I. Introduction

Prostaglandin (PG) is well known as an important mediator of immune, inflammation and pain transmission. Cyclooxygenase (COX) is a catalytic enzyme which produces the prostaglandins from arachidonic acids (Smith et al., 1991). Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used for acute pain and inflammatory pain such as rheumatoid arthritis (Boni et al., 1999; Gordon et al., 2002; Tang et al., 2002) as well as many types of pain associated with surgery. In past decades, the characteristics of COX isoforms, COX-1 and COX-2 have been studied. Recently, COX-3, a variant of COX-1 is discovered (Schwab et al., 2003). COX-1 is constitutively expressed, and COX-2 is remarkably induced by inflammation, cytokine or mitogenic stimuli (Feng et al., 1993). Many researchers reported the analgesic effects of COX-2 inhibitors on the patients requiring analgesics continuously (Day et al., 2000; Matsumoto et al., 2002). However, the effect of NSAID on neuropathic pain is not controversial although NSAID can modulate the pain on tissue-injury pain model (Hempenstall and Rice, 2002; Ripamonti et al., 1996), animals following postoperative pain (Kroin et al., 2002; Yamamoto et al., 2000), with diabetes (Freshwater et al., 2002), and following a partial sciatic nerve ligation (Lashbrook et al., 1999), as well as for pain induced by formalin (Collins and Davies, 1998), spinal cord contusion (Hains et al., 2001), carrageenan (Buritova et al., 1996), and monoarthritis (Mazario et al., 2001).
Peripheral nerve injury induced the neuropathic pain (Kim and Chung, 1992). When the peripheral nerve is injured, the changes are neurodegeneration (Wallerian degeneration) following initial inflammation according to nerve injury (George et al., 2004; Ma and Eisenach, 2002; Ma and Eisenach, 2002). Injured peripheral afferent nerve increases the afferent signals to the spinal cord, and secondarily occurs in the spinal cord, which is regarded as central sensitization (Bennett and Xie, 1988; Kroin et al., 2002). The development and maintenance on neuropathic pain is associated with central and peripheral mechanism (Zimmermann, 2001). It is not also clarified that COX-2 is involved with neuropathic pain, although there are some evidences about that (Day et al., 2000; Hains et al., 2001; Suyama et al., 2004; Syriatowicz et al., 1999; Takahashi et al., 2004). Zhu and Eisenach (2003) reported that the COX-1 protein increased in the spinal cord 4 days after nerve injury. The intrathecal treatment of a nonselective inhibitor, indomethacin reduced the early development of tactile allodynia, not maintenance in spinal nerve injury (Zhao et al., 2000). The development of hypersensitivity following CCI model nerve injury can be attenuated with nonselective COX inhibitor, naproxen, but not a selective COX-2 inhibitor, rofecoxib (Padi and Kulkarni, 2004). Etodolac, a COX-2 inhibitor alleviated evoked hyperalgesia in the CCI model (Suyama et al., 2004). However, the analgesic effect of inhibitors of COX isoforms on neuropathic pain model and the action of COX in neuropathic pain is not clarified.

TNFα is an important cytokine associated with the development of neuropathic
pain. In human neuropathic pain, sTNFR1 was correlated with the pain behavior. Spinal nerve ligation induced expression of TNFR in the injured dorsal root ganglia (Schafers et al., 2003). This increase occurs within a narrow postoperative time window, the same time in which TNF antagonists are effective in reducing SNL-induced pain behavior and just prior to the development of tactile allodynia on the hindlimb. In chronic pain state, the neuron in the spinal cord and brain stem expressed persistent TNF until the maintenance of pain behavior (Convey et al., 2002). Exogenous TNF also induced hyperalgesia (Sorkin and Doom, 2000), and TNFα-induced hyperalgesia can be prevented partially by COX inhibition (Schafers et al., 2004).

In present study, we investigated the involvement of COX isoforms in the development of maintenance of neuropathic pain following the ligation of the L5 spinal nerve entering the dorsal root ganglia. We examined the change of COX isoforms and TNFα expression in lesioned spinal nerve and DRG, and also the changes of pain behavior following the administration of COX inhibitors such as aspirin, a preferential COX-1 inhibitor and nimesulide, a COX-2 selective inhibitor. To define whether the analgesic effect is due to the control of inflammation in spinal nerve ligation model or not, we compared between neuropathic model and neuritis model on the L5 DRG and spinal nerve.
Ⅱ. Materials and Methods

A. Experimental Animals

Young adult male Wistar Furth rats with 180 to 200 g body weight were purchased from Haran Sprague-Dawley Company and used for experiments. Animals were housed 2-3 per cage with free access to food and water in a reversed light-dark cycle. All animals were acclimated at least 5 day before any experimental manipulations. All experimental procedures were approved by the Animal Care and Use Committee of the Ajou University in accordance with the NIH guidelines for the care and use of laboratory animals.

B. Neuropathic pain model and neuritis model

For induction of neuropathic pain, rats were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg intraperitoneally). While the rats under deep anesthesia, the L5 spinal nerve was exposed by removing paraspinal muscles and transverse process of the L6 vertebra. Exposed the proximal portion of the L5 spinal nerve was tightly ligated with 6-0 silk. In sham group, the L5 spinal nerve was exposed without ligation.

For induction of neuritis, the epineurium in the exposed L5 spinal nerve was peeled off. A piece of adsorbable gelatin sponge (about 1 X 2 mm) soaked with 30-40 $\mu$l of 0.2% CFA was placed on the exposed nerve. The CFA was prepared by
mixing 40 mg of Mycobacterium butyricum (heat-killed Mycobacterium butyrium; DIFCO Laboratories, Detroit, MI, U.S.A.) in 10 ml of 100% peanut oil and then diluted with 10 ml of saline (a final concentration of 0.2%). After confirming hemostasis, the incision was closed, and rats were recovered from anesthesia under a warm light and then returned to their cage.

C. Behavioral testing

The foot withdrawal threshold to mechanical stimuli applied to the paw (mechanical threshold) was measured and used as an indicator of mechanical sensitivity of the affected paw. The mechanical thresholds were measured by using the up-and-down method following the procedures described in previous studies. In brief, rats were placed in a transparent plastic box on a metal wire mesh floor. A series of 8 von Frey (VF) filaments with approximately equal logarithmic incremental (0.22) VF values (3.56, 3.87, 4.10, 4.31, 4.52, 4.74, 4.92, and 5.16) were used to determine the threshold stiffness required for 50% paw withdrawal. Because VF values are logarithmically related to gram (g) values [VF=\log(1000 \times g)] , these chosen VF numbers are equivalent to 0.45, 0.74, 1.26, 2.04, 3.31, 5.50, 8.32, and 14.45 in gram value, respectively. Starting with filament 4.31, VF filaments were applied perpendicularly to the ventral surface of the proximal part of the third and fourth toe for 2-3 seconds. Whenever a positive response to a stimulus occurred, the next smaller VF hair was applied. Whenever a negative response
occurred, the next higher one was applied. The test was continued until the response of 6 stimuli after the first change in response had been obtained or the test reached either of the spectrums of the VF set. The 50% threshold value was calculated by using the formulated of Dixon: $50\%$ threshold = $X + kd$, where $X$ is value of the final VF hair used (in log units), $k$ is the tabular value for the pattern of positive/negative responses, and $d$ is the mean difference between stimuli in log units(0.22). In the cases in which continuous positive or negative responses were observed all the way out to the end of the stimulus spectrum, values of 3.54 or 5.27 were assigned, respectively, by assuming a value of ± 0.5 for $k$ in these cases. Outcome of behavioral data were expressed as VF values (maximum range, 3.54 to 5.27) and plotted in a linear scale. Because VF values are logarithmically related to gram values, plotting in gram values requires logarithmic plots, which can be deceptive. A few examples of conversion between 2 values are VF 4.0 = 1.0 g, VF 4.3 = 2.0 g, VF 4.7 = 5.0 g, and VF 5.0=10 g.

D. Effect of COX inhibitors on mechanical hyperalgesia

To test the effect of COX inhibitors on the mechanical hyperalgesia in spinal nerve-ligated animals or neuritis, aspirin (100 mg/kg) or nimesulide (10 mg/kg) was injected intraperitoneally everyday. The drug was injected under halothane anesthesia. The mechanical thresholds of the injected paw were measured just before the injection (basal mechanical threshold) and then 0.5, 1, 2, 4 and 24 hours
after each drug injection.

E. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

mRNAs for COX-1 and COX-2 were detected by the RT-PCR. Animals were sacrificed 6hr, 1day or 6 days after Freund’s complete adjuvant injection; their DRG were removed (n=5 each group). Total RNA was extracted from the samples with easy-blue kit (Intron, Seoul, Korea) and aliquots of total RNA (1 µg) were used in the RT reaction. First strand cDNA synthesis was performed using 1 µg total RNA and AMV reverse transcriptase (Boehringer Mannheim, Germany). The reaction was performed at 25 °C for 10min, 42 °C for 60min and heated at 97 °C for 5min. 2 µl from each RT reaction mixture was used for PCR amplification.

COX-1 primers were forward, 5’-ACTCACTCAGTTTGTTGAGTCATTC-3’; reverse, 5’-TTTGATTAGTACTGTAGGGTTAGTGTAATG-3’: which resulted in a PCR product of 450bp

COX-2 primers were forward, 5’-TGCACTGTGGCTGTGGATGTCATAAC-3’; reverse, 5’-CACTAAGACAGACCCGTCATCTCCA-3’: which resulted in a PCR product of 583bp.

COX-3 primers were forward, 5’-GCGTTGCTCATTCCATCTACT 3’; reverse, 5’-AGGGAT AGTACAGTTGGGGC-3’: which resulted in a PCR product of 300 bp.

GAPDH primers were forward, 5’-GTGAAGGTCGGTGTGAACGATT-3’; reverse, 5’-CACAGTCTTCTGAGTGCCAGTGAT-3’: which resulted in a PCR product of 553bp.

The PCR reaction was performed with the following cycle parameters: COX-1
and GAPDH; 94 ºC, 1 min; 60 ºC, 30 sec; 72 ºC, 30 sec, 30 cycles; and 72 ºC, 10 min. COX-2; 94 ºC, 30 sec; 58 ºC, 30 sec; 72 ºC, 90 sec, 38 cycles; and 72 ºC, 10 min. COX-3; 94 ºC, 30 sec; 58 ºC, 30 sec; 72 ºC, 90 sec, 34 cycles. Reaction products were then separated on a 2% agarose gel, stained with ethidium bromide, and photographed. The optical density of the bands was determined by a Gel doc system (Bio-Rad, Hercules, CA, U.S.A.). Measurements were normalized to the optical density of the GAPDH band used as an internal standard.

F. Immunostaining of the dorsal root ganglia and the spinal nerve.

For immunostaining, rats were perfused with saline, followed by a fixative containing 4% paraformaldehyde and 0.1% picric acid in 0.1 mol/L phosphate buffer, pH 7.2. The DRG was isolated, and an approximately 5-6 mm length of the L5 spinal nerve was removed from the region of ligation and from the same region on the contralateral side. The tissues were stored in the perfusion fixatives for 2-4 hours and then changed into 30% sucrose until equilibrium. The tissue was cryosectioned at 16 um thickness in a longitudinal plane and mounted on gelatin-coated slides. The sections were first incubated with primary antibodies to COX-1, COX-2 (rabbit origin; Santa Cruz, CA, U.S.A.) at a dilution of 1:500 or TNF (rabbit origin; R & D Systems, Minneapolis, MN, U.S.A.) at a dilution of 1:1000 and then with rhodamine conjugated goat-antirabbit immunoglobulin G (1:200 dilution; Chemicon International, Temecula, CA, U.S.A.). After rinsing and air drying, tissues were
observed under a compound light microscope equipped for epifluorescence. Photomicrographs of the immunostained spinal nerve sections were taken with a Spot RT digital camera system (Diagnostic Instrument Inc, Sterling Heights, MI, U.S.A.) and saved as image files. With an Image-Pro Plus image analysis system (Media Cybernetics, Silver Spring, MD, U.S.A.), the density of the immunostaining density and exposure times of photomicrographs were varied in different sets of tissue, the relative ratio of density of the experimental nerve (ipsilateral side) by that of the control nerve (contralateral side) of the same animal.

G. Statistical Treatments

Data are expressed as the mean ± standard error of the mean (SEM). Differences in mechanical thresholds at various times after a certain manipulation were tested with one-way repeated measures analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test with the premanipulation value as a control. Changes between groups were tested by unpaired t-test. P values less than 0.05 were considered to be significant.
Ⅲ. Results

A. Neuropathic Pain Model

1. Ligation of left L5 spinal nerve causes mechanical hyperalgesia in the affected hind paw

In normal naive rats, the 50% withdrawal threshold of the hind paw to VF filament stimuli (mechanical threshold) is usually a value 5.27 (18.62 g), which is assigned maximum value when none of VF filaments produce any response. After a ligation of the L5 spinal nerve, the mechanical thresholds of the hind paw on the ipsilateral side significantly decreased to 4.04 ± 0.07 (mean ± SEM) by 3 days after the operation (Fig. 1), thus showing mechanical hyperalgesia. On average, this severe hyperalgesia lasted over 5 weeks. In the sham-operated rats (Sham), mechanical thresholds did not change the threshold.
Fig. 1. The time course of mechanical hyperalgesia in rats with L5 spinal nerve ligation. * Values are significantly different from the control value (0 d) (p<0.05, by one-way repeated measures ANOVA followed by Dunnett’s test).
2. Nimesulide, a COX-2 selective inhibitor alleviated neuropathic mechanical hyperalgesia, but not aspirin, a preferential COX-1 inhibitor

After ligation of L5 spinal nerve, the affected paw not only showed mechanical hyperalgesia (SNL group) but also responded to intraperitoneally injected COX inhibitors such as aspirin (5 mg/kg) and nimesulide (5 mg/kg) by transiently recovering mechanical thresholds. For instance, from 1 day after operation, the mean basal mechanical thresholds were decreased to 3.80 ± 0.26 (SNL, 3d), thus showing mechanical hyperalgesia. Furthermore, injection of nimesulide reversed lowered mechanical threshold leading to alleviation of pain. Fig. 2 showed the effect of vehicle or COX inhibitors intraperitoneally treated once everyday on spinal nerve-ligated neuropathic pain by measuring the mechanical threshold in pre-administration of drugs.

The injection of nimesulide was repeated every day up to 15 days, and the behavioral test was done every other day. Until 11 days after operation, mechanical hyperalgesia gradually decreased in nimesulide group compared with vehicle group, however, after then the nimesulide group could not show more analgesic effect than vehicle group. In aspirin group, the mechanical hyperalgesia did not subside significantly until 15 days. At time progressed, the mechanical threshold in aspirin group increased, and the mechanical threshold was lower than nimesulide group later than 13days, but that is statistically insignificant. In other words, nimesulide(5
mg/kg, once everyday, i.p.), not aspirin (5 mg/kg, once everyday, i.p.) could abolish the neuropathic mechanical hyperalgesia until 11 days, and after then COX inhibitors did not affect the neuropathic mechanical hyperalgesia.

Typically, the immediate effect of COX inhibitors was first detected at 30 minutes after injection (the earliest time tested after drug injection), sustained a significantly level of the mechanical threshold for approximately 2-4 hours, and regained the pre-administration level (Fig. 3). The average of these repeated measuring values from 7-9 operated rats in each group are presented as data of the group in the form of mean ± SEM. The analgesic effect of nimesulide is more potent than aspirin group. The analgesic effect of aspirin lasted for 2 hours, and that of nimesulide for 4 hours. However, a time showing the peak response of analgesia was variable in even same animal.
Fig. 2. Effect of COX inhibitors on the maintenance of neuropathic pain. In rats with spinal nerve ligation, mechanical thresholds were measured (SNL) after vehicle and nimesulide injection. The data are presented as mean ± SEM. Basal: control baseline before nerve ligation. * Value is significantly different from the control baseline value (p<0.05, one-way repeated measures ANOVA followed by Dunnett’s test). # Value is significantly different between the vehicle and nimesulide injection (p<0.05, unpaired t-test).
Fig. 3. The immediate effect of COX inhibitors on neuropathic pain. The % of response presents percentage of changing the thresholds from pre-treated state. The data are presented as mean ± SEM. H: hour; 0H means pretreated state before drug injection. * Value is significantly different from the pretreated value (p<0.05, one-way repeated measures ANOVA followed by Dunnett’s test). # Value is significantly different between the vehicle and drug injection group (p<0.05, unpaired t-test).
3. Ligation of L5 spinal nerve increased expression of COX isoforms in the ipsilateral DRG and lesioned spinal nerve

In PCR, COX isoforms such as COX-1, COX-2 and COX-3 mRNA in neuropathic state was increased significantly after 1 day when the injected side and the contralateral side of DRG were compared. In 3 days, COX-2 mRNA was slightly increased, whilst COX-1 and COX-3 mRNA was recovered to the control. In 7 days, COX-1 mRNA was slightly increased (Fig. 4A and 4B).

In immunohistochemistry, COX-1 and COX-2 proteins in the dorsal root ganglia increased 7 days after operation. COX-1 normally expressed in the neuron, the satellite cells wrapping the neuron and glial cells including Schwann cells. COX-2 expressed mainly in glial cells and some neurons. After operation, COX-1 and COX-2 immunoreactivity in the DRG is increased in both ganglial neurons and glial cells (Fig. 5A and 5B).
Fig. 4. Expression of COX isoforms mRNA in the dorsal root ganglia with spinal nerve ligation. The both sides of DRG of L5 spinal nerve-ligated animal 1, 3 and 7 days after operation were examined. CTL: control group, SNL: spinal nerve ligated group, L: left (ipsilateral) side of the DRG, R: right (contralateral) side of the DRG., SHAM: sham or right (contralateral) side of the DRG. * Value is significantly different between the sham and SNL group (p<0.05, unpaired t test).
Fig. 5. Immunohistochemical photograph of COX-1 and COX-2 expression in spinal nerve-ligated DRG(SNL). A. COX-1 expression of control and lesioned DRG. CON, contralateral side of the DRG; In lesioned DRG, the expression of COX-1 increased in neurons and glial cells around the ganglion neuron, called satellite cells. B. COX-2 expression of control and lesioned DRG. In lesioned DRG, the expression of COX-2 increased especially in neurons. In other sections, expression of COX-2 increased in the glial cells around the ganglion neuron, called satellite cells. Scale Bar = 50 µM.
The L5 spinal nerves of 28 rats with spinal nerve ligated, 4 rats with sham operation, and 4 normal naive rats, were sampled and immunostained for COX-2. The density of COX-2 immunostaining was measured from the L5 spinal nerves of both sides from each rat. The spinal nerves were sampled at 3 days, 1 week, or 3 weeks after the operation. At the time of tissue sampling, the mechanical thresholds eliciting withdrawal response in the animals were variable. The segment dissected for immunostaining is proximal to ligation site. The diameter of the spinal nerve in ipsilateral side was enlarged 1.5-2 times compared to contralateral side.

The level of COX-2 immunoreactivity was almost undetectable in the L5 spinal nerves of normal rats except along the blood vessels and epineurial sheath. This pattern did not change in the control spinal nerves (contralateral side) of neuropathic animals as well as sham-operated rats. Thus, the density of COX-2 immunoreactivity of the experimental spinal nerve (ipsilateral to operation) was compared to that of the control (contralateral) spinal nerve in the same animal (Fig. 6A). The COX-2 immunoreactivity in control nerve was 66.48 ± 2.00 (mean ± SEM, n=10). The immunoreactivity means the arbitrary units of fluorescent intensity. This value was increased to 86.26 ± 3.61 (n=10) in the spinal nerve-ligated rats that showed the neuropathic pain at the time of tissue sampling. The COX-2 immunoreactivity of the experimental nerve to that of the control nerve was 132.03 ± 7.18 % (n=10) in spinal nerve-ligated rats (Fig. 6B).
Fig. 6. Expression of COX-2 in lesioned spinal nerve. A. Immunohistochemical photograph of COX-2 expression in the spinal nerve. SNL, spinal nerve section with lesion; CON, contralateral spinal nerve without lesion. In lesioned nerve, the expression of COX-2 increased in Schwann cells and some cells around the blood vessels. Scale Bar = 100 µM. B. Changes of COX-2 immunoreactivity in injured spinal nerve (SNL). Sham: contralateral side or shamed-operated nerve. Y axis represents % of control value. * Value is significantly different between the sham and SNL group (p<0.05, unpaired t-test).
The results showed that COX-2 immunoreactivity is significantly higher in the spinal nerves of rats with ligation as compared to the spinal nerves without ligation. The correlation between the immunoreactivity of COX-2 in the spinal nerve and mechanical threshold showing withdrawal response to VF filaments was measured (Fig. 7). The data suggest that the increased expression of COX-2 in previously ligated L5 spinal nerve might be related to the neuropathic pain lowering the paw mechanical threshold.

![Fig. 7. Correlation between the COX-2 immunoreactivity and neuropathic pain behavior.](image)

X-axis presents the mechanical threshold showing withdrawal response to Von Frey filaments on the ipsilateral paw of the spinal nerve-ligated animals.
4. The mechanical hyperalgesia induced by neuropathy is related to the increased expression of TNFα in the lesioned nerve.

The spinal nerve for TNFα immunostaining was sampled from L5 spinal nerves of 28 rats with the left spinal nerve ligated, 2 rats with sham operation, and 2 normal naive rats. The immunoreactivity of TNFα was measured from the L5 spinal nerves of both sides from each rat. The spinal nerves were sampled at 3 days, 1 week, or 3 weeks after the operation. At the time of tissue sampling, the mechanical thresholds eliciting withdrawal response in the animals were variable. The level of TNFα immunoreactivity was almost undetectable in the L5 spinal nerves of normal rats. 7 days after operation TNFα immunoreactivity in the ipsilateral nerve was markedly increased, in contrast to the scarceness in the spinal nerve of contralateral side (Fig. 8A).

The TNFα immunoreactivity in control nerve was 56.26 ± 3.13% (mean ± SEM, n=10). The immunoreactivity means the arbitrary units of fluorescent intensity. This value was increased to 95.62 ± 4.30% (n=10) in the spinal nerve-ligated rats that showed the neuropathic pain at the time of tissue sampling. The TNFα immunoreactivity of the experimental nerve to that of the control nerve was 179.97 ± 12.66% (n=10) in spinal nerve-ligated rats (Fig. 8B).
Fig. 8. Expression of TNFα in lesioned spinal nerve. A. Immunohistochemical photograph of TNFα expression in spinal nerve. CON: contralateral spinal nerve without lesion, SNL: spinal nerve section with lesion. In lesioned nerve, the expression of TNFα increased in Schwann cells and some cells around the blood vessels. Scale Bar = 100 μM. 8B. Changes of TNFα immunoreactivity in injured spinal nerve (SNL). Sham: contralateral side or shamed-operated nerve. Y axis presents % of control value. * Value is significantly different between the sham and SNL group (p<0.05, unpaired t-test).
The correlation between the immunoreactivity of TNFα in the spinal nerve and mechanical threshold showing withdrawal response to VF filaments was measured (Fig. 9). The results showed that TNFα immunoreactivity is significantly higher in the spinal nerves of rats with ligation as compared to the spinal nerves without ligation. The data suggested that the increased expression of TNFα in previously ligated L5 spinal nerve, which most likely indicated persistent neuropathic pain, might be related to the neuropathic pain lowering the paw mechanical threshold.

![Fig. 9. Correlation between the TNFα immunoreactivity and neuropathic pain behavior. X-axis presents the mechanical threshold showing withdrawal response to Von Frey filaments on the ipsilateral paw of the spinal nerve-ligated animals.](image)
B. Neuritis model

1. Inflammation of left L5 spinal nerve causes mechanical hyperalgesia in the affected hind paw

After inflammation of the L5 spinal nerve, the mechanical thresholds of the hind paw on the ipsilateral side significantly decreased to 4.39 ± 0.08 (mean ± SEM) by 3 days after the operation (Fig. 10), thus showing mechanical hyperalgesia. On average, this hyperalgesia lasted until 2 weeks, after then hyperalgesic pain subsided.

**Fig. 10. The time course of mechanical hyperalgesia in rats with neuritis.** The 50% paw withdrawal thresholds to VF filament stimuli (mechanical threshold) were measured before operation and a various times after inflammation. In the inflammed rats(INF), mechanical hyperalgesia developed within 3 days after operation and lasted for 2 weeks. In the sham-operated rats (SHAM), mechanical thresholds did not change the threshold. * Values are significantly different from the control value (p<0.05).
2. Nimesulide did not alleviate the mechanical hyperalgesia in neuritis model.

After inflammation of L5 spinal nerve, the affected paw not only showed mechanical hyperalgesia (INF group), but also responded to intraperitoneally injected nimesulide by transiently recovering mechanical thresholds. However, injection of nimesulide did not reverse the lowered mechanical threshold. Fig. 11 showed the effect of nimesulide (5 mg/kg) treated once everyday on neuritis pain by measuring mechanical thresholds before drug treatment. The injection of nimesulide was repeated every day up to 15 days, and the behavioral test was done every other day. As time progressed, mechanical hyperalgesia gradually decreased both in vehicle group and nimesulide group. However, treatment of nimesulide did not alleviate the mechanical hyperalgesia (Fig. 11).

Although administration of nimesulide did not decrease the maintenance of neuritis-induced mechanical hypersensitivity measured 24 hr after injection of nimesulide, nimesulide showed immediate analgesic effects. The immediate effect of nimesulide was first detected at 30 minutes after injection (the earliest time tested after nimesulide injection), sustained a significantly level of the mechanical threshold for approximately 2-4 hours, and regained the pre-nimesulide level (Fig. 12). The average of these values from 9 operated rats are presented as data of nimesulide in the form of mean ± SEM.
Fig. 11. Effect of nimesulide on the maintenance of inflammatory pain. In rats with inflammation, mechanical thresholds were measured (INF) after vehicle and nimesulide injection. The data are presented as mean ± SEM. Basal: control baseline before inflammation. * Value is significantly different from the control baseline value (p<0.05, one-way repeated measures ANOVA followed by Dunnett’s test). # Value is significantly different between the vehicle and nimesulide injection (p<0.05, unpaired t-test).
Fig. 12. The immediate effect of nimesulide on inflammatory pain. The % of response presents percentage of changing the thresholds from pre-treated state. Until 4 hours alleviating effect of nimesulide was shown. The data are presented as mean ± SEM. 0H means pretreated state before nimesulide injection. * Value is significantly different from the pretreated value (p<0.05, one-way repeated measures ANOVA followed by Dunnett’s test). # Value is significantly different between the vehicle and nimesulide injection group (p<0.05, unpaired t-test).
3. FCA-induced nerve inflammation increased expression of COX isoforms in the ipsilateral DRG and the inflammed spinal nerve

In PCR, mRNA of COX isoforms, such as COX-1 and COX-2 in inflammatory state was not increased after 6h and 1 day in the ipsilateral side of DRG. However, 6 days after induction, COX-1 mRNA was slightly increased and some rats showed markedly increased (Fig. 13A and 13B).
Fig. 13. Expression of COX isoforms mRNA in DRG with inflammation. A. The both sides of DRG with inflammation 6 days after injection were examined. B. The both sides of DRG with inflammation 6hr, 1day and 6 day after injection of left paw were examined. FCA: left (ipsilateral) side of the DRG with FCA-induced inflammation, Veh: vehicle or right (contralateral) side of the DRG. * Value is significantly different between the sham and FCA group (p<0.05, unpaired t test).
In immunohistochemistry, COX-1 and COX-2 proteins in the DRG increased 7 days after inflammation. COX-1 increased significantly in the neurons and glial cells around the ganglion neuron, called satellite cells, and COX-2, in particular, in the glial cells markedly increased after inflammation (Fig. 14A and 14B).

A.

![Immunohistochemical photograph of COX-1 expression](CON) magnification X200. The scale bar represents 50 µm.

B.

![Immunohistochemical photograph of COX-2 expression](INF) magnification X200. The scale bar represents 50 µm.

**Fig. 14. Expression of COX isoforms in L5 spinal nerve inflamed DRG (INF).** A. Immunohistochemical photograph of COX-1 expression CON: contralateral side of the DRG. magnification X200. The scale bar represents 50 µm. B. Immunohistochemical photograph of COX-2 expression in L5 spinal nerve inflamed DRG (INF). CON: contralateral side of the DRG. magnification X200. The scale bar represents 50 µm.
The L5 spinal nerves of 10 rats with spinal nerve inflammation and 4 rats with sham operation were sampled and immunostained for COX-2. The density of COX-2 immunostaining was measured from the L5 spinal nerves of both sides from each rat. The spinal nerves were sampled at 3 days, 1 week or 2 weeks after the operation. At the time of tissue sampling, the mechanical thresholds eliciting withdrawal response in the animals were variable.

Compared with the ligated spinal nerve, which was significantly increased over 21 days after operation, the COX-2 immunoreactivity in inflamed L5 spinal nerve did not increase except 3 days after operation. At 7 days when neuritis-induced pain behavior showed, the increase of COX-2 immunoreactivity was not significant. At 3 days COX-2 immunoreactivity in control nerve was 85.20 ± 7.48 (mean ± SEM, n=10). The immunoreactivity means the arbitrary units of fluorescent intensity. The immunoreactivity in the inflammatory rat was increased to 106.99 ± 10.71 (n=10) but this value is not significant. The COX-2 immunoreactivity of the experimental nerve to that of the control nerve were 132.80 ± 14.73% (mean ± SEM, n=10) in inflamed nerve 3 days after operation. 7 and 14 days after operation, the COX-2 immunoreactivity of the experimental nerve to that of the control nerve were 116.49 ± 10.51% and 96.13 ± 8.65 (mean ± SEM, n=10) in inflamed nerve, and the increase was not significant (Fig. 15).
Fig. 15. Expression of COX-2 in lesioned or inflamed spinal nerve. A. COX-2 immunoreactivity in the spinal nerve 7 days after operation. B. COX-2 immunoreactivity in the inflamed spinal nerve 3, 7 and 14 days after operation. Sham: contralateral side or shamed-operated nerve. % of COX-2 immunoreactivity means % of control immunoreactivity. * Value is significantly different between the sham and SNL or INF group (p<0.05, paired t-test).
The results showed that COX-2 immunoreactivity is not significantly higher in the spinal nerves of rats with inflammation, whilst significantly increased in the spinal nerve ligated rats. The correlation between the immunoreactivity of COX-2 in the spinal nerve and mechanical threshold showing withdrawal response to VF filaments in neuritis rats was measured (Fig. 16). The data suggest that the expression of COX-2 in neuritis model might not be related to the neuritis-induced pain lowering the paw mechanical threshold after 14 days (r=0.10). Fig.16 showed COX-2 immunoreactivity in inflammatory group after 3 and 14 days. In inflammatory state, the COX-2 immunoreactivity in early time (3days) tended to correlate with pain behavior.

![Graphs showing correlation between COX-2 immunoreactivity and mechanical threshold](image)

**Fig. 16. Correlation between the COX-2 immunoreactivity and inflammatory pain behavior.** The X-axis presents mechanical threshold showing withdrawal response to Von Frey filaments on the ipsilateral paw of the spinal nerve-neuritis animals.
4. The mechanical hyperalgesia induced by neuritis is rather related to the increased expression of TNFα in inflamed nerve

The density of TNFα immunostaining was measured from the L5 spinal nerves of both sides from each rat. The spinal nerves were sampled at 3d, 1weeks, or 2 weeks after the operation. At the time of tissue sampling, the mechanical thresholds eliciting withdrawal response in the animals were variable.

The level of TNFα immunoreactivity was almost undetectable in the L5 spinal nerves of normal rats. This pattern did not change in the control spinal nerves (contralateral side) of neuropathic animals as well as sham-operated rats. Thus, the density of TNFα immunoreactivity of the experimental spinal nerve (ipsilateral to operation) was highly increased compared to that of the control (contralateral) spinal nerve in the same animal (Fig. 17A). In inflamed nerve, the expression of TNFα increased in Schwann cells lining the axons.

The TNFα immunoreactivity of the experimental nerve to that of the control nerve was 169.41 ± 18.18 % (mean± SEM, n=10) in inflamed nerve 7 days after operation. In both neuritis model and spinal nerve-ligated model, TNFα immunoreactivity in the ipsilateral spinal nerve was significantly increased compared to the contralateral side (Fig. 17B).
Fig. 17. Expression of TNFα in lesioned and inflamed spinal nerve. A. Immunohistochemical photograph of TNFα expression in the inflamed nerve. INF: spinal nerve section with inflammation, CON: contralateral spinal nerve without inflammation. magnification X200. The scale bar represents 100 µm. B. Changes of TNFα immunoreactivity in the lesioned spinal nerve (SNL) and neuritis (INF). Sham: contralateral side or shamed-operated nerve. The immunoreactivity means the arbitrary units of fluorescent intensity. * Value is significantly different between the sham and SNL or INF group (p<0.05, unpaired t-test).
The correlation between the immunoreactivity of TNFα in the spinal nerve and mechanical threshold showing withdrawal response to VF filaments was measured (Fig. 18). The data suggested that the increased TNFα in neuritis had quite a correlation, however in spinal nerve ligated group the correlation was higher (Fig. 9). However, the TNF immunoreactivity and the correlation with TNF expression and pain behavior tended to increase according to time progress, whilst COX-2 immunoreactivity increased early time (Fig. 18). Fig. 19 showed the relationship between COX-2 immunoreactivity and TNFα immunoreactivity of the spinal nerves 3 days and 14 days after inflammation. Early time the COX-2 immunoreactivity increased compared with TNFα, and 14 days, at that time the pain behavior subsided, the TNFα immunoreactivity compared with COX-2 increased and more correlated with the pain behavior (Fig. 18 and 19). Therefore, COX-2 might initiate and sustain the pain response, whilst TNFα might be implicated more or less to develop a chronic pain response.
Fig. 18. Correlation between TNFα immunoreactivity and pain behavior in the L5 spinal inflamed model. The X-axis presents mechanical threshold showing withdrawal response to Von Frey filaments on the ipsilateral paw of the spinal nerve-ligated or inflamed animals.

Fig. 19. Correlation between TNFα immunoreactivity and COX-2 immunoreactivity of the spinal nerve in neuritis group. Close circle (●) represents the COX-2 and TNFα immunoreactivity of the spinal nerves 3 days after operation, and open circle (○) represents the COX-2 and TNFα immunoreactivity of the spinal nerves 14 days after operation,
IV. Discussion

The present study demonstrated that when the L5 spinal nerve in the rat was ligated with a silk thread, mechanical hyperalgesia developed on the affected paw within 1 day and then lasted for 5 weeks. Intraperitoneally administered nimesulide, a COX-2 selective inhibitor significantly alleviated the maintenance of neuropathic pain, however, the analgesic effect of nimesulide on neuropathic pain lasted until 11 days, whereas the effect of nimesulide did not show after then. However, a COX-1 preferential inhibitor, acetylsalicylic acid did not significantly alleviate the neuropathic pain, although late phase after injury acetylsalicylic acid tended to alleviate the pain. The immunoreactivity of COX-2 in the ligated spinal nerve was increased 132% compared to the contralateral spinal nerve. The pain behavior and COX-2 immunoreactivity were closely correlated. Thus, COX-2 might be participated in development and maintenance of neuropathic pain.

Prostaglandins are derived from neuronal and nonneuronal cell pools. COX-1 expressed constitutively, generates prostaglandins for maintaining the normal physiological function, while inducible COX-2 generates huge quantities of prostaglandins in pathological conditions. In seizures, cerebral ischemia or Alzheimer’s disease related to inflammation, COX-2 was markedly induced, and also increased prostaglandins such as PGE$_2$, PGD$_2$, and PGF$_2\alpha$ (Baik et al., 1999; Nogawa et al., 1997; Lukiw and Bazan, 1997). In the normal brain, COX-2 was expressed
constitutively and also associated with neuronal excitability such as synaptic excitation (Adams et al., 1996). The inducible COX-2 expression might act as the immediate early gene and is thought to be an important modulatory factor of the nociceptive transmission (Hay and Belleroche, 1997; Hay et al., 1997; Zhao et al., 2000). In nociceptive transmission, rofecoxib, a selective COX-2 inhibitor did not reduce neuropathic hyperalgesia, but significantly reduced the inflammatory hypersensitivity (Broom et al., 2004). In other neuropathic injury model, the treatment of a COX-2 inhibitor was effective only in early treatment (Schafers et al., 2004). The COX-3, a variant of COX-1 is recently known, and the function of COX-3 in the nociceptive processes including inflammatory pain remains to be clarified (Schwab et al., 2003).

The role of prostaglandin in transmission of nociceptive information was well known, especially in the sensitization of nociceptors (Cohen and Pearl, 1988). In peripheral inflammation, COX-2 mRNA and the release of PGI\textsubscript{2} and PGE\textsubscript{2} in the spinal cord were increased (Hay et al., 1997). The spinal administration of prostaglandins induced hyperalgesia to noxious stimuli and alldynia to innocuous stimuli (Minami et al., 1994). However, the mechanisms underlying prostaglandin-induced nociception are unclear. One possibility is that prostaglandins may enhance the release of glutamate and substance P from the primary afferent terminals. In addition, arachidonic acid and leukotrienes, via an influence upon protein kinase activity in primary afferent terminals, may retrogradely enhance glutamate/substance
P release (Collins and Davies, 1998). The hyperalgesic agents such as PGE$_2$, serotonin, and adenosine modulate TTX-resistant Na current in sensory neurons (Gold, 1999), and capsaicin-receptors in sensory neurons may be activated by arachidonic metabolites such as products of lipoxygenase (Hwang et al., 2000).

There are the analgesic effect of selective COX-2 inhibitor on neuropathic pain is controversial. In other study (Lui and Lee, 2004), the preemptive analgesic effect of i.t. tenoxicam (a nonselective COX-1 and COX-2 inhibitor) on the inhibition of thermal hyperalgesia and allodynia after nerve injury was more effective than that of an equimolar dose of NS-398. NS-398, a selective COX-2 inhibitor, is a sulfonamide NSAID which has no effect on COX-1 activity even at high concentration (Futaki et al., 1994). These results also are in agreement with other studies showing a persistent accumulation of COX-1 expressing cells in the DRG after spinal nerve injury. As a corollary, blocking both COX-1 and COX-2 is more effective for the suppression of these painful behaviors than selectively blocking a single one. This notion was supported by the work of Schwab in which short-term treatment with the blockade of COX-2 alone was suggested to be an insufficient approach to suppress the local synthesis of prostanoids.

Nimesulide is not selective to COX-2 than NS-398, and can inhibit COX-1 activity at high dosage though the dosage used in the present study does not affect COX-1 activity. Systemically administered nimesulide showed analgesic effect on neuropathic pain model, but also affect the maintenance of the pain until 11 days
after operation. We used single dosage of nimesulide each day, and did not know the effect of nimesulide with multiple use or higher dosage; however, the analgesic effect of nimesulide was shown in early phase but not in late phase. It is conceivable that preemptive analgesia may reduce the risk of developing chronic postoperative pain in humans. Patients with high intensity of pain scores after surgery have a higher risk of establishing a chronic pain state (Poobalan, 2003). Preemptive analgesia could only be observed when the blockade of noxious stimuli was complete and extended into the initial postoperative period (Kissin, 2000). Therefore, the complete use of analgesics during initial stage would be needed.

Systemic administrations of COX inhibitors are only partially effective in the treatment of neuropathic pain. Evidence is also far from clearness regarding the efficacy of intrathecal administration of COX inhibitors in humans. Anecdotal cases were reported regarding the use of lysine acetylsalicylic acid in providing pain relief in patients with neuropathic pain (Devoghel, 1983). Intrathecal injection of diclofenac or tenoxicam was also reported to provide analgesia for hours in a few patients with cancer pain (Lauretti et al., 1998). In Ma’s notion (Ma et al., 2002), a single intrathecal injection of COX-1 preferring inhibitor ketorolac, but not a nonselective inhibitor, piroxicam significantly reversed tactile allodynia. Inhibition of spinal COX may be an important mechanism of action in treating some patients with neuropathic pain following peripheral nerve injury.
Some studies showed that specific inhibitors of COX-2 inhibits edematous changes at the inflammatory site, and also produces analgesic effect other than neuropathic pain (Boni et al., 1999). However, we want to know whether the analgesic effect of nimesulide on neuropathic pain is due to control the inflammation or neurogenic effect. To discriminate these, we used neurtis-evoked pain model. In neuritis, COX-2 inhibition could alleviate the pain for a few hours, but could not affect the maintenance of the pain. The COX-2 immunoreactivity of the inflamed nerve on neurtitis was not correlated with pain behavior. However, the pain behavior in neuritis, which was milder and shorter than neuropathic pain, was different from neuropathic pain. This can be supported by COX-2 immunoreactivity in neuritis at 3 days tended to be higher than contralateral side, however the tendency is disappeared at 14 days. Takahashi et al (2004) also showed the biphasic increase of COX-2, the first increase of COX-2 positive cells with coexpressed in Schwann cell marker S-100 one day after L5 spinal nerve injury, and second increase after 7-14 days and these cells co-expressed the macrophage marker ED-1. In spinal nerve injury, COX-2 in Schwann cells impinged on development and maintenance of neuropathic pain.

The typical inflammatory cytokine, TNFα was also increased in the lesioned spinal nerve and correlated well with mechanical pain behavior in the neuropathic pain model. So we could speculate some role of TNFα in the neuropathic pain mechanism. The expression of TNFα is known to be implicated in the initiation of neuropathic pain (Schafners et al., 2003). Expression of COX-2 in neuropathic pain
lasts over 3 wks, and that is closely correlated with pain. In neuritis model, the expression of COX-2 in inflamed nerve disappeared after 3-7 days, and that time the mechanical hyperalgesia remained highly. The expression of TNFα compared with COX-2 increased until 14 days and that time mechanical hyperalgesia was sustained. This suggests COX-2 initially expressed by inflammation might trigger the expression of TNFα in the nervous system. The expression of COX-2 and TNFα in the nervous tissue might play an important role on the persistent chronic pain.
V. Conclusion

In the L5 spinal nerve-ligated neuropathic pain model, COX isoforms mRNA’s and proteins in the dorsal root ganglia and the lesioned spinal nerve were changed in time. The COX-2 selective inhibitor, nimesulide (5 mg/kg) administered intraperitoneally once every day significantly alleviated the mechanical hyperalgesia until 11 days after operation. The COX-1 preferential inhibitor, acetylsalicylic acid (5 mg/kg) did not significantly alleviate the mechanical hyperalgesia in neuropathic pain, however, in late phase the mechanical hyperalgesia tended to decrease by COX-1 inhibitor. The immunoreactivity of COX-2 and TNFα in lesioned nerve was remarkably increased, and both were closely correlated with the mechanical behavior.

In spinal nerve-inflammation model, COX isoforms mRNA’s and proteins in the dorsal root ganglia and the inflamed nerve were changed. The mechanical hyperalgesia in nerve inflammation model was milder and shorter than that of neuropathic model. The COX-2 expression was not significantly increased in inflamed nerve, except 3 days after inflammation; however TNFα expression in inflamed nerve was significantly increased. COX-2 or TNFα expression in the inflamed nerve was little correlated with nerve inflammation-induced pain behavior.

In conclusion, though there are some limitations for the present study, we suggest that COX-2 might participate in the development and maintenance of neuropathic
pain, regarding the selective COX-2 inhibitor, nimesulide’s alleviating effect on the mechanical hyperalgesia in the early period of neuropathic pain model and the correlation between the COX-2 immunoreactivity and the mechanical hypersensitivity. The Expression of TNFα and COX-2 in lesioned nerve probably plays an important role in the early development and maintenance of mechanical hyperalgesia in neuropathic-specific condition beyond the inflammatory states.
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말초 신경병증성 통증모델에서의 Cyclooxygenase isoforms의 역할

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신경손상에 의해 발생하는 신경병증성 통증은 손상부위 영역에 기계적 자극에 대한 과민을 비롯하여 일상생활에 지장을 초래하며 많은 고통을 초래한다. 이에 대한 진통 효과를 나타낼 수 있는 약물의 개발이 되어 있지 않으며 이는 신경병증성 통증에 대한 기전이 확립되어 있지 않은 상태에서 그 해결책이 시급함에도 불구하고 많은 연구가 갖춰질 필요가 있는 실정이다. 이를 연구하는 실험모델로 환자와의 L5 척수신경을 결찰하면 수일내에 그 신경이 자극하는 부위에 기계적과민이 발생하면서 인간에게 나타나는 유사한 증상을 초래한다. 본 연구는 통증전달에 중요한 인자로 알려진 prostaglandin을 합성하는 효소인 cyclooxygenase isoforms들이 이러한 척수신경손상에 따른 통증반응에서의 역할을 규명하고자 하였다.

실험동물은 환자를 마취하고 왼쪽 L5 척수신경을 노출하여 척수신경절 근처에서 결찰한 후 발바닥에 기계적 자극을 가하여 통증으로 인한 withdrawal 반응을 보이는 역치를 측정한 결과 그 역치가 현저히 감소하는 기계적 과민은 양상을 나타내었고 이는 5 주이상 지속하였다. 선택적 COX-2 억제제인 nimesulide (5 mg/kg, i.p)를 매일 1회씩 투여한 결과 기계적과민이 현저히 감소하였고 알 수 있었던 반면, COX-1에 비교적 선택적인 억제제인 acetylsalicylic acid (5 mg/kg, i.p)는 그러한 기계적 과민을 억제시키지 못하였다. 감각정보가 척수로 들어가는 척수신경절에서 COX isoforms mRNA와 단백질의 발현을 관찰한 결과 신경경과에 따라 이들 COX 들이 작용하고 있음을 알 수 있었다. 결찰된 쪽의 척수신경절에서도 COX-2 단백질의 발현이 증가함을 관찰할 수 있었으며, 특히 이들 단백질의 발현정도가 기계적 자극의 역치와 과민성 지표와 상관관계가 있음을 나타내고 있었다. 이는 신경계의 COX isoform 들이...
말초신경 손상으로 발생되는 신경병증성 통증의 전달에 관여하고 있음을 알 수 있다. 또한 말초신경 손상 부위에서 TNFα의 발현 역시 크게 증가하였으며 그 증가 정도는 신경손상후 기계적 과민통의 정도와 좋은 상관관계를 보였다.

본 연구에서는 신경에 염증을 유발시킨 신경염모델을 이용하여 신경병증성 통증발생과정에서의 염증반응의 역할을 실험하여 보았다. 신경염에 따른 기계적 과민성은 신경손상에 따른 통증에 비해 약하고 기간이 짧게 나타났다. COX-2 선택적 억제제인 nimesulide 에 의해 일시적인 진통효과를 보였으나 통증의 발생과 진행에는 전혀 영향을 미치지 못하였다. 염증이 유발된 척수신경에서 대조군에 비해 TNFα의 발현이 증가된 반해 COX-2 변화는 염증유발후 3일이내에서만 증가하였다. 그리고 이러한 척수신경손상 모델에서는 TNFα나 COX-2 발현과 통증반응의 상관관계와는 달리 염증모델에서는 그들의 상관관계가 적었다. 아마도 신경병증성 통증모델에서의 손상신경조직에 나타나는 TNFα나 COX-2 증가는 염증작용으로 나타난 효과라기 보다는 신경병증에 나타나는 만성적 통증의 발생과 유지에 중요한 의미가 있는 것을 보여주고 있다.