A proportion of patients with diabetic neuropathy report aberrant sensations that may range from exaggerated perception of sensory stimuli to spontaneous paresthesias and pain. Morphometric analyses of peripheral nerves from patients with painful diabetic neuropathy have been unable to find clear associations with either nerve fiber degeneration or regeneration (1,2). In the absence of simple structural correlates in the peripheral nerves, it is plausible that neurochemical abnormalities at the peripheral, spinal, or supraspinal levels could contribute to painful diabetic neuropathy.

Diabetic rats also display evidence of altered sensory processing, as illustrated by behavioral studies in which nocifensive responses to normally nonpainful stimuli (alldynia) and exaggerated responses to stimuli that usually produce mild nocifensive responses (hyperalgesia) were reported. Thus, alldynia to light touch (3) or mechanical pressure (4) and hyperalgesia after paw formalin injection (5,6) all develop within weeks of the onset of hyperglycemia and can be corrected by instituting a protracted period of tight glycemic control (3,7). Rats with short-term (4 weeks) experimental diabetes do not exhibit marked fiber degeneration or regeneration in their peripheral (8) or cutaneous (9) nerves. They may therefore allow investigation of the contribution of neurochemical disorders to the behavioral indexes of hyperalgesia and also provide a model for the assessment of potential therapeutic agents.

Of the behavioral tests in which diabetic rats show alldynia or hyperalgesia, the formalin test has the advantage of exhibiting a prolonged response period that facilitates experimental investigation and intervention. The relevance of this test is supported by observations that gabapentin, which alleviates painful diabetic neuropathy (10), also reduces hyperalgesia during the formalin test in diabetic rats (11). Typically, distinctive flinching and attention behaviors to the afflicted paw begin immediately after injection of formalin and persist for 60 min or longer (12). The responses to formalin are biphasic with the two phases of activity separated by an intervening quiescent period. The first phase directly represents afferent activity in response to the peripheral injury, whereas the second phase also incorporates modulation of primary afferent input by spinal release of factors, including prostaglandins (13,14) and nitric oxide (15). In diabetic rats, the frequency of flinching responses to paw formalin injection is increased, most notably during the otherwise quiescent period and during phase 2 (15,16). The absence of markedly increased phase 1 activity, together with our recent finding of reduced formalin-evoked release of the primary afferent-derived neuropeptide substance P in the spinal cord of diabetic rats (17), suggests that hyperalgesia in this test is not likely to arise from increased primary afferent responses to the insult. The present studies were initiated to investigate the potential role of spinal modulation of sensory processing in diabetes-induced hyperalgesia with particular focus on the role of locally produced prostaglandin E2 (PGE2).

**RESEARCH DESIGN AND METHODS**

**Animals.** These studies were performed with the approval of the local institutional animal care committee and used adult female Sprague-Dawley rats (Harlan, San Diego, CA). After an overnight fast, rats were made diabetic

**Diabetic rats display exaggerated hyperalgesic behavior in response to noxious stimuli that may model aspects of painful diabetic neuropathy.** This study examined the contribution of spinal prostaglandin production to this exaggerated hyperalgesic behavior. Rats were implanted with spinal dialysis probes and received noxious stimulation to the hind paw by subcutaneous injection of 0.5% formalin solution. Prostaglandin E2 (PGE2) was measured in dialysates of lumbar spinal cerebrospinal fluid concurrent with behavioral responses to formalin injection. In separate experiments, formalin-evoked behavioral responses were measured after intrathecal delivery of either a cyclooxygenase inhibitor or an EP1 receptor antagonist, and cyclooxygenase protein was measured in spinal cord homogenates. Diabetic rats exhibited exaggerated behavioral responses to paw formalin injection and a concurrent prolongation of formalin-evoked PGE2 release. Formalin-evoked behavioral responses were dose-dependently reduced in diabetic rats by spinal delivery of a cyclooxygenase inhibitor or an EP1 receptor antagonist. Protein levels of cyclooxygenase-2 were elevated in the spinal cord of diabetic rats, whereas cyclooxygenase-1 protein was reduced. Hyperalgesic behavior in diabetic rats is associated with both increased cyclooxygenase-2 protein and cyclooxygenase-mediated PGE2 release. Spinal delivery of selective inhibitors of cyclooxygenase-2 or antagonists of prostaglandin receptors may have therapeutic potential for treating painful diabetic neuropathy. *Diabetes* 51:2249–2255, 2002

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by a single intraperitoneal injection of streptozotocin (50 mg/kg freshly dissolved in sterile 0.9% saline), and hyperglycemia was confirmed 4 days later using blood taken by tail prick and a strip-operated reflectance meter (Glucostix and Glucochek; Bayer, Elkhart, IN). Blood samples were also obtained at the conclusion of each study, 4–6 weeks after onset of hyperglycemia, and plasma glucose levels were determined by spectrophotometric assay (Glucose Trinder kit; Sigma, St. Louis, MO). Only animals with a blood glucose concentration of ≥15 mmol/l at the start and end of the study were included in the diabetic groups. All animals 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 or dialysis probe implantation, animals were isolated one per cage to prevent cage mates from chewing implants.

**Spinal microdialysis**. While under 2% halothane anesthesia, rats were implanted with a loop-formed intrathecal microdialysis probe, constructed as described elsewhere (17) from a dialysis fiber with an approximate cutoff of 10 kDa (GPE-11; Gambro, Lund, Sweden) and with polyethylene (PE)-10 tubing at the inflow and outflow ends. The probe was inserted through an incision in the atlanto-occipital membrane after exposure by skin incision and separation of the overlying muscle and then inserted caudally into the subarachnoid space such that the active segment of the probe was at the level of the lumbar enlargement. Patency of flow was confirmed before implantation by flushing with sterile 0.9% saline, and both ends were plugged with wire to prevent leakage. After implantation, the probe was held in place with a 6-0 Vicryl suture (Ethicon, Peterborough, Canada) attached to the adjacent muscle. The skin incision was closed with wound clips, allowing transcutaneous projection of ~10 mm of both PE-10 arms of the probe, and the animal was allowed to recover. Rats with dialysis fiber implants were monitored daily, and only those exhibiting normal motor and sensory behaviors were used in dialysis studies.

Dialysis studies were performed 3 days after implantation of the dialysis probe in conscious unrestrained animals using a procedure described in detail elsewhere (18). Briefly, artificial cerebrospinal fluid (CSF) (151.1 mmol/l Na+/H11001), 2.6 mmol/l K+ (9.0 mmol/l Mg2+), 1.3 mmol/l Ca2+, 122.7 mmol/l Cl−, 21.0 mmol/l HCO3−, 2.5 mmol/l HPO42−, and 3,5 mmol/l glucose) was infused at a rate of 5 μl/min via a PE-50 catheter connecting a microinfusion pump (BAS, West Lafayette, IN) to the inflow arm of the dialysis fiber. Before infusion, the solution was gassed with a 95% O2/5% CO2 mixture to adjust pH to 7.2 and then filtered through a sterile 0.2-μm cellulose nitrate filter (Nalgene, Rochester, NY). Outflow was directed to storage tubes on ice via PE-50 tubing. Experiments began with a 30-min wash-out period to accustom the animal to the experimental conditions, establish perfusion equilibrium, and ensure that there was no leakage from the system. This was followed by collection of three fractions, each of 10-min duration, to represent basal concentrations. The animal was then manually restrained and 50 μl of 0.5% formalin solution injected into the dorsal surface of the right hind paw before returning it to the observation chamber. Thereafter, dialysate was collected in 10-min fractions for up to 60 min, making allowance for the transit time of dialysate from spinal cord to collection vial. Each sample was immediately transferred to liquid nitrogen and then stored at −70°C until assay.

**PGE2 assays**. PGE2 immunoactivity for the 0.5% formalin dialysis experiment was measured using a commercial enzyme immunoassay kit (titerZyme kit; PerSeptive Biosystems, Framingham, MA), which does not cross-react with PGA, PGB, PGF2α, 5-, 12-, or 15-HETET, TxB2, or arachidonic acid.

**Behavioral assessment**. After formalin injection into the hind paw, defined flinches of the injected paw were counted per minute at 5-min intervals during the subsequent 60 min. Paw thickness was measured before and 60 min after paw formalin injection using a thickness gauge (Mitutoyo, Kawasaki, Japan).

**Treatments**. The nonsteroidal anti-inflammatory drug (NSAID) indomethacin (Sigma), a nonselective inhibitor of both isoforms of cyclooxygenase (COX-1 and -2), was dissolved in a vehicle of 20 mmol/l NaOH and 280 mmol/l D-glucose in distilled water. The EP1 receptor antagonist ONO 8711 (6-(2S,3S)-3-(4-chloro-2-methylphenylsulfonylamino)methyl)bicyclo[2.2.2]octan-2-yl)-5Z-hexanoic acid; Ono Pharmaceutical, Osaka, Japan) was dissolved in a vehicle of 20 mmol/l NaOH and 280 mmol/l D-glucose and then 100 ml of the solution was gassed with a 95% O2/5% CO2 mixture to adjust pH to 7.2 and then filtered through a sterile 0.2-μm cellulose nitrate filter (Nalgene, Rochester, NY). Outflow was directed to storage tubes on ice via PE-50 tubing. Experiments began with a 30-min wash-out period to accustom the animal to the experimental conditions, establish perfusion equilibrium, and ensure that there was no leakage from the system. This was followed by collection of three fractions, each of 10-min duration, to represent basal concentrations. The animal was then manually restrained and 50 μl of 0.5% formalin solution injected into the dorsal surface of the right hind paw before returning it to the observation chamber. Thereafter, dialysate was collected in 10-min fractions for up to 60 min, making allowance for the transit time of dialysate from spinal cord to collection vial. Each sample was immediately transferred to liquid nitrogen and then stored at −70°C until assay.

**Results**

Formalin-evoked behavior and spinal PGE2 release. Four weeks after streptozotocin injection, diabetic rats were hyperglycemic (32.0 ± 1.8 mmol/l) and had lower body weight (181 ± 8 g) compared with age-matched controls (5.2 ± 0.3 mmol/l and 240 ± 7 g; n = 6/group). Injection of 0.5% formalin into the hind paw produced a biphasic response in control rats with the active phases exaggerated within minutes of the injection of formalin, and this was maintained throughout the monitoring period (sum flinches counted over the 60-min observation period, 73 ± 8) (Fig. 1). In diabetic rats, flinching was exaggerated within minutes of the injection of formalin, and this was maintained throughout the monitoring period (sum flinches counted over the 60-min observation period, 110 ± 8; P < 0.01).
0.05 vs. controls by unpaired t test). Spinal microdialysis before paw formalin injection in these same animals showed no significant difference in basal PGE2 levels in the spinal CSF (controls, 2.9 ± 1.6 and diabetic rats, 3.3 ± 1.3 nmol/l). In the first 10 min after paw formalin injection of control rats, there was a marked increase in PGE2 levels in the spinal CSF dialysates to 234 ± 12% of basal levels, which then subsided toward basal levels (Fig. 2). Diabetic animals showed a similar initial increase in PGE2 (239 ± 17% basal levels), but unlike controls, this persisted into the subsequent 10-min period (227 ± 5% basal levels) before subsiding.

**Formalin-evoked behavior and prostaglandin inhibitors.** At the conclusion of these studies, diabetic rats exhibited weight loss and hyperglycemia (Tables 1 and 2). Paw thickness of diabetic rats was significantly (P < 0.001) lower than that of controls before formalin injection (Tables 1 and 2). Paw thickness increased by a similar amount in both control and vehicle-treated diabetic rats 60 min after formalin injection. This paw swelling was dose-dependently attenuated in diabetic rats treated systemically with indomethacin (Table 1) but did not change after intrathecal delivery of indomethacin (Table 2).

Diabetic rats treated intraperitoneally (i.p.) (Fig. 3) or intrathecally (i.t.) (Fig. 4) with vehicle before paw formalin injection displayed hyperalgesia, as indicated by a significant (P < 0.01) increase in the sum of flinches counted during the 60 min after formalin injection. Systemic delivery of the NSAID indomethacin before paw formalin injection produced a dose-dependent reduction in the hyperalgesia of diabetic rats (Fig. 3). Hyperalgesia in diabetic rats was also significantly attenuated (P < 0.05 vs. vehicle-treated diabetic rats) by intrathecal delivery of indomethacin (Fig. 4) or the EP1 receptor antagonist ONO 8711 (vehicle-treated diabetic rats, 96 ± 14; ONO 8711-treated diabetic rats, 53 ± 8 sum flinches per 60-min period, n = 6 and 10, respectively; P < 0.05) (Fig. 5).

**Cyclooxygenase protein in the spinal cord.** Portions of lumbar spinal cord were removed from control and diabetic rats (n = 5/group). The amount of COX-1 protein was significantly (P < 0.05) reduced in diabetic spinal cord compared with controls (Fig. 6), whereas there was a threefold increase (P < 0.01 vs. control) in levels of COX-2 protein. Densitometric data for COX-2 represent the

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Control rats</th>
<th>Diabetic rats</th>
<th>Diabetic + 6.7 mg/kg NSAID</th>
<th>Diabetic + 26.7 mg/kg NSAID</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>250 ± 3</td>
<td>195 ± 10*</td>
<td>210 ± 8*</td>
<td>173 ± 5*</td>
</tr>
<tr>
<td>Plasma glucose (nmol/l)</td>
<td>3.3 ± 0.3</td>
<td>26.6 ± 1.3*</td>
<td>21.5 ± 1.3*</td>
<td>23.5 ± 2.2*</td>
</tr>
<tr>
<td>Paw thickness: pre-formalin (mm)</td>
<td>3.40 ± 0.04*</td>
<td>2.90 ± 0.08*</td>
<td>2.93 ± 0.09*</td>
<td>2.73 ± 0.07*</td>
</tr>
<tr>
<td>Absolute change (mm)</td>
<td>1.07 ± 0.18</td>
<td>0.88 ± 0.12†</td>
<td>0.72 ± 0.09</td>
<td>0.45 ± 0.1†</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.001; †P < 0.05 vs. control rats.
bined doublet, and densitometry values for both COX-1 and -2 were normalized to total protein.

**DISCUSSION**

Injection of a dilute formalin solution into the paw has become a widely studied means of inducing a prolonged noxious stimulus, the magnitude of which can be assessed by measuring nocifensive responses that correlate with the concentration of formalin used. The protracted nature of the response has proven particularly useful for studying the electrophysiological and neurochemical characteristics of peripheral and spinal nociceptive processing, along with the efficacy of pharmacological interventions. Spinal microdialysis studies have shown that there is an immediate release of the neurotransmitters glutamate, aspartate, and substance P in the spinal cord after injection of formalin, but not vehicle, into the hind paw (14,17). This immediate PGE2 release is dependent on normal function of capsaicin-sensitive neurons and modulates flinching behavior during phase 2 of the test (28). Diabetes, which increased flinching behavior during the quiescent

![Diagram](image)

**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>Control rats</th>
<th>Control + 66.7 µg NSAID</th>
<th>Diabetic rats</th>
<th>Diabetic + 66.7 µg NSAID</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>245 ± 6</td>
<td>249 ± 3</td>
<td>193 ± 9*</td>
<td>182 ± 8*</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>4.2 ± 0.3</td>
<td>3.8 ± 0.2</td>
<td>25.3 ± 2.0*</td>
<td>25.2 ± 1.8*</td>
</tr>
<tr>
<td>Paw thickness: pre-formalin (mm)</td>
<td>3.70 ± 0.05*</td>
<td>3.65 ± 0.10*</td>
<td>3.00 ± 0.07*</td>
<td>3.07 ± 0.07*</td>
</tr>
<tr>
<td>Absolute change (mm)</td>
<td>1.07 ± 0.19</td>
<td>1.18 ± 0.22</td>
<td>0.79 ± 0.10</td>
<td>0.80 ± 0.24</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.001 vs. control rats.

 hypotheses that the increase in formalin-evoked nocifensive behavior seen in diabetic rats was secondary to increased primary afferent activity after paw formalin injection. However, formalin-evoked release of substance P (17) and glutamate (27) in the spinal cord was depressed rather than exaggerated in diabetic rats, presenting the apparent anomaly of depressed peripheral input to the spinal cord after noxious stimulation but exaggerated behavioral responses. This raised the possibility that spinal or supra-spinal mechanisms may be involved in amplification of sensory processing during hyperalgesia in the diabetic rat and prompted our current investigation of spinal prostaglandins.

Using a submaximal stimulus of 0.5% formalin in control rats, we confirmed our previous finding of a single burst of PGE2 release that immediately follows the injection (28). This immediate PGE2 release is dependent on normal function of capsaicin-sensitive neurons and modulates flinching behavior during phase 2 of the test (28). Diabetes, which increased flinching behavior during the quiescent
and second phases of the formalin test, did not alter the magnitude of PGE₂ release after paw formalin injection, but the period of release was extended. The source of the additional PGE₂ release is not known and could include any cells that either express the PGE₂-forming enzyme COX constitutively or in which it may be induced, including primary afferent neurons, spinal neurons that are stimulated by primary afferents, or spinal glia (29,30).

To test the association between protracted spinal PGE₂ release and exaggerated formalin-evoked flinching in diabetic rats, we pretreated diabetic rats systemically with the nonselective COX-1/COX-2 inhibitor indomethacin before formalin stimulation. This produced a dose-dependent inhibition of flinching, suggesting that COX activity contributes to the hyperalgesic state after paw formalin injection. However, because indomethacin also attenuated paw swelling arising from inflammation at the injection site, it was plausible that the effect was related to inhibition of the peripheral stimulus rather than modulation of spinal nociceptive processing. We have previously shown that spinal delivery of a COX inhibitor can suppress both formalin-evoked PGE₂ release and flinching behavior in normal rats (14). Confirmation of the role of spinal COX-derived prostaglandin release in the hyperalgesia seen in diabetic rats after paw formalin injection was provided by the efficacy of indomethacin when delivered spinally at doses that did not have peripheral effects on paw swelling. Further, the efficacy of a spinally delivered EP₁ receptor antagonist suggests that spinally released prostaglandins exert their effect in part via local EP₁ receptors in the spinal cord. Messenger RNA for EP₁ receptors is found within both dorsal root ganglia and spinal cord (31); therefore, the site of action of spinally released PGE₂ could include primary afferent terminals or cells of the spinal cord such as interneurons, glia, or vascular cells.

The prolongation of formalin-evoked spinal PGE₂ release in diabetic rats occurs in the context of diminished release of substance P and glutamate, so it is unlikely to result from protracted primary afferent input. Plausible alternative mechanisms to explain extended PGE₂ release include an increase in spinal receptors that are coupled to arachidonic acid release, thereby providing additional substrate for COX to produce PGE₂ after receptor stimulation, changes in the amount or activity of enzymes in the pathway that produces PGE₂, or a decrease in PGE₂ breakdown. To begin to investigate these possibilities, we measured the amount of COX protein isoforms in the spinal cord of normal and diabetic rats. Of the two isoforms, COX-1 is constitutive to many cell types, whereas COX-2 is generally considered inducible with gene expression triggered by external events, including exposure to proinflammatory cytokines such as interleukin-1β (32). However, there is recent evidence of constitutive expression of COX-2 in both neurons (33) and glia (34,35) of the spinal cord but not the dorsal root ganglia (33). It therefore appeared appropriate to examine levels of both isoforms of the enzyme.

Four weeks of diabetes induced a small decrease in COX-1 protein levels, which is consistent with a previous report of decreased COX-1 mRNA in peripheral nerve of diabetic rats (36). More strikingly, there was a threefold increase in the amount of spinal COX-2 protein in animals that were not used for formalin testing, indicating an increase in constitutive expression. We have not identified the cell types in which this increase occurs, and it is plausible that there may be either increased expression in cells that constitutively express COX-2, such as spinal neurons and glia, or induction of the enzyme in other cell types, such as primary afferent neurons that have central terminals within the dorsal horn of the spinal cord. COX-2 gene expression is known to be under a number of
regulatory mechanisms. The COX-2 gene promoter region contains consensus sequences for several nuclear transcription factors, thereby providing potential mechanisms for induction in response to extracellular stress events (37). In diabetic animals, these could include hyperglycemia per se or metabolic consequences of hyperglycemia, such as osmotic and oxidative stress. Interestingly, such insults have been reported to induce intracellular signaling through the p38 MAPK pathway (38,39), and p38 activation has been associated with induction of COX-2 gene expression in both neuronal and glial cells (40,41). Alternatively, because COX-2 undergoes suicide inactivation (42), it is plausible that the increase in COX-2 protein represents an accumulation of inactivated enzyme and reflects increased flux through the PGE$_2$ synthesis pathway arising from events upstream of COX-2.

We have found that diabetic rats have elevated COX-2 protein levels in their spinal cord and that hyperalgesia after a noxious stimulus is accompanied by protracted spinal PGE$_2$ release and can be alleviated by spinal delivery of a COX inhibitor or EP$_1$ receptor antagonist. This adds to a growing list of neurochemical abnormalities found in the spinal cord of diabetic rodents that may have an impact on spinal nociceptive processing (43–47) and highlights a developing appreciation that the spinal cord is a site of injury in diabetic patients (48,49). It also highlights a developing appreciation that the spinal cord is a site of injury in diabetic patients (48,49). It also

**ACKNOWLEDGMENTS**

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