Pathogenesis of spinally mediated hyperalgesia in diabetes

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Running title: Spinal COX-2, aldose reductase and pain

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ABSTRACT
Hyperalgesia to noxious stimuli is accompanied by increased spinal cyclooxygenase-2 (COX-2) protein in diabetic rats. The present studies were initiated to establish causality between increased spinal COX-2 activity and hyperalgesia during diabetes, and to assess the potential involvement of polyol pathway activity in the pathogenesis of spinally-mediated hyperalgesia. Rats with one, two or four weeks of streptozotocin-induced diabetes exhibited significantly increased levels of spinal COX-2 protein and activity, along with exaggerated paw flinching in response to 0.5% paw formalin injection.

Increased flinching of diabetic rats was attenuated by intrathecal pre-treatment with a selective COX-2 inhibitor immediately prior to formalin injection, confirming the involvement of COX-2 activity in diabetic hyperalgesia. Chronic treatment with insulin or ICI222155, an aldose reductase inhibitor (ARI) previously shown to prevent spinal polyol accumulation and formalin-evoked hyperalgesia in diabetic rats, prevented elevated spinal COX-2 protein and activity in diabetic rats. In contrast, the ARI IDD676 had no effect on spinal polyol accumulation, elevated spinal COX-2, or hyperalgesia to paw formalin injection. In the spinal cord, aldose reductase immunoreactivity was present solely in oligodendrocytes, which also contained COX-2 immunoreactivity. Polyol pathway flux in spinal oligodendrocytes provides a pathogenic mechanism linking hyperglycemia to hyperalgesia in diabetic rats.
A proportion of diabetic patients experience chronic pain that severely degrades quality of life. The pathogenesis of painful diabetic neuropathy is unclear and treatment options are limited to palliatives including opioids, anticonvulsants, and tricyclic antidepressants that do not target a pathogenic mechanism specific to painful diabetic neuropathy and frequently provide limited efficacy before side effects become intolerable (1). Experimental models of diabetic neuropathy can spur development of novel therapies by providing an understanding of how diabetes alters sensory processing.

Diabetic rats display abnormal pain-associated behaviors, measured as exaggerated responses to painful stimuli (hyperalgesia) or nocifensive responses to normally innocuous stimuli (allodynia). Hyperalgesia to paw formalin injection (2,3) and allodynia to tactile stimulation of the paw (4) develop within four weeks of induction of hyperglycemia. As such short-term diabetes does not induce overt structural pathology in peripheral nerves, attention has focused on biochemical abnormalities that could exaggerate nociceptive processing in painful diabetic neuropathy.

The formalin test is used to investigate spinal sensitization in animals (5). Diabetic rats exhibit increased nocifensive behavior during periods of the formalin test associated with prostaglandin-mediated spinal sensitization (6,7) in concert with paradoxically decreased spinal release of excitatory neurotransmitters (8,9). These findings suggest that hyperalgesia to paw formalin injection in diabetic rats is not caused by increased activation of primary afferents. Moreover, the observation that direct delivery of substance P to the spinal cord induces a protracted thermal hyperalgesia in diabetic rats (10) has lead us to investigate the spinal cord as a potential site of amplification of sensory processing in diabetes.

We have previously shown that diabetic rats exhibit increased spinal cyclooxygenase-2 (COX-2) protein and a prolonged spinal release of prostaglandin E2 (PGE2) in response to paw formalin injection. Further, hyperalgesia to paw formalin injection was attenuated by intrathecal pre-treatment with a PGE2 receptor antagonist or a non-selective COX inhibitor (11). These observations suggest a role for PGE2-mediated spinal sensitization in the hyperalgesia observed in diabetic rats that may be related to increased local COX-2 protein. The present studies were designed to extend these findings by investigating whether increased spinal activity of the COX-2 isozyme contributes to hyperalgesia in diabetic rats and to determine the primary pathogenic mechanism linking hyperglycemia with hyperalgesia.

**RESEARCH DESIGN AND METHODS**

**Animals.**

These studies were approved by the University of California, San Diego, Institutional Animal Care and Use Committee and used adult female Sprague-Dawley rats (Harlan, San Diego, CA). Following an overnight fast, rats were made diabetic with one intraperitoneal injection of streptozotocin (50 mg/kg freshly dissolved in sterile saline; Sigma, St. Louis, MO). Hyperglycemia was confirmed two days later and at the
conclusion of each study using blood taken by tail-prick and a strip-operated reflectance meter (LifeScan, Milpitas, CA). Only rats with a blood glucose concentration $\geq 15$ mmol/l at the beginning and end of a study were included in diabetic groups. Rats were monitored daily, weighed weekly, and any with a body weight below 200 g was treated with sufficient insulin to increase body weight without affecting hyperglycemia (12). At the conclusion of each study, any rats showing lethargy and/or general poor health were excluded from analysis. Rats were maintained 2-3 per cage under standard vivarium conditions with 12:12 h light/dark cycle and free access to standard rat chow and tap water. After catheter implantation, rats were housed individually to prevent cage-mates from chewing implants.

**Paw thermal response latency.**
This test was used to measure small sensory fiber function, and was performed as described in detail elsewhere (10). The mobile heat source was calibrated to heat at 1°C per second and this was confirmed on each day of testing.

**Sciatic nerve conduction velocity.**
This test was used to measure peripheral motor and large sensory fiber function. Rats were anesthetized with 3% isoflurane and measurements were made as described in detail elsewhere (10).

**Formalin test.**
50 µl of 0.5% formalin solution was injected into the dorsum of the hind paw. Defined flinches of the injected paw were counted per minute at 5 min intervals for 60 min, with phases defined as flinches counted during the following time bins: phase I, 1-2 min and 5-6 min post-injection; quiescent phase, 10-11 min and 15-16 min post-injection; and phase II, 20-21 min, 25-26 min, 30-31 min, 35-36 min, 40-41 min, 45-46 min, 50-51 min, and 55-56 min post-injection. Comparisons of behavior during each phase were made by summing the flinches recorded at measurement points within the phase.

**Intrathecal catheterization.**
Lumbar intrathecal PE-10 polyethylene catheters were implanted under 3% isoflurane anesthesia as described in detail elsewhere (13). Rats were allowed to recover for 3-5 days before use. Any rats exhibiting neurological dysfunction were removed from the study.

**Intrathecal drug delivery.**
The non-steroidal anti-inflammatory drug (NSAID) indomethacin (Cayman Chemical, Ann Arbor, MI), a non-selective inhibitor of COX-1 and COX-2, was dissolved in 20 mmol/l NaOH and 280 mmol/l D-glucose in distilled water. The selective COX-2 inhibitor SC-58125 (Cayman Chemical) was dissolved in 10% dimethylformamide and 5% Tween-80 in saline. Drugs or vehicles were administered intrathecally in a volume of 10 µl followed by 10 µl of saline to flush the catheter. Intrathecal injections were given 10 min before paw formalin injection.

**Chronic treatments.**
A group of diabetic rats was treated from the onset of hyperglycemia with the aldose reductase inhibitor (ARI) ICI222155 (4-amino-2,6-dimethylphenyl-sulphonyl nitromethane, Zeneca Pharmaceuticals, Macclesfield, UK), suspended in water and Tween-20, once daily by oral gavage at 20 mg/kg. ICI222155, and its dose and treatment regimen, were chosen because of published observations that it penetrates the spinal cord and prevents the development of formalin hyperalgesia in
diabetic rats (6). A second group of diabetic rats was treated from the onset of hyperglycemia with the ARI IDD676 (3-[(4,5,7-trifluorobenzothiazol-2-yl)methyl]indole-N-acetic acid, Institute for Diabetes Discovery, Branford, CT), suspended in water and Tween-20, once daily by oral gavage at 10 mg/kg. IDD676, and its dose and treatment regimen, were chosen based on prior studies showing efficacy in blocking accumulation of the polyol pathway metabolites fructose and sorbitol in peripheral nerve (14). A third group of diabetic rats was treated with insulin from the onset of hyperglycemia to restore and maintain euglycemia, using slow-dissolving insulin pellets that deliver approximately 2-4 U insulin per day (Linshin, Scarborough, Ontario, Canada). Pellets were implanted subcutaneously and blood glucose was checked daily. When blood glucose levels increased above 15 mmol/l, implants were replaced.

**Cyclooxygenase immunoblot and activity assay.**

Spinal cords were obtained from rats by hydraulic extrusion, and levels of COX-2 protein were measured by Western blotting as previously described (11). Because studies involved substantial numbers of rats, multiple gels were required for each study, with samples from control rats repeated on each gel. Density per unit protein was calculated for each sample by dividing the measured intensity of the COX-2 band by the measured intensity of the β-actin band. To plot samples from multiple gels on one graph, the mean of the control samples on each gel was calculated, and ratios of these means were used to scale all samples to the mean of controls on one randomly chosen gel.

To study COX-2 activity, freshly harvested spinal cords were rinsed with 1 mmol/l Tris buffer containing 0.16 mg/ml heparin, and homogenized in 0.1 mol/l Tris buffer containing 1 mmol/l EDTA. COX-2 enzymatic activity was then measured using a COX activity assay (Cayman Chemical) according to manufacturer instructions. Multiple microplates were used with samples from control rats repeated on each microplate. To reduce inter-assay variability, the mean of the control samples on each microplate was calculated and set to 100%. This mean was then used to calculate S.E.M.s for the control samples, and to normalize activity levels in all other samples to % control expression.

**Gas Chromatography.**

Twenty-four hours after the last ARI treatment, portions of spinal cord and sciatic nerve were removed and stored at -20°C until determination of sugar and polyol content exactly as described elsewhere (6).

**Immunohistochemistry.**

Rats were anesthetized via intraperitoneal injection of a cocktail (2 ml/kg) containing pentobarbital (12.5 mg/ml) and diazepam (1.25 mg/ml) in sterile saline. After transcardial perfusion with saline, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), the lumbar spinal cord was dissected and post-fixed in 4% paraformaldehyde at 4°C for 2 hours, cryoprotected by immersion in 0.1 M PBS containing 30% sucrose for 48 hours at 4°C, embedded in OCT medium, and stored at -20°C. Tissue was cut into 14 µm-thick sections for double immunofluorescence staining. After blocking, sections were incubated with mouse antibodies against NeuN (Chemicon, MAB377, Temecula, CA),
GFAP (Chemicon, MAB360), CD11b (Biosource, ARS1122, Camarillo, CA), or APC-Ab7 (Calbiochem, OP80, San Diego, CA), followed by goat anti-mouse Alexa-594 (Molecular Probes, Eugene, OR). Afterwards, sections were incubated with rabbit antibodies against COX-2 (Cayman Chemical, 160126) or aldose reductase, followed by goat anti-rabbit Alexa-488 (Molecular Probes). Coverslips were mounted with Prolong anti-fade mounting medium (Molecular Probes), and sections were evaluated using a Zeiss LSM 510 Axiovert 100 confocal microscope.

**Data presentation and statistical analyses.**

All data are presented as group mean ± S.E.M except blood glucose measurements, which are presented as group medians because many diabetic rats had blood glucose levels exceeding the 33.3 mmol/l upper limit of detection of the strip-operated reflectance meter. Any such rat was assigned a blood glucose measurement of 33.3 mmol/l for determining median blood glucose within a group of diabetic rats. Where appropriate, between-group comparisons were made by unpaired *t*-test or one-way ANOVA followed by Dunnett’s or Student-Neuman-Keuls’ post-hoc test.

**RESULTS**

**Time course of changes in spinal COX-2 protein and enzymatic activity and in formalin-evoked behavior.**

COX-2 protein levels are elevated in the spinal cord of rats with four weeks of diabetes (11). We therefore initially measured spinal COX-2 protein levels in rats with shorter durations of diabetes to establish the duration of hyperglycemia necessary to upregulate COX-2. We confirmed that COX-2 protein levels were doubled in the spinal cord of rats with four weeks of diabetes, as compared with age-matched controls (*P* < 0.05, Fig. 1A and B). Rats with one or two weeks of diabetes also exhibited a two-fold increase of spinal COX-2 protein (*P* < 0.05 and *P* < 0.01 vs. age-matched controls, respectively, Fig. 1B). Because COX-2 is an enzyme that undergoes use inactivation (15), we next measured levels of spinal COX-2 activity. Compared with age-matched control rats, rats with one week of diabetes exhibited a significant increase in spinal COX-2 activity (*P* < 0.05) that was maintained through four weeks of diabetes (Fig. 1C). To determine if elevated spinal COX-2 and hyperalgesia to paw formalin injection are co-incident, we measured behavioral responses to 0.5% formalin injection in a subset of this cohort of rats. Formalin-evoked hyperalgesia was evident after only one week of diabetes, as indicated by a significant (*P* < 0.05 vs. age-matched controls) increase in the sum of flinches counted during the 60 min after paw formalin injection, and the magnitude of this hyperalgesia increased with duration of diabetes (Fig. 2). All diabetic rats used in these studies exhibited blood glucose levels above 20 mmol/l (median of 33.3 mmol/l), while the median blood glucose of control rats was 5.7 mmol/l. Significant weight loss was observed only in rats with four weeks of diabetes (235 ± 8 g vs. 258 ± 4 g for age-matched controls; *P* < 0.05).

**Formalin-evoked behavior and spinal cyclooxygenase inhibition.**

Having demonstrated that diabetes-induced hyperalgesia to paw formalin injection and elevated spinal COX-2 are co-incident, we next investigated whether intrathecal treatment with the selective COX-2 inhibitor SC-58125
would attenuate hyperalgesia to paw formalin injection. These studies used rats with four weeks of diabetes and age-matched controls. The cohort of diabetic rats exhibited weight loss (211 ± 3 g vs. 240 ± 8 g for controls; \( P < 0.0001 \)) and blood glucose levels above 20 mmol/l (median blood glucose 32.3 mmol/l vs. 5.6 mmol/l for controls). There was no significant difference in weights or blood glucose levels between the various sub-groups of diabetic rats (data not shown). Untreated diabetic rats exhibited significant hyperalgesia to paw formalin injection during phase II of the formalin test (\( P < 0.001 \) vs. controls, Fig. 3A). Intrathecal treatment with the non-selective COX inhibitor indomethacin or the selective COX-2 inhibitor SC-58125, delivered 10 min before paw formalin injection, attenuated hyperalgesia of diabetic rats during phase II of the formalin test (\( P < 0.05 \) for each inhibitor vs. respective vehicle-treated rats, Fig. 3B and C). There was no statistically significant difference in the number of flinches counted during phase I or the quiescent phase between any of the groups, indicating that diabetes selectively potentiated the spinally-mediated phase II and that none of the treatments affected acute pain behavior.

**Effect of ICI222155 on spinal cyclooxygenase-2.**

Previous work has shown that treating diabetic rats systemically with the ARI ICI222155 prevents the development of hyperalgesia to paw formalin injection, blocks the accumulation of polyol pathway metabolites in the sciatic nerve, and crosses the blood-brain-barrier (BBB) to also block polyol accumulation in the spinal cord (6). These findings, along with our work associating elevated spinal COX-2 with hyperalgesia to paw formalin injection, prompted us to test whether treating diabetic rats systemically with ICI222155 would prevent the induction of elevated spinal COX-2 protein and activity levels. A group of diabetic rats treated with insulin to restore and maintain euglycemia was included to exclude the possibility that increased spinal COX-2 expression was the result of direct streptozotocin toxicity. ICI222155-treated and insulin-treated diabetic rats, along with untreated diabetic rats and control rats, were maintained for four weeks.

At the conclusion of these studies, untreated and ICI222155-treated diabetic rats exhibited weight loss and hyperglycemia, whereas insulin-treated diabetic rats had normal body weights and tended to have blood glucose levels lower than those of controls (Table 1). Efficacy of ICI222155 treatment was verified by analyzing sugar and polyol levels in sciatic nerves. Untreated diabetic rats accumulated glucose, sorbitol, and fructose in the sciatic nerve, which was associated with depletion of myo-inositol (Table 1). ICI222155 treatment prevented the accumulation of sorbitol and fructose in the sciatic nerve, without significantly affecting glucose levels, and also prevented myo-inositol depletion (Table 1). Insulin-treated diabetic rats had normal levels of glucose, sorbitol, fructose, and myo-inositol in the sciatic nerve (Table 1).

Untreated diabetic rats showed significantly elevated levels of spinal COX-2 protein and activity (\( P < 0.001 \) and \( P < 0.05 \), respectively, vs. controls, Fig. 4). Treatment with insulin or ICI222155 prevented the increase in COX-2 protein (\( P < 0.001 \) and \( P < 0.01 \), respectively, vs. untreated diabetic rats, Fig. 4A) and COX-2 activity (\( P < 0.05 \)
for both, vs. untreated diabetic rats, Fig. 4B).

**Effect of IDD676 treatment on hyperalgesia and spinal cyclooxygenase-2.**

Given the spinal sensitization underlying phase II of the formalin test and that diabetic rats exhibit hyperalgesia during this phase of the test, we hypothesized that in order for any ARI to prevent hyperalgesia to paw formalin injection in diabetic rats, it must cross the BBB for access to spinal sites of action. Because the ARI IDD676, unlike ICI222155, does not prevent exaggerated flinching in response to paw injection of 0.2% formalin in diabetic rats (14), we further hypothesized that IDD676 does not cross the BBB and therefore would not block exaggerated polyol pathway flux or elevated COX-2 in the spinal cord of diabetic rats. These hypotheses were tested by studying the efficacy of systemic IDD676 treatment against accumulation of polyol pathway metabolites in the spinal cord and sciatic nerve, and determining whether IDD676 treatment would affect a range of diabetes-induced disorders in the PNS and spinal cord. Untreated diabetic rats, IDD676-treated diabetic rats, and control rats were maintained for four weeks.

At the conclusion of these studies, untreated and IDD676-treated diabetic rats exhibited weight loss and hyperglycemia (Table 2). IDD676 treatment prevented the accumulation of polyol pathway products in the sciatic nerve but not the spinal cord (Table 2). These data indicate that IDD676 was effective in the PNS, but did not cross the BBB sufficiently to block exaggerated polyol pathway flux in the CNS. IDD676 treatment also alleviated nerve myo-inositol depletion, nerve conduction velocity slowing, and thermal hyperalgesia in diabetic rats (Table 2), consistent with efficacy against exaggerated polyol pathway flux in the PNS. However, IDD676 treatment had no effect on elevated spinal COX-2 protein (Fig. 5A) and did not prevent hyperalgesia to 0.5% formalin (Fig. 5B), suggesting that polyol product accumulation in the PNS does not underlie these abnormalities.

**Spinal localization of cyclooxygenase-2 and aldose reductase.**

Having identified the importance of spinal COX-2 and aldose reductase (AR) in hyperalgesia to paw formalin injection in diabetic rats, we examined the spinal localization of these two proteins. Spinal cord tissue from control rats was also analyzed to determine if diabetes alters the normal distribution of spinal COX-2 and AR protein. Immunohistochemistry performed on spinal cord tissue from control and diabetic rats showed that diabetes does not induce COX-2 expression in cell types that do not express COX-2 under normal conditions. In spinal cord tissue from control and diabetic rats, we confirmed that COX-2 is expressed in dorsal horn neurons and motor neurons (16; data not shown) and we observed COX-2-immunoreactivity (COX-2-IR) in oligodendrocytes, as indicated by the colocalization of COX-2-IR with APC-IR (Fig. 6A). COX-2-IR was not detected in astrocytes or microglia, as indicated by the lack of colocalization of COX-2-IR with GFAP-IR or CD11b-IR, respectively (data not shown). AR-IR was seen only in oligodendrocytes in spinal cord tissue from both control and diabetic rats, as indicated by the colocalization of AR-IR with APC-IR (Fig. 6B), whereas AR-IR did not co-localize with NeuN-IR (neurons), GFAP-IR...
DISCUSSION
One week of diabetes induced a two-fold increase in spinal COX-2 protein and activity levels, and both remained elevated through at least four weeks of diabetes. These findings are important as they demonstrate that elevated COX-2 protein measured in the spinal cord of diabetic rats is enzymatically active and is not residual use-inactivated protein. Measurement of behavioral responses to paw formalin injection revealed that rats with one week of diabetes exhibited exaggerated flinching, and this hyperalgesia increased with duration of diabetes. These data indicate that the onset of formalin hyperalgesia and of up-regulated spinal COX-2 activity and protein in diabetic rats occur in parallel, and prompted us to investigate whether there is a causal link between elevated spinal COX-2 and formalin hyperalgesia in diabetic rats. To do this we pre-treated the spinal cord of diabetic rats with indomethacin or SC-58125, with doses chosen for these drugs based on previous studies in which they were given intrathecally to alleviate hyperalgesia in rats (11,17). Both drugs attenuated exaggerated paw flinching during phase II of the test, indicating that hyperalgesia to paw formalin injection in diabetic rats is spinally mediated and at least partly due to activity of the COX-2 isozyme.

We next tested the hypothesis that exaggerated polyol pathway flux in the CNS causes diabetes-induced increased spinal COX-2 and hyperalgesia to paw formalin injection. Previous work indicated that systemic treatment with the ARI ICI222155 prevented the development of formalin hyperalgesia in diabetic rats, and also blocked the accumulation of fructose and sorbitol in both the sciatic nerve and spinal cord (6). Our current experiments showed that ICI222155 also prevented the increase of spinal COX-2 protein and activity in diabetes. In contrast, systemic treatment with the ARI IDD676 blocked accumulation of polyol pathway metabolites in the sciatic nerve but not in the spinal cord, and while it protected peripheral nerve function in diabetic rats, it did not affect formalin hyperalgesia or elevated spinal COX-2 levels. These data indicate that protecting the PNS alone from polyol product accumulation does not impact spinal disorders and suggest that exaggerated spinal polyol pathway flux may cause increased spinal COX-2 and formalin hyperalgesia at short durations of diabetes. Exaggerated polyol pathway flux in the brain may also affect nociceptive processing and this possibility awaits future investigation.

Having identified the potential importance of spinal COX-2 and AR in hyperalgesia to paw formalin injection in diabetic rats, the localization of COX-2 and AR protein in the spinal cord of diabetic and control rats was examined. The expression of COX-2 protein in naïve rat spinal cord has been profiled, with agreement that it is expressed in dorsal and ventral horn neurons (16), and some debate regarding its presence in radial glia (18), astrocytes (19), or endothelial cells (20). In our experiments, we saw that spinal cords from control and diabetic rats showed COX-2-immunoreactivity only in dorsal horn neurons, motor neurons, and oligodendrocytes, indicating that the increased COX-2 protein and activity observed in diabetes does not involve induction of COX-2 expression in novel
cell types. When studying AR expression, we found that spinal cord tissue from both control and diabetic rats showed AR-immunoreactivity only in oligodendrocytes. As ICI222155 treatment in diabetic rats prevented spinal accumulation of polyol pathway products, the increase of spinal COX-2 activity, and the development of formalin hyperalgesia (6, and present study) this suggests that exaggerated polyol pathway flux in spinal oligodendrocytes could be a primary initiating event for the increased spinal COX-2 and formalin hyperalgesia observed in diabetic rats.

These experiments raise the question of how a pathological process in spinal oligodendrocytes could affect pain processing and perception. One possibility is that exaggerated polyol pathway flux may stress oligodendrocytes, leading to local secretion of hyperalgesia-inducing substances. Indeed, oligodendrocytes in diabetic mice brains show enhanced expression of αB-crystallin, a heat shock protein upregulated under various pathological conditions (21), and oligodendrocytes in vitro secrete inflammatory lipid products, including PGE₂, in response to sub-lethal injury with complement complexes (22). In diabetes, exaggerated polyol pathway flux in oligodendrocytes may induce COX-2 activity, resulting in local secretion of PGE₂ that could readily diffuse to dorsal horn neurons and sensitize them to nociceptive input from primary afferents (23). Alternatively, exaggerated polyol pathway flux in spinal oligodendrocytes may disrupt myelination within the spinal cord, possibly causing pain via cross-excitation between abnormally myelinated axons (24,25). Interestingly, a significant portion of patients with the demyelinating disease multiple sclerosis experience pain that has been hypothesized to result from spinal cord lesions (26).

The stress to oligodendrocytes caused by exaggerated polyol pathway flux may alternatively activate other glia, such as astrocytes. Throughout the CNS, gap junctions couple astrocytes to oligodendrocyte cell bodies (27), providing an explanation of how a primary injury to oligodendrocytes could be communicated to astrocytes. We have observed a significant 40% increase in the expression of GFAP protein in spinal cords from diabetic rats (K.M. Ramos, unpublished observations), which is suggestive of astrogliosis. It is therefore possible that diabetes disturbs glial function, and the role of activated glial cells in various pain states is becoming increasingly appreciated (for review, see 28).

In summary, we have demonstrated that acute spinal inhibition of COX-2 significantly alleviated formalin hyperalgesia in diabetic rats, highlighting the significance of the spinal cord as a site of aberrant nociceptive processing in diabetes. Diabetes-induced upregulation of spinal COX-2 protein and activity was prevented by treatment with an ARI that crosses the BBB, while an ARI that does not cross the BBB had no effect. In the spinal cord, AR was expressed solely in oligodendrocytes, suggesting an important role for oligodendrocyte-localized exaggerated polyol pathway flux in the upregulation of COX-2 and hyperalgesia observed in diabetes. The relatively ineffective treatment of painful diabetic neuropathy by orally delivered NSAIDs or ARIs may be due to insufficient quantity of drug crossing the
BBB to gain access to spinal sites of action, and spinal targeting of such agents may therefore benefit patients with painful diabetic neuropathy.

ACKNOWLEDGMENTS
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REFERENCES


**TABLE 1**  
Physiological parameters and sciatic nerve sugars and polyols in control and untreated diabetic rats, and diabetic rats treated with ICI222155 or insulin.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic + ICI222155</th>
<th>Diabetic + insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>260 ± 4 (^a)</td>
<td>226 ± 8 (^b)</td>
<td>218 ± 6 (^b)</td>
<td>269 ± 5 (^a)</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>median = 5.9</td>
<td>All &gt; 20; median = 33.3</td>
<td>All &gt; 20; median = 33.3</td>
<td>median = 2.6</td>
</tr>
<tr>
<td>Nerve glucose</td>
<td>6.0 ± 0.4 (^a)</td>
<td>55.0 ± 3.6 (^b)</td>
<td>45.7 ± 3.2 (^b)</td>
<td>5.2 ± 1.1 (^a)</td>
</tr>
<tr>
<td>Nerve sorbitol</td>
<td>nd</td>
<td>0.3 ± 0.2</td>
<td>0.01 ± 0.01</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Nerve fructose</td>
<td>0.6 ± 0.1 (^a)</td>
<td>15.2 ± 0.8 (^b)</td>
<td>1.6 ± 0.3 (^a)</td>
<td>0.4 ± 0.3 (^a)</td>
</tr>
<tr>
<td>Nerve myo-inositol</td>
<td>11.1 ± 1.2 (^c)</td>
<td>8.1 ± 0.6 (^d)</td>
<td>12.1 ± 1.2 (^c)</td>
<td>9.9 ± 0.5 (^c)</td>
</tr>
</tbody>
</table>

Sugar and polyol data are nmol/mg dry weight. \(^a\) vs. \(^b\) \(P < 0.001\); \(^c\) vs. \(^d\) \(P < 0.05\); One-way ANOVA with Newman-Keuls’ post-hoc test; \(n = 5-6\) per group. nd = not detected.
TABLE 2
Physiological parameters, indices of peripheral nerve function, and sugar and polyol levels measured in sciatic nerve and spinal cord, in control rats, untreated diabetic rats, and diabetic rats treated with IDD676.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic + IDD676</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>261 ± 4</td>
<td>223 ± 8</td>
<td>234 ± 11</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>median = 5.5</td>
<td>All &gt; 20; median = 33.3</td>
<td>All &gt; 20; median = 32.6</td>
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<tr>
<td>Paw withdrawal latency (sec)</td>
<td>8.6 ± 0.3</td>
<td>7.1 ± 0.4</td>
<td>9.2 ± 0.5</td>
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<tr>
<td>Motor nerve conduction velocity (m/s)</td>
<td>52.4 ± 1.3</td>
<td>46.9 ± 1.0</td>
<td>49.4 ± 1.6</td>
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<tr>
<td>Sensory nerve conduction velocity (m/s)</td>
<td>50.4 ± 1.2</td>
<td>46.2 ± 1.2</td>
<td>48.2 ± 1.0</td>
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<tr>
<td>Nerve glucose</td>
<td>4.2 ± 0.7</td>
<td>35.4 ± 2.8</td>
<td>47.1 ± 2.6</td>
</tr>
<tr>
<td>Nerve sorbitol</td>
<td>nd</td>
<td>3.3 ± 1.3</td>
<td>1.1 ± 0.6</td>
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<tr>
<td>Nerve fructose</td>
<td>0.4 ± 0.1</td>
<td>14.5 ± 1.2</td>
<td>1.1 ± 0.5</td>
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<tr>
<td>Nerve myo-inositol</td>
<td>13.6 ± 1.2</td>
<td>9.4 ± 0.8</td>
<td>14.0 ± 2.3</td>
</tr>
<tr>
<td>Spinal cord glucose</td>
<td>1.7 ± 0.3</td>
<td>12.5 ± 1.4</td>
<td>12.1 ± 1.3</td>
</tr>
<tr>
<td>Spinal cord sorbitol</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Spinal cord fructose</td>
<td>1.3 ± 0.2</td>
<td>3.1 ± 0.3</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>Spinal cord myo-inositol</td>
<td>28.8 ± 0.7</td>
<td>27.6 ± 1.0</td>
<td>29.4 ± 0.5</td>
</tr>
</tbody>
</table>

Sugar and polyol levels are presented as nmol/mg dry weight. a vs. b $P < 0.05$; a vs. c $P < 0.01$; d vs. a,c $P < 0.001$; One-way ANOVA with Newman-Keuls’ post-hoc test; n = 5-6 per group. nd = not detected.
FIGURE LEGENDS

FIG. 1.
A: Western blots of lumbar spinal cord from control rats and rats with four weeks of diabetes, immunostained for COX-2 and β-actin. B: Densitometric quantification of COX-2-immunostained Western blots of lumbar spinal cord and C: COX-2 activity levels measured in lumbar spinal cord from control and diabetic rats. One-way ANOVA with Dunnett’s post-hoc test, \( n=5-6 \) per group. Control (C); rats with one week (1WK D), two weeks (2WK D), or four weeks (4WK D) of diabetes.
FIG. 2.
A: Time course of formalin-evoked flinching in control and diabetic rats. B: Total flinches counted in 60 min after paw formalin injection in control and diabetic rats. One-way ANOVA with Dunnett’s post-hoc test, \( n=5-6 \) per group. Control (C); rats with one week (1WK D), two weeks (2WK D), or four weeks (4WK D) of diabetes.
FIG. 3. Flinches counted during phase I, the quiescent (Q) phase, and phase II of the formalin test. A: Untreated control rats and untreated diabetic rats. B: Diabetic rats treated intrathecally with 10 µl indomethacin vehicle (D+INDO V) or 10 µl vehicle containing 65 µg indomethacin (D+INDO). C: Diabetic rats treated intrathecally with 10 µl SC-58125 vehicle (D+SC-58125 V) or 10 µl vehicle containing 20 µg SC-58125 (D+SC-58125). For each panel, an unpaired t-test was used for each phase to compare matched groups, n=5-6 per group.
FIG. 4.
A: Densitometric quantification of COX-2-immunostained Western blots of lumbar spinal cord and B: COX-2 activity levels measured in lumbar spinal cord from control rats (C), untreated diabetic rats (D), and diabetic rats treated with ICI222155 (D+ICI) or insulin (D+I). One-way ANOVA with Student-Newman-Keuls’ post-hoc test, n=5-6 per group.
FIG. 5.
A: Densitometric quantification of COX-2-immunostained Western blots of lumbar spinal cord from control rats (C), untreated diabetic rats (D), and diabetic rats treated with IDD676 (D+IDD). B: Total flinches counted in 60 min after paw formalin injection in control rats (C), untreated diabetic rats (D), and diabetic rats treated with IDD676 (D+IDD). One-way ANOVA with Dunnett’s post-hoc test, n=5-6 per group.
FIG. 6.
Confocal imaging of COX-2-IR and AR-IR in the lumbar spinal cord. A: In white matter near the dorsal horn, an APC-IR-positive (red) oligodendrocyte (arrow) has a COX-2-IR-positive process (green; overlap appears yellow) that extends into the surrounding tissue. Individual oligodendrocyte processes (arrowheads) co-express APC-IR (red) and COX-2-IR (green; overlap appears yellow). B: Also in white matter near the dorsal horn, many oligodendrocyte cell bodies (arrows) and processes (arrowheads) are positive for both APC-IR (red) and AR-IR (green; overlap appears yellow).