalation followed by cervical dislocation, for specific end-point assessments.

Genotyping

For genotyping, genomic DNA was isolated from tail clipping using the DNEasy Tissue Kit from Quiagen. Polymerase chain reaction (PCR) was performed using the protocol obtained from Jackson Laboratories. In brief, two sets of primers were used. The first consists of 5′-CTTGGGTGGAGAGGCTATTC-3′ and 5′-AGGTGAGATGACAGGAGATC-3′. This set amplifies a 280-bp fragment of the Neo1 gene, which indicates a knocked-out gene. The second set consists of a 5′-CACCATAGAATCCAGTCCGG-3′ sequence and a 5′-ACCTCTGCGATGCTCTTCC-3′ sequence. This set of primers amplifies an 857-bp fragment and indicates a wild-type gene. The cycling conditions include a 3-min melt at 94°C, a cycle of 94°C for 35 s, 64°C for 45 s (reduced by 0.5°C per cycle), and 72°C for 45 s repeated 13 times, a cycle of 94°C for 35 s, 58°C for 30 s, and 72°C for 45 s repeated 26 times, finally followed by a final extension at 72°C for 2 min. The PCR reaction contained 20 m*M* Tris-HCl, 50 m*M* KCl, 3 m*M* $MgCl_2$, 0.2 m*M* dNTP, 1 μ*M* of each primer, 25 mU of Taq, and 2 μl of DNA.

PCR products were analyzed using gel electrophoresis with ethidium bromide on a 1.5% agarose gel (Fig. 1A).

Table 1. Experimental Conditions and Group Designations

Experimental groups and genotypes	*Group designation*
$COX\text{-}2^{+/+}$ control	$COX\text{-}2^{+/+}$ C
$COX\text{-}2^{+/+}$ STZ-D	$COX\text{-}2^{+/+}$ D
$COX\text{-}2^{+/-}$ control	$COX\text{-}2^{+/-}$ C
$COX\text{-}2^{+/-}$ STZ-D	$COX\text{-}2^{+/-}$ D
$COX\text{-}2^{-/-}$ control	$COX\text{-}2^{-/-}$ C
$COX\text{-}2^{-/-}$ STZ-D	$COX\text{-}2^{-/-}$ D

Western blot analysis

Heart tissue from $COX\text{-}2^{+/+}$, $COX\text{-}2^{+/-}$, and $COX\text{-}2^{-/-}$ diabetic (D) mice was lysed in homogenization buffer (5 mmol/L HEPES, pH 7.9, 26% glycerol, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 300 mmol/L NaCl) containing Complete Mini Protease Inhibitor (Roche) and centrifuged at 4°C at 15,000 *g* for 20 min three times. Protein concentrations were measured by the bicinchoninic acid protein assay kit (Pierce). Seventy-microgram aliquots of protein extract were separated by electrophoresis on a 7.5% sodium dodecyl sulfate Tris-HCl ReadyGel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane. The blots were then blocked in 5% nonfat dry milk in Tris-buffered saline for 1 h and incubated overnight at 4°C with anti-COX-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). After washing, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody and analyzed using enhanced chemiluminescence detection kits using Super Pico West (Pierce Biotechnologies). As expected, COX-2 protein expression was undetectable in the $COX\text{-}2^{-/-}$ mice as compared with $COX\text{-}2^{+/+}$ mice (Fig. 1B).

Functional studies

Sciatic MNCV. The mouse was anesthetized with a 3:1 ketamine xylazine mix at 80 mg/ kg. The mouse was maintained on a heating pad at ~37°C until sufficiently anesthetized. A halogen lamp was then placed in close proximity to the rear of the mouse to maintain the nerve's temperature. The mouse left sciatic nerve was stimulated proximally at the sciatic notch and distally at the ankle via bipolar electrodes with supramaximal stimuli (8 V) at 20 Hz. The latencies of the compound muscle action potentials were recorded via bipolar electrodes from the first interosseous muscle of the hind paw and measured from the stimulus artifact to the onset of the negative M-wave deflection. MNCV was calculated by subtracting the distal latency from the proximal latency, and the result was divided into the distance between the stimulation and recording electrode.

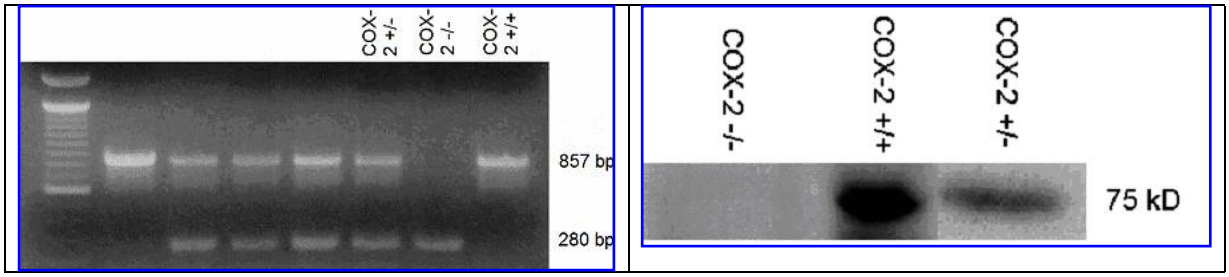

FIG. 1. (**A**) PCR analysis of genomic DNA obtained from COX-$2^{+/+}$ (wild type), COX-$2^{+/-}$, and COX-$2^{-/-}$ was performed as described in Materials and Methods. The 280-bp band indicates a knocked-out gene, and the 857-bp band indicates a wild-type gene. (**B**) Western blot analysis of COX-$2^{+/+}$, COX-$2^{+/-}$ and COX-$2^{-/-}$ was performed as described in Materials and Methods. The 75-kDa band designates the presence of the COX-2 protein.

Digital SNCV. SNCV measurements were done as previously described (43). In brief, hind-limb SNCV was recorded in the digital nerve to the second toe by stimulating with a square-wave pulse of 0.05-ms duration using the smallest intensity current that resulted in a maximal amplitude response. The sensory nerve action potential was recorded behind the medial malleolus. Sixteen responses were averaged to obtain the position of the negative peak. The maximal SNCV was calculated by measuring the latency to the onset/peak of the initial negative deflection and the distance between stimulating and recording electrodes.

Measurements of markers of oxidative stress in the mouse peripheral nerve

Measurements of nerve malondialdehyde plus 4-hydroxyalkenals (MDA + 4-HA) and glutathione (GSH). These measurements were performed independently using commercially available kits (Oxis Research), as previously described (43). In brief, mouse sciatic nerves were homogenized in 125 µl of 0.1 mol/L sodium phosphate buffer, pH 6.5. Twenty microliters of the mouse sciatic nerve homogenate at 15% weight to buffer was used for measurements of MDA + 4-HA performed in accordance with the manufacturer's instructions with modifications to account for the reduced mass of tissue collected from the mice. Another 20–30 µl was used for GSH measurements.

Antioxidative defense enzyme activity. Superoxide dismutase (SOD) and catalase were measured in the mouse sciatic nerve using commercially available kits (Oxis Research) as previously described (35). Mouse sciatic nerves were homogenized in 1 ml of 0.1 mol/L sodium phosphate buffer, pH 6.5, and centrifuged at 20,000 *g* for 20 min. The supernatants were used for spectrophotometric measurements of SOD and catalase in accordance with the manufacturer's instructions.

PG determination. Thromboxane (TX) B_2 (stable metabolite of TXA_2), PGE_2, $PGF_{2\alpha}$, and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$; stable metabolite of PGI_2) were measured in the mouse sciatic nerve by standard enzyme immunoassay (ELISA) using commercially available kits (Cayman Chemicals, Ann Arbor, MI, U.S.A.) and following the manufacturer's instruction.

Chemicals

Unless otherwise stated, all non–PCR-related chemicals were of reagent-grade quality and were purchased from Fisher Scientific (Hanover Park, IL, U.S.A.) and Sigma (St. Louis, MO, U.S.A.). All PCR-related reagents including primers were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Kits for measurements of MDA + 4-HA, GSH, and SOD were purchased from Oxis International (Portland, OR, U.S.A.).

Statistical analysis

Data are expressed as means ± SEM. Differences among experimental groups were determined by ANOVA, and the significance of between-group differences assessed by Student–Newman–Keuls 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

Effects of COX-2 gene inactivation and STZ-D on body weight and blood glucose levels

Body weights (BW) were similar in all experimental groups at baseline. All control (C) mice, including the COX-$2^{-/-}$, gained significantly more weight during the experiments than the D mice (31% to 55% increase from baseline; $p < 0.05$; Table 2), and there was no difference in the final BW in all groups of C mice. COX-$2^{-/-}$ D mice had significantly lower final BW as compared with the other D mice ($p < 0.05$; Table 2). Blood glucose (BG) concentration was increased threefold in D mice compared with C mice. Importantly, COX-2 gene inactivation did not significantly affect glucose levels compared with wild-type D mice (Table 2).

Effects of COX-2 gene inactivation and STZ-D on MNCV

MNCV was unaffected in the COX-$2^{+/-}$ and COX-$2^{-/-}$ C mice compared with COX-$2^{+/+}$ C mice, indicating that inactivation of the COX-2 gene did not affect the function of the

TABLE 2. EFFECTS OF COX-2 GENE INACTIVATION AND STZ-D ON BG AND BW

	BG (mg/dl)		*BW (g)*	
Genotype	*Initial*	*Final*	*Initial*	*Final*
COX-$2^{+/+}$ C	93 ± 5	97 ± 24	19.8 ± 1	26.2 ± 1
COX-$2^{+/+}$ D	81 ± 4	329 ± 13	17.3 ± 1	19.7 ± 0.7*
COX-$2^{+/-}$ C	88 ± 6	120 ± 7	18.0 ± 0.8	23.6 ± 1
COX-$2^{+/-}$ D	81 ± 4	318 ± 8	17.1 ± 0.6	21 ± 1*
COX-$2^{-/-}$ C	74 ± 11	111 ± 8	15.9 ± 0.6	24.8 ± 0.8
COX-$2^{-/-}$ D	77 ± 8	327 ± 18	15.1 ± 0.9	20.1 ± 1.9*

BG was measured using tail-vein puncture as described in Materials and Methods. Data are expressed as means ± SEM.

*$p < 0.05$ versus C.

nerve under normal conditions (Fig. 2). Six weeks of STZ-D significantly decreased MNCV in the COX-$2^{+/-}$ and COX-$2^{+/+}$ mice versus their corresponding C mice by 24% and 23%, respectively ($p < 0.01$). However, this decrease was partially prevented in the COX-$2^{-/-}$ D mice (13% reduction as compared with their respective C mice) ($p < 0.05$ versus COX-$2^{+/-}$ and COX-$2^{+/+}$ D mice).

Effects of COX-2 gene inactivation and STZ-D on SNCV

Consistent with MNCV data, COX-2 gene inactivation did not affect SNCV in the C mice (Fig. 3). After 6 weeks of STZ-D, SNCV was decreased by 19% in the COX-$2^{+/-}$ mice ($p < 0.01$ versus corresponding C mice), but this reduction was less ($p = 0.09$) than that produced by 6 weeks of diabetes in the COX-$2^{+/+}$ mice (25% decrease, $p < 0.001$ versus corresponding C mice). However, in the COX-$2^{-/-}$ mice, the SNCV deficits induced by STZ-D were completely prevented ($p = 0.973$ versus respective C mice, $p < 0.001$ versus COX-$2^{+/+}$ D mice, $p < 0.05$ versus COX-$2^{+/-}$ D mice).

Effects of COX-2 gene inactivation and STZ-D on nerve GSH content

Under nondiabetic conditions, there was no difference in the level of nerve GSH of knockout and wild-type mice (15.1 ± 2 μ*M*/g wet tissue wt versus 14.9 ± 0.8 μ*M*/g wet tissue wt versus 16.1 ± 0.7 μ*M*/g wet tissue wt, respectively; Fig. 4). D COX-$2^{+/+}$ and COX-$2^{+/-}$ mice showed a significant reduction in the sciatic nerve GSH level (2.4- and 3.1-fold reduction, respectively, versus C mice, $p < 0.01$), whereas in D COX-$2^{-/-}$ mice, the nerve GSH level was similar to that in C mice ($p = 0.9$).

Effects of COX-2 gene inactivation and STZ-D on nerve lipid peroxidation product (MDA + 4-HA) concentration

Sciatic nerve lipid peroxidation product MDA + 4-HA concentration was similar among all C mice and was increased 2.4- and 2.3-fold, respectively, in the COX-$2^{+/+}$ and COX-$2^{+/-}$ D mice compared with the corresponding C mice ($p < 0.05$; Fig. 5). COX-2 gene inactivation completely prevented this increase in D mice.

Effects of COX-2 gene inactivation and STZ-D on nerve SOD activity

There was no difference in the nerve SOD activity in all C mice (Fig. 6). SOD activity was significantly decreased in the nerve of D COX-$2^{+/+}$ mice compared with C mice ($p < 0.01$). SOD activity tended to decrease in the COX-$2^{+/-}$ D mice, but

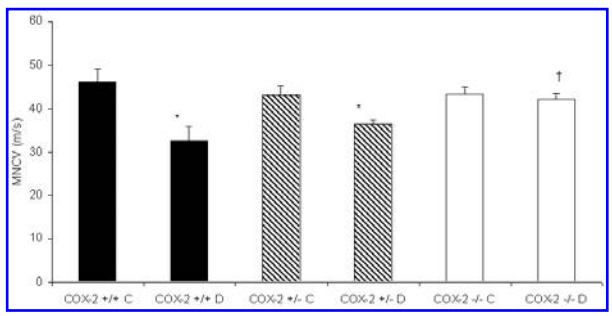

FIG. 2. MNCV was measured in the mouse left sciatic nerve as described in Materials and Methods after 6 weeks. Data are expressed as means ± SEM. *$p < 0.05$ versus C; †$p < 0.05$ versus other D.

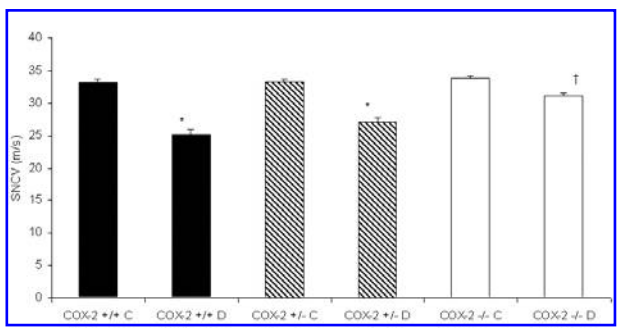

FIG. 3. SNCV was measured in the mouse left sciatic nerve as described in Materials and Methods after 6 weeks. Data are expressed as means ± SEM. *$p < 0.05$ versus C; †$p < 0.05$ versus other D.

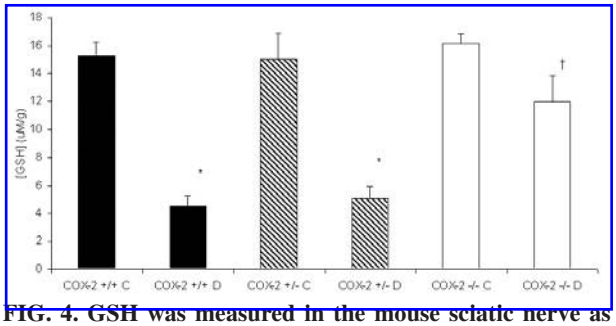

FIG. 4. GSH was measured in the mouse sciatic nerve as described in Materials and Methods after 6 weeks. Data are expressed as means ± SEM. *$p < 0.05$ versus C; †$p < 0.05$ versus other D.

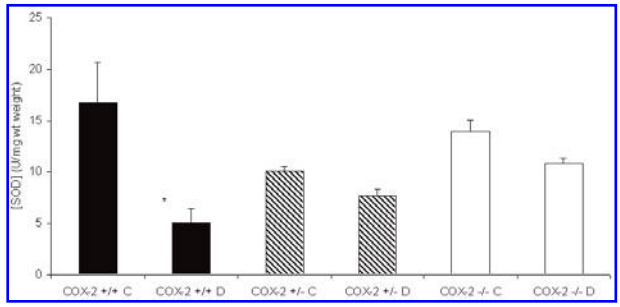

FIG. 6. SOD was measured in the mouse left sciatic nerve as described in Materials and Methods after 6 weeks. Data are expressed as means ± SEM. *$p < 0.05$ versus C; †$p < 0.05$ versus other D.

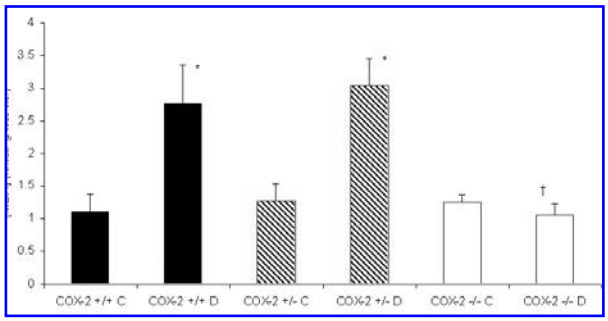

FIG. 5. MDA was measured in the mouse sciatic nerve as described in Materials and Methods after 6 weeks. Data are expressed as means ± SEM. *$p < 0.05$ versus C; †$p < 0.05$ versus other D.

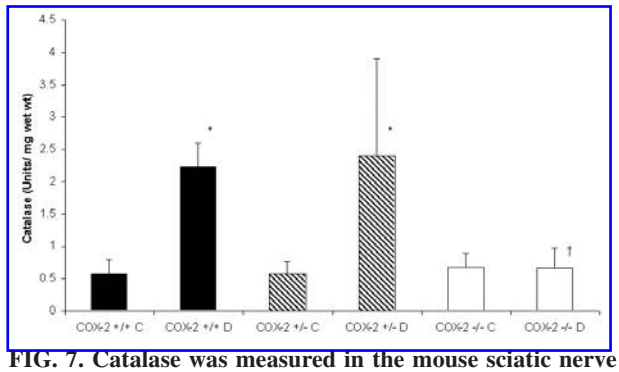

FIG. 7. Catalase was measured in the mouse sciatic nerve as described in Materials and Methods after 6 weeks. Data are expressed as means ± SEM. *$p < 0.05$ versus C; †$p < 0.05$ versus other D.

the difference between D and C groups did not achieve statistical significance ($p = 0.4$) and remained unchanged in the D COX-$2^{-/-}$ as compared with C mice ($p = 0.9$).

Effects of COX-2 gene inactivation and STZ-D on nerve catalase activity

There was no difference in nerve catalase activity in the C mice, irrespective of their genotype (Fig. 7). Nerve catalase was increased 3.7- and 4.3-fold in COX-$2^{+/-}$ and COX-$2^{+/+}$ D mice versus their respective C mice ($p < 0.01$) and remained completely unchanged in the COX-$2^{-/-}$ mice.

Effects of COX-2 gene inactivation and STZ-D on nerve PG production

The sciatic nerve levels of vasoconstricting prostanoids PGE_2, PGF2α, and TXB_2 were similar in all C mice irrespective of their genotype and were increased 2.4- to 5-fold in the COX-$2^{+/+}$ and COX-$2^{+/-}$ D mice as compared with C mice (Table 3). Levels remained unchanged in the COX-$2^{-/-}$ D mice.

TABLE 3. EFFECTS OF COX-2 GENE INACTIVATION AND STZ-D ON PG LEVELS

Genotype	*PGF2α (pg/mg wet wt)*	*PGE_2 (pg/mg wet wt)*	*TXB_2 (pg/mg wet wt)*
COX-$2^{+/+}$ C	11.5 ± 5.0	0.62 ± 0.18	13.4 ± 5.9
COX-$2^{+/+}$ D	34.9 ± 11.6*	3.6 ± 1.6*	34.0 ± 10.2*
COX-$2^{+/-}$ C	10.7 ± 4.3	0.56 ± 0.28	12.1 ± 4.6
COX-$2^{+/-}$ D	32.3 ± 9.7*	3.17 ± 1.2*	32.8 ± 11.3*
COX-$2^{-/-}$ C	7.6 ± 4.7	0.64 ± 0.26	13.2 ± 5.9
COX-$2^{-/-}$ D	11.1 ± 3.6†	0.55 ± 0.21†	15.6 ± 1.2†

PGs were measured in the mouse sciatic nerve as described in Materials and Methods after 6 weeks. Data are expressed as means ± SEM.

*$p < 0.05$ versus C.

†$p < 0.05$ versus other D.

DISCUSSION

In this study, we demonstrate for the first time that COX-2 gene inactivation has a protective effect against nerve functional and biochemical deficits in experimental diabetes in a murine model. Our experiments also provide support for a major contribution of the COX-2 pathway activation-enhanced oxidative stress relationship in experimental diabetic neuropathy.

Several lines of evidence support these conclusions.

COX-$2^{+/+}$, COX-$2^{+/-}$, and COX$2^{-/-}$ mice demonstrated different susceptibility to hyperglycemia-induced neuropathy. After 6 weeks, D COX-$2^{+/+}$ and COX-$2^{+/-}$ mice developed motor and sensory nerve conduction deficits similar to those found in rats with STZ-D (37, 38, 43). In contrast, D COX-$2^{-/-}$ mice preserved normal SNCV after 6 weeks (Fig. 3). D COX-$2^{+/+}$ mice developed significant markers of increased oxidative injury in the peripheral nerve, manifested by accumulation of lipid peroxidation products, depletion of GSH, down-regulation of SOD activity, and up-regulation of catalase activity. D COX-$2^{-/-}$ mice preserved normal nerve antioxidant defense and lipid peroxidation levels. These effects were not due to alleviation of hyperglycemia, because all D mice had comparable final BG concentrations regardless of their genotype. Interestingly, although the D COX-$2^{+/-}$ mice showed similar deficits in MNCV, nerve lipid peroxidation, GSH levels, and catalase activity as the D COX-$2^{+/+}$ mice, they demonstrated a partial protection toward the SNCV slowing and SOD down-regulation. C mice did not develop these functional abnormalities regardless of their genotype. These studies confirmed that, acutely, COX-2 gene-deficient STZ-D mice demonstrate differential protection against selected biochemical and functional markers of experimental diabetic neuropathy and that the extent of this protection appears to be dependent on the degree of COX-2 gene deficiency. We have also found that in COX-$2^{+/+}$ and COX-$2^{+/-}$ D mice, the production of PGE_2 and TXB_2 was significantly increased and the production of 6-keto-$PGF_{1\alpha}$ was significantly decreased after 6 weeks as compared with C mice, whereas COX-$2^{+/-}$ D mice were protected against the imbalanced PG production. Our data provide therefore a molecular support to several prior observations, including our own, that have implicated an overproduction of vasoconstricting PG in diabetes-induced vascular dysfunction (47), suggesting therefore a differential regulation of the synthesis of various PGs by COX-2/COX-1 pathways in diabetes.

A proposed paradigm of the effects of diabetes on the COX pathway is detailed in Fig. 8. PGs are generated by COX from arachidonic acid. Two isoforms of the enzyme, encoded by distinct genes, have been isolated in mammalian cells (48). COX-1 is constitutively expressed in most tissues and is involved in maintenance of cellular homeostasis, including regulation of vascular tone (48). In contrast, under normal conditions, COX-2 is expressed at low or undetectable levels, but is readily up-regulated by inflammatory, mitogenic, and physical stimuli (27, 49) and oxidative stress (23). Hyperglycemia, through increased autooxidation and activation of the AR pathway, is proposed to generate ROS and promote oxidative stress (40). In addition, hyperglycemia may induce oxidative stress through increased mitochondrial ROS production (29)

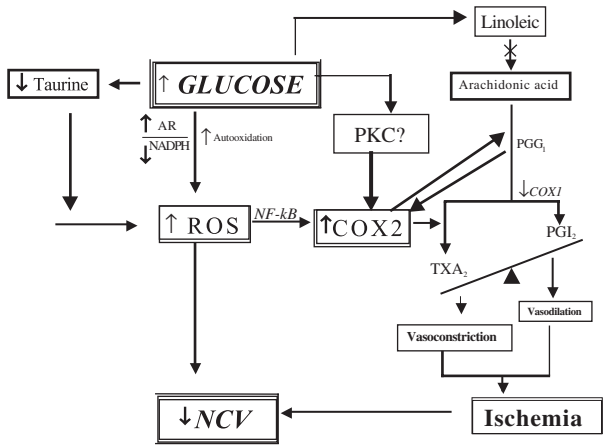

FIG. 8. Hyperglycemia through increased autooxidation, and activation of AR pathway, with secondary NADPH and taurine depletion, generates ROS and increased oxidative stress. Alternatively, glucose-induced increase of PKC activity induces increased O_2^- production, further promoting increased oxidative stress. Increased ROS, via modulation of NF-κB, and PKC signaling induce COX-2 expression, which regulates the conversion of arachidonate to vasoconstricting and proinflammatory PG. This precipitates the imbalance in TXA_2/PGI_2 ratio, favoring therefore vasoconstriction and ischemia. Reciprocally, COX-2 up-regulation increases the rate of PGG_2 to PGH_2 conversion and ROS generation, further exacerbating oxidative stress. Increased oxidative stress and endoneurial dysfunction may result in NCV slowing.

and PKC activation (17). This results in nuclear factor-κB (NF-κB) activation (4, 22), COX-2 mRNA induction (17, 29) and COX-2 protein expression. COX- 2 up-regulation leads to an altered PG profile with an increased production of vasoconstricting PGH_2 (39), TXA_2 (44), and $PGF_{2\alpha}$ and reduction in vasodilatory PGI_2 (26), favoring therefore vasoconstriction and ischemia. Reciprocally, COX-2 up-regulation increases the rate of PGG_2 to PGH_2 conversion and ROS generation, further exacerbating oxidative stress.

In experimental DPN, the effects of COX inhibition on neurovascular and functional deficits have been inconsistent. Initial reports suggested that nonselective COX inhibition could ameliorate certain specific deficits of nerve function, (36, 51), but later reports could not confirm any beneficial effect (9, 12). Consistent with these later observations, we have reported (38) that nonselective inhibition of the COX pathway with flurbiprofen in nondiabetic rats replicated, and in STZ-D rats potentiated, many of the biochemical and physiological defects of experimental DPN. In contrast, selective COX-2 inhibition was without affect on NCV and NBF in nondiabetic rats and completely prevented NCV slowing and NBF deficits in STZ-D rats. We have therefore postulated that the apparent inconsistency of the effects of different COX inhibitors on both NCV and NBF deficits in STZ-D rats may reflect differences in the relative degree of inhibition of the two COX pathways (or other unspecified metabolic effects) and that the COX-1 pathway may play a tonic role in the regulation of nerve metabolism and the maintenance of NBF (38).

The studies described herein confirm our hypothesis that although the production of PGE_2 and TXA_2 is not unique to the COX-2 pathway in normal conditions, in proinflammatory conditions, such as diabetes, their formation occurs mainly via COX-2. Several other reports support our findings. For instance, there is evidence that in inflammation COX-2 is the major pathway accounting for 75% of PGE_2 production (25, 30). In addition, COX-2 appears to be coregulated and biochemically coupled with the inducible gene product microsomal PGE_2 synthase, a dominant generator of PGE_2 under certain inflammatory conditions (5). Another recent report showed that high glucose, via PKC, induces oxidative stress and up-regulation of COX-2, resulting in reduced nitric oxide availability and an altered PG profile consistent with a higher TXB_2 and a decreased prostacyclin release (17).

In concert with several other independent groups of investigators (24, 31, 43), these data provide additional evidence of hyperglycemia-induced oxidative stress and of its link to peripheral nerve dysfunction. The central role played by oxidative stress in the pathogenesis of diabetic neuropathy has been extensively documented. In animal models of diabetic neuropathy, which show similar neurophysiological and morphometric alterations to those observed in humans, various antioxidants were shown to improve nerve microcirculation and protect against neurovascular dysfunction (14, 43). Alternatively or in addition, free radicals have been shown to impair endothelium-dependent vasodilatation either by changes in the generation and bioactivity of nitric oxide or by an increased synthesis of vasoconstricting PG (16, 17). It has been also shown that, in the rabbit aorta, impaired relaxation was restored by nonselective COX blockade, prostanoid receptor antagonists, as well as SOD, suggesting that vasoconstricting PG and ROS are the underlying cause of endothelial dysfunction (45, 46). The results we report herein are consistent with these data. Our findings that COX-$2^{-/-}$ D preserved normal oxidative status and normal levels of vasoconstricting PG, being protected therefore against reductions of NCV, substantiate that in diabetes, oxidative stress via various mechanisms (4, 17, 22) upregulates the COX-2 pathway. It also suggests that in a diabetic milieu, COX-2 pathway–oxidative injury interplay is more complex, because ROS-induced activation of COX-2 can further increase ROS production (21, 27) and close therefore a vicious circle (Fig. 8). Prevention of these deficits in the COX-$2^{-/-}$ D mice probably contributes to the acute preservation of their neurovascular function.

In summary, our findings suggest that, acutely, COX-2-deficient D mice appear to be preconditioned to buffer D-induced oxidative stress and to be protected against an increased production of vasoconstrictory PG in diabetes. This confirms our hypothesis that the diabetes state is a proinflammatory condition promoting the production of inflammatory and proaggregation PG. These interesting data provide new insights into the mechanisms responsible for buffering oxidative stress and PG metabolism imbalance in experimental diabetes and clearly have implications for therapeutic approaches in man. Therefore, COX-2 inactivation might represent a novel approach for the prevention and/or treatment of DPN, although further studies with additional and longer end points are needed to verify whether it is really the case.

ABBREVIATIONS

AR, aldose reductase; BG, blood glucose; BW, body weight; C, control; COX, cyclooxgenase; D, diabetic; DPN, diabetic peripheral neuropathy; GSH, glutathione; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; MDA + 4HA, malondialdehyde plus 4-hydroxyalkenals; MNCV, motor nerve conduction velocity; NBF, nerve blood flow; NCV, nerve conduction velocity; NF-κB, nuclear factor-κB; PCR, polymerase chain reaction; PG, prostaglandin; PKC, protein kinase C; ROS, reactive oxygen species; SNCV, sensory nerve conduction velocity; SOD, superoxide dismutase; STZ streptozotocin; STZ-D, streptozotocin-induced diabetes; TX, thromboxane.

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REFERENCES

1. [No authors listed] The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group [see comments]. *N Engl J Med* 329: 977–986, 1993.
2. [No authors listed] Effect of intensive diabetes treatment on nerve conduction in the Diabetes Control and Complications Trial. *Ann Neurol* 38: 869–880, 1995.
3. [No authors listed] Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group [see comments]. *Lancet* 352: 837–853, 1998.
4. Adderley SR and 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.
5. Brock TG, McNish RW, and Peters-Golden M. Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E2. *J Biol Chem* 274: 11660–11666, 1999.
6. Cameron NE and Cotter MA. Comparison of the effects of chronic aldose reductase inhibitor and acetyl-L-cysteine treatment on peripheral nerve blood flow and function in anesthetized diabetic rats. *J Physiol (Lond)* 483P: 142P, 1995.
7. Cameron NE and Cotter MA. Comparison of the effects of ascorbyl-gamma-linolenic acid and gamma-linolenic acid

in the correction of neurovascular deficits in diabetic rats. *Diabetologia* 39: 1047–1054, 1996.
8. Cameron NE and Cotter MA. Metabolic and vascular factors in the pathogenesis of diabetic neuropathy. *Diabetes* 46(Suppl 2): 31S–37S, 1997.
9. Cameron NE, Cotter MA, Dines KC, Robertson S, and Cox D. The effects of evening primrose oil on nerve function and capillarization in streptozotocin-diabetic rats: modulation by the cyclo-oxygenase inhibitor flurbiprofen. *Br J Pharmacol* 109: 972–979, 1993.
10. Cameron NE, Cotter MA, and Maxfield EK. Anti-oxidant treatment prevents the development of peripheral nerve dysfunction in streptozotocin-diabetic rats. *Diabetologia* 36: 299–304, 1993.
11. Cameron NE, Cotter MA, Dines KC, Maxfield EK, Carey F, and Mirrlees DJ. Aldose reductase inhibition, nerve perfusion, oxygenation and function in streptozotocin-diabetic rats: dose–response considerations and independence from a myo-inositol mechanism. *Diabetologia* 37: 651–663, 1994.
12. Cameron NE, Cotter MA, and Hohman TC. Interactions between essential fatty acid, prostenoid, polyol pathway and nitric oxide metabolism in the neurovascular deficit of diabetic rats. *Diabetologia* 39: 172–182, 1996.
13. Cameron NE, Cotter MA, Jack A, and Hohman TC. Inhibition of protein kinase C corrects nerve conduction and blood flow deficits in diabetic rats. *Diabetologia* 40: A115, 1997.
14. Cameron NE, Cotter MA, Horrobin DH, and Tritschler HJ. Effects of alpha-lipoic acid on neurovascular function in diabetic rats: interaction with essential fatty acids. *Diabetologia* 41: 390–399, 1998.
15. Cameron NE, Cotter MA, Jack AM, Basso MD, and Hohman TC. Protein kinase C effects on nerve function, perfusion, Na^+,K^+-ATPase activity and glutathione content in diabetic rats. *Diabetologia* 42: 1120–1130, 1999.
16. Cosentino F, Barker JE, Brand MP, Heales SJ, Werner ER, Tippins JR, West N, Channon KM, Volpe M, and Luscher TF. Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice. *Arterioscler Thromb Vasc Biol* 21: 496–502, 2001.
17. Cosentino F, Eto M, De Paolis P, van der Loo B, Bachschmid M, Ullrich V, Kouroedov A, Delli Gatti C, Joch H, Volpe M, and 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.
18. Cotter MA and Cameron NE. Effects of dietary supplementation with arachidonic acid rich oils on nerve conduction and blood flow in streptozotocin-diabetic rats. *Prostaglandins Leukot Essent Fatty Acids* 56: 337–343, 1997.
19. De Vriese AS, Verbeuren TJ, Van de Voorde J, Lameire NH, and Vanhoutte PM. Endothelial dysfunction in diabetes. *Br J Pharmacol* 130: 963–974, 2000.
20. Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffee BD, Covington MB, Contel NR, Eng VM, Collins RJ, Czerniak PM, *et al.* Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. *Nature* 378: 406–409, 1995.
21. Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, and Lipsky PE. Cyclooxygenase in biology and disease [see comments]. *FASEB J* 12: 1063–1073, 1998.
22. Faux SP and Howden PJ. Possible role of lipid peroxidation in the induction of NF-kappa B and AP-1 in RFL-6 cells by crocidolite asbestos: evidence following protection by vitamin E. *Environ Health Perspect* 105 Suppl 5: 1127–1130, 1997.
23. Feng L, Xia Y, Garcia GE, Hwang D, and 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.
24. Greene DA, Stevens MJ, Obrosova I, and Feldman EL. Glucose-induced oxidative stress and programmed cell death in diabetic neuropathy. *Eur J Pharmacol* 375: 217–223, 1999.
25. Gross G, Imamura T, Vogt SK, Wozniak DF, Nelson DM, Sadovsky Y, and Muglia LJ. Inhibition of cyclooxygenase-2 prevents inflammation-mediated preterm labor in the mouse. *Am J Physiol Regul Integr Comp Physiol* 278: R1415–R1423, 2000.
26. Harrison HE, Reece AH, and Johnson M. Decreased vascular prostacyclin in experimental diabetes. *Life Sci* 23: 354–356, 1978.
27. Herschman HR, Reddy ST, and Xie W. Function and regulation of prostaglandin synthase-2. *Adv Exp Med Biol* 407: 61–66, 1997.
28. Kihara M, Schmelzer JD, Poduslo JF, Curran GL, Nickander KK, and Low PA. Aminoguanidine effects on nerve blood flow, vascular permeability, electrophysiology, and oxygen free radicals. *Proc Natl Acad Sci U S A* 88: 6107–6111, 1991.
29. Kiritoshi S, Nishikawa T, Sonoda K, Kukidome D, Senokuchi T, Matsuo T, Matsumura T, Tokunaga H, Brownlee M, and 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.
30. Langenbach R, Loftin CD, Lee C, and Tiano H. Cyclooxygenase-deficient mice. A summary of their characteristics and susceptibilities to inflammation and carcinogenesis. *Ann N Y Acad Sci* 889: 52–61, 1999.
31. Low PA, Nickander KK, and Tritschler HJ. The roles of oxidative stress and antioxidant treatment in experimental diabetic neuropathy. *Diabetes* 46(Suppl 2): 38S–42S, 1997.
32. Morham SG, Langenbach R, Loftin CD, Tiano HF, Vouloumanos N, Jennette JC, Mahler JF, Kluckman KD, Ledford A, Lee CA, and Smithies O. Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* 83: 473–482, 1995.
33. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, and Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404: 787–790, 2000.
34. Obrosova I, Fathallah L, Stockert C, Kennedy A, Frank R, and Stevens M. Early upregulation of vascular endothelial

growth factor (VEGF) in the diabetic rat retina: attenuation by taurine. *Diabetes* A172, 2000.
35. Obrosova IG, Fathallah L, and Greene DA. Early changes in lipid peroxidation and antioxidative defense in diabetic rat retina: effect of DL-alpha-lipoic acid. *Eur J Pharmacol* 398: 139–146, 2000.
36. Parry GJ and Kozu H. Piroxicam may reduce the rate of progression of experimental diabetic neuropathy. *Neurology* 40: 1446–1449, 1990.
37. Pop-Busui R, Sullivan KA, Van Huysen C, Bayer L, Cao X, Towns R, and Stevens MJ. Depletion of taurine in experimental diabetic neuropathy: implications for nerve metabolic, vascular, and functional deficits. *Exp Neurol* 168: 259–272, 2001.
38. Pop-Busui R, Marinescu V, Van Huysen C, Li F, Sullivan K, Greene DA, Larkin D, and 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.
39. Shimizu K, Muramatsu M, Kakegawa Y, Asano H, Toki Y, Miyazaki Y, Okumura K, Hashimoto H, and Ito T. Role of prostaglandin H2 as an endothelium-derived contracting factor in diabetic state. *Diabetes* 42: 1246–1252, 1993.
40. Sies H. Oxidative stress: oxidants and antioxidants. *Exp Physiol* 82: 291–295, 1997.
41. Sima AAF, Ristic H, Merry A, Kamijo M, Lattimer SA, Stevens MJ, and Greene DA. The primary preventive and secondary interventionary effects of acetyl-L-carnitine on diabetic neuropathy in the BB/W-rat. *J Clin Invest* 97: 1900–1907, 1996.
42. Stevens MJ, Dananberg J, Feldman EL, Lattimer SA, Kamijo M, Thomas TP, Shindo H, Sima AA, and Greene DA. The linked roles of nitric oxide, aldose reductase and (Na^+,K^+)-ATPase in the slowing of nerve conduction in the streptozotocin diabetic rat. *J Clin Invest* 94: 853–859, 1994.
43. Stevens MJ, Obrosova I, Cao X, Van Huysen C, and 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.
44. Subbiah MTR and Deitemeyer D. Altered synthesis of prostaglandins in platelet and aorta from spontaneously diabetic Wistar rats. *Biochem Med* 23: 231–235, 1980.
45. Tesfamariam B. Free radicals in diabetic endothelial cell dysfunction. *Free Radic Biol Med* 16: 383–391, 1994.
46. Tesfamariam B, Brown ML, Deykin D, and Cohen RA. Elevated glucose promotes generation of endothelium-derived vasoconstrictor prostanoids in rabbit aorta. *J Clin Invest* 85: 929–932, 1990.
47. Tesfamariam B, Brown ML, and Cohen RA. Elevated glucose impairs endothelium-dependent relaxation by activating protein kinase C. *J Clin Invest* 87: 1643–1648, 1991.
48. Williams CS and DuBois RN. Prostaglandin endoperoxide synthase: why two isoforms? *Am J Physiol* 270: G393–G400, 1996.
49. Wu KK. Inducible cyclooxygenase and nitric oxide synthase. *Adv Pharmacol* 33: 179–207, 1995.
50. Yasuda H, Sonobe M, Yamashita M, Terada M, Hatanaka I, Huitian Z, and Shigeta Y. Effect of prostaglandin E1 analogue TFC 612 on diabetic neuropathy in streptozocin-induced diabetic rats. Comparison with aldose reductase inhibitor ONO 2235. *Diabetes* 38: 832–838, 1989.
51. Zochodne DW and Ho LT. The influence of sulindac on experimental streptozotocin-induced diabetic neuropathy. *Can J Neurol Sci* 21: 194–202, 1994.

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3. Rodica Pop-Busui, Anders Sima, Martin Stevens. 2006. Diabetic neuropathy and oxidative stress. *Diabetes/Metabolism Research and Reviews* **22**:4, 257-273. [CrossRef]
4. Martin J. Stevens . 2005. Redox-Based Mechanisms in DiabetesRedox-Based Mechanisms in Diabetes. *Antioxidants & Redox Signaling* **7**:11-12, 1483-1485. [Citation] [PDF] [PDF Plus]