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Cyclooxygenase (COX) -2 Activation and Diabetic Neuropathy

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Effect of Cyclooxygenase (COX)-2 Activation on Diabetic Neuropathy

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INTRODUCTION

Currently more than 21 million Americans, or 9.6% of people 20 yr of age and older, have diabetes and the incidence is increasing by 5% per year (www.diabetes.org; www.cdc.gov). Diabetic neuropathy is the most common complication of diabetes and occurs in both type 1 and type 2 diabetes. A generally accepted overall prevalence rate is 50%, although estimates vary from 10 to 100% depending on the diagnostic criteria used (Feldman et al., 2005; Pop-Busui et al., 2007; Stevens et al., 2002; Thomas and Tomlinson, 1993).

Diabetic peripheral neuropathy (DPN) is the leading cause of non-traumatic lower limb amputations and diabetes-related hospital admissions, while diabetic autonomic neuropathy significantly increases cardiovascular morbidity and mortality (1995; 1998a; DCCT, 1993). Additionally, DPN impacts the affected individual’s work capacity and overall quality of life (Padua et al., 2005; Vileikyte et al., 2005). The estimated annual cost for the care of DPN patients in the USA is up to $22 billion (Gordois et al., 2003)(www.diabetes.org).

Cardiac autonomic neuropathy (CAN) is also a serious complication is associated with increased cardiovascular (CVD) risk and mortality (Navarro et al., 1991; Rathmann et al., 1993; Sampson et al., 1990), particularly in patients with sympathetic CAN. The loss of heart rate (HR) variability, an index of CAN, is an independent predictor of mortality after an acute myocardial infarction (Bigger et al., 1992; Kleiger et al., 1987). Loss of HR variability has been demonstrated in diabetic populations (Ziegler et al., 1992) and is associated with increased mortality (Vinik et al., 2003). It also has been shown that diabetic subjects with alterations of cardiac sympathetic innervation, tone
and/or responsiveness exhibit abnormal myocardial blood flow regulation (Stevens et al., 1998b), which may increase mortality associated with myocardial ischemia. In this respect, increased systemic oxidative stress was linked to early alterations in cardiac sympathetic tone and responsiveness in subjects with type 1 diabetes which in concert with impaired perfusion may contribute to the eventual development of myocardial injury (Pennathur and Heinecke, 2004; Pop-Busui et al., 2004).

In spite of the enormous health care burden resulting from DPN, an effective treatment, other than tight glycemic control, is not yet available. This is due to the complexity of the mechanisms involved in its pathogenesis such as increased production of reactive oxygen species by mitochondrial and non-mitochondrial mechanisms, non-enzymatic glycation, increased glucose flux through the polyol pathway, PKC activation, and neurovascular deficits (Brownlee, 2001; Ho et al., 2006; King et al., 1997; Newsholme et al., 2007; Song et al., 2003; Yagihashi et al., 2001). Glucose-mediated upregulation of cyclooxygenase (COX)-2 pathway activity also has been implicated in the pathogenesis of DPN. COX is the rate-limiting enzyme in the production of prostaglandins (PGs), catalyzing the conversion of arachidonic acid to PGG2 and subsequently to PGH2. Two isoforms of COX, encoded by distinct genes, have been isolated in mammalian cells (Williams and DuBois, 1996). Cyclooxygenase-1 is constitutively expressed in most tissues and is involved in maintenance of cellular homeostasis, including regulation of vascular tone (Williams and DuBois, 1996). In contrast, under normal conditions, COX-2 is expressed at low or undetectable levels but is readily up regulated by inflammatory, mitogenic, physical stimuli (Herschman et al., 1997; Wu, 1995), and oxidative stress (Feng et al., 1995), and in several disease states
such as mechanical injury (Broom et al., 2004; Dudhgaonkar et al., 2007) including carpal tunnel syndrome (Talmor et al., 2003), Guillain-Barre syndrome (Miyamoto et al., 2002), chronic inflammatory demyelinating polyradiculoneuropathy (Kawasaki et al., 2001), and vasculitic neuropathy (Hu et al., 2003).

Upregulation of COX-2 leads to impaired production and function of PGs and subsequent activation of downstream inflammatory reactions. Various pathways linking hyperglycemia to COX-2 activation have been described, including increased reactive oxygen species (ROS) generation through both mitochondrial and non-mitochondrial sources (Cosentino et al., 2003; Kiritoshi et al., 2003; Sies, 1997b), activation of the receptor for advanced glycated end-products (RAGE), increased PKC activation, and increased flux through the aldose reductase (AR) pathway have been described. In addition, data from animal studies and from epidemiologic investigation have demonstrated that prostaglandins contribute to the maintenance of cardiovascular tone and function. These data provide support for the hypothesis that COX-2 activation in diabetes plays an important role in the pathogenesis of diabetic peripheral neuropathy and myocardial dysfunction.
Diabetic Neuropathy

Peripheral Neuropathy

The overall accepted classification of DPN includes distal symmetric sensorimotor polyneuropathy, autonomic neuropathy, and focal and multifocal neuropathies. Diabetic peripheral neuropathy is the leading cause of non-traumatic lower limb amputations and diabetes-related hospital admissions, while diabetic autonomic neuropathy significantly increases cardiovascular mortality (Airaksinen, 2001; Ambepityia et al., 1990). In addition, DPN impacts the affected individual’s work capacity and overall quality of life (Padua et al., 2005; Vileikyte et al., 2005).

The hallmark presentation of DPN is a progressive damage and loss of the large and small myelinated and unmyelinated nerve fibers, which accounts for a broad spectrum of sensory deficits and symptoms in a typical “stocking and glove” distribution. These symptoms can vary in intensity and can present as numbness, sensory loss, gait ataxia, weakness, and pain. Sensory loss leads to painless injuries which are often complicated by infection, leading to amputations (Little et al., 2007). Pain associated with DPN is described as sharp, stabbing, burning, or aching. Painful diabetic neuropathy is present in approximately 30% of patients with DPN (Quattrini et al., 2007).

Diabetic peripheral neuropathy usually presents symmetrically and is usually length-dependent (i.e. longer nerves in the body are affected first). The latter has led many to postulate metabolic and structural alterations affecting that portion of the nerve most distal to the cell body and therefore at risk; examples include distal symmetrical and autonomic neuropathies.
Early reports of nerve pathology in diabetes were controversial, some citing an underlying neuritis and others emphasizing lesions in the spinal cord. Eventually, after extensive histological studies of autopsy and nerve biopsy material from patients with diabetic neuropathy, a consensus was reached that the hallmark pathologic findings in diabetic neuropathy involve peripheral nerve axons and consist of progressive damage to and loss of large and small myelinated and unmyelinated nerve fibers. The most commonly described lesions are: Wallerian degeneration; segmental and paranodal demyelination; and proliferation of endoneurial connective tissue, including thickening and reduplication of the basement membranes of nerve fibers, endoneurial blood vessels, and the perineurium, and platelet aggregation resulting in vessel occlusion (Feldman et al., 2001; Greene et al., 1990; Sima and Chakrabarti, 1992; Stevens et al., 2002). The proximal-to-distal increase in morphologic abnormalities, and the topographic and temporal distribution of neurological signs, and symptoms in the distal symmetric polyneuropathy of diabetes, suggests a primary axonopathy preferentially involving longer myelinated axons. Axonal loss may progress to the dorsal spinal roots. If dorsal root ganglion cells are affected, secondary degeneration of the posterior column of the spinal cord may also occur.

In spite of the enormous health care burden resulting from DPN, an effective treatment, other than tight glycemic control, is not yet available. A potential reason is the complexity of the mechanisms involved in its pathogenesis such as: increased production of reactive oxygen species by mitochondrial and non-mitochondrial mechanisms, non-enzymatic glycation, increased glucose flux through the polyol pathway, PKC activation, and neurovascular deficits (Brownlee, 2001; Ho et al., 2006; King et al., 1997;
Newsholme et al., 2007; Song et al., 2003; Yagihashi et al., 2001) as shown in Figure 1. Glucose-mediated upregulation in cyclooxygenase (COX)-2 pathway activity, with subsequent impaired production and function of derived prostaglandins (PGs) and activation of downstream inflammatory reactions, also has been implicated in the pathogenesis of DPN.

Figure 1 Proposed Paradigm Linking Glucose to Peripheral Nerve Dysfunction

Hyperglycemia can induce nerve dysfunction through a variety of pathways. These include increased flux through the AR pathway, increased production of AGE, increased PKC activity, increased PARP activity, increased nuclear factor-kappa B (NF-kB) activity, and increased COX-2 activity, as well as through other pathways. One mechanism common to a majority of the pathways is increased oxidative stress.
Cardiac Autonomic Neuropathy (CAN)

Diabetic autonomic neuropathy, although greatly overlooked, may affect virtually any sympathetic or parasympathetic function (1995; 1998b; DCCT, 1993). Cardiac autonomic neuropathy is the most prominent focus of autonomic dysfunction because of the life-threatening consequences of this complication, such as sudden cardiac death and silent myocardial ischemia. It is well established that patients with diabetes die of CVD at rates two to four times higher than non-diabetic populations of similar demographic characteristics (Baynes and Thorpe, 2000). Such an increased CVD risk in diabetic patients is independent of the presence of any other traditional associated CVD risk factors (Haffner et al., 1998). Longitudinal studies of CAN subjects have shown 5 yr mortality rates between 16-50% (Ewing et al., 1980; Navarro et al., 1991; O'Brien et al., 1991; Page and Watkins, 1977; Rathmann et al., 1993; Sampson et al., 1990), with particularly high mortality rates in patients with sympathetic CAN. In addition, loss of HR variability is an independent predictor of mortality after an acute myocardial infarction (Bigger et al., 1992; Kleiger et al., 1987). It has been shown that diabetic subjects with alterations of cardiac sympathetic innervation, tone and/or responsiveness (Stevens et al., 1998b) exhibit abnormal myocardial blood flow regulation (Stevens et al., 1998b), which may increase mortality associated with myocardial ischemia.

There is also ample evidence demonstrating that diabetes is able to induce cardiomyopathy and myocardial dysfunction before detectable atherosclerotic plaques (Korte et al., 2005). Alterations of diastolic (Fang et al., 2003; Francis, 2001; Frustaci et al., 2000) and systolic (Vered et al., 1984) function are widely reported in healthy diabetic subjects and often predate the development of other chronic diabetic
complications, suggesting that elevated glucose and the associated downstream effects are sufficient to induce cardiomyopathy (Fang et al., 2003; Korte et al., 2005).

The contribution of hyperglycemia to the pathogenesis of microvascular complications both in type 1 (DCCT, 1993; Low et al., 1997b) and most recently in type 2 diabetic subjects (1995) is now beyond dispute. However, the mechanisms underlying CAN development and its links to increased cardiac risk are complex and still poorly understood.

Oxidative stress and chronic inflammation play an important role in the progression of diabetic complications including CAN and peripheral neuropathy (Baynes and Thorpe, 2000; Pop-Busui, 2005; Stevens et al., 1998a). It has been demonstrated that elevations of inflammation-sensitive plasma proteins are linked with increase development of cardiovascular events (Lobbes et al., 2005; Ridker et al., 2001). It also has recently been demonstrated that systemic oxidative stress is increased and cardiac sympathetic tone and responsiveness is altered early in the course of type 1 diabetes (Pop-Busui et al., 2004).

In addition, it has been shown that hyperglycemia-induced increased oxidative stress and subsequent altered myocardial substrate metabolism can lead to myocardial ischemia and myocardial apoptosis (Robertson et al., 2004). In the vascular endothelium, hyperglycemia and oxidative stress can promote apoptosis (Baumgartner-Parzer et al., 1995; Wu et al., 1999) and impair the action of vasodilatory agents (Tesfamariam and Cohen, 1992; Ward et al., 1989) which could further contribute the development to microangiopathy and cardiomyopathy (Delanty and Dichter, 1998). An enhanced COX-2 expression also has been involved in ischemia-induced apoptosis (Matsuoka et al., 1999).
In addition, regional cardiac sympathetic hyperactivity is associated with malignant arrhythmogenesis and cardiac death in humans and animals particularly when accompanied by reduced parasympathetic tone and myocardial ischemia (Lown and Verrier, 1976; Schwartz et al., 1987; Willich et al., 1993). Elevations of norepinephrine in the heart, reflecting sympathetic hyperactivity (Felten et al., 1982; Paulson and Light, 1981) may increase mitochondrial ROS production (Givertz et al., 2001; Scacco et al., 2000) and calcium-dependent apoptosis (Communal et al., 1998; Iwai-Kanai et al., 1999). In addition ROS-induced activation of the apoptosis pathway (Patel and Gores, 1997; Shimizu et al., 1998; Verhaegen et al., 1995), has been proposed as an alternative pathogenic mechanism for the autonomic neurons dysfunction in diabetes (Luo et al., 1998; Park et al., 1998; Russell and Feldman, 1999; Russell et al., 1999; Srinivasan et al., 2000). Since the etiology of diabetic complications, including CAN and cardiomyopathy, is considered to have a vascular component, prevention of oxidative stress and inflammation in the vasculature might be an important defense against their development.

Oxidative Stress

There is convincing experimental and clinical evidence that the generation of ROS is increased in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress. Hyperglycemia is reported to induce oxidative stress through multiple pathways such as: redox imbalance secondary to enhanced AR activity (Yagihashi et al., 2001), increased production of advanced glycation end products (AGE) (Brownlee et al., 1988), increase in PKC, especially the β-isoform (Cameron et al., 1999), prostanoid imbalances (Kellogg and Pop-Busui, 2005; Kellogg et al., 2007; Pop-Busui et al., 2002), and mitochondrial overproduction of superoxide (Brownlee, 2003; Nishikawa
et al., 2000). All these pathways converge in producing oxidative stress. This has been
documented in the peripheral nerve (Cameron et al., 1999; Obrosova et al., 1998; Song et
al., 2003; Stevens et al., 2000), the dorsal root and sympathetic ganglia (Low et al.,
1997b) and the vasculature (Coppey et al., 2003) of the peripheral nervous system,
therefore contributing to nerve blood flow and conduction deficits, impaired neurotrophic
support, changes in signal transduction and metabolism, and to the morphological
abnormalities characteristic of DPN.

Oxidative stress in essence refers to the situation of a serious imbalance between the
production of free radicals and the antioxidant defense mechanisms, leading to potential
tissue damage (Halliwell, 1995). The term “free radical species” summarizes a variety of
highly reactive molecules that can be divided into different categories, e.g. oxygen ROS,
reactive nitrogen species (RNS) and reactive chlorine species (RCS). The most
prominent members of such categories include superoxide (O$_2^-$), hydroxyl radical (OH$^-$),
peroxyl radical (ROO$^-$) in the ROS group, and nitric oxide NO$^-$ in the RNS group, and are
summarized in Table I.
Table I. Various Reactive Species generated from Oxygen, Nitrogen, and Chlorine Including Both Radicals and Non-radicals.

<table>
<thead>
<tr>
<th>Reactive Oxygen Species (ROS)</th>
<th>Reactive Nitrogen Species (RNS)</th>
<th>Reactive Chlorine Species (RCS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Radicals</strong></td>
<td><strong>Radicals</strong></td>
<td><strong>Radicals</strong></td>
</tr>
<tr>
<td>Superoxide, O$_2^-$</td>
<td>Nitric Oxide (nitrogen monoxide), NO</td>
<td>Atomic Chlorine, Cl$^-$</td>
</tr>
<tr>
<td>Hydroxyl, OH$^-$</td>
<td>Nitrogen dioxide, NO$_2^-$</td>
<td></td>
</tr>
<tr>
<td>Peroxy, RO$_2^-$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkoxyl, RO$^-$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydperoxyl, HO$_2^-$</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Non-Radicals</strong></td>
<td><strong>Non-Radicals</strong></td>
<td><strong>Non-Radicals</strong></td>
</tr>
<tr>
<td>Hydrogen Peroxide, H$_2$O$_2$</td>
<td>Hydrochlorous Acid, HOCl</td>
<td>Hypochlorous Acid, HOCl</td>
</tr>
<tr>
<td>Hydroxyl Acid, HO$_2$</td>
<td>Hypobromous Acid, HOBr</td>
<td>Ozone, O$_3$</td>
</tr>
<tr>
<td>Peroxy, RO$_2^-$</td>
<td>Ozone, O$_3$</td>
<td>Singlet Oxygen $^1\Delta g$</td>
</tr>
<tr>
<td>Hydroperoxyl, HO$_2$-$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singlet Oxygen $^1\Delta g$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitroso (nitrogen monoxide), NO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrous Acid, HNO$_2$</td>
<td>Nitrogen (nitryl) Cation, NO$_2^+$</td>
<td></td>
</tr>
<tr>
<td>Nitrosyl Cation, NO$^+$</td>
<td>Nitronium (nitryl) Chloride, NO$_2$Cl</td>
<td></td>
</tr>
<tr>
<td>Nitrous Acid, HNO$_2$</td>
<td>Peroxynitrite, ONOO$^-$</td>
<td></td>
</tr>
<tr>
<td>Nitroso Cation, NO$^+$</td>
<td>Peroxynitrates, ONOOH</td>
<td></td>
</tr>
<tr>
<td>Nitrosyl Anion, NO$^-$</td>
<td>Nitronium (nitryl) Cation, NO$_2^+$</td>
<td></td>
</tr>
<tr>
<td>Nitrous Acid, HNO$_2$</td>
<td>Nitronium (nitryl) Cation, NO$_2$</td>
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<tr>
<td>Nitroso Cation, NO$^+$</td>
<td>(e.g. as nitryl chloride, NO$_2$Cl)</td>
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<td>Nitronium (nitryl) Cation, NO$_2^+$</td>
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<td>Nitronium (nitryl) Cation, NO$_2$</td>
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<td>Nitroso Cation, NO$^+$</td>
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Free radicals and other reactive species are involved in many human diseases, as their increased formation often accompanies tissue injury (Bendich, 1990; Halliwell and Gutteridge, 1998; Sies, 1997a; Southorn and Powis, 1988) and there is emerging evidence that free radicals are involved in the progression of diabetes and its complications (Cohen, 1993; Jones et al., 1988; Lyons, 1991; Pieper and Gross, 1998; Tesfamariam, 1994; Tilton et al., 1997). Additionally, ROS-induced damage to proteins may also be important in vivo because it affects the function of receptors, enzymes, transport proteins, etc., and it contributes to secondary damage of other biomolecules, e.g., by inactivating antioxidant defense enzymes or repair enzymes (Aruoma et al., 1989).
Incubation of endothelial and smooth muscle cells with increasing concentrations of glucose initiates the formation of ROS (Diedrich et al., 1994; Du et al., 1999; Giardino et al., 1998; Graier et al., 1996; Rosen et al., 1998; Tesfamariam et al., 1990; Wascher et al., 1994), a significant increase being already observed at glucose concentrations as low as 10 mM (Du et al., 1999). In addition, advanced AGE have been shown to stimulate the formation of ROS by a receptor-mediated process (Mullarkey et al., 1990; Sakurai and Tsuchiya, 1998; Schmidt et al., 1994) and Wolff et al. (Wolff, 1993) has reported that the auto-oxidation of glucose leads to the formation of ROS. Recently Giardino et al. (Giardino et al., 1998) have shown that the intracellular formation of AGE and the lipid peroxidation are closely dependent processes, the inhibition of lipid peroxidation preventing the formation AGE products. Furthermore, the regeneration of glutathione is delayed in the presence of high glucose causing an impairment of the antioxidant defense (Kashiwagi et al., 1994).

Conversely, Baynes (Baynes, 1991) has presented evidence that oxidative stress may not occur early in the disease process of diabetes, but may rather be an underlying pathogenic factor in the progression of the disease.

Malondialdehyde (MDA), a degradation product of lipid hydroperoxide, 4-hydroxynonenyl and carbonyl derivatives of protein side chains, are common markers of oxidative stress in biological systems (Amici et al., 1989; Davis and Dean, 1997; Levine et al., 1990). Lipid peroxidation, has been shown to correlate closely with all diabetic complications in vivo and strongly contribute to the development of atherosclerosis (Esterbauer et al., 1992; Heinecke, 1997; Steinberg et al., 1989). Increased levels of
MDA have been reported in both experimental (Karpen et al., 1982) and human diabetes (Sato et al., 1979).

In order to avoid excess levels of free radicals, antioxidants are present in tissues to neutralize them as they are produced (Bendich, 1990; Halliwell, 1996; Halliwell et al., 1992; Sies, 1997a). Antioxidants are metabolic intermediates or substrates which protect biological tissues from free radical damage, and are able to be recycled or regenerated by biological reductants (Packer and Tritschler, 1996). A variety of compounds, including glutathione (GSH), several antioxidant defense enzymes such as: catalase, superoxide dismutase, and glutathione peroxidase, as well as various other products such as: vitamin E (RRR-\(\alpha\)-tocopherol), vitamin C (ascorbate), \(\alpha\)-lipoic acid (thioctic acid), taurine, and flavonoids have been described.

Glutathione is by far the most important antioxidant in most mammalian cells. The most significant role of GSH is as a water-soluble antioxidant since toxic lipid peroxides combine with two molecules of GSH under the control of GSH peroxidase to form an inert lipid hydroxyl group and a GSH disulfide (GSSG). In addition, GSH is involved in the maintenance of functionally important protein thiol groups in reduced form, in amino acid transport, deoxyribonucleotide synthesis, and conjugation with toxic compounds such as xenobiotics under the control of glutathione-S-transferase to promote their elimination from the cell (Hayes and McLellan, 1999; Lowndes et al., 1994). After participation in redox reactions, GSH is regenerated from GSSG by the enzyme GSSG reductase using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Many cells can synthesize GSH de novo by gamma-glutamylcysteine synthetase. However, neurons do not contain this enzyme and so require the dipeptide to be secreted.
from glial cells (Iwata-Ichikawa et al., 1999; Keelan et al., 2001; Vincent et al., 2004). Depletion of GSH in the cell and mitochondria increases their susceptibility to oxidative injury (Rizzardini et al., 2003). In contrast, loading the cell, and particularly the mitochondria, with GSH can prevent neuronal apoptosis produced by ischemia (Li et al., 2002) and excitotoxicity (Kobayashi et al., 2000).

Besides antioxidant molecules, there are antioxidant defense enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (Sies, 1993) that detoxify free radical entities in cells, tissues, and extracellular fluids.

One of the most ubiquitous of these is SOD. The three major isoforms of SOD are: cytosolic CuZn-SOD (SOD1), mitochondrial SOD (SOD2), and extracellular SOD. Extracellular SOD is similar in structure to SOD1 but is localized in the extracellular space. SOD converts $\text{O}_2^-$ to $\text{H}_2\text{O}_2$ and oxygen. Decreased expression of SOD2 leads to decreased mitochondrial GSH and increased oxidative stress (Williams et al., 1998). Complete knockout of SOD2 is lethal within days of birth due to renal dysfunction (Lebovitz et al., 1996).

Catalase is a cytosolic enzyme that converts $\text{H}_2\text{O}_2$ to water, and therefore its activity needs to be present when SOD is active to convert the $\text{H}_2\text{O}_2$ produced by SOD.

**DPN Therapies- Where Do We Currently Stand?**

Several landmark trials have shown that strict glycemic control reduces the incidence and progression of diabetic microangiopathic complications including DPN (1995; 1998b; Ohkubo et al., 1995). However, this approach alone does not completely eliminate its occurrence. Thus, the development of new drugs to prevent or delay DPN remains a high priority.
As discussed above, oxidative stress plays an important role in the pathogenesis of DPN (Cameron et al., 1994a; Coppey et al., 2000). Therefore, the use of antioxidants was considered a rational approach for DPN treatment. Many animal studies evaluating the effects of various antioxidants such as probucol, vitamin E (Kunisaki et al., 1995), vitamin C (Jariyapongskul et al., 2007), and especially alpha-lipoic acid (Cameron et al., 1994a; Low et al., 1997a; Stevens et al., 2000) in several models of experimental DPN, showed beneficial effects of anti-oxidant therapy on most indices of DPN including nerve conduction, nerve blood flow, and quantitative sensory testing (Cameron et al., 2001; Low et al., 1997a; Stevens et al., 2000). However, in humans, the results are rather disappointing, with most of these trials failing to show any effects on hard end points of nerve function and only marginal benefits on reducing neuropathic pain (Ametov et al., 2003; Ziegler et al., 2006).

Diabetes-induced aldose reductase pathway activation is another important mechanism contributing to DPN (Song et al., 2003; Yagihashi et al., 2001) and the use of aldose reductase inhibitors (ARIs) has been extensively evaluated in animal and human trials as a potential therapeutic approach for DPN. Along the line with the data reported with antioxidants, animal studies utilizing ARIs showed significant promise (Cameron et al., 1994b; Dvornik, 1987; Obrosova et al., 2002; Sima et al., 1990; Tomlinson et al., 1982). However, in humans weak efficacy and unacceptable toxic effects were demonstrated by earlier agents. Newer agents such as ranirestat and fidarestat, which are currently being tested in clinical trials in Japan and the U.S., have thus far shown some modest benefit (Bril, 2005; Hotta et al., 2001). Recently, it also has been reported that long term treatment with epalrestat (Ono Pharmaceuticals, Osaka, Japan), the only ARI
currently available commercially and approved for use in Japan, is well tolerated and can effectively delay the progression of DPN ameliorating the associated symptoms, particularly in subjects with good glycemic control and limited DPN (Hotta et al., 2006).

Acetyl-L-carnitine, through its effect on fatty acid beta-oxidation, also has emerged as a potential DPN therapeutic agent, demonstrating significant improvement in nerve conduction in animal studies (Kamiya et al., 2006; Pop-Busui et al., 2002; Stevens et al., 1996). Data in human studies are controversial with some reporting no effect (Windebank and Feldman, 2001) and more recent data reporting a beneficial effect in human DPN (Quatraro et al., 1995; Sima et al., 2005).

Studies with either nerve growth factor or PKC inhibitors were disappointing, showing unfortunately no effect on indices of peripheral nerve dysfunction in human trials (Apfel et al., 2000; Windebank and Feldman, 2001), in spite of positive effects on animal studies (Apfel et al., 1994; Ishii et al., 1996; Nakamura et al., 1999).

Control of DPN pain remains challenging. Two drugs, duloxetine and pregabalin, are currently FDA approved specifically for DPN pain (Terneus, 2007). A variety of other compounds are also used to manage neuropathic pain in diabetic patients, such as: non-steroidal anti-inflammatory drugs (NSAIDs) (Kingery, 1997), opiates (Watson and Babul, 1998), tricyclic antidepressants (Kingery, 1997), and antiepileptics (Backonja, 2001), all of which have important side effects that limit their usefulness (Backonja and Glanzman, 2003).

**Cyclooxygenase Pathway**

Cyclooxygenase, also known as prostaglandin H synthase, is the rate-limiting enzyme in PGs synthesis from arachidonic acid (Cohn and Tognoni, 2001; Herschman et
Cyclooxygenase exists as two isoforms, COX-1 and COX-2, encoded by distinct genes (Williams and DuBois, 1996). Cyclooxygenase-1 is the constitutively expressed isoform that is present in most tissues and acts as a “housekeeping” protein, responsible for maintaining cellular homeostasis, including regulation of vascular tone (Williams and DuBois, 1996). In contrast, under normal conditions, COX-2 is expressed at low or undetectable levels in most tissues and is readily upregulated by inflammatory cytokines, growth factors, tumor promoters, PKC activation, and oxidative stress (Cosentino et al., 2003; Feng et al., 1995; Harris, 2007; Herschman et al., 1997; Wu, 1995; Xu and Shu, 2007). Recently a third COX isoform, COX-3, has been identified (Chandrasekharan et al., 2002). However, its clinical relevance is still a matter of controversy (Kis et al., 2005).

Prostaglandins modulate a wide array of biological functions and exaggerated or unbalanced PGs production play essential roles in a large number of pathophysiologic conditions including inflammation. Therefore, there was an intense interest in the development of drugs targeted specifically to treat these processes. For instance, one of the largest classes of pharmaceutical agents, NSAIDs, exerts most of their biologic effects by inhibiting COX–dependent PGs production. The majority of the initially developed NSAIDSs, however, have significant side effects such as gastrointestinal ulcers, limiting their use.

It was postulated that most of the NSAIDs side effects (e.g., gastrointestinal ulceration and bleeding and platelet dysfunctions) are caused by a suppression of COX-1 activity (Hernandez-Diaz and Rodriguez, 2002; MacDonald et al., 1997), whereas inhibition of COX-2 derived PGs facilitates the anti-inflammatory, analgesic, and
antipyretic effects. This was confirmed in several *in vitro* and *in vivo* models of disease. For instance, studies in human endothelial cell cultures demonstrated that COX-2 pathway activation in response to high glucose was associated with increased thromboxane A$_2$ (TXA$_2$) production (Cosentino et al., 2003). In addition, accumulating evidence indicates that COX-2 derived prostaglandin E$_2$ (PGE$_2$) expression is increased in models of osteoarthritis, rheumatoid arthritis (Clemett and Goa, 2000), gastric and colon tumorigenesis (Mutoh et al., 2004; Oshima et al., 2006), and that the use of selective COX-2 inhibitors reduced the production of COX-2 derived PGE$_2$ (Sheng et al., 1997). Also, a COX-2 dependent imbalanced PGs response favoring PGE$_2$ has been shown to promote oxidative stress and related neuronal damage in a model of Alzheimer disease via PGE$_2$ receptor signaling (Quilley and Chen, 2003).

Thus, selective COX-2 inhibitors, known also as coxibs, have emerged within the last decade as important pharmacological tools for treatment of pain, arthritis, and inflammation. The coxibs selectively inhibit COX-2 activity and clinical evidence shows that their use is associated with a lower incidence of gastrointestinal ulcers (Marshall, 2006; Schnitzer et al., 2004). A crystal structure of the COX-2 isoform bound to a selective inhibitor is presented in Figure (2). Various selective COX-2 inhibitors and their associated COX-2 selectivity are shown in Figure (3).
Figure 2. Crystal Structure of COX-2.

Crystal structure of COX-2 bound to the selective COX-2 inhibitor SC-558.
Figure 3. Molecular Structure of Selected Coxibs.

The molecular structures of the selective COX-2 inhibitors meloxicam (Pairet et al., 1998), rofecoxib (Gierse et al., 2005), celecoxib (Gierse et al., 2005), valdecoxib (Di Nunno et al., 2004), parecoxib (Talley et al., 2000), and lumiracoxib (Esser et al., 2005) are depicted here. The associated IC$_{50}$ for each compound is included.

It has been demonstrated that COX-2 upregulation has tissue specific consequences on downstream PGs profile and its neurovascular involvement with specific emphasis on DPN will be discussed below.

**COX-2 and Diabetic Neuropathy**

In contrast with most of the peripheral tissues, in the central nervous system, COX-2 is expressed under normal conditions and contributes to fundamental brain functions, such as synaptic activity, memory consolidation, and functional hyperemia (Minghetti, 2004). The constitutive neuronal COX-2 expression should, however, be regarded as “dynamically” regulated, since it is dependent on normal synaptic activity and it is rapidly increased during seizures or ischemia (Yamagata et al., 1993) and in
response to peripheral inflammation (Ichitani et al., 1997; Kyrkanides et al., 2002).
Interestingly, in spite of the emerging evidence of a physiological role for COX-2 in brain development and function, COX-2 knockout mice show no gross abnormalities of brain anatomy. In addition, over-expression of COX-2 has been associated with neurotoxicity and is thought to be instrumental in several chronic neurodegenerative processes such as Parkinson disease (Sanchez-Pernaute et al., 2004; Teismann et al., 2003), Alzheimer disease (Nagano et al., 2004), multiple sclerosis (Bezzi et al., 1998), amyotrophic lateral sclerosis (ALS) (Yasojima et al., 2001) and Creutzfeldt-Jakob disease (Minghetti et al., 2002). It also has been shown that various selective COX-2 inhibitors are able to reach therapeutic levels in the central nervous system (Dembo et al., 2005).
Selective COX-2 inhibition prevents dopamine neuron loss in animal models of Parkinson’s disease (Sanchez-Pernaute et al., 2004) and protects motor neurons, delaying ALS onset by reducing spinal neurodegeneration in superoxide dismutase 1 transgenic mice (Drachman et al., 2002).

On the contrary, in most of the peripheral nervous system, COX-2 expression is undetectable under normal conditions, but is upregulated in several disease states such as mechanical injury (Broom et al., 2004; Dudhgaonkar et al., 2007) including carpal tunnel syndrome (Talmor et al., 2003), Guillain-Barre syndrome (Miyamoto et al., 2002), chronic inflammatory demyelinating polyradiculoneuropathy (Kawasaki et al., 2001), and vasculitic neuropathy (Hu et al., 2003). Findings from animal and human studies of peripheral nerves injuries suggest that COX-2 metabolites play a role in processes associated with Wallerian degeneration, nerve regeneration and persistent pain (Durrenberger et al., 2004). It also has been shown that COX-2 is upregulated and
enhances pain behavior in painful DPN (Freshwater et al., 2002; Ramos et al., 2007). Furthermore, selective COX-2 inhibition has been shown, by some, to be therapeutic in treating neuropathic pain in rats (Dudhgaonkar et al., 2007; Suyama et al., 2004) and in humans (Clemett and Goa, 2000; Laborde et al., 2005). It was also shown that intrathecal administration of selective COX-2 inhibitors suppressed allodynia in rats in a nerve ligation model of painful neuropathy (Zhao et al., 2000). However, other data on the efficacy of selective COX-2 inhibitors in treating neuropathic pain conflict with these reports. For instance, selective COX-2 inhibition showed no effect on hyperalgesia in tumor necrosis factor alpha (TNF-α) or chronic constriction injury induced models of neuropathic pain (Padi and Kulkarni, 2004; Schafers et al., 2004).

Our laboratory has previously demonstrated a link between hyperglycemia and COX-2 activation in the pathogenesis of experimental DPN (Pop-Busui et al., 2002) and we confirmed in several experiments that COX-2 pathway activation plays a critical role in mediating peripheral nerve dysfunction in diabetes. For instance, our laboratory has demonstrated that COX-2 expression is elevated in the peripheral nerves in experimental diabetes (Pop-Busui et al., 2002) which is consistent with data reported by other groups (Bagi et al., 2005; Guo et al., 2005). We also have demonstrated that selective COX-2 inhibition in rats (Pop-Busui et al., 2002) is protective against diabetes induced peripheral nerve dysfunction. In an early study, we reported that after 4 wk of streptozotocin induced diabetes, rats treated with the selective COX-2 inhibitor meloxicam were protected against diabetes induced nerve conduction slowing and against diabetes-induced total and endoneurial blood flow deficits (Pop-Busui et al., 2002). In contrast, the non-selective COX inhibitor flurbiprofen had no beneficial effects on nerve
conduction or nerve blood flow in diabetic rats and induced significant deterioration in nerve conduction velocity in non-diabetic animals (Pop-Busui et al., 2002).

Vascular mechanisms associated with either PG imbalance and/or reduced nitric oxide (NO) availability (Cosentino et al., 2003) with effects on endoneurial perfusion could also be involved. For instance, human aortic endothelial cells exposed to high glucose demonstrate an increased COX-2 expression, with subsequent increase in the TXA2 and a decrease in the PGI2 levels (Cosentino et al., 2003). These findings are line with our observation in the peripheral nerves and might explain the observed increased response to vasoconstriction signals and decreased response to vasodilatation signals (Choudhary et al., 2007). This is possibly linked to an altered PG profile in diabetes that favors vasoconstriction, explaining the decrease in the endoneurial blood flow induced by diabetes as well as the beneficial effects observed with the selective COX-2 inhibition in preventing these deficits (Pop-Busui et al., 2002).

However, a reduced bioavailability of NO, due to its rapid inactivation by excess superoxide radical (Laight et al., 2000; Moncada et al., 1991; Nishikawa et al., 2000) with impaired endothelium-dependent vasodilatation and early nerve dysfunction, could also be involved. In this respect, a direct beneficial NO-mediated vascular effect of the COX-2 selective inhibition is supported by recent findings that reported an increased coronary vasodilatation and coronary blood flow with a selective COX-2 inhibitor, celecoxib, which were mediated through the NO/cyclic guanidine monophosphate signaling pathway (Klein et al., 2007). However, other selective COX-2 inhibitors, such as rofecoxib, parecoxib or lumiracoxib, had a completely different vascular action, indicating molecule-specific properties on vascular targets (Klein et al., 2007).
Cyclooxygenase-2 pathway activation also stimulates a peroxidase-dependent conversion of prostaglandin G₂ to prostaglandin H₂, resulting in a further increase in superoxide production, subsequent lipid peroxidation, and protein nitrosylation (Consilvio et al., 2004). In diabetes, an increased superoxide production-NO interaction induces increased formation of the highly reactive oxidant, peroxynitrite (Chen et al., 2006), resulting in nitrosative stress (Cosenzi et al., 2002; Onozato et al., 2002). Nitrosative stress has been shown to induce detrimental down-stream consequences including protein nitration and nitrosylation (Drel et al., 2007), mitochondrial dysfunction (Escames et al., 2007), poly(ADP-ribose) polymerase (Garcia Soriano et al., 2001) and mitogen-activated protein kinases activation, leading to induction of COX-2, inducible nitric oxide synthase, cell adhesion molecules and various inflammatory mediators (Mabley and Soriano, 2005). All these pathways have direct relevance to diabetic vascular and peripheral nerve dysfunction. On the other hand, recent evidence suggest that the pharmacological manipulation of the COX-2 metabolites also may prevent the accumulation of oxidative/nitrosative mediators in various tissues exposed to pathological conditions similar to diabetes (Garcia-Bueno et al., 2005), which could therefore imply a bidirectional feed-back loop between nitrosative/oxidative stress and COX-2 activation.

In addition, COX-2 has a nuclear factor-kappa B (NF-kB) binding site in its promoter region and the NF-kB pathway directly modulates cellular inflammatory processes within the nervous system (Consilvio et al., 2004). It has been shown that while PGE₂ upregulates NF-kB activity, which subsequently increases inflammation, other PGs such as prostaglandin D₂ and prostaglandin J₂ suppress NF-kB activation and
decrease inflammation (Consilvio et al., 2004). Nuclear factor-kappa B can be further activated by increased mitochondrial superoxide (Brownlee, 2001), non-mitochondrial reactive oxygen species production (Newsholme et al., 2007), TNF-α upregulation (Jobin et al., 1998; Moller, 2000), and receptor for advanced glycated end-products (RAGE) signaling in diabetes (Cipollone et al., 2003; Cosentino et al., 2003). Our data indicate that the NF-kB derived TNF-α (Consilvio et al., 2004) is elevated by diabetes in the peripheral nerves and that either COX-2 gene inactivation or pharmacological COX-2 blockade prevents this increase. Therefore, in diabetes, COX-2 derived PGE₂ in concert with increased oxidative/nitrosative stress and RAGE activation may create a positive feedback loop that results in NF-kB activation and subsequent inflammation.

Other molecular possibilities also may explain different cellular effects involving the interaction between PGs, inflammation, and COX-2 in the diabetic peripheral nerves. For instance, studies in monocyte cell cultures from diabetic and nondiabetic patients described elevated RAGE expression in the vasculature of diabetic patients that was associated with increased NF-kB and COX-2 expression and subsequent increased PGE₂ production (Cipollone et al., 2003). In addition, selective COX-2 inhibition was shown to prevent the increased RAGE expression in experimental models associated with increased inflammation (Yamamoto et al., 2006). Such data suggest a complex interplay between COX-2 and RAGE signaling pathways (Cipollone et al., 2003) with associated downstream effects and a possible vicious cycle linking the increased expression of both RAGE and COX-2 in response to hyperglycemia.

Direct interactions between hyperglycemia-induced PKC or AR activation, both important mechanisms in inducing peripheral nerve dysfunction, and COX-2
upregulation also were described (Cosentino et al., 2003; Ramos et al., 2007). For instance, several studies have reported that an increased AR flux mediates downstream COX-2 activation which can be prevented by AR inhibitors in models of experimental diabetes and human colon cancer (Pladzyk et al., 2006; Ramos et al., 2007; Tammali et al., 2007). However, other reports described that selective COX-2 inhibition reduced AR expression and prevented AR-metabolites accumulation in renal inner medulla cells (Moeckel et al., 2003), which suggests that pharmacological manipulation of the COX-2 pathway appears to modulate AR gene expression and downstream metabolites generation as well.

All this evidence makes a further case for the broad spectrum of the molecular targets associated with diabetic microvascular complications in general, and neuropathy in particular, that can be modulated by COX-2 inactivation. A proposed model linking hyperglycemia to COX-2 activation in inducing peripheral nerve dysfunction in diabetes is shown in Figure 4.
Hyperglycemia activates COX-2 expression through increased auto-oxidation and activation of aldose reductase (AR) pathway with secondary NADPH and NAD$^+$ depletion, increased protein kinase C (PKC) activity, activation of receptor for advanced glycated end-products (RAGE) by AGE, and increased mitochondrial and non-mitochondrial reactive oxygen species production. Increased reactive oxygen species, via modulation of nuclear factor-kappa B (NF-kB), and PKC signaling, induce COX-2 expression in hyperglycemia.

Cyclooxygenase-2 pathway metabolites also have been directly linked to the development of painful diabetic neuropathy (Freshwater et al., 2002; Matsunaga et al., 2007). Freshwater et al. reported increased COX-2 expression and activity, as measured...
by PGE₂ content, in the spinal cords of diabetic rats as compared to controls (Freshwater et al., 2002). The increased COX-2 expression was associated with enhanced pain behavior and hyperalgesia in this model and the use of a selective COX-2 inhibitor reduced markers of hyperalgesia in a dose-dependent manner (Freshwater et al., 2002; Ramos et al., 2007). Furthermore, selective COX-2 inhibition has been found to be therapeutic in treating allodynia and hyperalgesia in rats (Dudhgaonkar et al., 2007; Suyama et al., 2004) and treating neuropathic pain in humans (Clemett and Goa, 2000; Laborde et al., 2005). Intrathecal administration of selective COX-2 inhibitors suppressed allodynia in rats in a nerve ligation model of painful neuropathy (Zhao et al., 2000). Additionally, intrathecal injection of selective COX-2, but not COX-1 or COX-3, inhibitors prevents mechanical hyperalgesia in streptozotocin-induced diabetes in rats (Matsunaga et al., 2007). However, other data on the efficacy of selective COX-2 inhibitors in treating neuropathic pain conflict with these reports. For instance, selective COX-2 inhibition showed no effect on hyperalgesia in models of neuropathic pain induced by TNF-α or chronic constriction injury (Padi and Kulkarni, 2004; Schafers et al., 2004).

The mechanisms by which increased COX-2 activity leads to pain in diabetic neuropathy remain unknown. Since it was described that COX-2 expression can be induced by interleukins in DRG neurons (Inoue et al., 1999; Vanegas, 2002), one can speculate that this interleukin-dependent pathway could increase the expression of substance P in DRG neurons and thus induce pain (Inoue et al., 1999).

Therefore, since pain is one of the dominant symptoms associated with human diabetic neuropathy, with high morbidity and direct impact on quality of life, a successful
pharmacological manipulation of the COX-2 pathway could also represent a potential beneficial treatment option for painful diabetic neuropathy.

**Safety Concerns Regarding the Use of COX-2 Inhibitors**

Evidence from animal and clinical trials showed that, compared with traditional NSAIDs, COX-2 inhibitors are associated with a reduced rate of serious gastrointestinal events such as bleeding, perforation, obstruction, and nuisance symptoms such as dyspepsia, and with a reduced requirement for concomitant gastroprotective therapies. However, recent concerns about the cardiovascular and renal safety of selective COX-2 inhibitors have questioned and limited their use. For instance, the Vioxx Gastrointestinal Outcomes Research (VIGOR) study reported a five-fold increase in myocardial infarction with rofecoxib as compared with naproxen among participants with rheumatoid arthritis (Bombardier et al., 2000), although initially it was thought that this difference might have occurred, at least in part, because high dose naproxen inhibits platelet aggregation (Bombardier et al., 2000). However, a later study, the Adenomatous Polyp Prevention on Vioxx (APPROVe), which was the first relatively large trial comparing a selective COX-2 inhibitor with placebo on the reoccurrence of colon polyps, indicated that rofecoxib increased the risk of cardiovascular events by about two-fold compared to placebo (Bresalier et al., 2005). These findings eventually resulted in rofecoxib being withdrawn from the market and prompted scientists to review the cardiovascular-safety results of similar trials with other COX-2 inhibitors.

Numerous meta-analyses have been performed to evaluate the risk of cardiovascular events associated with all COX-2 inhibitors. The results of these meta-analyses have reported significant differences in the cardiovascular outcomes with
various agents within this class (Bombardier et al., 2000; Levesque et al., 2005; Mukherjee et al., 2001; Pratico and Dogne, 2005; Solomon et al., 2005). For instance, results from a recent systematic review of controlled observational studies with COX-2 inhibitors and nonselective NSAIDs, confirmed the elevated risk with rofecoxib, indicating that it is dose-related (McGettigan and Henry, 2006). It also showed that in doses of 200 mg/d, celecoxib was not associated with an increased risk, although an increased risk with higher doses was not excluded (McGettigan and Henry, 2006). Of the other nonselective NSAIDs, the highest risk was seen with diclofenac (McGettigan and Henry, 2006). Another recent large meta-analysis including patient’s databases derived from studies in osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, low back pain, and Alzheimer’s disease analyzed the incidence of cardiovascular events in patients treated with celecoxib, placebo, or nonselective NSAIDs using defined Antiplatelet Trialists’ Collaboration end points of nonfatal myocardial infarction, nonfatal stroke, and CV death. This meta-analysis of 7,462 patients exposed to celecoxib 200 to 800 mg/day for 1,268 patient-years compared with 4,057 patients treated with placebo for 585 patient-years, failed to demonstrate an increased cardiovascular risk with celecoxib compared with placebo or with other nonselective NSAIDs (White et al., 2007). No increase in myocardial infarction was apparent, even in patients not taking aspirin who were candidates for secondary prophylaxis for myocardial infarction. Overall, in clinical trials involving > 41,000 patients, no significant increase in CV events (nonfatal myocardial infarction, nonfatal stroke, and CV death) was found when patients treated with the COX-2 selective inhibitor celecoxib were compared with patients who received nonselective NSAIDs or placebo for up to 1 yr (White et al., 2003). Studies comparing
the renal safety of COX-2 inhibitors suggest similar effects on renal function with all NSAIDs when used at comparable doses. In addition, a recent trial specifically designed to explore cardiovascular risk outcomes in subjects with type 2 diabetes randomized to either 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 wk (Sowers et al., 2005).

Thus, these significant differences in cardiovascular outcomes reported with various COX-2 inhibitors suggest a drug and not a class specific effect.

Nevertheless, another interesting possibility which might also explain the different relative risk of cardiovascular outcomes seen with the selective COX-2 inhibitors is the potential interaction between their use and specific COX-2 gene polymorphisms associated with increased or decreased CV risk. For instance it is reported that increased COX-2 activity is associated with an increased production of macrophage metalloproteinases (MMPs) and with increased inflammation, both critical elements in the cascade of events that results in plaque fissuring (Cipollone et al., 2001).

It has also been reported that patients with a 765G->C polymorphism in the COX-2 promoter region have significantly decreased promoter activity and decreased plasma levels of C-reactive protein (Cipollone and Patrono, 2002; Papafili et al., 2002). In addition, a recent case-control study involving 864 patients with first myocardial infarction or atherothrombotic ischemic stroke and 864 hospitalized matched controls, found that the same polymorphism in the COX-2 promoter was associated with a decreased risk of myocardial infarction and stroke due to a significantly lower COX-2 and MMPs expression in atherosclerotic plaques (Cipollone et al., 2004). Consistent with
these observations, another study reported that among patients undergoing a coronary artery bypass graft, those carrying the 765G->C polymorphism had significantly lower C-reactive protein level after the surgery compared with patients homozygous for the 765G polymorphism (Papafili et al., 2002). Therefore, further studies might identify additional polymorphisms in the COX-2 gene and its pathway that are associated with risk of myocardial infarction and stroke.
Peripheral Nerve Dysfunction in Experimental Diabetes is Mediated by Cyclooxygenase-2 and Oxidative Stress

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Running title: COX-2 and Diabetes Neuropathy

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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 EDN, using a COX-2 knockout mouse model. Here we provide evidence that COX-2 inactivation had a protective effect from 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 SNCV and MNCV deficits in EDN. These findings also suggest that COX-2 pathway seems to be an important modulator of oxidative stress in EDN.

**Key words:** Cyclooxygenase-2, oxidative stress, experimental diabetes, mouse, nerve conduction velocity, peripheral neuropathy.

**Abbreviations:** 6-keto-prostaglandin F1a (6-keto-PGF1a), Aldose Reductase (AR), body weight (BW), control (C), cyclooxygenase (COX), cyclooxygenase-2 (COX-2), diabetic (D), diabetic peripheral neuropathy (DPN), experimental diabetic neuropathy (EDN), glutathione (GSH), horseradish peroxidase (HRP), malondialdehyde plus 4-hydroxyalkenals (MDA+4He), motor nerve conduction velocity (MNCV), mouse sciatic
nerve (MSN), nerve blood flow (NBF), nerve conduction velocity (NCV), non-diabetic (ND), nuclear factor-kappa B (NF-kB), polymerase chain reaction (PCR), polyvinylidene difluoride (PVDF), prostaglandin (PG), prostaglandin E2 (PGE2), prostaglandin F2a (PGF2a), prostaglandin G2 (PGG2), prostaglandin H2 (PGH2), Protein Kinase C (PKC), reactive oxygen species (ROS), sensory nerve conduction velocity (SNCV), streptozotocin induced diabetes (STZ-D), superoxide dismutase (SOD), thromboxane B2 (TXB2)
INTRODUCTION

Diabetic peripheral neuropathy (DPN) affects most patients with diabetes and is the leading cause of non-traumatic lower limb amputations, accounting for ~85,000 amputations/year in the US. 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 in type 1 (1; 18) and most recently in type 2 diabetic subjects (2) is now beyond dispute. Although mounting evidence provides support for a microvascular basis of DPN (7; 11; 14), the pathophysiology of DPN is still quite poorly understood.

Increased glucose-induced oxidative stress (14; 24; 31), impaired protein kinase C (PKC) activity, redox imbalance secondary to enhanced aldose reductase (AR) activity, impaired nitric oxide synthesis (7; 10; 12; 13; 42) and endothelial dysfunction (19; 33), have all been identified as critical changes 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; 10; 37; 41) in the pathogenesis of experimental DPN at a neurovascular level. Some pharmacological studies of experimental DPN in the streptozotocin induced-diabetes (STZ-D) rat model attribute reduced nerve blood flow (NBF) (5; 6; 31) to reactive oxygen species (ROS) -induced vascular dysfunction (7; 11; 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, prostaglandins and aminoguanidine (8; 10;
We have recently reported (37) 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.

However, since 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 knock-out 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, Maine) and subsequently bred at our institution.

Homozygous and heterozygous (COX-2^{-/-}, COX-2^{+/-}) -deficient male mice and littermate, wild-type (+/+) (WT) male mice were rendered diabetic (STZ-D) by 5 consecutive i.p. injections of 40 mg/kg STZ (Upjohn, Kalamazoo, MI) in 0.2 ml of 10 mM citrate buffer pH 4.5. Diabetes was defined as a non-fasting plasma glucose ≥250 mg/dl in tail vein blood (One Touch II, Lifescan, Inc., Milpitas, CA) ~48 hours after the last STZ injection. Animals were subsequently randomly assigned to the experimental groups listed in Table 1, and maintained for 6 wk 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 CO₂ 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. PCR was performed using the protocol obtained from Jackson
Laboratories. Briefly, two sets of primers were used. The first consists of 5’-
CTTGGGTGGAGAGGCTATTC-3’ and 5’-AGGTGAGATGACAGGAGATC-3’. This
set amplifies a 280bp fragment of the Neo1 gene, which indicates a knocked-out gene.
The second set consists of a 5’-CACCATAAGAATCCAGTCCGG-3’ sequence and a 5’-
ACCTCTGCGATGCTCTTCC-3’ sequence. This set of primers amplifies an 857bp
fragment and indicates a wild-type gene. The cycling conditions include a 3 minute melt
at 94 °C, a cycle of 94 °C 35 sec, 64 °C 45 sec (reduced by 0.5 °C per cycle), and 72 °C
45 sec repeated 13 times, a cycle of 94 °C 35 sec, 58 °C 30 sec, and 72 °C 45 repeated 26
times, finally followed by a final extension at 72 °C for 2 min. The PCR reaction
contained 20 mM Tris-HCl, 50 mM KCl, 3 mM MgCl2, 0.2 mM dNTP, 1 uM of each
primer, 25 mU Taq, and 2 uL of DNA.
PCR products were analyzed using gel electrophoresis with ethidium bromide on a 1.5%
agarose gel (Fig. 1A).

**Western Blot Analysis**

Heart tissue from COX-2+/+, COX-2+/-, and COX-2-/- D mice and was lysed in
homogenization buffer (HEPES 5mmol/L, pH 7.9, glycerol 26%, MgCl2 1.5 mmol/L,
EDTA 0.2 mmol/L, NaCl 300 mmol/L) containing Complete Mini Protease Inhibitor
(Roche) and centrifuged at 4°C at 15,000 x g for 20 minutes three times. Protein
concentrations were measured by bicinchoninic acid protein assay kit (Pierce).
Seventy-microgram aliquots of protein extract were separated by electrophoresis on a
7.5% SDS Tris-HCl ReadyGel (Bio-Rad) and transferred to a polyvinylidene difluoride
(PVDF) 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).
After washing, membranes were incubated with the appropriate horseradish peroxidase (HRP)-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-2<sup>−/−</sup> mice as compared with COX-2<sup>+/+</sup> 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 approximately 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.

**Digital SNCV.**

SNCV measurements were done as previously described (43). Briefly, 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-hydroxynonenals (MDA + 4-HA) and glutathione (GSH).

These measurements were performed independently using commercially available kits (Oxis Research), as previously described (44). Briefly, mouse sciatic nerves were homogenized in 125 μl of 0.1 mol/l sodium-phosphate buffer, pH 6.5. 20 μl of the mouse sciatic nerve homogenate at 15% weight to buffer were 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 were 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 20,000g 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\(_2\)\(_a\) and 6-keto-PGF\(_{1\,a}\) (stable metabolite of PGI\(_2\)) were measured in the mouse sciatic nerve (MSN) by standard enzyme immunoassay (ELISA) using commercially available kits (Cayman Chemicals, Ann Arbor, MI) 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) and Sigma (St. Louis, MO). All PCR related reagents including primers were purchased from Invitrogen (Carlsbad, CA). Kits for measurements of MDA+4-HA, GSH and SOD were purchased from Oxis International (Portland, OR).

Statistical Analysis

Data are expressed as mean ± 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 which 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 (Table 2).

Body weights (BW) were similar in all experimental groups at baseline. All 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 concentration was increased 3-fold in diabetic mice compared with C. Importantly; COX-2 gene inactivation did not significantly affect glucose levels compared to wild type D mice (Table 2).

Effects of COX-2 Gene Inactivation and STZ-D on MNCV (Fig.2)

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 nerve under normal conditions. 6 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 (Fig.3)

Consistent with MNCV data, COX-2 gene inactivation did not affect SNCV in the C mice. After 6 weeks of STZ-D, SNCV was decreased by 19% in the COX-2\(^{+/+}\) mice (p < 0.01 vs. 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 vs. corresponding C mice). However, in the COX-2+/− mice the SNCV deficits induced by STZ-D were completely prevented (p=0.973 vs. respective C mice, p<0.001 vs. COX-2+/+ D mice, p< 0.05 vs. COX-2+/− D mice).

**Effects of COX-2 Gene Inactivation and STZ-D on Nerve GSH Content (Fig.4)**

Under non-diabetic condition there was no difference in the level of nerve GSH of knockout and WT mice (15.1±2 μM/g wet tissue wt vs. 14.9 ±0.8 μM/g wet tissue wt vs. 16.1 ±0.7 μM/g wet tissue wt, respectively). 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 vs. C mice, p< 0.01), whereas in D COX-2−/− mice the nerve GSH level was similar with C mice (p=0.9).

**Effects of COX-2 Gene Inactivation and STZ-D on Nerve Lipid Peroxidation Product (MDA + 4-HA) Concentration (Fig.5)**

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). COX-2 gene inactivation completely prevented this increase in D mice.

**Effects of COX-2 Gene Inactivation and STZ-D on Nerve SOD Activity (Fig.6)**

There was no difference in the nerve SOD activity in all C mice. SOD activity was significantly decreased in the nerve of D COX-2+/+ mice compared with C (p< 0.01). SOD activity tended to decrease in the COX-2+/− -D mice, but 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 (Fig.7)

There was no difference in nerve catalase activity in the C mice, irrespective of their genotype. Nerve catalase was increased 3.7 and 4.3 fold in COX-2+/- and COX-2+/+ D mice vs. 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 (Table 3).

The sciatic nerve levels of vasoconstricting prostanoids PGE$_2$, PGF$_2\alpha$, and TXB2 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. COX-2-/- -D mice did.
DISCUSSION

In this study we demonstrate for the first time that COX-2 gene inactivation has a protective effect against nerve conduction 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 COX2 -/- 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-induced diabetes (37; 38; 44). 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, since all D mice had comparable final blood glucose 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 towards 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 EDN and
that the extent of this protection appears to be dependent to the degree of COX-2 gene deficiency. We have also found that in COX-2 \(^{+/+}\), COX-2 \(^{+/-}\) -D mice the production of PGE\(_2\) and TXB\(_2\) was significantly increased and the production of 6-keto-PGF\(_{1a}\) 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 provides 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 PG by COX-2/ COX-1 pathways in D.

A proposed paradigm of the effects of diabetes on COX pathway is detailed in Fig. 8.

Prostaglandins are generated by COX from arachidonic acid. Two isoforms of the enzyme, encoded by distinct genes, have been isolated in mammalian cells (49). COX-1 is constitutively expressed in most tissues and is involved in maintenance of cellular homeostasis, including regulation of vascular tone (49). In contrast, under normal conditions, COX-2 is expressed at low or undetectable levels but is readily up regulated by inflammatory, mitogenic, physical stimuli (27; 50), and oxidative stress (23). Hyperglycemia, through increased auto-oxidation 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) and PKC activation (16). This results in nuclear factor (NF)- \(\kappa\)B activation (3; 22), COX-2 mRNA induction (16; 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\(_2\)
(45), 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 non-selective COX-inhibition could ameliorate certain specific deficits of nerve function, (36; 52), but later reports could not confirm any beneficial effect (9; 10). Consistent with these later observations, we have reported (37) that nonselective inhibition of the COX pathway with flurbiprofen in non-diabetic (ND) 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 ND 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 COX-1 pathway may play a tonic role in the regulation of nerve metabolism and the maintenance of NBF (37).

The studies described herein confirm our hypothesis that although the production of PGE$_2$ and TXA$_2$ is not unique to COX-2 pathway in normal conditions, in pro-inflammatory conditions, such as diabetes, their formation occurs mainly via COX-2. Several other reports support our findings. For instance, there are evidences 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 co-regulated and biochemically coupled with the
inducible gene product microsomal PGE2 synthase, a dominant generator of PGE2 under
certain inflammatory conditions (4). Another recent report showed that high glucose, via
PKC, induces oxidative stress and up regulation of COX-2, resulting in reduced NO
availability and an altered PG profile consistent with a higher TXB2 and a decreased
prostacyclin release (16).

In concert with several other independent groups of investigators (24; 31; 44),
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 (11; 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 (15; 16). 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 (46;
48). The results we report herein are consistent with these data. Our findings that COX-2−/
DG 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 (3; 16; 22) up-regulates COX-2 pathway. It also suggest
that in a diabetic milieu, COX-2 pathway-oxidative injury interplay is more complex,
since ROS-induced activation of COX-2 can further increase ROS production (21; 27) and closing 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 D. This confirms our hypothesis that diabetes state is a pro-inflammatory condition promoting the production of inflammatory and pro-aggregation PG. These interesting data provide new insights into the mechanisms responsible for buffering oxidative stress and prostaglandin metabolism imbalance in experimental D 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.
Bibliography


<table>
<thead>
<tr>
<th>Experimental Groups and genotypes</th>
<th>Group Designation</th>
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<tr>
<td>COX-2 +/+ Control</td>
<td>COX-2 +/+ C</td>
</tr>
<tr>
<td>COX-2 +/+ STZ-D</td>
<td>COX-2 +/+ D</td>
</tr>
<tr>
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<td>COX-2 +/- C</td>
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<tr>
<td>COX-2 -/- STZ-D</td>
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Table 1
Experimental conditions and group designations
Table 2. Effects of COX-2 gene inactivation and STZ-D on BG and BW.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>BG (mg/dl)</th>
<th>BW (g)</th>
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<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>COX-2 +/+ C</td>
<td>93±5</td>
<td>97±24</td>
</tr>
<tr>
<td>COX-2 +/+ D</td>
<td>81±4</td>
<td>329±13</td>
</tr>
<tr>
<td>COX-2 +/- C</td>
<td>88±6</td>
<td>120±7</td>
</tr>
<tr>
<td>COX-2 +/- D</td>
<td>81±4</td>
<td>318±8</td>
</tr>
<tr>
<td>COX-2 -/- C</td>
<td>74±11</td>
<td>111±8</td>
</tr>
<tr>
<td>COX-2 -/- D</td>
<td>77±8</td>
<td>327±18</td>
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Table 3. Effects of COX-2 gene inactivation and STZ-D on PG levels.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>PGF2α (pg/ mg wet wt.)</th>
<th>PGE2 (pg/ mg wet wt.)</th>
<th>Thromboxane B2 (pg/ mg wet wt.)</th>
</tr>
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<tbody>
<tr>
<td>COX-2 +/+ C</td>
<td>11.5±5.0</td>
<td>0.62±0.18</td>
<td>13.4±5.9</td>
</tr>
<tr>
<td>COX-2 +/+ D</td>
<td>34.9±11.6*</td>
<td>3.6±1.6*</td>
<td>34.0±10.2*</td>
</tr>
<tr>
<td>COX-2 +/- C</td>
<td>10.7±4.3</td>
<td>0.56±0.28</td>
<td>12.1±4.6</td>
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<tr>
<td>COX-2 +/- D</td>
<td>32.3±9.7*</td>
<td>3.17±1.2*</td>
<td>32.8±11.3*</td>
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<tr>
<td>COX-2 -/- C</td>
<td>7.6±4.7</td>
<td>0.64±0.26</td>
<td>13.2±5.9</td>
</tr>
<tr>
<td>COX-2 -/- D</td>
<td>11.1±3.6†</td>
<td>0.55±0.21†</td>
<td>15.6±1.2†</td>
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Fig. 2. Effects of COX-2 gene inactivation and STZ-D on sciatic MNCV.
Fig. 3. Effects of COX-2 gene inactivation and STZ-D on sciatic SNCV.
Fig. 4. Effects of COX-2 gene inactivation and STZ-D on GSH.
Fig. 5. Effects of COX-2 gene inactivation and STZ-D on MDA levels.
Fig. 6. Effects of COX-2 gene inactivation and STZ-D on sciatic nerve SOD activity.
Fig. 7. Effects of COX-2 gene inactivation and STZ-D on catalase activity.
Fig 8. Proposed paradigm of glucose-induced neurotoxicity
**Legends**

**Table 2.** BG was measured using tail vein puncture as described in methods. Data are expressed as mean ± SEM. * p<0.05 vs C.

**Table 3.** PG’s were measured in the mouse sciatic nerve as described in Methods after 6 weeks. Data are expressed as mean ± SEM. * p<0.05 vs C, † p<0.05 vs other D

**Fig. 1A.** PCR analysis of genomic DNA obtained from COX-2+/+ (wild-type), COX-2+/− and COX-2−/− was performed as described in Methods. This approach yielded a 280bp band which indicates a knocked-out gene and an 857bp fragment which indicates a wild-type gene.

**Fig. 1B. Western Blot Analysis of COX-2+/+, COX-2+/−, and COX-2−/−** was performed as described in Methods. The 75 kD band is approximately the same size as the COX-2 protein band.

**Fig. 2.** MNCV was measured in the mouse left sciatic nerve as described in Methods after 6 weeks. Data are expressed as mean ± SEM. * p<0.05 vs C, † p<0.05 vs other D

**Fig. 3.** SNCV was measured in the mouse left sciatic nerve as described in Methods after 6 weeks. Data are expressed as mean ± SEM. * p<0.05 vs C, † p<0.05 vs other D
Fig. 4. GSH was measured in the mouse sciatic nerve as described in Methods after 6 weeks.  
Data are expressed as mean ± SEM. * p<0.05 vs C, † p<0.05 vs other D

Fig. 5. MDA was measured in the mouse sciatic nerve as described in Methods after 6 weeks.  
Data are expressed as mean ± SEM. * p<0.05 vs C, † p<0.05 vs other D

Fig. 6. SOD was measured in the mouse sciatic nerve as described in Methods after 6 weeks.  
Data are expressed as mean ± SEM. * p<0.05 vs C, † p<0.05 vs other D

Fig. 7. Catalase was measured in the mouse sciatic nerve as described in Methods after 6 weeks.  
Data are expressed as mean ± SEM. * p<0.05 vs C, † p<0.05 vs other D

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-kB, and PKC signaling, induce COX-2 expression, which regulates the conversion of arachidonate to vasoconstricting and pro-inflammatory PG. This precipitates the imbalance in TXA2/PGI2 ratio, favoring therefore vasoconstriction and ischemia.
Reciprocally, COX-2 up regulation increases the rate of PGG2 to PGH2 conversion and ROS generation, further exacerbating oxidative stress. Increased oxidative stress and endoneurial dysfunction may result in NCV slowing.
Diabetes

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

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Running title:
Cyclooxygenase-2 and Diabetic Neuropathy

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ABSTRACT

Objective. Activation of the cyclooxygenase (COX) pathway with secondary neurovascular deficits are implicated in the pathogenesis of experimental diabetic peripheral neuropathy.

The aim of this study was to explore the interrelationships between hyperglycemia, activation of the COX-2 pathway, 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 indices of oxidative stress, prostaglandins content, markers of inflammation, and intraepidermal nerve fibers (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 as compared with nondiabetic mice. Diabetic COX-2−/− mice were protected against functional and biochemical deficits of experimental DPN and against nerve fibers 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 diabetic neuropathy.
Key words: Cyclooxygenase-2, oxidative stress, experimental diabetes, peripheral neuropathy, inflammation, mouse, rat, nerve conduction velocity, intraepidermal nerve fibers density.
Diabetic peripheral neuropathy (DPN) commonly complicates diabetes and is the leading cause of non-traumatic lower limb amputations in the U.S. (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, non-enzymatic glycation, increased glucose flux through the polyol pathway, protein kinase C (PKC) activation, and neurovascular dysfunction have all been proposed (6-10).

We have previously demonstrated a link between hyperglycemia and COX-2 activation in the pathogenesis of experimental DPN (11). We postulated that up-regulation of COX-2, by inflammatory stimuli (12; 13), PKC 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, 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 fibers loss in long term experimental diabetes. The effects of a selective COX-2 inhibitor, celecoxib (CEL) (Pfizer, Incorporated), on nerve electrophysiology, markers of oxidative stress, PG production and inflammatory cytokines were also investigated in diabetic (D) rats.
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 ELISA 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 "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<sup>tm1Jed</sup>) on a C57B6:129S7 strain were purchased from Jackson Laboratories (Bar Harbor, Maine) and subsequently bred at our institutions. COX-2<sup>−/−</sup> and littermate COX-2<sup>+/+</sup> male mice were rendered diabetic by streptozotocin (STZ-D) as previously described (16). Genotype and phenotype were confirmed through PCR and western blot as previously published (16). At least 8 animals per experimental group were maintained for 6 months for end-point measurements.

Rat experiments: Male Wistar rats (200-250g) were purchased from Charles River (Wilmington, MA). After fasting overnight, rats were rendered STZ-D as previously described (11). Experimental groups comprised of nondiabetic (ND) and D rats (≥8/group) were randomly assigned to either no treatment or treatment with the selective
COX-2 inhibitor (IC50 4.8 nM) CEL (50 mg/kg/day in drinking water) and maintained for up to 6 months.

After all electrophysiological measurements were obtained; animals were euthanized by CO2 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 (MNCV) and Digital Sensory NCV (SNCV).**

MNCV and 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 ul of 0.1 mol/l sodium-phosphate buffer, pH 6.5. Five mMol butylated-hydroxy toluene was added to each sample to prevent further lipid peroxidation. Twenty ul of homogenate at ~15% weight to buffer were used for spectrophotometric measurements of MDA performed in accordance with the manufacturer’s instructions.
GSH was measured by using a ~15% w/v homogenate using 5% meta-phosphoric acid, which prevents enzymatic GSH regeneration, as the homogenizing buffer. GSH was assessed spectrophotometrically in accordance with manufacturer’s instructions.

**PG and Inflammatory Cytokines**

Thromboxane (TX) B₂, PGE₂, 6-keto-PGF₁α (stable metabolite of prostacycline), and tumor necrosis factor-alpha (TNF-a) were measured by ELISA in the sciatic nerve using commercially available kits.

**Western Blot Analysis**

Nerve tissue from treated and untreated ND and D rats were analyzed as previously described (11). Rabbit anti-COX-1 and COX-2 antibodies 1:100 (Santa Cruz, CA) or mouse anti-GAPDH 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 (Santa Cruz, rabbit) and S-1001:50 (Santa Cruz, goat) overnight at 4°C. Slides were stained with fluorescent secondary antibodies (Donkey anti-goat 488 and Donkey anti-rabbit 594, Invitrogen, Carlsbad, CA). Cover slips were mounted with Prolong Gold mounting media, which contains the nuclear stain DAPI.

**IENF Density**

IENF density was examined in hind paw foot pads using previously reported techniques (18). Briefly, foot pads were collected from the plantar surface of the hind paws before
animal perfusion, fixed in Zamboni’s fixative (2% paraformaldehyde, 0.2% picric acid, 0.1 M sodium phosphate buffer) for 14 hours, and cryoprotected through a series of sucrose gradients and embedded in OCT. Free-floating 30 um foot pad sections were stained with anti-PGP 9.5 antibody (a pan-axonal marker) (19). Analysis of IENF density was performed using an Olympus FluoView 500 laser scanning confocal microscope. FITC fluorescence was excited with a 488 nm blue argon laser and emission was measured through a 505-525 nm barrier filter. Samples were scanned on an Olympus IX-71 inverted microscope using a 20X 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 mm.

**Biomarker Cluster Analysis**

Hierarchical clustering was performed on a subset of data evaluating biomarkers of oxidative stress and inflammation using Gene Pattern (www.broad.mit.edu/cancer/software/genepattern) specifically adapted with the support of the National Center for Integrative Biomedical Informatics (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 (SI), a measure of the variability within a cluster vs. the variability between clusters, was used to evaluate the separation between defined sample groups (20).
**Statistical Analysis**

Data are expressed as mean ± SEM. Differences among experimental groups were determined by ANOVA using SPSS and Prism3 software, and the significance of between-group differences assessed by Tukey multiple range test. Significance was defined as p≤0.05. If the variances for the variables were found to differ significantly, a logarithmic transformation was performed which corrected the unequal variances. All analyses were then performed on the transformed data.
**Results**

In ND animals COX-2 gene inactivation/COX-2 selective inhibition did not affect body weight or blood glucose levels compared to 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), six months of STZ-D significantly reduced MNCV and SNCV in D COX-2+/+ , as compared with ND (p<0.01), whereas D COX-2−/− mice were completely protected against diabetes-induced MNCV and SNCV slowing (p=0.9 and p=0.9 vs. COX-2−/− ND, p<0.01 vs. COX-2+/+ D) (Fig 1A).

As expected, in untreated rats, diabetes induced a significant slowing in both MNCV and SNCV as compared with ND rats and the deficits in both variables progressed significantly with three and six months duration of STZ-D (p<0.001 for both vs. ND rats) (Fig. 1B, C). CEL treatment prevented both MNCV and SNCV slowing in D rats at each time point without affecting either variable in ND rats (p=1.0 and 0.977 respectively vs. ND 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 six months
of STZ-D by 2.2 fold in COX-2+/+ mice (p<0.05 vs. ND mice) and by 3.1 fold in untreated D rats (p<0.01 vs. ND rats) (Fig 2 A, C). In contrast, D COX-2/- mice and CEL treated D rats exhibited similar MDA levels to ND COX-2/- mice (p=0.8) and rats (p=1.0).

GSH was significantly reduced in the sciatic nerves of D COX-2+/+ mice (p<0.01 vs. ND COX-2+/+) and untreated D rat (p<0.05 vs. ND). COX-2/- D mice preserved similar nerve GSH levels with nondiabetic mice (Fig 2 B, D) and in D rats, CEL prevented nerve GSH depletion.

Measurements of various PGs were performed in peripheral nerves to determine trends of PGs changes induced by diabetes and the effects of interventions. Under ND conditions, the production of TXB2 in the peripheral nerve was not different between COX-2/- and COX-2+/+ mice at any time point. However, ND COX-2/- mice had significantly higher nerve 6-keto-PGF1a levels as compared with ND COX-2+/+ mice (p<0.01). Sciatic nerve TXB2 was significantly increased and 6-keto-PGF1a was significantly decreased after 6 months of diabetes in COX-2+/+ mice (p<0.001 for both vs. COX-2+/+ ND) whereas D COX-2/- mice were completely protected against this imbalance in PG synthesis (p=0.8 and 0.7 respectively vs. ND COX-2/- mice) (Table 2). Likewise in rats, diabetes induced a significant increase in nerve PGE2 and TXB2 and a significant decrease in 6-keto-PGF1a levels at all time points (p<0.01 vs. ND rats). CEL protected D rats against the increase in the vasoconstrictor and the decrease in the vasodilator PGs levels (Table 2).

To further examine the link between COX-2 metabolites and inflammation in the peripheral nerve, we measured levels of NF-kB derived cytokines. Sciatic nerve TNF-
alpha levels were not different in ND COX-2−/− and COX-2+/+ mice or any ND rats. Diabetes induced a significant increase in nerve TNF-alpha levels in COX-2+/+ mice (p<0.05 vs. ND mice), and rat nerve TNF-alpha in untreated rats (p<0.01 vs. ND), whereas D COX-2−/− mice and CEL treated D rats were protected against the increase in TNF-alpha (Fig 3 A, 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 Methods. As shown in Fig. 4, cluster analysis of these biomarkers identified specific diabetes signatures and shows that COX-2−/− D mice and CEL treated D rats clusters with respective ND animals for markers of oxidative stress, PG production, and inflammation (SI =0.85 and SI=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 ND rats, regardless of treatment group. However, COX-2 protein expression was increased in the sciatic nerve of all D rats and this increase was not affected by treatment with CEL. In contrast, D had no effect of COX-1 protein expression (Fig 5A).

Immunofluorescence of mouse sciatic nerves and DRG demonstrates a diffusely increased COX-2 immunoreactivity within the axons and DRG of COX-2+/+ D compared with COX-2+/+ ND mice, as confirmed by labeling serial adjacent sections with antibody to S-100 and DAPI. No significant COX-2 immunofluorescence was found in the axons or DRG of COX-2−/− D mice (Fig 5 B, C).
COX-2 Gene Inactivation Attenuates Diabetes-Induced Nerve Fiber Loss

COX-2 gene inactivation had no effect on IENF density in ND mice. After 6 months of diabetes, IENF density was significantly decreased in COX-2$^{+/+}$ mice as compared to respective ND mice (p<0.05). In contrast, D COX-2$^{--}$ mice were completely protected against D-induced nerve fibers loss (p=1.0 vs. ND COX-2$^{--}$) (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, CEL, replicates these effects in D rats.

Several mechanisms may explain these beneficial effects of COX-2 inhibition on experimental diabetic neuropathy. 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 NO 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 D COX-2+/+ mice and D rats. However, D COX-2-/- mice or CEL-treated D 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 D COX-2\(^{+/+}\) mice and untreated D rats, whereas D COX-2\(^{-/-}\) mice and CEL-treated D 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-kB 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 up-regulated 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 5A). We
have now extended these studies to show that COX-2 up-regulation subsequently induces a progressive PG imbalance due to increased levels of vasoconstrictor TXB2 and pro-inflammatory PGE2 and decreased prostacyclin in the peripheral nerves, which was maintained during long term diabetic conditions in both our animal models (Table 2). This imbalance, in turn, may, contribute to the observed oxidative and neurovascular deficits. These findings are consistent with several other studies reporting a similar shift in the PG balance induced by diabetes (28-30). A COX-2 dependent imbalanced PG response favoring PGE2, has been shown to promote oxidative stress and related neuronal damage in a model of Alzheimer disease via PGE2 receptor signaling (31). In addition, COX-2 up-regulation stimulate a peroxidase-dependent conversion of PGG2 to PGH2, 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 other reports demonstrate a direct relationship between COX-2 and ROS production (32; 33).

Our data indicate that additional markers of inflammation, such as the NF-kB-derived TNF-alpha (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-kB binding site in its promoter region and NF-kB pathway directly modulates cellular inflammatory processes within the nervous system (32). It has been shown that while PGE2 upregulates NF-kB activity, with subsequent increasing inflammatory response, other PGs such as PGD2, and PGJ2 suppress NF-kB activation
and decrease inflammation (32). Therefore, in diabetes, COX-2 derived PGE2 in concert with oxidative stress generate a positive feed-back loop resulting in NF-kB 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 D COX-2++ mice or untreated D rats and their COX-2-/- and CEL-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 PG’s have the ability to antagonize one 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 ND
COX-2-/- mice, which had consistently significantly higher nerve 6-keto-PGF$_{1\alpha}$ levels as compared with ND wild type mice support the hypothesis that formation of the vasodilator PG occurs mainly by a mechanism independent of the COX-2 pathway. CEL treatment had no effect on COX-2 protein expression in ND or D rats, suggesting that the inhibition of the COX-2 activity is responsible for the beneficial effects observed with CEL (Fig 5A).

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 D COX-2$^{+/+}$ mice have a marked decrease in IENF density compared to their nondiabetic littermates whereas the D 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 fibers impairment. On the contrary, foot pad 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 have recently questioned the cardiovascular safety of COX-2 selective inhibitors (44-46). One of the potential mechanisms involved includes a predominant decrease in PGI2, but not TXB2, 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 either 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: a) loss of sensory nerve function assessed by electrophysiological measurements; b) increased oxidative stress and inflammation in the
peripheral nerves and c) *nerve fiber loss* measured by IENF density in footpads. Selective COX-2 inhibition may therefore be useful for preventing or delaying diabetic neuropathy.

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References


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<tr>
<th>Experimental Group</th>
<th>Body Weight (g)</th>
<th>Blood Glucose (mmol/L)</th>
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<tr>
<td></td>
<td>Start</td>
<td>End</td>
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<tr>
<td><strong>Mice</strong></td>
<td>*</td>
<td>*</td>
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<td>258±4</td>
<td>743±12</td>
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<td>D</td>
<td>260±3</td>
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<tr>
<td>D CEL</td>
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Table 2. Effects of STZ-D and COX-2 Gene Inactivation or Treatment on PG Production

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<thead>
<tr>
<th>Experimental Group</th>
<th>TXB2 (pg/mg wet wt)</th>
<th>6-keto-PGF1a (pg/mg wet wt)</th>
<th>PGE2 (pg/mg wet wt)</th>
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LEGENDS

Fig 1. Effects of STZ-D and COX-2 Gene Inactivation or Treatment on Nerve Conduction. MNCV and SNCV were measured in mice after 6 months (A) and rats after three and six months (B and C) as described in Methods. Data are expressed as Mean ± SEM. *p<0.01 vs. ND †p<0.01 vs. other D. ND: nondiabetic, D: diabetic, COX-2+/+: wild type mice; COX-2−/−: knockout mice; CEL: celecoxib.

Fig 2. Effects of STZ-D and COX-2 Gene Inactivation or Treatment on Biomarkers of Oxidative Stress. MDA and GSH were measured in mice (A, B) and rats (C, D) after 6 months as described in Methods. Data are expressed as Mean ± SEM. *p<0.01 vs. ND, †p<0.01 vs. other D. ND: nondiabetic, D: diabetic, COX-2+/+: wild type mice; COX-2−/−: knockout mice; CEL: celecoxib.

Fig 3. Effects of STZ-D and Gene Inactivation or Treatment on Inflammation Biomarkers. TNF-α was measured in mice (A) and rats (B) after 6 months as described in Methods. Data are expressed as Mean ± SEM. *p<0.01 vs. ND, †p<0.01 vs. other D. ND: nondiabetic, D: diabetic, COX-2+/+: wild type mice; COX-2−/−: knockout mice; CEL: celecoxib.

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 (Fig 4 A) or rat (Fig 4 B) sciatic nerve as described in
Methods. Each row corresponds to a marker measured and each column represents an individual sample. Genotype (Fig 4 A) and treatment group (Fig 4 B) are listed above each corresponding column. Data were normalized to a mean of zero and a standard deviation of 1. Measures above the mean are shaded in red, those below the mean are shaded in blue. The color scale indicates the standard deviation above or below the mean. ND: nondiabetic, D: diabetic, COX-2+/+: wild type mice; COX-2−/−: knockout mice; CEL: celecoxib.

Fig 5. Western Blot Analysis and Immunofluorescence 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 Methods. GAPDH was used as a loading control. COX-2 immunofluorescence of mouse sciatic nerves (B) and DRG neurons (C) was performed as described in methods. S-100 was used as a Schwann cell anatomical marker in the sciatic nerve and DAPI as a nuclear stain in DRG. For panels B and C bar = 50 um. For panel B, arrows indicate COX-2 staining in axons and arrowheads indicate Schwann cells. For panel C, arrows indicate DRG and arrowheads indicate nuclei. Images were captured with an oil immersion 60X objective. S-100 (Green), COX-2 (Red), and DAPI (blue). All images were simultaneously enhanced to increase clarity. ND: nondiabetic, D: diabetic, COX-2+/+: wild type mice; COX-2−/−: knockout mice; CEL: celecoxib.
Fig 6. Effects of STZ-D and COX-2 Gene Inactivation on IENF Density

IENF density was measured in ND and D COX-2\textsuperscript{+/+} and COX-2\textsuperscript{-/-} mice after 6 months as described in Methods. Fiber density is expressed as number of fibers per linear mm epidermis (D). Bar = 100 um. White circles indicate individual nerve fibers. Images were acquired with an oil immersion 20X objective. Data are Means ± SEM.*p<0.01 vs. ND, †p<0.01 vs. other D. ND: nondiabetic, D: diabetic, COX-2\textsuperscript{+/+}: wild type mice; COX-2\textsuperscript{-/-}: knockout mice.

Table 1. Initial and Final Body Weights and Blood Glucose Concentrations in Various Experimental Groups. Data are expressed as Mean ± SEM. *p<0.05 vs. ND. ND: nondiabetic, D: diabetic, COX-2\textsuperscript{+/+}: wild type mice; COX-2\textsuperscript{-/-}: knockout mice; CEL: celecoxib.

Table 2. Sciatic Nerve PG Production.

Sciatic nerve PG’s were measured in mice after 6 months and in rats after 3 and 6 months as described in Methods. Data are expressed as Mean ± SEM.*p<0.01 vs ND, †p<0.01 vs other D. ND: nondiabetic, D: diabetic, COX-2\textsuperscript{+/+}: wild type mice; COX-2\textsuperscript{-/-}: knockout mice; CEL: celecoxib.
Figure 1

A

B

C

NVC (m/s)

MNCV
SNCV

ND COX-2−/−
D COX-2−/−
ND COX-2+/−
D COX-2+/−

ND D ND CEL D CEL

MNCV
SNCV

ND D ND CEL D CEL

NVC (m/s)

MNCV
SNCV

ND D ND CEL D CEL

100
Figure 2

A

B

C

D

MDA (nmol/dg wet wt)

GSH (umol/mg wet wt)

MDA (nmol/mg wet wt)

GSH (umol/dg wet wt)

C

D

C CEL

D CEL
Figure 5
Figure 6

A. Western Blot

COX-1
COX-2
GAPDH

B. Mouse Sciatic Nerve

ND COX-2^{+/+}
D COX-2^{+/+}
D COX-2^{-/-}

C. Mouse DRG

ND COX-2^{+/+}
D COX-2^{+/+}
D COX-2^{-/-}
Effects of Cyclooxygenase-2 Gene Inactivation on Cardiac Autonomic and Left Ventricular Function in Experimental Diabetes.

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Running Title
Cyclooxygenase-2 and Diabetic Heart

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Word Count: 5,999
Subject Codes: 189, 130
Abstract

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Running Title

Cyclooxygenase-2 and Diabetic Heart

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Word Count: 5,992

Subject Codes: 189, 130
Abstract

Background: Glucose-mediated oxidative stress and upregulation of cyclooxygenase (COX)-2 pathway activity have been implicated in the pathogenesis of several vascular complications of diabetes including peripheral neuropathy. However, in non-diabetic subjects the cardiovascular safety of selective COX-2 inhibition is controversial.

Methods and Results: The aim of this study was to explore the links between hyperglycemia, oxidative stress, activation of the COX-2 pathway, cardiac sympathetic integrity and the development of left ventricular (LV) dysfunction in experimental diabetes. R-R interval and parameters of LV function measured by echocardiography, LV sympathetic nerve fiber density, and collagen content as well as markers of myocardial oxidative stress, inflammation, and prostaglandins content were assessed after 6 months in control and diabetic COX-2 deficient (COX-2−/−) and littermate, wild-type (COX-2+/+) mice. There were no differences in blood glucose, LV echocardiographic measures, collagen content, sympathetic nerve fiber density and markers of oxidative stress and inflammation between nondiabetic COX-2+/+ and COX-2−/− mice at baseline and thereafter. After 6 months, diabetic COX-2+/+ mice developed significant deteriorations in the R-R interval and signs of LV dysfunction. These were associated with a loss of LV sympathetic nerve fiber density, increased LV collagen, and a significant increase in myocardial oxidative stress and inflammation as compared with nondiabetic mice. Diabetic COX-2−/− mice were protected against these functional and biochemical deficits and against LV sympathetic nerve fiber loss.
Conclusions: These data suggests that in experimental diabetes, selective COX-2 inactivation confers protection against sympathetic denervation and LV dysfunction by reducing intramyocardial oxidative stress, inflammation and myocardial fibrosis.

Key Words: cyclooxygenase-2, diabetes, sympathetic function, left ventricular function
Introduction

Diabetes is a complex metabolic disorder associated with an increased risk of cardiovascular disease (CVD) events independent of the presence of any other risk factors. The increased CVD risk is mediated by a wide array of factors including: increased oxidative stress, alterations in myocardial substrate metabolism\(^1,2\), diabetes induced myocardial ischemia, and myocardial fibrosis and apoptosis\(^1\) contributing to alterations of diastolic\(^1,2\) and systolic function.

Cardiac autonomic neuropathy (CAN) is a serious complication of diabetes, which also associates with increased CVD risk and mortality\(^3\). The loss of heart rate (HR) variability -an index of CAN- is an independent predictor of mortality after an acute myocardial infarction\(^4\) or in patients with type 1 and type 2 diabetes\(^5\). It has been also shown that resting HR is another measure of cardiac autonomic function in animal models\(^6\) and in humans\(^7\). Increased systemic oxidative stress is linked to early alterations in cardiac sympathetic tone and responsiveness and with diastolic dysfunction in young subjects with type 1 diabetes. These in concert with impaired perfusion may contribute to the eventual development of myocardial injury\(^8\), abnormal myocardial blood flow regulation\(^9\), and electrical instability and ultimately increase mortality associated with myocardial ischemia.

In addition, prostaglandins (PGs), produced from arachidonic acid by the cyclooxygenase (COX) pathway, play a wide range of regulatory roles in the cardiovascular system. Two isoforms of the enzyme, COX-1 and COX-2, encoded by distinct genes and with significantly different functions but mutually interactive, have been isolated in mammalian cells\(^10\). There is ample evidence of glucose-mediated
upregulation of COX-2 pathway activity with resulting downstream inflammatory reactions and vascular dysfunction in diabetes complications-prone tissues and of the beneficial effects of COX-2 selective inhibition under these conditions \(^{11,12}\).

However, the cardiovascular safety of using selective COX-2 inhibitors has been questioned. Several meta-analyses of prospective clinical trials and observational studies performed to date have reported significant differences in the cardiovascular outcomes among the various agents within this class, suggesting a drug- and not class-specific effect \(^{13,14}\).

Therefore, the aim of this study was to determine the effects of COX-2 activation on cardiac sympathetic integrity and LV function in experimental diabetes. Thus we assessed indices of LV function as assessed by echocardiography, markers of cardiac sympathetic innervation and collagen production, oxidative stress, PGs production, and inflammation in a COX-2 gene knock-out diabetic mouse model.
Materials and Methods

Animal Model

The experiments were approved by the University of Michigan’s Committee on Use and Care of Animals. Mice heterozygous for disruption of the COX-2 gene (Ptgs2<sup>tm1Jed</sup>) on a B6;129S7 strain were purchased from Jackson Laboratories (Bar Harbor, Maine) and subsequently bred at our institution. Homozygous (COX-2<sup>−/−</sup>) deficient and littermate, wild-type (COX-2<sup>+/+</sup>) male mice were rendered diabetic (D) as previously described<sup>15</sup>. Genotype and phenotype were confirmed as previously published<sup>15</sup>. 10-14 animals/experimental group were maintained for 6 months, at which time end-point measurements were assessed. The animals were fed standard chow with ad libitum access to water.

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 myocardial malondialdehyde plus 4-Hydroxyalkenals (MDA) and glutathione (GSH) were purchased from Oxis International (Portland, OR). PGs and tumor necrosis factor-alpha (TNF-α) ELISA kits were purchased from Cayman Chemical (Ann Arbor, MI).

Echocardiography

Echocardiographic indices were obtained according to the recommendations of the American Society of Echocardiography in collaboration with the Center for Integrative Genomics at the University of Michigan(http://www.med.umich.edu/cig). Transthoracic echocardiography was performed in nondiabetic (ND) and D mice at 6 months by blinded
observer with Vivid 7 Ultrasound System. Mice were initially anesthetized using 3% isoflurane and maintained with 1% isoflurane. Wall thickness and LV dimensions were obtained from a short axis view. LV mass was calculated using the formula LV mass=1,047*[(LV in diastole+Posterior wall in diastole+Interventricular Septum in diastole)*3-(LV diastole*3)] where 1,047 is the specific gravity of muscle. Maximal early diastolic peak velocity (E) and late peak velocity (A) were derived from mitral flow. Isovolumetric relaxation time (IVRT) was derived from aortic valve closure to the onset of mitral flow. Systolic measures include fractional shortening (FS%), ejection fraction (EF%), and stroke volume (SV). R-R interval was calculated by averaging 3 consecutive R-R intervals as measured by echocardiography. Sample echocardiographic images are shown in Figure 1.

**Measurements of Myocardial Oxidative Stress**

MDA and GSH measurements were performed as previously described\(^\text{15}\).  

**Myocardial PGs and Inflammatory Cytokines**

Thromboxane (TX)B\(_2\), 6-keto-PGF\(_{1\alpha}\) (stable metabolite of prostacycline), and TNF-\(\alpha\) were measured by ELISA in the myocardium as previously described\(^\text{15}\).

**Myocardial Collagen Determination**

Total collagen content of the 0.1% picrosirius red and 0.25% Fast Green FCF stained sections was measured under bright field according to previously published methods\(^\text{16}\) and quantified by digital image analysis\(^\text{17}\). Briefly, mice were perfused with 4% paraformaldehyde, hearts were removed, cryoprotected, and frozen in Optimum Cutting Temperature compound. Tissue sections of 5 um thickness were obtained from the LV. Images were captured using a Nikon microscope using a 100X oil-immersion objective.
and SPOT software. ImageJ (http://rsb.info.nih.gov/ij/) was used to calculate the percentage area of collagen staining versus total area. For each sample, at least 10 fields were measured.

**Immunohistochemistry (IHC)**

Immunohistological staining for LV sympathetic fibers was carried out in perfused mice using Santa Cruz rabbit anti-Tyrosine Hydroxylase (TH) (1:100)\textsuperscript{18} with an Invitrogen donkey anti-rabbit 594 (1:500) fluorescent secondary. Images were captured using an Olympus IX-71 inverted microscope with 40X oil-immersion objective utilizing Fluoview Software (version 5). ImageJ was used to measure the area of the LV and calculate the number of fibers/100 um\textsuperscript{2}. 3 slides per sample were prepared and 3 random images were captured from each slide.

**Biomarker Cluster Analysis**

Hierarchical clustering was performed on a subset of data evaluating indices of LV function, biomarkers of oxidative stress and inflammation using Gene Pattern (http://www.broad.mit.edu/cancer/software/genepattern) specifically adapted with the support of the National Center for Integrative Biomedical Informatics (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 (SI), a measure of the variability within a cluster vs. the variability between clusters, was used to evaluate the separation between defined sample groups\textsuperscript{19}.

**Statistical Analysis**

Data are expressed as mean ± sem. Differences among experimental groups were determined by ANOVA, and the significance of between-group differences 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 which corrected the unequal variances. All analyses were then performed on the transformed data using Prism (version 3.00, Graphpad Inc., San Diego, CA). Pearson correlation coefficients comparing R-R, E/A, EF%, FS%, and sympathetic nerve fiber density were calculated using Prism.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.
**Results**

COX-2 gene inactivation did not affect body weight or blood glucose compared to respective wild-type mice (Table 1). As expected, diabetic mice exhibited significantly increased blood glucose (p<0.01) and decreased body weight (p<0.01) regardless of genotype.

For all echocardiography, oxidative stress, inflammation, IHC, and histology measures ND COX-2−/− mice did not differ significantly compared to ND COX-2+/+ mice. Consistent with our previous observations, in wild type mice, myocardial COX-2 protein expression was upregulated in the STZ-D mice as compared with ND mice (Fig.2E) whereas COX-2 was undetectable in the COX-2 gene knockout mice15.

**Effects of Diabetes and COX-2 Gene Inactivation on Myocardial Function and Structure**

LV systolic and diastolic functions were assessed non-invasively by transthoracic echocardiography. Six months of STZ-D, induced a significant deterioration in FS%, EF%, and SV by 39%, 25%, and 19% respectively in the COX-2+/+ mice compared to ND COX-2+/+ (p<0.01 for all; Table 2) consistent with LV systolic dysfunction. In contrast the D COX-2−/− mice preserved normal indices of systolic function after 6 months, which were similar with the ND COX-2−/− mice (p=0.5, 0.9, and 0.4 respectively). In addition, D COX-2+/+ mice presented a significant decrease in the E/A ratio (Fig.1A,B; Table 2) and a significant increase in IVRT as compared to ND COX-2+/+ mice (p<0.01 for both) consistent with diastolic dysfunction, (Table 2) whereas diastolic function was preserved in D COX-2−/− mice (p=0.6 and 0.7 respectively vs. ND COX-2−/− mice).
Since changes in the collagen composition influence the passive mechanical properties of the myocardium and thus are important for cardiac hemodynamics, we analyzed the total LV collagen content. After 6 months of STZ-D, COX-2+/+ mice demonstrated a 4-fold increase in total collagen content as compared to ND COX-2+/+ (p<0.05) (Fig. 3). In agreement with a superior contractility in the D COX-2+/+ vs. D COX-2+/+ mice as demonstrated by preserved FS% and EF%, COX-2 gene inactivation blunted diabetes-induced interstitial fibrosis as evidenced by prevention of the increased collagen staining observed in the D COX-2+/+ mice (p=0.7 vs. ND COX-2-/-) (Fig. 3D). In concordance with these data, we observed a 30% increase in LV mass/body weight ratio and 24% increase in RW in D COX-2+/+ mice as compared to ND COX-2+/+ (p<0.01 for both). These structural changes were not observed in D COX-2-/- mice (p=0.3 and 0.4 vs ND COX-2-/- respectively).

**Effects of Diabetes and COX-2 Gene Inactivation on Indices of CAN**

The presence of CAN was explored by measuring the R-R interval, as a measure of the resting HR, and LV sympathetic nerve fiber density. As shown in Table 2, after 6 months of experimental diabetes, the R-R interval was significantly decreased in D COX-2+/+ mice compared to ND COX-2+/+ (p<0.05) consistent with a higher resting HR. In contrast, D COX-2-/- mice preserved a similar resting HR with the ND COX-2-/- (p=0.8) (Fig.1C,D, Table 2). In addition, D COX-2+/+, but not D COX-2-/-, mice showed a significant 64% reduction in the LV sympathetic nerve fiber staining compared to respective control mice (p<0.01) (Fig 2D). A Pearson correlation analysis showed significant correlations between R-R interval and sympathetic nerve fiber density.
Effects of Diabetes and COX-2 Gene Inactivation on Myocardial Oxidative Stress, PGs Production, and Inflammation

Oxidative stress was assessed by measurements of myocardial MDA and GSH. As seen in Fig. 5A, MDA was increased by 5.9-fold and GSH was decreased by 5.3-fold in the D COX-2+/+ mice (Fig.5B) as compared with ND COX-2+/+ (p<0.01 for both). In contrast, D COX-2−/− mice were protected against this increase in the oxidative stress markers (Fig.5A,B).

Measurement of vasoconstricting and vasodilating PGs were performed to assess the effects of diabetes and COX-2 gene inactivation on the overall myocardial PGs content. We found that ND COX-2−/− mice demonstrated an 1.6-fold increase in the myocardial 6-keto-PGF1α as compared to ND COX-2+/+ mice (p<0.05). However, there was no difference in the myocardial TXB2 production between ND COX-2+/+ and ND COX-2−/−. After 6 months of STZ-D, we observed a 7-fold increase in the myocardial TXB2 and a 3.2-fold decrease in 6-keto-PGF1α in COX-2+/+ mice (p<0.01 vs. ND mice for both). This imbalance in the myocardial PGs production was not observed in D COX-2−/− mice (p=0.7 and p=0.8 respectively) (Fig.5C).

To further examine the effects of COX-2 inhibition and experimental diabetes on myocardial inflammation, we have also measured levels of the nuclear factor-kappa B (NF-kB) derived TNF-α. Myocardial TNF-α was significantly increased in D COX-2+/+ by 5.3-fold after 6 months (p<0.01 vs. ND COX-2+/+), whereas D COX-2−/− demonstrated similar levels of TNF-α as compared to ND COX-2−/− (p=0.6; Fig. 5D)
Lastly, we have applied methods commonly used for high-throughput gene expression analysis, specifically adapted for our analysis, to evaluate the clustering pattern in the change of several markers of LV function, oxidative stress, and inflammation induced by diabetes and/or COX-2 gene inactivation\textsuperscript{12}. Cluster analysis of these markers (Fig.6) demonstrate that D COX-2\textsuperscript{+/+} mice clustered independently of all ND mice and D COX-2\textsuperscript{-/-} mice, identifying a strong diabetes-specific phenotype pattern (SI=0.481).
Discussion

Our findings suggest that, in experimental diabetes, COX-2 pathway activation in concert with associated increased myocardial oxidative stress and inflammation is instrumental in mediating CAN and changes in LV systolic and diastolic function. We also demonstrate that COX-2 gene inactivation is protective against indices of CAN, oxidative stress, PGs imbalance, and inflammation, and prevents LV dysfunction and myocardial fibrosis in long-term experimental diabetes.

While historically it has been assumed that atherosclerotic vascular disease is responsible for all diabetes-induced deficits on the heart, more recent data obtained in both animal and human studies have shown that the increased CVD risk in diabetes is also associated with the development of a putative cardiomyopathy comprising a wide array of pathological changes in the diabetic heart. Among these myocardial interstitial and perivascular fibrosis\(^1\), myocardial apoptosis\(^1\), increased oxidative stress\(^20\), and impaired cardiac metabolism were all described\(^21\). These often predate the development of other chronic diabetic complications, suggesting that elevated glucose and the associated downstream effects are sufficient to induce LV systolic and diastolic dysfunction\(^1,2\). Impairments in myocardial function have been observed as early as 7 weeks after STZ-D in animal models\(^22\) and alterations of systolic and diastolic\(^1,2\) function are widely reported in otherwise healthy diabetic subjects.

Consistent with these reports\(^2,23\), our echocardiography data demonstrate the presence of diastolic and systolic dysfunction in the diabetic COX-2\(^{-/-}\) mice as documented by the decreased E/A ratio and EF\%. These findings correlate with increased intramyocardial collagen content and increased LV mass/body weight ratio in
the D COX-2+/+ mice, which may, at least partly be attributable to the increased interstitial fibrosis and the resulting stiffening of the LV walls since changes in the collagen content and composition influence the passive mechanical properties of the myocardium. Although there are some conflicting data regarding the changes in LV mass in response to diabetes, with some researchers reporting a decreased LV mass, or no change, our findings are in agreement with many other reports that have shown an increase in the heart mass/body weight ratio or increased myocardial collagen content associated with LV dysfunction in various rodents models of experimental diabetes.

Both alterations in the LV function and the associated structural changes were prevented in the D COX-2−/− which indicates that COX-2 inactivation is not associated with a detrimental effect on cardiac function, but is protective against cardiomyopathy in experimental diabetes which is a novel finding. This raises questions as to the possible mechanisms that may explain the beneficial effects of COX-2 inactivation. One possibility is that reduction of diabetes–induced myocardial inflammation may be involved in the anti-fibrotic effects of COX-2 inactivation, since it has been well documented that myocardial fibrosis in experimental diabetic cardiomyopathy is partly mediated by the upregulation of cytokines that have a pro-fibrotic action, including TNF-α and IL-1β and that inhibition of the TNF-α pathway in a STZ-D rat model prevented the increase myocardial fibrosis induced by diabetes. Consistent with these reports we found that COX-2 inactivation prevented the increase in myocardial TNF-α associated with diabetes.

In addition, strong evidence demonstrates that increased intramyocardial apoptosis in diabetes also contributes to the development and progression of cardiac
dysfunction\(^1\). Although we did not assess the presence of myocardial apoptosis, we have found significant increases in markers of myocardial oxidative stress in the D COX-2\(^{+/+}\) mice, as indicated by the reduced myocardial GSH and the increased lipid peroxidation, which was prevented in D COX-2\(^{-/-}\).

Increased oxidative stress has been shown to promote both myocardial apoptosis and impaired myocardial metabolism\(^20\) that contribute to LV dysfunction\(^29\). It has been also shown that anti-oxidants have beneficial effects in preventing diabetes-induced cardiomyopathy\(^27\). Additionally, since hyperglycemia and oxidative stress can promote apoptosis in the myocardial vascular endothelium\(^30\), this could further contribute to the development of microangiopathy and LV dysfunction. Therefore one can speculate that COX-2 inactivation prevented the development of LF dysfunction in this model of experimental diabetes through modulation of oxidative stress.

Our data demonstrating that COX-2 inactivation prevents the myocardial oxidative stress associated with diabetes in our model are consistent with our previous findings in diabetic peripheral nerves\(^12\). Several possibilities could explain these results. We and others have shown that COX-2 expression and pathway activity are upregulated in response to diabetes with specific downstream effects on PGs synthesis\(^15,31\). It has been also shown that in the presence of COX-2 pathway activation, the conversion of PGG\(_2\) to PGH\(_2\) is peroxidase-dependent, resulting in a further increase in superoxide production. In diabetes, an increased superoxide production induces both subsequent lipid peroxidation and protein nitrosylation\(^32\), resulting in nitrosative stress\(^33\). Nitrosative stress has been shown to induce detrimental down-stream consequences including mitochondrial dysfunction\(^34\), poly(ADP-ribose) polymerase\(^35\) and mitogen-activated
protein kinases activation, leading to induction of inducible nitric oxide synthase, cell adhesion molecules and various inflammatory mediators\(^36\). All these pathways have direct relevance to diabetic vascular tone. Therefore it is possible that blocking some of these pathways would contribute to preventing an increased oxidative stress in the diabetic heart as well.

In addition, consistent with our findings in the peripheral nerves, we found that diabetes induces a shift in the myocardial PGs production favoring the vasoconstricting TXB\(_2\) over prostacyclin. Diabetes promotes increased inflammation, as measured by the NF-kB pathway derived TNF-\(\alpha\) in the D COX-2\(^{+/+}\) mice. Since it has been also shown that the COX-2 derived PGE\(_2\) production is increased in response to diabetes\(^12\) and that increased PGE\(_2\) results in increased NF-kB activity\(^32\), we can assume that preventing these various imbalances in myocardial PGs in the diabetic COX-2\(^{-/-}\) mice could have contributed to the observed beneficial cardiovascular effect of COX-2 inactivation in experimental diabetes.

A large body of evidence has invoked CAN as an important mediator of increased CVD risk in diabetes and we provide evidence that diabetic mice developed an important deterioration in the resting HR and in LV sympathetic innervation, which were prevented in the D COX-2\(^{-/-}\) mice.

Considering the high average resting HR of mice and the difficulty in obtaining reliable data in conscious mice using non-invasive methods, we were unable to perform HR variability (HRV) analysis, considered to be the test of choice in evaluating CAN. However, we were able to measure the resting HR (as assessed by the R-R interval), which although not as sensitive as the HRV studies, can be a useful \textit{in vivo} marker of
CAN and also associates with peripheral neuropathy\textsuperscript{37}. HR is a result of the balance between parasympathetic and sympathetic tone\textsuperscript{38}, with a decreased parasympathetic tone resulting in an increased HR and decreased sympathetic tone resulting in an decreased HR\textsuperscript{39}. Diabetic wild type mice demonstrated a significantly higher resting HR as compared to ND mice and this increase was not observed in the diabetic COX-2\textsuperscript{2/-} mice.

To further assess the extent of diabetes-induced CAN, we have also used TH immunohistochemistry, which is a sensitive marker for LV sympathetic fibers. The extent of the LV sympathetic nerve fiber loss after 6 months of diabetes in the wild-type mice, is consistent with our previous observations of loss of intraepidermal nerve fibers in this model\textsuperscript{12} and it is probably mediated by the increased oxidative stress and inflammation. Due to its anatomical distribution and the relative paucity of parasympathetic nerve fibers in the LV\textsuperscript{40}, a direct evaluation of the myocardial parasympathetic nerve fibers in this study was not possible. However, since it is generally accepted that parasympathetic denervation occurs earlier than sympathetic denervation in diabetic CAN \textsuperscript{41}, we can assume that the loss of parasympathetic innervation was at least as extensive as the disruption to sympathetic integrity. This is consistent with other reports showing a significant decrease in the LV sympathetic innervation as measured by positron emission tomography with [\textsuperscript{11}C]hydroxyephedrine in long term STZ-D in rats\textsuperscript{42}.

The protection against CAN observed in the in the D COX-2\textsuperscript{2/-} mice, as documented by the preservation in the resting HR and LV sympathetic innervation could further explain the conservation of LV function, since CAN contributes to diabetes-induced LV dysfunction\textsuperscript{3}. This is also in line with our previously reported data showing
that the presence of CAN is associated with an early diastolic dysfunction in type 1 diabetic patients without CAD\textsuperscript{8}.

The favorable effects of COX-2 gene inactivation in experimental diabetes are also underlined by the clustering in the D COX-2\textsuperscript{-/-} mice and the nondiabetic mice for markers of LV function, CAN, oxidative stress, and inflammation (Fig 6).

Emerging data from several clinical trials have recently questioned the cardiovascular safety of COX-2 selective inhibitors\textsuperscript{13} and these concerns eventually resulted in certain selective COX-2 inhibitors being withdrawn from the market. One of the potential mechanisms invoked included a predominant decrease in PGI\textsubscript{2}, but not TXB\textsubscript{2}, production with an associated increase in blood pressure\textsuperscript{13}.

However, the results of numerous meta-analyses evaluating the risk of cardiovascular events associated with COX-2 inhibitors have shown significant differences in the cardiovascular outcomes among the various agents within this class\textsuperscript{13,14}. For instance, rofecoxib use was associated with a significantly increased risk of cardiovascular events in numerous trials\textsuperscript{13}, while this risk was not reproduced with celecoxib\textsuperscript{14}. A recent trial exploring cardiovascular risk markers in patients with type 2 diabetes randomized to celecoxib, rofecoxib, or naproxen, reported a significant increase in systolic blood pressure with rofecoxib while no increase was observed in patients treated with celecoxib after 12 weeks\textsuperscript{43}. Additionally, a recent study reported an increased coronary vasodilatation and coronary blood flow mediated through the nitric oxide/cyclic guanidine monophosphate signaling pathway when guinea-pig hearts were treated with celecoxib, indicating a direct beneficial nitric oxide-mediated vascular
These data demonstrate that increased cardiovascular risk may be a drug-, and not a class-, specific effect.

There are some limitations to our studies. The first is that our model utilizes a type 1 model of diabetes. Therefore, it is possible that COX-2 gene inactivation may not provide the same protection in a model of type 2 diabetes. However, it has recently been reported that COX-2 is also upregulated in type 2 diabetes with similar metabolic and inflammatory changes as observed in type 1 diabetes. Therefore it seems reasonable to speculate that inhibition of COX-2 may also prevent LV dysfunction in type 2 diabetes. Another limitation is that we utilized a prevention model, as COX-2 gene inactivation was present from birth in our mice. It is possible therefore that an intervention after the induction of diabetes or the development of LV dysfunction may not provide the same beneficial effect. Finally, although we did not measure the blood pressure in our mice, studies have shown no significant change in blood pressure in mice in response to diabetes for up to 6 months, therefore it is unlikely that the observed changes in LV function are induced by differences in blood pressure.

In summary, these data suggests that in experimental diabetes, selective COX-2 inactivation confers protection against sympathetic denervation and LV dysfunction by reducing intramyocardial oxidative stress, inflammation and myocardial fibrosis. Further studies are needed to prove if similar findings are true in humans.
Acknowledgments

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References


Legends

Figure 1. **Sample Echocardiographic Measurements for Measurement of E/A ratio and R-R interval**. Sample echocardiographic images of E/A (A,B) and R-R interval (C,D) measurements.

Figure 2. **Effects Of COX-2 Gene Inactivation And STZ-D On Sympathetic Neuron Staining**. A-C, after 6 months, TH was stained in the LV as described in Methods. D, fibers/ 100 um2 was determined as described in Methods. White arrows indicate TH positive neurons in the LV. Western Blot of COX-2 protein in myocardium of ND COX-2+/+ and D COX-2+/+, (E). Data are expressed as mean±sem. *p<0.05 vs. C, †p<0.05 vs. D.

Figure 3. **Effects Of COX-2 Gene Inactivation And STZ-D On Collagen Content**. A-C, after 6 months, collagen was stained in the LV as described in Methods. D, percentage of collagen was determined as described in Methods. Black arrows indicate collagen staining in the LV. Data are expressed as mean±sem. *p<0.05 vs. C, †p<0.05 vs. D.

Figure 4. **Pearson Correlation Analysis of LV Sympathetic Innervation and E/A or R-R interval**. Pearson Correlation Coefficient and p values were calculated for LV Sympathetic Innervation and E/A (A) and LV Sympathetic Innervation and R-R interval (B) using Prizm as described in Methods.
Figure 5. Effects Of COX-2 Gene Inactivation And STZ-D On Markers Of Oxidative Stress, PG Production, and Inflammation. After 6 months MDA (A) and GSH (B), TXB2 and 6-keto-PGF1a (C), and TNF-α (D) were assessed in whole myocardium as described in Methods. Data are expressed as mean±sem. *p<0.05 vs. C, †p<0.05 vs. D.

Figure 6. Cluster Analysis of the effects of STZ-D and Gene Inactivation on Cardiac Function, Oxidative Stress, PG production, and Inflammation. Clusters were generated using datasets for oxidative stress, PG production, and markers of inflammation obtained from mouse myocardium as described in Methods. Each row corresponds to a marker measured and each column represents an individual sample. Data was normalized to a mean of zero and a standard deviation of 1. Measures above the mean are shaded in red; those below the mean are shaded in blue. The scale indicates the standard deviation above or below the mean.

Table 1.
Data are expressed as Mean±SEM. *p<0.05 vs. ND

Table 2.
Echocardiographic markers were measured 6 months described in Methods. Data are expressed as Mean±SEM.*p<0.01 vs. ND, †p<0.01 vs. other D.
Table 1  Effects of STZ-D and COX-2 Gene Inactivation on Body Weight and Blood Glucose.

<table>
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<th>Experimental Group N ≥ 8</th>
<th>Body Weight (g)</th>
<th>Blood Glucose (mmol/L)</th>
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<tr>
<td>Mice</td>
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<tr>
<td>ND COX-2+/+</td>
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<tr>
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<tr>
<td>D COX-2--</td>
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Table 2  Effects of STZ-D and COX-2 Gene Inactivation on Echocardiography Measures.

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<tr>
<th>Variables</th>
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<tr>
<td>R-R</td>
<td>167±3</td>
<td>151±2*</td>
<td>164±2</td>
<td>166±1†</td>
</tr>
</tbody>
</table>
Figure 1

ND COX-2+/+

E = 1682.98 mm/s

A = 499.35 mm/s

D COX-2+/+

E = 1324.19 mm/s

A = 1043.08 mm/s

ND COX-2+/+

Time R-R =
161.27 ms

D COX-2+/+

Time R-R =
130.00 ms
Figure 2

A. ND COX-2+/+

B. D COX-2+/+

C. D COX-2–

D. 

E. 

<table>
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<th>Condition</th>
<th>COX-2</th>
<th>GAPDH</th>
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<tr>
<td>D COX-2+/+</td>
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<tr>
<td>ND COX-2–</td>
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<tr>
<td>D COX-2+</td>
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</table>
Figure 3

A. ND COX-2+/+

B. D COX-2+/+

C. D COX-2−/

D. Collagen (%)

![Images of tissue sections with bar graphs showing collagen levels for different conditions.](image-url)
Figure 4

A.  
\[ r = 0.8477, p < 0.0001 \]

B.  
\[ r = 0.8401, p < 0.0001 \]
Figure 5

A.

B.

C.

D.
Figure 6
DISCUSSION

COX-2 is involved in the generation of PG from arachidonic acid by catalyzing the conversion arachidonic acid into PGG₂ and subsequently PGH₂. Under basal conditions, COX-2 is undetectable in most tissues, but is up regulated by a variety of factors, including: cytokines, growth factors, tumor promoters, hyperglycemia, and oxidative stress (Cosentino et al., 2003; Feng et al., 1995; Harris, 2007; Herschman et al., 1997; Wu, 1995; Xu and Shu, 2007). Cyclooxygenase-2 has been demonstrated to be involved in inflammatory processes.

In our first manuscript, we provide a potential mechanism linking hyperglycemia to neural dysfunction through COX-2 pathway activation. Hyperglycemia is reported to induce oxidative stress through multiple pathways such as: redox imbalance secondary to enhanced AR activity (Yagihashi et al., 2001), increased production of AGE (Brownlee et al., 1988), increase PKC, especially the β-isoform (Cameron et al., 1999), prostanoid imbalances (Kellogg and Pop-Busui, 2005; Kellogg et al., 2007; Pop-Busui et al., 2002), and mitochondrial overproduction of superoxide (Brownlee, 2003; Nishikawa et al., 2000), resulting in an increased expression and activity of COX-2 (Pop-Busui et al., 2002). Cyclooxygenase-2 upregulation with associated PG imbalance and/or reduced nitric oxide (NO) availability (Cosentino et al., 2003) with effects on endoneurial perfusion may be involved in neural dysfunction. For instance, human aortic endothelial cells exposed to high glucose demonstrate an increased COX-2 expression, with subsequent increase in the TXA₂ and a decrease in the PGI₂ levels (Cosentino et al., 2003).
In addition, although the etiology of myocardial injury and dysfunction in diabetes is still not completely understood, detrimental consequences of myocardial ischemia, oxidative stress, alterations in myocardial substrate metabolism (Fang et al., 2003; Francis, 2001; Frustaci et al., 2000) and a putative diabetic cardiomyopathy comprising myocardial interstitial and perivascular fibrosis (Francis, 2001) and apoptosis (Frustaci et al., 2000) have been linked to the development of impaired LV function and an enhanced CVD risk. There is ample evidence demonstrating that diabetes is able to induce cardiomyopathy and myocardial dysfunction before detectable atherosclerotic plaques (Korte et al., 2005). Indeed, alterations of diastolic (Fang et al., 2003; Francis, 2001; Frustaci et al., 2000) and systolic (Vered et al., 1984) function are widely reported in healthy diabetic subjects and often predate the development of other chronic diabetic complications, suggesting that elevated glucose and the associated downstream effects are sufficient to induce cardiomyopathy (Fang et al., 2003; Korte et al., 2005).

We have demonstrated that COX-2 gene inactivation/selective inhibition has preventative effects on experimental diabetes induced deficits of nerve electrophysiology and specific biomarkers of peripheral nerve function in the short and long-term, as well as protection against loss of intra-epidermal nerve fiber density.

We also have presented data showing the COX-2 gene inactivation is protective against experimental diabetes induced deficits of left ventricular function, cardiac autonomic neuropathy, and morphological changes.

Several mechanisms may explain these beneficial effects of COX-2 inhibition on experimental diabetic neuropathy. Vascular mechanisms associated with either PG imbalance and/or reduced NO availability (Cosentino et al., 2003) with effects on
endoneurial perfusion are involved. For instance, our laboratory has reported that treatment with a selective COX-2 inhibitor prevented diabetes-induced endoneurial nerve blood flow deficits (Pop-Busui et al., 2002). In addition, interactions between COX-2 and RAGE signaling pathway were recently described (Cipollone et al., 2003). This is possibly linked to an altered PG profile in diabetes that favors vasoconstriction, explaining the decrease in the endoneurial blood flow induced by diabetes as well as the beneficial effects observed with the selective COX-2 inhibition in preventing these deficits (Pop-Busui et al., 2002).

Our data indicate that additional markers of inflammation, such as the NF-kB-derived TNF-alpha (Consilvio et al., 2004), 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-kB binding site in its promoter region and NF-kB pathway directly modulates cellular inflammatory processes within the nervous system (Consilvio et al., 2004). It has been shown that while PGE2 upregulates NF-kB activity, with subsequent increasing inflammatory response, other PGs such as PGD2, and PGJ2 suppress NF-kB activation and decrease inflammation (Consilvio et al., 2004). Therefore, in diabetes, COX-2 derived PGE2 in concert with oxidative stress generate a positive feed-back loop resulting in NF-kB 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
uni-directional since COX-2 activation not only results from, but also leads to increased oxidative stress and inflammation in the diabetic peripheral nerves.

Concerns regarding the cardiovascular safety of COX-2 inhibitors were recently raised. However, multiple subsequent meta-analyses have concluded that the current evidence does not support the hypothesis that increased cardiovascular risk is a “class effect” of all COX-2 inhibitors.

In summary, COX-2 over-expression was described in a wide variety of disease states, including diabetes. In the central nervous system, over-expression of COX-2 has been associated with neurotoxicity and is thought to be instrumental in several chronic neurodegenerative processes. Our data demonstrates that in experimental diabetes COX-2 is upregulated in the peripheral nerves and dorsal root ganglia, and is associated with increased oxidative stress, imbalanced PGs production, inflammation, broad electrophysiological deficits and intra-epidermal nerve fiber loss. We have also demonstrated that in rodents, COX-2 gene inactivation/COX-2 selective inhibition is protective against various DPN deficits including nerve conduction slowing and intra-epidermal nerve fiber loss. Therefore, selective COX-2 inhibition may be a potentially useful treatment for preventing or delaying diabetic neuropathy and LV dysfunction.
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ABSTRACT

Diabetic peripheral and autonomic neuropathies are debilitating complications of disease, leading to increased risk of amputation, hyperalgesia and allodynia or hypoalgesia, and increased morbidity and mortality due to myocardial infarctions. We propose that activation of the COX-2 pathway by hyperglycemia with secondary neurovascular deficits are implicated in the pathogenesis of experimental diabetic peripheral sensory-motor and autonomic neuropathies. We further propose that selective COX-2 inhibition may provide a protective effect against the onset and/or progression of these diabetic neuropathies. This hypothesis was tested by the use of a COX-2 gene knockout mouse and a rat model using the selective COX-2 inhibitor celecoxib, using a type 1 diabetes model. Wild-type littermate diabetic mice and untreated diabetic rats developed the normal complications of diabetes such as decreased motor and sensory nerve conduction velocities, increased oxidative stress and inflammation, and alter PG production. Diabetic COX-2 gene knockout mice and rats treated with celecoxib demonstrated protection against deficits in nerve conduction velocity, sensory perception, oxidative stress, and inflammation and maintained normal PG production balance.