Transient Up-regulation of Spinal Cyclooxygenase-2 and Neuronal Nitric Oxide Synthase following Surgical Inflammation

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Background: Surgery induces pain and hyperalgesia postoperatively. The products of cyclooxygenases and nitric oxide synthase (NOS) have been implicated in the development of inflammatory pain and hyperalgesia experimentally, and the use of drugs clinically that modify cyclooxygenase activity has been advocated in the management of perioperative pain. However, regulation of these enzymes following surgery has not been studied.

Methods: Adult female sheep (n = 12) undergoing a midline laparotomy for collection of ova were used in this study. Lumbar and cervical spinal cord tissue was collected from animals euthanized 1 day and 6 or 7 days after surgery and processed for cyclooxygenase (cyclooxygenase-1 and cyclooxygenase-2), neuronal NOS mRNA expression using reverse-transcription polymerase chain reaction and *in situ* hybridization. Tissues were also processed for NADPH-diaphorase staining and cyclooxygenase-1 and cyclooxygenase-2 protein expression by immunohistochemistry and Western blotting.

Results: No alteration in cyclooxygenase-1 or cyclooxygenase-2 mRNA or protein concentrations were detected in spinal cord by reverse-transcription polymerase chain reaction and Western blotting, respectively, at 1 day or 6 or 7 days after surgery. However, using techniques that localize mRNA and protein expression (*in situ* hybridization and immunohistochemistry, respectively), increases in cyclooxygenase-2 were identified in lamina V dorsal horn neurons in lumbar spinal cord 1 day after surgery. A significant increase in neuronal NOS mRNA was observed in lumbar spinal cord 1 day after surgery, localized to laminae I–II and lamina V neurons, which returned to baseline concentrations by 6 to 7 days. NADPH-diaphorase staining was significantly increased in laminae I–II in lumbar spinal cord 1 day after surgery but not after 6 to 7 days.

Conclusions: Spinal cyclooxygenase and neuronal NOS pathways are differentially altered following surgical inflammation. The early and transient nature of these changes suggests that these enzymes are implicated in postoperative pain and hypersensitivity.

SURGICAL pain is one of the most common causes of acute pain in humans. Pain from surgery occurs at rest and is exacerbated by patient movement and mechanical stimulation.[1] Mechanical hyperalgesia, increased responsiveness to noxious mechanical stimuli, has been observed in humans following surgery[1–3] and in compara-

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ble animal models,[4–8] which lasted for several days. Both peripheral nociceptor and spinal neuronal sensitization contribute to postinjury pain hypersensitivity.[9] Central sensitization is dependent on activation of dorsal horn *N*-methyl-D-aspartate (NMDA) receptors,[10,11] which have been linked to production of prostaglandins and nitric oxide (NO) through activation of cyclooxygenase and NO synthase (NOS) enzymes, respectively.[12–14] Treatment with nonsteroidal antiinflammatory drugs, which inhibit prostaglandin production,[15] attenuates postoperative pain in humans and pain and hyperalgesia in animals.[6,16] Two isoforms of cyclooxygenase exist (cyclooxygenase-1 and cyclooxygenase-2), both of which are constitutively expressed in spinal cord.[17,18] Evidence suggests that cyclooxygenase-2 is the dominant isoform associated with central pain transmission.[14,19,20] Cyclooxygenase-2 mRNA is induced in neurons in rat brain by intense neuronal activity[21] and in spinal cord following chemically induced peripheral inflammation[17,22,23] and spinal nerve ligation.[24] However, there is conflicting and sparse information regarding the laminar arrangement of cyclooxygenase isoenzyme mRNA expression in spinal cord and its modulation by peripheral inflammation, and there is no information regarding the expression following a surgical insult.

Nitric oxide, derived from L-arginine by the enzyme NOS, is a neuronal messenger involved in spinal nociceptive processing.[25] Three isoforms of NOS exist, constitutively expressed neuronal NOS (nNOS) and endothelial NOS, which are both Ca^{2+} calmodulin-dependent, and an inducible isoform that is Ca^{2+} calmodulin-independent. nNOS is present in the superficial dorsal horn of the spinal cord, suggesting a role in nociceptive information processing.[26] Pharmacologic studies have supported such a role for spinal NOS in nociceptive processing in normal animals[27] and following chemically induced peripheral inflammation,[28–30] and while the other isoforms of NOS, endothelial NOS and inducible NOS, are known sources of NO in spinal cord, nNOS has been shown to be dynamically regulated in response to inflammation in models of both acute and chronic pain.[31–34]

The NOS and cyclooxygenase pathways share a number of similarities, and a dynamic interaction between these two pathways has been identified.[35–38] The current study aimed to examine modulation of cyclooxygenase-1, cyclooxygenase-2, and nNOS mRNA expression in spinal cord by surgical inflammation, and to
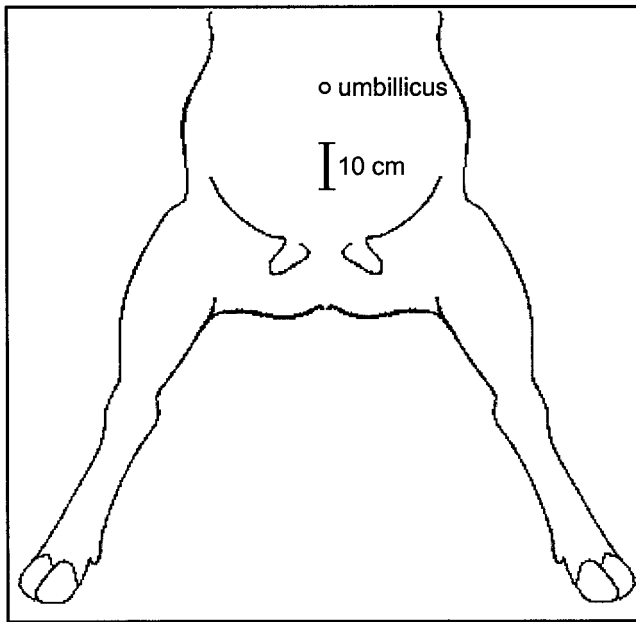
Fig. 1. Schematic diagram of abdominal region of sheep in dorsal recumbency, showing the midline incision.

identify if changes were associated with changes in protein expression or activity.

Materials and Methods

This study was conducted on animals as part of a study undertaken at the Roslin Institute, East Lothian, and was approved by the Institute's Ethics and Welfare committee and conducted under the UK Animal Scientific Procedures Act.

Animals

Spinal cord tissue was collected from adult female sheep (n = 12) undergoing a midline laparotomy for collection of ova (fig. 1). Tissue was taken from the cervical region (C3–C7) and from the lumbar region (L1–L4) of the spinal cord. Animals underwent standard superovulatory treatment according to methodology previously described.[39] Briefly, intravaginal progesterone sponges (medroxyprogesterone acetate [Veramix]; Genus Express, Falkirk, United Kingdom) were implanted 14 days before surgery, followed by twice-daily subcutaneous injections of follicle stimulating hormone (Ovagen; Synergy Products Ltd., Melksham, Wiltshire, United Kingdom) for 4 days before the removal of intravaginal sponges. Finally, on day 14, animals were injected intramuscularly with GnRH (Receptal; Hoechst Roussel Vet, Milton Keynes, Bucks, United Kingdom). Animals were allocated into two groups and were euthanized 1 day or 6 or 7 days after surgery. Spinal cord tissues were obtained in addition from two control groups of animals: (1) from superovulated sheep that did not undergo surgery (superovulated control), euthanized 1 day (n = 5)

and 7 days after treatment (n = 5); and (2) from untreated healthy female sheep (n = 6) that underwent no drug treatment or surgery. Once spinal cord was removed, tissue for use in mRNA expression studies and Western blotting was wrapped in parafilm, rapidly frozen, and stored at −70°C. Tissue processed for immunohistochemistry and diaphorase staining was placed in 4% paraformaldehyde overnight and then cryoprotected in 30% sucrose before freezing at −70°C.

Reverse-Transcription Polymerase Chain Reaction

Whole transverse sections of spinal cord were cut for use in these studies. Total RNA was extracted from lumbar and cervical spinal cord using RNAzol B™ (Biogenesis Ltd., Poole, Dorset, United Kingdom) and reverse transcribed using random hexamers (Promega, Southampton, Hampshire, United Kingdom) and Moloney-murine leukemia virus reverse transcriptase (Gibco-BRL, Paisley, United Kingdom). Polymerase chain reaction (PCR) was performed with Reddy-Load PCR Mix (ABgene, Epsom, Surrey, United Kingdom) and 200 nM each of 5′ and 3′ primers. PCR products were quantified by adding [^{32}P]dCTP (3 μCi/tube; ICN Pharmaceuticals, Basingstoke, Hampshire, United Kingdom) to the reaction mixture. The sequences of primers for PCR amplification were based on known sheep sequences (Genbank, National Center for Biotechechnology Information, Bethesda, MD): for cyclooxygenase-1 the sense primer was 5′-tcatcggggagaccatcaag and antisense primer was 5′-accaatccggcctgcaggct (product size 298 base pairs); for cyclooxygenase-2 the sense primer was 5′-ggtgcagcaaatccttgctg and antisense primer was 5′-gtgatcttcgacgtcaacac (product size 276 base pairs); for nNOS the sense primer was 5′-aaatccagtcgatcgaccatc and antisense primer was 5′-tgataaccccaaagccgtgt (product size 211 base pairs); and for β-actin the sense primer was 5′-tccttcctgggcatggaatc and antisense primer was 5′-gggcgcgatgatcttgatct (product size 204 base pairs). Where possible, primers were selected from two exons separated by one or more intron regions to prevent amplification of contaminating genomic DNA. The final 30-μl reaction mixture was heated at 95°C for 2 min followed by 22–34 cycles at 95°C for 35 s, 55°C for 1 min, and 72°C for 1 min. PCR products were electrophoresed on an agarose gel (1%) containing ethidium bromide. Gels were dried and bands cut out guided by ethidium bromide staining and radioactivity counted in a scintillation counter. Concentrations of mRNA were measured semiquantitatively relative to β-actin mRNA by measuring the quantity of radiolabeled nucleotide incorporated during the exponential phase of amplification.[40] Determination of the exponential phase was achieved by running duplicate tubes and terminating the reaction at sequential cycles.

Table 1. Oligonucleotide Probes Designed for *In situ* Hybridization Studies

Probe	Sequence (5′-3′)	Target Site	Accession No.
COX-1	GGTCTATGTTCCTACCCCCACCAATCCGGCCTGCAGGCTGGCG	1290–1332	M18243
COX-2	AAGTTGGCGGACTCTCAATCAAATGTGATCTCGACGTCAACAC	385–427	U68486
nNOS	GCGGCTGAGGAGTCGAGCGGCTGAGACCCGCTTCTTGTGGACA	3–45	U76739

nNOS = neuronal nitric oxide synthase.

In Situ *Hybridization*

In situ hybridization was conducted as described by Wisden and Morris.[41] Spinal cords were sectioned at 15 μm on a cryostat at $-20°$C. Spinal cord sections from an untreated control, a superovulated control, and surgical animal were thaw mounted on poly-L-lysine–coated slides and air dried before being fixed in ice-cold 4% paraformaldehyde for 5 min. Slides were then rinsed for a few minutes in 1 $\times$ phosphate-buffered saline (PBS), 70% ethanol, and 95% ethanol, and transferred to 100% ethanol where they were stored until required. *In situ* hybridization was performed with 43/44mer cRNA oligonucleotide probes (5 ng/μl) directed against cyclooxygenase-1, cyclooxygenase-2, and nNOS (table 1), based on known sheep sequences (Genbank, National Center for Biotechechnology Information). Oligonucleotides were labeled with 10 pmol of [α-^{35}S]dATP (NEN Life Sciences Products, Hounslow, United Kingdom) using terminal deoxynucleotidyl transferase (25 U/ml; Roche Diagnostics, Lewes, East Sussex, United Kingdom). Labeled probes were diluted in hybridization buffer (50% deionized formamide, 4 $\times$ SSC, 10% dextran sulfate, 100 μl/μl polyadenylic acid, 25 μM sodium phosphate, pH 7, and 1 μM sodium pyrophosphate) to a concentration of 0.3 pmol/5,000 μl. Hybridization solution was applied to air-dried slides, which were then covered with parafilm and incubated overnight at 42°C. Slides were washed briefly in 1 $\times$ SSC at room temperature followed by 1 $\times$ SSC at 60°C for 30 min, then passed through a series of room temperature rinses (1 $\times$ SSC, 0.1 $\times$ SSC, 70% ethanol, and 95% ethanol). Slides were air dried and exposed to film (Kodak X-Omat; Sigma, Poole, Dorset, United Kingdom) for 2 weeks before being dipped in Illford K5 emulsion diluted to a 1:1 ratio with prewarmed water–0.5% glycerol. After exposure for 8–12 weeks at 4°C, the dipped slides were allowed to warm to room temperature and were developed in D19 developer (Kodak, Sigma), rinsed in deionized water, and fixed. Finally, sections were counterstained using a standard hematoxylin and eosin histologic technique, dehydrated in an ascending ethanol series, transferred to histoclear, and coverslipped. Nonspecific labeling was identified using the same labeled probe in the presence of 50-fold excess unlabeled probe on adjacent sections.

The mean number of silver grains per neuron (a minimum of 4–10 cells from each area) was calculated for neurons in laminae I–II, lamina V, and ventral horn motoneurons. From these data, the mean number of silver grains per neuron was calculated for each animal (four sections per animal). The area of each cell was also measured using a computerized image analysis system (Scion Image, v5.2; Scion Corporation, Frederick, MD).

Western Blotting

Western blots were performed on protein extracts from superovulated control and surgical animal spinal cord tissue, and on cyclooxygenase-1 and cyclooxygenase-2 (ovine) electrophoresis standards (Cayman Chemical, Ann Arbor, MI) to show specificity of antisera. Spinal cord samples were homogenized in 1 ml 0.1 M PBS using Ribolyser matrix tubes and a Ribolyser (Thermo Hybaid, Ashford, Middlesex, United Kingdom). The lysate was transferred to a fresh tube and spun at 12,500 rpm for 2 min. The supernatant was removed and the pellet washed two times in ice-cold PBS and spun at 12,500 rpm for 2 min. PBS was aspirated, and the pellet was resuspended in ice-cold lyses buffer (1% Nonidet P-40; 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in PBS) with freshly added protease inhibitors (Complete Cocktail; Roche Diagnostics, Lewes, East Sussex, United Kingdom), and incubated for 30 min on ice. Tubes were centrifuged at 12,500 rpm for 20 min, and supernatants containing the protein lysates were collected and aliquots stored frozen at $-70°$C. Supernatants were diluted in PBS to a final protein content of 0.1 μg/μl (determined using the BCA method according to the method described by Smith *et al.*[42]) and incubated for 10 min at 95°C. Protein lysates (10 μg) were loaded onto NuPage Novex 4–12% BisTris gels (Invitrogen) and run for 45 min at 200 V. Proteins were transferred onto PVDF membranes (Invitrogen) at 30 V for 1 h using a semidry blotting system. Membranes were blocked in PBS–5% skimmed milk–0.1% Tween20 for 1 h at room temperature, followed by overnight incubation at 4°C with cyclooxygenase-1 ovine polyclonal (1:1,000) or cyclooxygenase-2 murine polyclonal (1:1,000) primary antisera (Cayman Chemical) diluted in blocking buffer. Immunoblots were then incubated for 1 h at room temperature with horseradish–peroxidase linked secondary antibody diluted 1:1,000 in blocking buffer. Immunoblots were developed by enhanced chemiluminescence ECL (Amersham Biosciences, Little Chalfont, Bucks, United Kingdom) and visualized on Kodak BIOMAX MR X-ray film (Sigma). Immunoreactivity was quantified using densitometric analysis on Scion Image v.5.2 (Scion Corporation) software.

Fig. 2. Reverse-transcription polymerase chain reaction amplification of β-actin, neuronal nitric oxide synthase (nNOS), and cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) mRNA extracted from spinal cord from an untreated control animal (*A*) and 1 day after surgery (*B*) resulted in polymerase chain reaction products of 204, 211, 276 and 298 base pairs, respectively, as expected. The reaction was run in quadruplicate and terminated at sequential cycles to determine the exponential phase of amplification.
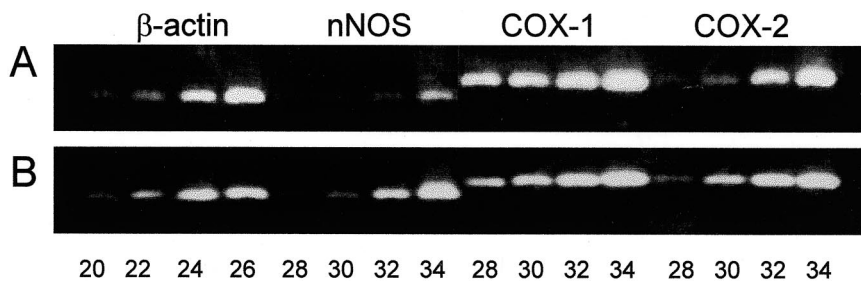
Immunohistochemistry

Lumbar and cervical spinal cord was sectioned (30 μm) and processed for cyclooxygenase-1 and cyclooxygenase-2 immunoreactivity using standard biotin-avidin techniques (ABC Elite kit; Vector Laboratories, Southgate, Peterborough, United Kingdom). Superovulated control and surgical animal spinal cord sections were slide-mounted and postfixed in 4% paraformaldehyde at 4°C and pretreated with 0.1 M PBS and 20% normal goat serum (Sigma) for 1 h. Sections were incubated overnight at 4°C in PBS containing 0.1% Triton X100, 20% normal goat serum, and cyclooxygenase-1 ovine polyclonal (1:10,000) or cyclooxygenase-2 murine polyclonal (1:10,000) primary antisera (both Cayman Chemical), followed by incubation at room temperature for 1 h in biotinylated secondary antisera (1:1,000) and avidin-coupled peroxidase (Vector Laboratories). All incubations were preceded by three rinses in PBS. Finally, sections were treated with Vectastain ABC elite kit and visualized using 3,3'-diaminobenzidine substrate kit (Vector Laboratories). After rinsing in PBS, slides were dehydrated and coverslipped for examination. Control sections were generated using the above protocol but omitting the primary antiserum. Immunopositive cells in laminae I–II and lamina V were counted on each section (four sections per animal), and the mean number of immunopositive cells per lamina was calculated for each animal.

NADPH-Diaphorase Staining

Spinal cord sections, lumbar and cervical (30 μm), from superovulated control, untreated control, and surgical animals were slide-mounted and postfixed in 4% paraformaldehyde at 4°C. Slides were rinsed in 0.1 M PBS containing 0.25% Triton X100, incubated for 5 min at 25°C, and then overnight at 37°C in the dark in PBS–Triton X100 containing 5 μM NADPH and 0.25 μM nitroblue tetrazolium (Sigma), as described previously by Morris *et al.*[43] After rinsing in PBS, slides were dehydrated and coverslipped for examination. The mean optical density of tissue staining in lamina I–II and in cells in lamina V was measured (four sections per animal) using Scion image v.5.2 (Scion Corporation). The mean optical density of unstained background area was measured and subtracted from all subsequent measurements.

Statistical Analysis

Statistical analyses of data were conducted using either one-way analysis of variance or analysis of variance adopting a general linear model routine, with *post hoc* Tukey test (Minitab, v. 12.1; Minitab Inc., State College, PA). Treatment (control or surgical), laminae (lamina I–II or V) and time (1 or 6 or 7 days) were considered fixed factors, and animals were considered as a random factor. A significance level of 5% was applied.
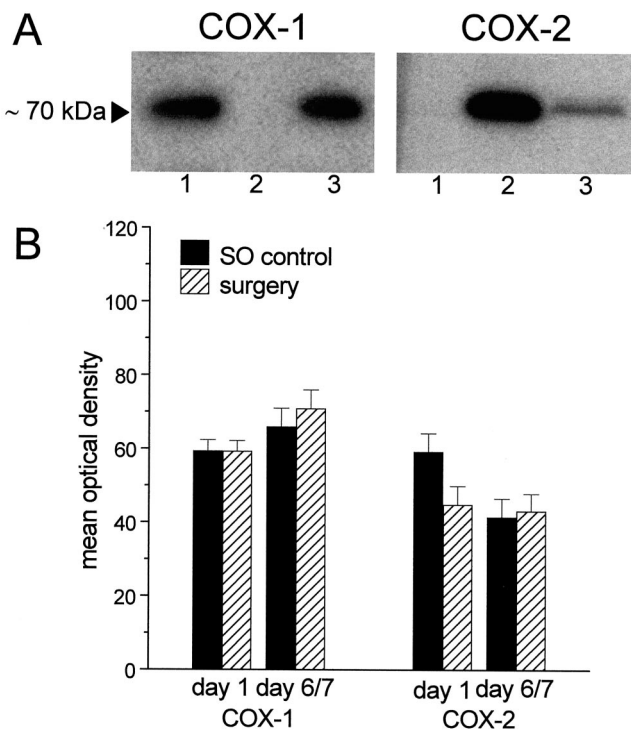
Fig. 3. (*A*) Western blots for cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) using 30 ng purified ovine cyclooxygenase-1 (1), 100 ng purified ovine cyclooxygenase-2 (2), and 10 μg protein extracted from lumbar spinal cord of an untreated control animal (3). (*B*) Densitometric quantification of cyclooxygenase-1 and cyclooxygenase-2 immunoreactivity on Western blots of proteins from lumbar spinal cord 1 and 6 or 7 days after surgery, and from superovulated (SO) control animals. Bars represent band optical density ± SEM.

Results

Expression of Cyclooxygenase

Reverse transcription (RT) PCR on tissue from untreated control animals indicated that cyclooxygenase-1 and cyclooxygenase-2 mRNAs are constitutively expressed in sheep spinal cord (fig. 2). *In situ* hybridization studies revealed that both cyclooxygenase isoforms displayed a widespread but heterogeneous pattern of expression within the gray matter in normal sheep. Cyclooxygenase-2–labeled neurons were most commonly observed in superficial (laminae I–II) and deep (lamina V) layers of the dorsal horn, as well as in large motoneurons in the ventral horn. More widespread distribution of cyclooxygenase-1–labeled cells was observed in all laminae in dorsal horn as well as in neurons in the ventral

horn and in lamina X, surrounding the central canal. There was no difference in cyclooxygenase-1 or cyclooxygenase-2 distribution between cervical and lumbar spinal cord. The diameters of cyclooxygenase mRNA-positive cells measured 8–15 μm in laminae I–II and 10–30 μm in lamina V. These are consistent with neuronal cells. Superovulatory treatment had no effect on cyclooxygenase mRNA isoform expression measured by RT-PCR or *in situ* hybridization. There was no change in cyclooxygenase-2 mRNA or protein concentrations measured by RT-PCR (fig. 4) and Western blotting (fig. 3) in homogenized lumbar spinal cord tissue 1 day or 6 or 7 days after surgery compared with untreated control or superovulated control animals. However, the use of *in situ* hybridization to measure cyclooxygenase-2 mRNA expression in spinal cord tissue sections indicated a
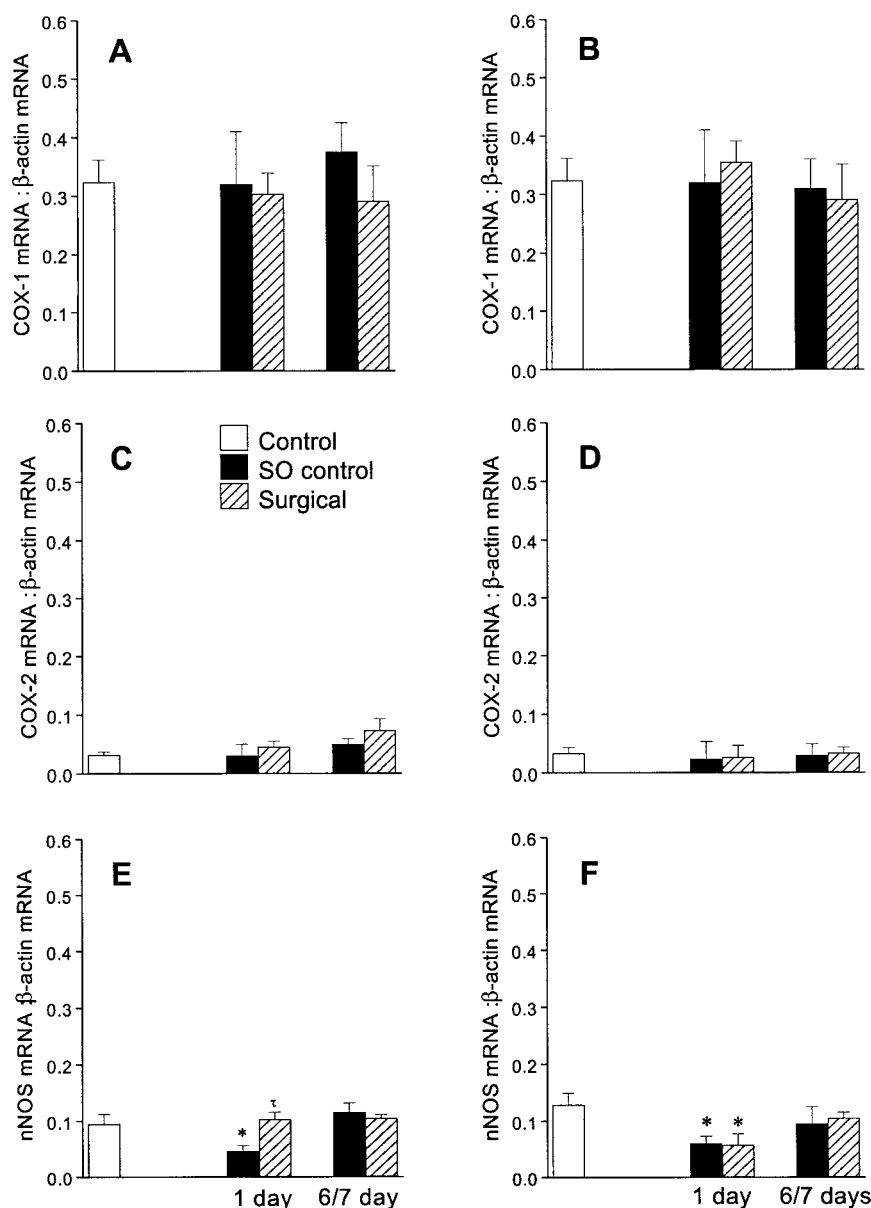

Fig. 4. Semiquantitative measurement of cyclooxygenase-1 (COX-1; *A*, *B*), cyclooxygenase-2 (COX-2; *C*, *D*), and neuronal nitric oxide synthase (nNOS; *E*, *F*) mRNA in lumbar (*left*) and cervical (*right*) spinal cord from sheep 1 day and 6 or 7 days after surgery compared with two control groups (superovulated [SO] control and untreated control animals) using reverse-transcription polymerase chain reaction. Concentrations of mRNA are represented relative to expression of the housekeeping gene β-actin. Each bar represents the mean ± SEM. Significantly different from SO control animals ($^{\tau}P$ < 0.05) and from healthy animals (*P < 0.05).

Fig. 5. Photomicrographs of dipped nuclear emulsion sections counterstained using standard hematoxylin and eosin histology, showing hybridization signals for cyclooxygenase-1 mRNA in lamina V lumbar spinal cord from a superovulated control animal (*A*), an untreated control animal (*B*), 1 day after surgery (*C*), and a negative control hybridization experiments conducted in the presence of 50-fold excess unlabeled probe (*D*). Scale bar = 20 μm.
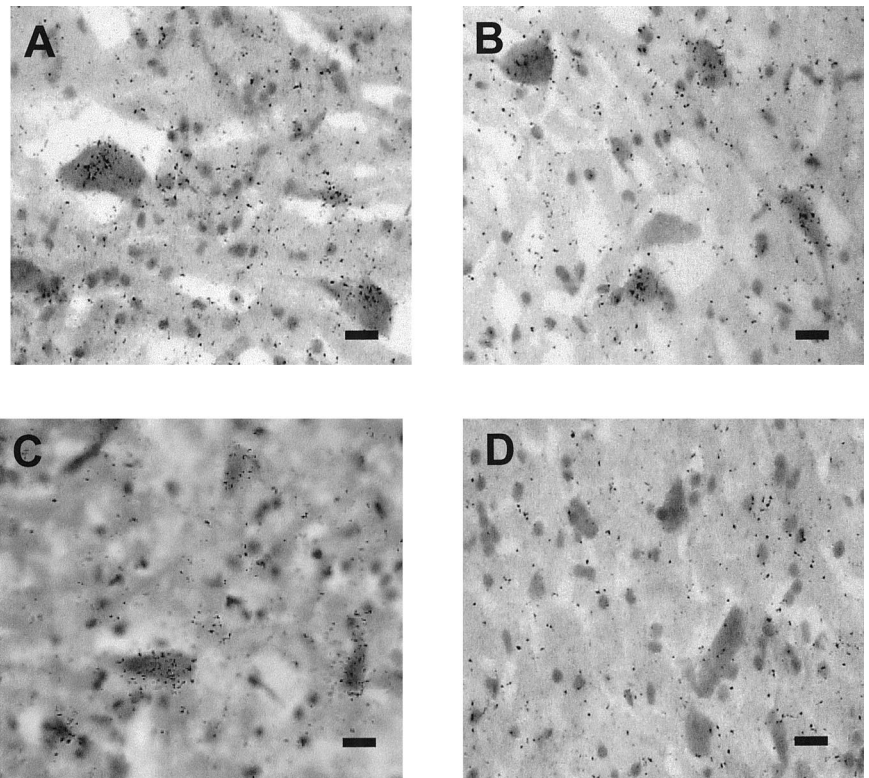
significant localized increase in lamina V neurons 1 day after surgery (twofold increase; $P < 0.001$; fig. 6), but not in neurons in laminae I–II or in ventral horn motoneurons (table 2). Changes in cyclooxygenase-2 mRNA expression were bilateral. On day 6 or 7, there was no difference in cyclooxygenase-2 mRNA in lamina V or in any other neurons compared with control concentrations from superovulated control and untreated control animals. A similar localized increase in cyclooxygenase-2 protein was detected in lumbar spinal cord sections by immunohistochemistry. The mean number of immunopositive neurons in lamina V increased from 10 ± 1 in

Fig. 6. Photomicrographs of dipped nuclear emulsion sections counterstained using standard hematoxylin and eosin histology, showing hybridization signals for cyclooxygenase-2 mRNA in lamina V lumbar spinal cord from a superovulated control animal (*A*), an untreated control animal (*B*), and 1 day after surgery (*C*), and a negative control hybridization experiment conducted in the presence of 50-fold excess unlabeled probe (*D*). Scale bar = 10 μm.
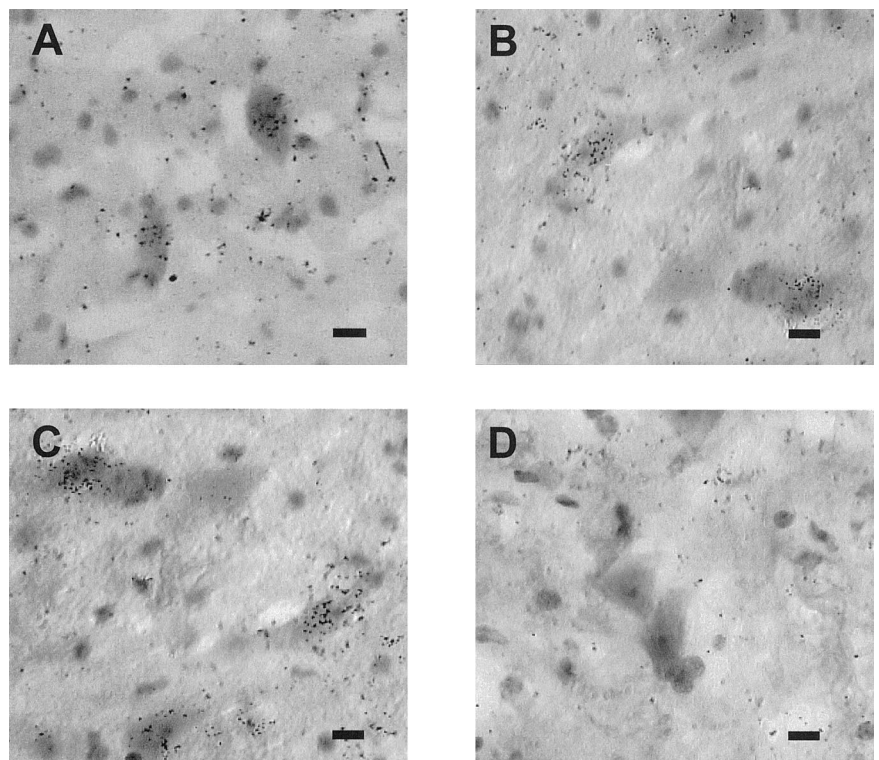
Table 2. Expression of COX-1, COX-2, and Neuronal Nitric Oxide Synthase Messenger RNA in Lumbar Spinal Cord Measured by *In situ* Hybridization

	COX-1			COX-2			nNOS		
	Untreated Control	SO Control	1 Day After Surgery	Untreated Control	SO Control	1 Day After Surgery	Untreated Control	SO Control	1 Day After Surgery
Laminae I–II	7 ± 1	8 ± 1	8 ± 1	10 ± 1	12 ± 1	9 ± 1	8 ± 2	7 ± 1	16 ± 2†
Lamina V	35 ± 10	46 ± 9	38 ± 6	23 ± 3	27 ± 4	57 ± 12*	31 ± 8	26 ± 5	74 ± 9†
Motoneuron	65 ± 14	104 ± 26	89 ± 9	43 ± 6	58 ± 7	53 ± 4	33 ± 8	37 ± 6	53 ± 10

Data shown are mean number of silver grains per cell ± SEM for 5 or 6 animals.

*$P < 0.05$, †$P < 0.01$; significantly different from SO control and from untreated control animals.

nNOS = neuronal nitric oxide synthase; SO = superovulatory.

superovulated control animals to 16 ± 0.8 neurons 1 day after surgery ($P < 0.01$). There was no change in the number of immunopositive cells in laminae I–II 1 or 6 or 7 days after surgery. The percentage increase in cyclooxygenase-2–immunopositive cells after surgery relative to superovulatory controls are plotted in figure 7.

No change in cyclooxygenase-2 mRNA (RT-PCR; fig. 4) or protein expression (immunohistochemistry; fig. 7) in cervical spinal cord was detected on day 1 or day 6 or 7 after surgery.
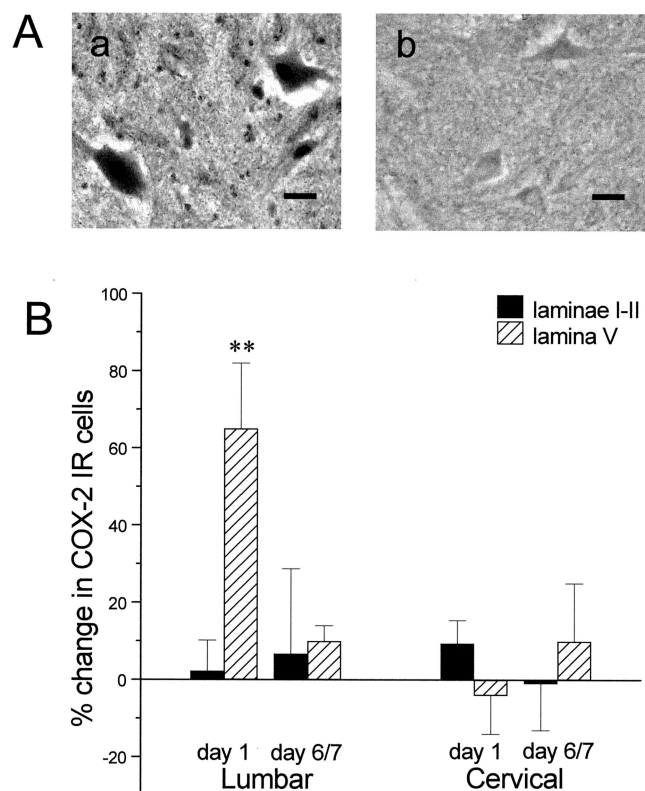

Fig. 7. (*A*) An example of cyclooxygenase-2–immunoreactive staining in lamina V neurons in spinal cord from an untreated control animal (a) and in the absence of primary antibody (b). Scale bar = 20 μm. (*B*) Mean number of cyclooxygenase-2 (COX-2) immunoreactive (IR) cells ± SEM in laminae I–II and lamina V lumbar and cervical spinal cord 1 day (n = 6) and 6 or 7 days (n = 6) after surgery are plotted as the percentage change relative to superovulatory control animals (n = 6). Significant increase from control: **$P < 0.01$.

Cyclooxygenase-1 mRNA (RT-PCR, fig. 4; *in situ* hybridization, fig. 5) and protein expression (Western blotting, fig. 3; immunohistochemistry) did not change in lumbar or cervical spinal cord on day 1 or day 6 or 7 after surgery compared with untreated control animals.

Expression of Neuronal Nitric Oxide Synthase

Neuronal NOS PCR products were detected in all spinal cord tissue examined, and *in situ* hybridization studies revealed expression in neurons throughout the spinal cord gray matter but most prominent in the superficial dorsal horn region. Superovulatory treatment alone markedly decreased nNOS mRNA expression in lumbar and cervical spinal cord 1 day after treatment compared with untreated control animals (fig. 4). This difference was not present on day 6 or 7 after superovulatory treatment. Animals that underwent surgery had significantly increased nNOS mRNA expression in lumbar but not cervical spinal cord 1 day after surgery compared with superovulated control animals (RT-PCR, twofold increase; $P < 0.01$) but not compared with untreated control animals (fig. 4). The increase in nNOS mRNA in lumbar spinal cord 1 day after surgery detected by RT-PCR was confirmed by *in situ* hybridization (table 2 and fig. 8). Localization and quantification of spinal nNOS mRNA by *in situ* hybridization revealed that, 1 day after surgery, nNOS mRNA was markedly increased in lamina I–II neurons (2.5-fold increase; $P < 0.05$) and in lamina V neurons (fourfold increase; $P < 0.001$) compared with both superovulated control and untreated control animals. These changes were bilateral.

NADPH-diaphorase staining was present in somata and fibers of neurons and surrounding neuropil in lamina I–II and in motoneurons in the ventral horn in lumbar and cervical spinal cord. Superovulatory treatment alone markedly decreased NAPDH-diaphorase staining in lumbar and cervical spinal cord 1 day after treatment compared with untreated control animals (decrease 30.0 ± 10% and 28.3 ± 13%, respectively; both $P > 0.05$). A significant increase in NADPH-diapahorase staining was detected in laminae I–II in lumbar spinal cord 1 day after surgery relative to superovulatory control animals (in-
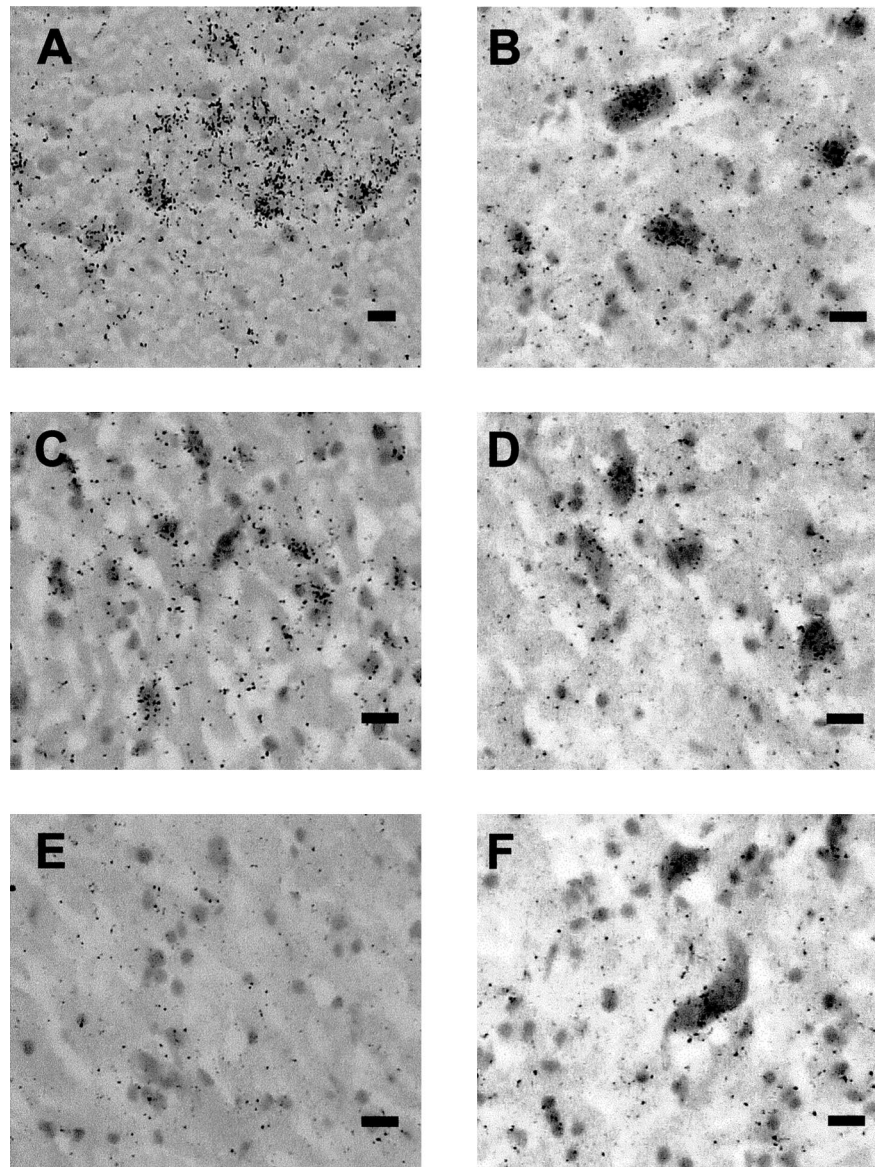
Fig. 8. Photomicrographs of dipped nuclear emulsion sections counterstained using standard hematoxylin and eosin histology, showing hybridization signals for neuronal nitric oxide synthase (nNOS) mRNA in laminae I–II (*left*) and lamina V (*right*) lumbar spinal cord 1 day after surgery (*A*, *B*) compared with a superovulated control animal (*C*, *D*), an untreated control animal (*F*), and a negative control hybridization experiments conducted in the presence of 50-fold excess unlabeled probe (*E*). Scale bar = 20 μm.

crease $43.3 \pm 9\%$; $P < 0.01$) and untreated control animals (increase $17.4 \pm 4\%$; $P < 0.05$). By day 6 or 7 after surgery, NADPH-diaphorase staining intensity was similar to control levels. No change was detected in NADPH-diaphorase staining in cervical spinal cord compared with superovulatory and untreated control animals.

Discussion

Pathologic pain has been classified into inflammatory and neuropathic pain, which frequently differ in their character and manifestation.[44] Inflammatory pain can take many forms but may be characterized as acute or chronic. Many models of inflammation have been developed to study the mechanisms of induction and maintenance of inflammatory pain and the associated hyperalgesia and allodynia. Chemical irritants such as carrageenan and complete Freund adjuvant have been

studied,[45,46] and while they have contributed to our current understanding of inflammation-induced alterations in nociceptive pain processing, they do not mimic surgical inflammation and pain. Some studies have focused on incisional pain and hyperalgesia[5-8]; however, the spinal mechanisms that contribute to development of behavioral hypersensitivity observed postoperatively are less well understood.

The innervation of the surgical site and the viscera under manipulation is served by the lumbar spinal cord (L1–L4) with some small input from T13,[47] and the lumbar spinal cord tissues harvested were from this region. The cervical cord was used as a "within-animal" control tissue to determine the specificity of neuronally induced changes in spinal cord activity and to differentiate these from those induced by mechanisms other than peripheral nerve activity, *e.g.*, factors mediated *via* the circulation.

Constitutive expression of both cyclooxygenase-1 and cyclooxygenase-2 mRNA was observed in spinal cord, confirming previous results in sheep[18] and in spinal cord[17,48] and brain[21] from normal rats. Cyclooxygenase-2 protein expression has also been identified in rat spinal cord, localized to superficial and deep dorsal horn laminae and in lamina IX motoneurons in untreated animals.[48,49] No change was observed in constitutive concentrations of cyclooxygenase-1 mRNA or protein expression in response to surgery. This is in agreement with previous studies, which have failed to detect any alteration in cyclooxygenase-1 following inflammatory stimulation with chemical agents.[17,22,48] Up-regulation in cyclooxygenase-2 mRNA and protein in lamina V neurons was detected bilaterally 1 day after surgery, restricted to lumbar spinal cord. The bilateral effect observed was clearly caused by the location of the incision, on the midline of the abdomen and bilateral handling of the reproductive organs. The localized changes were not detected by RT-PCR or Western blotting, which measured mRNA and protein concentrations in whole transverse homogenized spinal cord sections. Given the discrete and highly localized up-regulation in lamina V, this is perhaps not unexpected. Localization of cyclooxygenase-2 to lamina V neurons in lumbar spinal cord is consistent with cyclooxygenase products being involved in nociceptive processing, particularly in the development of altered sensitivity to Aβ or Aδ fiber activation, and with visceral handling. Laminae I–II and V are intimately associated with nociceptive processing. Central terminals of primary afferent nociceptors terminate in ordered spatial locations within the spinal cord, with high-intensity thinly myelinated (Aδ-) and unmyelinated (C-) fiber input terminating in laminae I–II, while lower threshold, large-diameter myelinated (Aβ-) fibers terminate in laminae III–V.[50] Lamina V neurons also receive input from Aδ-fibers supplying mechanical nociceptors, and from afferent fibers in laminae I–II *via* dendrites reaching into superficial layers.[51] Neurons in lamina V are also activated by mechanical stimuli that damage the skin[52] and are sensitized by plantar incision.[53] It is therefore interesting that cyclooxygenase-2 concentrations were unchanged in laminae I–II neurons on day 1. Previous evidence suggests that cyclooxygenase-2 induction is a rapid, transient event. For example, significant increases in spinal cyclooxygenase-2 mRNA were observed 2–6 h after adjuvant-induced inflammation,[22,23] 4–6 h after carrageenan-induced inflammation,[54] and 2 h after spinal cord injury.[55] Thus, changes in cyclooxygenase-2 expression in laminae I–II neurons may have occurred in this study at an earlier time point and returned to basal concentrations within 24 h of surgery. This temporal differentiation in spatial expression in dorsal horn has been described for the immediate early gene following inflammation and long-term stimulation.[56–58] These findings suggest that, although both superficial and deep layers are involved in nociceptive processing, they may be maximally involved at different stages during the inflammatory response.

A recent study reported widespread induction of cyclooxygenase-2 mRNA and protein in lumbar and cervical spinal cord and other regions of the central nervous system following inflammation, and suggested a nonneuronal (*e.g.*, circulating cytokine) mechanism for induction of cyclooxygenase.[59] However, in the current study, no alteration in cyclooxygenase-2 mRNA or protein expression was observed in cervical spinal cord, suggesting that localized cyclooxygenase-2 induction is a consequence of altered peripheral nerve input from the surgical region and is likely to be important in the development of perioperative pain and or hyperalgesia. The marked up-regulation of cyclooxygenase-2 observed in lumbar spinal cord 1 day after surgical inflammation suggests that this isoenzyme is responsible for increased spinal prostanoid release and associated activity-dependent plasticity following surgical inflammation.[14] Previous work in rodents supports the role of spinal cyclooxygenase enzymes in the development of hyperalgesia,[13] and a study in sheep undergoing the same surgical procedure as reported here indicated that cyclooxygenase inhibitors (nonselective) were effective in attenuating the development of perioperative hyperalgesia,[4] suggesting that these inhibitors may have a spinal site of action.

In spinal cord tissue obtained from superovulated sheep that did not undergo surgery, there was a marked reduction in nNOS mRNA concentrations and in NADPH-diaphorase staining, a measure of dynamically active NOS,[43] indicating an effect of the superovulatory treatment. In the neuroendocrine system, NO has been shown to regulate gonadotropin releasing hormone (GnRH) secretion from the hypothalamus,[60] and luteinizing hormone secretion from the pituitary.[61] In agreement with these studies, nNOS expression has been detected in the anterior pituitary[61] and hypothalamus.[62] Part of the superovulatory program involved treating the animals with GnRH. Interestingly, we have recently identified GnRH and GnRH mRNA receptor expression in sheep spinal cord (Dolan S, Glasgow, United Kingdom, unpublished observations, 2002), and while further work is required, these data suggest that an interaction between GnRH and NO may exist in spinal cord.

Relative to superovulated controls, there was a bilateral increase in nNOS mRNA observed in lumbar spinal cord dorsal horn 1 day after surgery. These data suggest that nNOS activity is involved in the early spinal changes that occur following surgery. NADPH-diaphorase staining was also increased, supporting the functional significance of the nNOS mRNA data. Although diaphorase staining is not specific for nNOS (it also reflects endothelial NOS and inducible NOS activity), the parallel effects of surgery on diaphorase and nNOS mRNA are strongly supportive of increased nNOS activity. Up-reg-

ulation of nNOS mRNA was localized to laminae I–II and lamina V neurons, laminae intimately associated with nociceptive processing. Previous studies have reported up-regulation in nNOS immunoreactivity in superficial dorsal horn neurons in acute formalin-induced inflammation in rodents.[30,63] In contrast, bilateral decreases in NOS activity following peripheral nerve injury[64] and in nNOS immunoreactivity[18] in lumbar spinal cord have been described in models of chronic inflammation. Down-regulation of nNOS is thought to be a consequence of the chronicity of the inflammatory stimulus, and in a review by Callsen-Cencic *et al.*,[65] it was hypothesized that nNOS expression is regulated by the type, frequency, and intensity of the afferent stimulus. In our study, nNOS mRNA concentrations and NADPH-diaphorase staining had returned to normal by 6 or 7 days after surgery, indicating that the stimulus is not long-lasting and is not intense. The rapid up-regulation of nNOS in nociceptive signaling pathways suggests that production of NO may be significant in the development of a postsurgical inflammatory response, although intrathecal administration of a NOS inhibitor, L-NAME, was reported to have no effect on mechanical hyperalgesia induced by plantar incision in rat.[66] The link between this response and behavioral hyperalgesia previously documented in this model[4] will be the subject of future work.

The interrelationship between NOS and cyclooxygenase enzymes is of interest. Neuronal NOS and cyclooxygenase-2 induction in lamina V neurons in response to surgical inflammation would lead to increased NO and prostaglandin production. Reports have shown that cyclooxygenase enzymes are regulated by NO production (see review by Salvemini[38]), and prostaglandin E_2 induces NO release in spinal cord slice cultures, which is dependent on NMDA receptor activation.[67] In addition, cyclooxygenase-2 is up-regulated in hippocampus in response to NMDA receptor activation,[21] and previous work in this laboratory has shown that NMDA-induced allodynia is dependent on spinal cyclooxygenase-2 and NOS activity.[27] This evidence suggests that, upon activation of spinal nociceptive pathways, for instance, following surgical inflammation, the NO and prostaglandin signaling systems interact to regulate the spinal responses to the inflammatory process. The differential up-regulation (NOS activity in laminae I–II and V and cyclooxygenase-2 activity in lamina V) is worthy of further investigation. The use of cyclooxygenase-2 and nNOS selective inhibitors may be indicated in the management of postoperative pain and hypersensitivity. Given the spinal induction of the enzymes, epidural and intrathecal modes of delivery should be the subject of further experimental studies.

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