CHAPTER 3:

ROLE OF CYCLOOXYGENASE ISOFORMS ON THE DEVELOPMENT AND MAINTENANCE OF HYPERSENSITIVITY IN MONONEUROPATHIC RATS
3.1. SELECTIVE INHIBITION OF CYCLOOXYGENASE-1 ATTENUATES THE DEVELOPMENT OF NEUROPATHIC PAIN IN NERVE-INJURED RATS

3.1.1. INTRODUCTION

Neuropathic pain is a common and chronically debilitating condition characterized by persistent pain, dysesthesia, hyperalgesia and allodynia (Zimmerman, 2001). It is generally agreed that both peripheral and central mechanisms have been involved in the pathogenesis of neuropathic pain. Peripheral nerve injury is associated with Wallerian degeneration and significant neuroplastic changes in the spinal cord that include changes in the expression and upregulation of mRNA for neurotransmitters, neuromodulators, and neuroimmune activation in the dorsal root ganglia and spinal cord and subsequent increase in excitability of primary afferent neurons and alterations of the afferent signals to the spinal cord (peripheral sensitization) (DeLeo and Yezierski, 2001; Taylor, 2001; Watkins et al., 2001; Zimmerman, 2001). The central mechanisms include central sensitization, reorganization of neuronal circuits in dorsal horn, and changes in the descending pain facilitation and pain inhibition (Taylor, 2001; Zimmerman, 2001).

Prostaglandins, potent inflammatory and pronociceptive mediators, are thought to play an important role in peripheral and central sensitization at peripheral sites and in the spinal cord (Willingale et al., 1997; Beiche et al., 1998). It has been reported that the intrathecal administration of PGE2 and PGF2α results in spontaneous agitation, hyperalgesia and tactile allodynia in mice and rats (Minami et al., 1994; Turnbach et al., 2002). Further, COX inhibitors significantly attenuate the PGE2 and PGF2α-induced hyperalgesia and allodynia (Taiwo and Levine, 1988; Park et al., 2000). Recently, Zhu and Eisenach (2003) have shown that a greater number of COX-1 immunoreactivity profile in spinal cord is up-regulated 4 days and 2 weeks after spinal nerve ligation. In contrast, recent studies indicate that COX-2 plays an important role in maintenance of neuropathic pain as increased COX-2 expression without any change in COX-1 expression in the spinal nerve is observed from 2 weeks onwards following peripheral nerve injury (Ma and Eisenach, 2002, 2003a). Moreover, NSAIDs are generally considered to be ineffective in the treatment of neuropathic pain (Max et al., 1988; Weber et al., 1993). However, one study in diabetic neuropathic patients reports clinical efficacy of ibuprofen (Cohen and Harris,
1987). Despite these conflicting results, the effect of COX inhibitors during the development of hypersensitivity following peripheral nerve injury remains unclear. Further, the relative role of COX-1 and COX-2 in the development of neuropathic pain following peripheral nerve injury is not fully understood and has received little attention.

In light of these, the present study was undertaken to determine the effects of acute and chronic systemic treatment with a nonselective COX-1/COX-2, selective COX-1 or COX-2 inhibitor on the development of neuropathic pain following peripheral nerve injury in rats. Further, intrathecal and perineural administration of COX inhibitors were also employed to differentiate site specific effects of COX inhibitors on the development of hypersensitivity to nerve injury in rats.

3.1.2. MATERIALS AND METHODS

3.1.2.1. Experimental animals

As per 1.2.1. Male Wistar rats weighing 150-180 g at the start of the surgery were randomly divided into various groups and each consisted of 6 - 8 animals. Following surgery, the animals were housed 3 per cage and provided with food and water ad libitum.

3.1.2.2. Chronic constriction nerve injury (CCI)

The unilateral mononeuropathy was produced according to the method described by Bennett and Xie (1988). Briefly, the rats were anesthetized using 40 mg/kg sodium pentobarbital intraperitoneally (i.p.) and the common sciatic nerve of the left hind paw was exposed at the level of the middle of the thigh by blunt dissection through the biceps femoris muscle. Proximal to the sciatic trifurcation, approximately 7-mm of nerve was freed and 4 ligatures of 4-0 chromic gut were placed around the sciatic nerve with 1-mm intervals. Great care was taken not to interrupt epineural blood flow during tying the ligatures. In sham-operated rats, the same surgical procedure was followed, the connective tissue was freed, and no ligatures were applied. After surgery, all animals received gentamicin (5 mg/kg, i.p.) to prevent sepsis.

3.1.2.3. Assessment of neuropathic pain

Allodynia (heightened response to normally non-noxious stimuli) and hyperalgesia (decreased threshold to noxious stimuli) were evaluated in sham and sciatic nerve injured rats, respectively.

3.1.2.3a. Cold allodynia
Cold allodynia was evaluated as the withdrawal latency to thermal, non-noxious stimuli of the left and right hind paws (ipsilateral and contralateral to nerve injury, respectively) when dipped in water bath maintained at 10 ± 0.5°C (Attal et al., 1990). Baseline latency of paw withdrawal to thermal stimulation was established thrice, 5 min apart, and averaged. A cut-off time of 15 sec was imposed.

3.1.2.3b. Mechanical hyperalgesia

The withdrawal threshold of the ipsilateral (nerve injured) and contralateral (nerve uninjured) paw was measured using an analgesymeter (Ugo Basile, Italy) by applying noxious pressure to hind paw and expressed in grams as per 1.2.2.3b.

3.1.2.4. Drugs and treatment schedule

Resveratrol (Archer Chem, India), naproxen and rofecoxib (both from Panacea Biotec Ltd., India) were used in the present study.

3.1.2.4.1. Systemic administration

All the drug solutions were freshly prepared by suspending them in one or two drops of Tween 80 in normal saline and administered intraperitoneally (1 ml/100 g body weight). For intrathecal (i.t.) and perineural (p.n.) administration, the drugs were dissolved in a vehicle containing 70% dimethyl sulfoxide (DMSO) and 30% normal saline and administered small volume (10 µl).

3.1.2.4.2. Spinal administration

For intrathecal (i.t.) administration, all doses of the drugs were delivered in a total volume of 10 µl via lumbar puncture at the L4/L5 level in restrained animals. The intrathecal administration of a 10 µl of 1% solution of methylene blue in 70% DMSO followed by dissection in a separate set 3 control animals confirmed the correct position of the injection and the spread of the dye in the intrathecal space.

3.1.2.4.3. Perineural administration

Perineural (p.n.) injection was performed as per the method described earlier (Thalhammer et al., 1995). In brief, the rat was restrained and held in lateral recumbency with the limb to be injected forming a right angle with the longitudinal axis of the trunk. On an imaginary line from the greater trochanter to the ischial tuberosity, about one third of the distance caudal to the greater trochanter, a 26-gauge injection needle was advanced from dorsolateral direction at a 45° angle until the tip encountered the ischium. Drugs for perineural administration were mixed such that all doses were delivered in a maximum volume of 0.2 ml at the site of nerve injury.
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3.1.2.4.4. Treatment schedule

All animals were acclimatized to laboratory environment for at least 2 h before testing. All the rats were subjected to these two pain tests on day 0 before performing surgery. To evaluate the effect of COX inhibition at the time of nerve injury, rats were administered with a single dose of saline/tween mixture, resveratrol, a selective COX-1 inhibitor (10 or 30 mg/kg, i.p.), naproxen, a non-selective COX inhibitor (10 or 30 mg/kg, i.p.) or rofecoxib, a selective COX-2 inhibitor (3 or 10 mg/kg, i.p.) 2 h before surgery and the response to behavioral tests was tested on day 1 and thereafter once a week for 4 weeks following nerve injury.

Similarly, to determine the effect of COX inhibitors during the development of hypersensitivity, various doses of saline/tween mixture, resveratrol (10 or 30 mg/kg, i.p.), naproxen (10 or 30 mg/kg, i.p.) or rofecoxib (3 or 10 mg/kg, i.p.) were administered on day 7 to separate groups of rats and the development of allodynia and hyperalgesia was tested on day 7, 2 h before and after treatment, and once a week thereafter for 4 weeks after nerve injury.

Further, separate groups of rats received 70% DMSO, resveratrol (100 or 300 µg i.t. or 3 or 10 mg/kg, p.n.), naproxen (100 or 300 µg i.t. or 3 or 10 mg/kg, p.n.) or rofecoxib (30 or 100 µg i.t. or 1 or 3 mg/kg, p.n.) intrathecally or perineurally and the animals were tested for behavioral response 2 h before and 0.5, 1, 2, 4 and 24 h after the treatment on day 7 following peripheral nerve injury in rats. Motor function was evaluated by the placing/stepping reflex 15 min after intrathecal or perineural administration of 70% DMSO in rats.

To evaluate the effect of chronic administration of COX inhibitors on the development of neuropathic pain, saline/tween mixture, resveratrol (3, 10 or 30 mg/kg, i.p.), naproxen (3, 10 or 30 mg/kg, i.p.) or rofecoxib (1, 3 or 10 mg/kg, i.p.) were administered 2 h before surgery and continued once daily for 7 days post-nerve injury. The paw withdrawal response to thermal and mechanical stimulation was tested on day 1 and 7 following 2 h after treatment and once a week thereafter for 4 weeks after nerve injury.

3.1.2.5. Statistical analysis

All the values were expressed as mean ± S.E.M. for 6 - 8 animals per group. The significance of the difference in the mean values of paw withdrawal latency to thermal and withdrawal threshold to mechanical stimulation from all groups were
analyzed by one-way analysis of variance with Dunnett’s test for multiple comparisons. \( P < 0.05 \) was considered statistically significant.

3.1.3. RESULTS

3.1.3.1 General behaviour of sham-operated and nerve injured animals

In the present series of experiments, the baseline paw withdrawal response in each test obtained on day 0 for each rat was relatively stable and showed no significant variation. The mean paw withdrawal latency to thermal stimulation was 14.18 ± 0.77 sec on the left and 14.09 ± 0.51 sec on the right, and withdrawal threshold to pressure was 201.5 ± 18.77 g and 207.44 ± 20.73 g, respectively, on day 0 before performing surgery. Following surgery, the animals kept their nerve injured paw elevated above the cage floor, but otherwise appeared healthy, exhibited normal grooming and feeding behavior, and gained weight normally. The paw withdrawal responses to thermal and mechanical stimulation in sham-operated rats remained unchanged from baseline values throughout the entire observation period. The ipsilateral paw withdrawal responses of all the vehicle-treated nerve injured rats were significantly less than that of sham-operated rats on day 7 onwards and reached steady state between days 14 and 28 after surgery indicating the development of allodynia and hyperalgesia in a time-dependent manner (Fig. 3.1.1A, 1B, 3.1.2A, 2B, 3.1.3A, and 3B).

3.1.3.2. Effect of acute systemic administration of COX inhibitors on neuropathic pain

Acute systemic administration of single dose of resveratrol (10 or 30 mg/kg, i.p.), naproxen (10 or 30 mg/kg, i.p.) or rofecoxib (3 or 10 mg/kg, i.p.) 2 h before nerve ligation on day 0 had no effect on the development of hypersensitivity as compared to saline/tween mixture treatment throughout the observation period (Fig. 3.1.2A and 3.1.3A). Further, drug treatment did not alter contralateral paw withdrawal responses to thermal and mechanical stimulation (Fig. 3.1.2B and 3.1.3B).

There was no significant difference in ipsilateral paw withdrawal responses between various groups of animals except sham-operated animals on day 7 before drug administration. Acute systemic administration of a single dose of resveratrol (10 or 30 mg/kg, i.p.) or naproxen (10 or 30 mg/kg, i.p.) to rats on day 7 following nerve injury significantly altered the decrease in ipsilateral paw withdrawal responses to
thermal and mechanical stimulation 2 h after the treatment as compared to saline/tween mixture treatment (Fig. 3.1.4A and 3.1.5A). However, it did not alter the development of hypersensitivity at later time points throughout the study period. Rats treated with rofecoxib (3 or 10 mg/kg, i.p.) showed paw withdrawal responses similar to saline/tween mixture-treated nerve injured rats (Fig. 3.1.4A and 3.1.5A).

3.1.3.3. Effect of spinal and perineural administration of COX inhibitors on neuropathic pain

Because systemic administration of resveratrol or naproxen showed antiallodynic and anti-hyperalgesic effects, intrathecal or perineural administration of COX inhibitors were employed to differentiate the possible site-specific effects of COX inhibitors on the attenuation of hypersensitivity on day 7 following peripheral nerve injury in rats. Intrathecal or perineural administration of 70% DMSO caused no detectable motor weakness as judged by placing/stepping reflexes. Intrathecally (100 or 300 μg/rat) or perineurally (3 or 10 mg/kg) administered resveratrol or naproxen was found to attenuate hypersensitivity in the ipsilateral paw for 4 h ($P < 0.05$ vs vehicle treated group), but the effect was not observed 24 h after the drug treatment on day 7. The peak antiallodynic and antihyperalgesic effect of resveratrol was observed 0.5 or 1 h, respectively after intrathecal administration (Fig. 3.1.6A and 3.1.7A) respectively whereas such peak effects were observed 1 or 2 h after perineural administration (Fig. 3.1.8A and 3.1.9A), respectively. However, perineural administration showed slow onset of action (3.1.8A and 3.1.9A). On the contrary, intrathecal (30 or 100 μg/rat) or perineural (1 or 3 mg/kg) treatment of rofecoxib did not show any significant difference in the cold alldynia (Fig. 3.1.6A and 3.1.8A) and mechanical hyperalgesia (3.1.7A and 3.1.9A) compared with vehicle (70% DMSO)-treated nerve injured rats.

3.1.3.4. Effect of chronic systemic administration of COX inhibitors on neuropathic pain

Chronic treatment with resveratrol (3, 10 or 30 mg/kg, i.p.) 2 h before and once daily for 7 days post-nerve injury significantly attenuated and the last two doses (10 or 30 mg/kg) further delayed the development of hypersensitivity in ipsilateral paw throughout the observation period as compared with saline/tween mixture-treated nerve injured rats (Fig. 3.1.10A and 3.1.11A). Similarly, chronic treatment with
naproxen (3, 10 or 30 mg/kg, i.p.) markedly attenuated and delayed the development of hypersensitivity in ipsilateral paw on day 7, 14 and 21, but not on day 28 as compared with saline/tween mixture-treated nerve injured rats (Fig. 3.1.12A and 3.1.13A). In contrast, rats administered rofecoxib (1, 3 or 10 mg/kg, i.p.) did not show any significant difference in the cold allodynia (Fig. 3.1.14A) and mechanical hyperalgesia (Fig. 3.1.15A) in ipsilateral paw compared with saline/tween mixture-treated nerve injured group. In all these experiments, following acute systemic administration on day 0, systemic, intrathecal or perineural administration on day 7 and chronic systemic administration for 7 days COX inhibitors had no effect on the contralateral paw withdrawal responses in these tests as compared to respective vehicle treatment (Fig. 3.1.2B to 3.1.15B).

3.1.4. DISCUSSION

In the present study, when resveratrol, a selective COX-1 inhibitor, naproxen, a nonselective COX inhibitor or rofecoxib, a selective COX-2 inhibitor were administered at the time of nerve injury, they did not affect the development of hypersensitivity indicating that there was no involvement of either isoforms of COX in the initiation of hypersensitivity. In contrast, resveratrol or naproxen, but not rofecoxib, significantly altered hypersensitivity when administered on day 7 (during the development of hypersensitivity) following nerve injury. However, such effect was observed on that day only, but it did not persist. Previous studies have shown that administration of COX inhibitors attenuate behavioral and neurochemical indices of tactile alldynia following spinal strychnine or bicuculline, which has been reported as PGs dependent (Hall et al., 1999; Zhang et al., 2001). Furthermore, COX inhibitors significantly attenuated the PGE2 and PGF2α-induced hyperalgesia and alldynia (Taiwo and Levine, 1988; Park et al., 2000). Because PGs are produced upon metabolism of arachidonic acid by COX, the rate-limiting enzyme that consists of two isoforms, COX-1 and COX-2, it seems likely that the alteration of hypersensitivity by naproxen could be due to preferential inhibition of COX-1, but not COX-2. This is further supported by the marked reversal of neuropathic pain by resveratrol. Thus, it is plausible that COX-1 might be involved in development of hypersensitivity.
Fig. 3.1.1. Ipsilateral and contralateral paw withdrawal responses to (A) thermal (cold allodynia) and (B) mechanical (hyperalgesia) stimulation were assessed prior to and thereafter once a week for 4 weeks following sham or chronic constriction nerve injury (CCI). Responses on day 0 represent baseline paw withdrawal responses. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group. n = 6-8 in each group.
Fig. 3.1.2. Effect of intraperitoneally administered resveratrol (Res; 10 or 30 mg/kg), naproxen (Nap; 10 or 30 mg/kg) or rofecoxib (Rof; 3 and 10 mg/kg) at 2 h before nerve ligation (on day 0) on (A) Ipsilateral and (B) contralateral paw withdrawal responses to cold stimulation in nerve injured rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated (one-way ANOVA followed by Dunnett’s test). (n = 6-8 in each group).
Fig. 3.1.3. Effect of intraperitoneally administered resveratrol (Res; 10 or 30 mg/kg), naproxen (Nap; 10 or 30 mg/kg) or rofecoxib (Rof; 3 or 10 mg/kg) at 2 h before nerve ligation (on day 0) on (A) Ipsilateral and (B) contralateral paw withdrawal responses to mechanical stimulation in nerve injured rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated (one-way ANOVA followed by Dunnett’s test). (n = 6-8 in each group).
Fig. 3.1.4. Effect of intraperitoneally administered resveratrol (Res; 10 or 30 mg/kg), naproxen (Nap; 10 or 30 mg/kg) or rofecoxib (Rof; 3 or 10 mg/kg) on day 7 following nerve injury on (A) ipsilateral and (B) contralateral paw withdrawal responses to thermal stimulation in nerve injured rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated and aP < 0.05 vs saline/tween mixture-treated nerve injured animals (one-way ANOVA followed by Dunnett’s test), (n = 6-8 in each group).
Fig. 3.1.5. Effect of intraperitoneally administered resveratrol (Res; 10 or 30 mg/kg), naproxen (Nap; 10 or 30 mg/kg) or rofecoxib (Rof; 3 or 10 mg/kg) on day 7 following nerve injury on (A) ipsilateral and (B) contralateral paw withdrawal responses to mechanical stimulation in nerve injured rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated and aP < 0.05 vs saline/tween mixture-treated nerve injured animals (one-way ANOVA followed by Dunnett’s test). (n = 6-8 in each group).
Fig. 3.1.6. Effect of intrathecally administered resveratrol (Res; 100 or 300 µg/rat), naproxen (Nap; 100 or 300 µg/rat) or rofecoxib (Rof; 30 or 100 µg/rat) on day 7 following nerve injury on (A) ipsilateral and (B) contralateral paw withdrawal responses to thermal stimulation in nerve injured rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M.
Fig. 3.1.7. Effect of intrathecally administered resveratrol (Res; 100 or 300 µg/rat), naproxen (Nap; 100 or 300 µg/rat) or rofecoxib (Rof; 30 or 100 µg/rat) on day 7 following nerve injury on (A) ipsilateral and (B) contralateral paw withdrawal responses to mechanical stimulation in nerve injured rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M.
Fig. 3.1.8. Effect of perineurally administered resveratrol (Res; 3 or 10 mg/kg), naproxen (Nap; 3 or 10 mg/kg) or rofecoxib (Rof; 1 or 3 mg/kg) on day 7 following nerve injury on (A) ipsilateral and (B) contralateral paw withdrawal responses to thermal stimulation in nerve injured rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M.
Fig. 3.1.9. Effect of perineurally administered resveratrol (Res; 3 or 10 mg/kg), naproxen (Nap; 3 or 10 mg/kg) or rofecoxib (Rof; 1 or 3 mg/kg) on day 7 following nerve injury on (A) ipsilateral and (B) contralateral paw withdrawal responses to mechanical stimulation in nerve injured rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M.
Fig. 3.1.10. Effect of intraperitoneally administered resveratrol (Res; 3, 10 or 30 mg/kg) on (A) ipsilateral and (B) contralateral paw withdrawal responses to thermal stimulation in nerve injured rats. Treatment was initiated 2 h before surgery and continued once daily for 7 days following nerve injury. Values are mean ± S.E.M. *P < 0.05 vs sham-operated and aP < 0.05 vs saline/tween mixture-treated nerve injured animals (one-way ANOVA followed by Dunnett’s test). (n = 6-8 in each group).
Fig. 3.1.11. Effect of intraperitoneally administered resveratrol (Res; 3, 10 or 30 mg/kg) on (A) ipsilateral and (B) contralateral paw withdrawal responses to mechanical stimulation in nerve injured rats. Treatment was initiated 2 h before surgery and continued once daily for 7 days following nerve injury. Values are mean ± S.E.M. *P < 0.05 vs sham-operated and #P < 0.05 vs saline/tween mixture-treated nerve injured animals (one-way ANOVA followed by Dunnett’s test). (n = 6-8 in each group).
Fig. 3.1.12. Effect of intraperitoneally administered naproxen (Nap; 3, 10 or 30 mg/kg) on (A) ipsilateral and (B) contralateral paw withdrawal responses to thermal stimulation in nerve injured rats. Treatment was initiated 2 h before surgery and continued once daily for 7 days following nerve injury. Values are mean ± S.E.M. *P < 0.05 vs sham-operated and aP < 0.05 vs saline/tween mixture-treated nerve injured animals (one-way ANOVA followed by Dunnett’s test). (n = 6-8 in each group).
Fig. 3.1.13. Effect of intraperitoneally administered naproxen (Nap; 3, 10 or 30 mg/kg) on (A) ipsilateral and (B) contralateral paw withdrawal responses to mechanical stimulation in nerve injured rats. Treatment was initiated 2 h before surgery and continued once daily for 7 days following nerve injury. Values are mean ± S.E.M. *P < 0.05 vs sham-operated and *P < 0.05 vs saline/tween mixture-treated nerve injured animals (one-way ANOVA followed by Dunnett’s test). (n = 6-8 in each group).
Fig. 3.1.14. Effect of intraperitoneally administered rofecoxib (Rof; 1, 3 or 10 mg/kg) on (A) ipsilateral and (B) contralateral paw withdrawal responses to thermal stimulation in nerve injured rats. Treatment was initiated 2 h before surgery and continued once daily for 7 days following nerve injury. Values are mean ± S.E.M. *P < 0.05 vs sham-operated and aP < 0.05 vs saline/tween mixture-treated nerve injured animals (one-way ANOVA followed by Dunnett’s test). (n = 6-8 in each group).
Fig. 3.1.15. Effect of intraperitoneally administered rofecoxib (Rof; 1, 3 or 10 mg/kg) on (A) ipsilateral and (B) contralateral paw withdrawal responses to mechanical stimulation in nerve injured rats. Treatment was initiated 2 h before surgery and continued once daily for 7 days following nerve injury. Values are mean ± S.E.M. *P < 0.05 vs sham-operated and aP < 0.05 vs saline/tween mixture-treated nerve injured animals (one-way ANOVA followed by Dunnett’s test). (n = 6-8 in each group).
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The most striking findings of the present study were those revealing that systemic administration of resveratrol or naproxen, but not rofecoxib for 7 days following nerve injury significantly attenuated cold allodynia and mechanical hyperalgesia. The effects of naproxen do not reflect hypoalgesic activity because contralateral paw withdrawal responses were not affected by systemic administration. It is unlikely that rofecoxib failed to reach threshold levels in the spinal cord to alleviate sufficient nociceptive response, because rofecoxib is known to readily cross the blood-brain barrier and sufficient levels to inhibit COX-2 in the spinal cord are achieved after systemic administration (Schwarz et al., 1999; Broom et al., 2004; Dembo et al., 2005). It is further supported by the antiallodynic and antihyperalgesic effects of resveratrol, an inhibitor of both COX and peroxidase reactions of COX-1 and weakly inhibits the peroxidase activity of COX-2 (Jang et al., 1997; Szewczuk et al., 2004). In addition, resveratrol readily cross the blood brain barrier following systemic administration (Wang et al., 2002). It has been reported that COX-2 expression is increased without any change in COX-1 expression in the spinal nerve, at nerve injury site and adjacent region from 2 weeks onwards after nerve injury indicating the time-dependent regulation of COX isozymes and lack of COX-2 involvement during the early stages of development of neuropathic pain (Ma and Eisenach, 2002, 2003a, b).

The antinociceptive activity of locally, perineurally or systemically administered COX inhibitors in alleviating established neuropathic pain has been well documented, however, greater efficacy was observed when administered locally or perineurally than systemically (Syriatowicz et al., 1999; Ma and Eisenach, 2002, 2003a,b). In the present study, all the drugs were also administered via intrathecal and perineural route to nerve injured rats to distinguish the potential site specific effects of COX inhibitors in attenuating the development of neuropathic pain since systemically administered drug is distributed throughout the body. Interestingly, intrathecally or perineurally administered resveratrol or naproxen, but not rofecoxib, attenuated hypersensitivity on that day indicating that PGs produced by COX-1 might be involved in the development of hypersensitivity following nerve injury. Furthermore, in one of the reported studies, a greater number of COX-1 immunoreactivity profiles were observed in the epidermis of the ipsilateral footpad of nerve injured rats suggesting the involvement of COX-1 generated peripheral PGs (Ma and Eisenach, 2002). Recently, perineural administration of indomethacin early after nerve injury
attenuated the development of allodynia (Takahashi et al., 2004). In addition, spinal administration of ketorolac, a COX-1 preferring inhibitor, on day 7 was effective in attenuating cold allodynia and thermal hyperalgesia in sciatic nerve injured rats indicating that spinal PGs were also involved (Parris et al., 1996). Thus it is plausible that COX-1 rather than COX-2 is involved in generating PGs, in both the spinal cord and sciatic nerve, and contributes to the development of neuropathic pain state.

After chronic constriction injury, Aβ-fibers, which normally terminate in deep lamina III and IV of the dorsal horn, have been shown to sprout into the superficial lamina I and II of the dorsal horn, where afferent Aδ- and C-fibers terminate and form a novel physiological synapse with transmission neurons (Chung et al., 1997). This presynaptic interaction between low threshold mechanoreceptors and C-fibers through these second order neurons, which normally code for nociceptive input, now appears to receive non-noxious input. Recently, it has been shown that a greater number of COX-1 immunoreactivity cells with glial morphology in the superficial laminae of the ipsilateral spinal dorsal horn L4-L6 of the spinal cord increased 4 days and 2 weeks after spinal nerve ligation (Zhu and Eisenach, 2003). Further, the time course of the activation and upregulation of COX-1 in those studies parallels the development of hypersensitivity in the present study. Indeed, the neuroanatomical sites for the increased COX-1 expression, termination of nociceptive afferent Aδ- and C-fibers, and second order neurons due to nerve sprouting are in superficial laminae I and II, which further supports that PGs produced by COX-1, but not COX-2, plays an important role in development of hypersensitivity following peripheral nerve injury.

Furthermore, chronic systemic treatment with resveratrol or naproxen, but not rofecoxib, markedly delayed the development of hypersensitivity in nerve injured rats suggesting that there is persistent generation of PGs following nerve injury. It has been previously reported that continuous administration of systemic or epidural NSAIDs relieved neuropathic pain in cancer patients indicating that PGs are continuously produced for longer periods in neuropathic pain (Ripamonti et al., 1996; Lauretti et al., 1998). Moreover, daily intraplantar injections of PGE₂ in rats for 14 days causing the development of persistent mechanical nociceptor hypersensitivity state for more than 30 days, was significantly attenuated by chronic treatment with indomethacin (Ferreira et al., 1990). Recent evidence suggests that COX-1 expression is not static, but changes in a time-dependent manner after peripheral nerve injury.
(Zhu and Eisenach, 2003). Together, the present findings suggest that the development of neuropathic pain is PGs dependent and a gradual and persistent increase in the production of PGs by COX-1 might be expected to last for several weeks in the periphery, injured sciatic nerve and spinal cord, and continuously contribute to the development of neuropathic pain. Although a more long term administration resveratrol or naproxen was not done, it cannot be excluded that long term administration of naproxen could result in a longer lasting decrease in sensitivity after nerve injury. Our findings are in general agreement with this, as chronic treatment with resveratrol, a selective COX-1 inhibitor or naproxen, which preferentially inhibits COX-1, but not rofecoxib that inhibits COX-2, started early before nerve injury for 7 days significantly attenuated the development of the neuropathic pain state following nerve injury.

In summary, the results suggest that there is no role for COX-2 is the development of neuropathic pain in this model of nerve injury. Chronic treatment with selective COX-1 inhibitors started early before nerve injury might prevent or at least delay the development of hypersensitivity.
3.2. SELECTIVE INHIBITION OF CYCLOOXYGENASE-2 ALLEVIATES HYPERSENSITIVITY IN NEUROPATHIC RATS

3.2.1. INTRODUCTION
Neuropathic pain associated with disease or physical damage to peripheral nerves is a physically and emotionally debilitating condition for which treatment is often inadequate. It is manifested as spontaneous pain, hyperalgesia and allodynia and has multiple etiologies (Jensen et al., 2001). Growing body of evidence indicates that the inflammatory response in injured nerves plays an important role in the pathogenesis of neuropathic pain following nerve injury (Tracey and Walker, 1995). Circulating macrophages are the major effector cells that invade the degenerating nerve, and these immune cells not only clear up degraded nerve debris but also produce and release various proinflammatory cytokines, growth factors, pronociceptive mediators (Cui et al., 2000; Sweitzer et al., 2001).

Recent evidence suggested the possible role of prostaglandins (PGs), potent inflammatory and pronociceptive mediators, in peripheral and central sensitization at peripheral sites and in the spinal cord (Willingale et al., 1997; Beiche et al., 1998). They are synthesized in tissues by cyclooxygenase (COX), which is the rate-limiting enzyme that catalyzes conversion of arachidonic acid to generate PGs. There are two isoforms of COX, namely COX-1 and COX-2. COX-1 is constitutively expressed in most cells while COX-2 is rapidly induced by inflammatory stimuli (Svensson and Yaksh, 2002; Simmons et al., 2004). Recently COX-3 has been identified but its physiological and pathological roles are yet to be characterized (Chandrasekharan et al., 2002; Snipes et al., 2005).

Accumulating data indicated differential role for COX isoforms in the hypersensitivity to nerve injury (Zhu and Eisenach, 2003; Ma and Eisenach, 2002, 2003a). Some studies found that systemic and spinal administration of COX inhibitors were ineffective (Lashbrook et al., 1999; Syriatowicz et al., 1999; Hefferan et al., 2003), while others detected significant antiallodynic and antihyperalgesic effects in alleviating experimental neuropathic pain (Ma and Eisenach, 2002, 2003a, b). The mechanisms that underlie neuropathic pain are poorly understood, but several animal models have been developed to probe these mechanisms. It has been reported that the efficacy of various agents in experimental neuropathic pain is dependent on the type of nerve injury and time and route of drug administration (Tal and Bennett 1994;
Martin and Eisenach 2001). Further, CCI of the sciatic nerve shows behavioral signs characteristic of clinical neuropathic pain conditions including allodynia and hyperalgesia (Bennett and Xie, 1988; Attal et al., 1990). In addition, the CCI of the sciatic nerve is one of the most widely used models of peripheral neuropathy and shows highest general sensitivity to various classes of drugs among the commonly used models of peripheral neuropathy (Kontinen and Meert, 2003).

Therefore, the present study was undertaken to determine the effect of acute as well as chronic systemic treatment with selective COX-1, non-selective COX-1/COX-2 or selective COX-2 inhibitors on attenuation of hypersensitivity following chronic constriction injury to the sciatic nerve in rats. Further, intrathecal and perineural injection of COX inhibitors were also employed to differentiate between spinal and peripheral mechanisms in the attenuation of neuropathic pain in nerve-injured rats.

3.2.2. MATERIALS AND METHODS

3.2.2.1. Experimental animals

As per 1.2.1.

Male Wistar rats (bred in Central Animal House of Panjab University, Chandigarh) weighing 130-170 g at the start of the surgery were randomly divided into various groups and each group comprised of 6 - 8 animals was used. Following surgery, the animals were provided with food and water ad libitum in groups of 3 in plastic cages with soft bedding.

3.2.2.2. Chronic constriction nerve injury (CCI)

As per 3.1.2.2.

3.2.2.3. Assessment of neuropathic pain

3.2.2.3.1. Cold allodynia

As per 3.1.2.3a.

3.2.2.3.2. Mechanical hyperalgesia

The withdrawal threshold of the ipsilateral (nerve injured) and contralateral (nerve uninjured) paw was measured using an analgesymeter (Ugo Basile, Italy) by applying noxious pressure to hind paw and expressed in grams as per 1.2.2.3b.

3.2.2.4. Drugs and treatment schedule

Resveratrol (Archer Chem, India), naproxen, ibuprofen, rofecoxib, and valdecoxib (all from Panacea Biotec Ltd., India) were used in the present study.

3.2.2.4.1. Systemic administration

As per 3.1.2.4.1.
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3.2.2.4.2. Spinal administration
As per 3.1.2.4.2.

3.2.2.4.3. Perineural administration
As per 3.1.2.4.3.

3.2.2.4.4. Treatment schedule
All animals were acclimatized to laboratory environment for at least 2 h before testing. All the rats were subjected to these two pain tests on day 0 before performing surgery. To evaluate the effect of COX inhibition on the existing hypersensitivity following nerve injury, rats were administered with single dose of vehicle (saline/tween mixture), a selective COX-1 inhibitor resveratrol (3, 10 or 30 mg/kg, i.p.), non-selective COX inhibitors naproxen or ibuprofen (each 3, 10 or 30 mg/kg, i.p.) or selective COX-2 inhibitors rofecoxib or valdecoxib (each 1, 3 or 10 mg/kg, i.p.) on day 14 following nerve injury and the response to behavioral tests was tested 2 h before, 0.5, 1, 2, 4, and 24 h after drug administration.

Similarly, to determine the effect of chronic administration of COX inhibitors on the maintenance of neuropathic pain, vehicle (saline/tween mixture), resveratrol, naproxen, ibuprofen (each 3 or 10 mg/kg, i.p.), rofecoxib or valdecoxib (each 1 or 3 mg/kg, i.p.) were administered on day 7 and continued once daily through day 14 post-nerve injury. The paw withdrawal response to thermal and mechanical stimulation was tested on day 14 following 2 h after treatment and once a week thereafter for 4 weeks after nerve injury.

Further, separate groups of rats were intrathecally (i.t.) or perineurally (p.n.) administered with 70% DMSO, resveratrol, naproxen, ibuprofen (each 100 or 300 µg i.t. or 3 or 10 mg/kg, p.n.), rofecoxib or valdecoxib (each 30 or 100 µg i.t. or 1 or 3 mg/kg, p.n.) and tested for behavioral response at 2 h before, 0.5, 1, 2, 4 and 24 h after treatment on day 14 following peripheral nerve injury in rats.

3.2.2.5. Statistical analysis
All the values were expressed as mean ± S.E.M. of 6-8 animals per group. The significance of the difference in the mean values of paw withdrawal latency to thermal and withdrawal threshold to mechanical stimulation were analyzed by repeated measures ANOVA for time courses of two or more groups with the within subject factor “time” and the between subject factor “treatment” followed by
3.2.3. RESULTS

3.2.3.1 General behavior of sham-operated and nerve injured animals

In the present series of experiments, the baseline paw withdrawal response in each test obtained on day 0 for each rat was relatively stable and showed no significant variation. The mean paw withdrawal latency to thermal stimulation was 14.21 ± 0.64 sec on the left and 13.98 ± 0.79 sec on the right, and withdrawal threshold to pressure was 175.87 ± 12.57 g and 172.93 ± 14.37 g, respectively, on day 0 before performing surgery. The body weights and general health of the animals were closely monitored. The animals kept their nerve injured paw elevated above the cage floor and the toes on that limb were held in a ventroflexed position, but otherwise appeared healthy, exhibited normal grooming, feeding behavior, and gained weight normally. The ipsilateral and contralateral paw withdrawal responses to thermal and mechanical stimulation in sham-operated rats remained unchanged from respective baseline values throughout the entire observation period. These values were closely paralleled those for the unoperated limb of the ligated animals under all behavioral testing conditions (Fig. 3.2.8 to 3.2.12).

3.2.3.2 Effect of acute systemic administration of COX inhibitors on neuropathic pain

There was no significant difference in ipsilateral paw withdrawal responses between various groups of animals except sham-operated animals on day 14 before drug administration. The ipsilateral paw withdrawal responses of all the vehicle (saline/tween mixture)-treated nerve injured rats were significantly less than that of sham-operated rats on day 7 onwards and reached steady state between days 14 and 28 after surgery indicating the development and maintenance of allodynia and hyperalgesia in a time-dependent manner (Fig. 3.2.8 to 3.2.12). Systemic administration of single dose of resveratrol (3, 10 or 30 mg/kg, i.p.) on day 14 following nerve ligation did not show any significant difference in the ipsilateral paw withdrawal responses to thermal and mechanical stimulation as compared to vehicle treatment (Fig. 3.1.1A and 1B). Similarly, administration of single dose of naproxen or ibuprofen (3, 10 or 30 mg/kg, i.p.) on day 14 following nerve ligation did not reverse hypersensitivity as compared to vehicle treatment (Fig. 3.2.2A, 2B, 3.2.3A, and 3B). On the contrary, administration of a single dose of rofecoxib or valdecoxib
(1, 3 or 10 mg/kg, i.p.) on day 14 following nerve injury significantly, dose- and time-
dependently reversed allodynia (Fig. 3.2.4A and 5A) and hyperalgesia (Fig. 3.2.4B
and 5B) up to 4 h after the treatment as compared to saline/tween mixture treatment. However, both the drugs did not alter hypersensitivity at 24 h after the treatment.

3.2.3.3. Effect of chronic systemic administration of COX inhibitors on
neuropathic pain
Saline-tween mixture treatment had no effect on ipsilateral paw withdrawal responses
to thermal (Fig. 3.2.8A, 9A, 10A, 11A, and 12A) and mechanical (Fig. 3.2.8B, 9B,
10B, 11B, and 12B) stimulation over a 24 h period of testing. Chronic treatment with
resveratrol (3 or 10 mg/kg, i.p., initiated from day 7 to day 14 post-nerve injury) in
nerve injured rats had no effect on cold allodynia (Fig. 3.2.8A) and mechanical
hyperalgesia (Fig. 3.2.8B). The lowest dose of naproxen (3 mg/kg, i.p., initiated from
day 7 to day 14 post-nerve injury) in nerve injured rats showed only weak
antiallodynic effect, however, the highest dose of naproxen (10 mg/kg, i.p.) showed
antiallodynic and antihyperalgesic effect on day 14 following 2 h after treatment. In
contrast, both the doses of naproxen on chronic administration did not alter the
maintenance of hypersensitivity at later time points throughout the study period as
compared with vehicle-treated nerve injured rats (Fig. 3.2.9A and 9B). The lowest
dose of ibuprofen (3 mg/kg, i.p., initiated from day 7 to day 14 post-nerve injury) in
nerve injured rats showed weak antiallodynic but not antihyperalgesic effect on day
14, however, it did not attenuate hypersensitivity through out the remaining study
period (Fig. 3.2.10A and 10B). In contrast, the highest tested dose ibuprofen (10
mg/kg, i.p.) significantly attenuated the maintenance of allodynia and hyperalgesia in
ipsilateral paw on day 14 and 21, however, it did not show any significant difference
in the ipsilateral paw withdrawal responses to thermal and mechanical stimulation as
compared to vehicle treatment on day 28 following nerve injury (Fig. 3.2.10A and
10B). Rats administered with rofecoxib (1 or 3 mg/kg, i.p., once daily for one week
starting on day 7 post-nerve injury) significantly and time-dependently attenuated
hypersensitivity in ipsilateral paw on day 14, 21, and 28 following nerve injury as
compared with vehicle-treated nerve injured rats (Fig. 3.2.11A and 11B). Similarly,
 systemic administration of valdecoxib (1 or 3 mg/kg, i.p., once daily for one week
starting on day 7 post-nerve injury) elicited a marked attenuation of hypersensitivity
in ipsilateral paw on day 14, 21, and 28 following nerve injury (Fig. 3.2.12A and
12B).
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3.2.3.4. Effect of spinal and perineural administration of COX inhibitors on neuropathic pain

Because systemic administration of nonselective or selective COX-2 inhibitors showed antiallodynic and antihyperalgesic effects, intrathecal or perineural administration of COX inhibitors were employed to differentiate between spinal and peripheral mechanisms, respectively, on the reversal of hypersensitivity on day 14 following nerve injury in rats. 70% DMSO treatment had no effect on ipsilateral paw withdrawal responses to thermal (Fig. 3.2.15A to 19A) and mechanical (Fig. 3.2.15B to 19B) stimulation over a 24 h period of testing. The intrathecal (100 or 300 μg/rat) or perineural (3 or 10 mg/kg) administration of resveratrol had no effect on ipsilateral paw withdrawal responses to thermal (Fig. 3.2.15A and 22A) and mechanical (Fig. 3.2.15B and 22B) stimulation as compared to vehicle treatment. Similarly, intrathecal (100 or 300 μg/rat) or perineural (3 or 10 mg/kg) administration of naproxen did not alter hypersensitivity in nerve-injured rats (Fig. 3.2.16A, 16B, 3.2.23A, and 23B). In contrast, the highest dose (300 μg/rat, i.t. and 10 mg/kg, p.n.) but not the lowest dose (100 μg/rat, i.t. and 3 mg/kg, p.n.) of ibuprofen showed significant, but weak antiallodynic or antihyperalgesic effect at 1 and 2 after intrathecal (Fig. 3.2.17A and 17B) and 2 h after perineural (Fig. 3.2.24A and 24B) administration, respectively. The intrathecal (30 or 100 μg/rat) or perineural (1 or 3 mg/kg) treatment of rofecoxib produced significant reversal of hypersensitivity for 4 h, however, such reversal was not observed 24 h after the treatment as compared to DMSO control animals (Fig. 3.2.18A, 18B, 3.2.25A, and 25B). Valdecoxib, administered intrathecally (30 or 100 μg/rat) or perineurally (1 or 3 mg/kg) significantly increased the ipsilateral paw withdrawal responses to thermal (Fig. 3.2.19A and 26A) and mechanical (Fig. 3.2.19BA and 26B) stimulation as compared to vehicle treatment for 4 h after drug administration. The spinal administration of selective COX-2 inhibitors showed immediate effect whereas perisciatic administration showed delayed antiallodynic and antihyperalgesic effect with a maximal effect was observed at 1 and 2 h following drug administration respectively.

In all these experiments, systemic, intrathecal or perineural administration of COX inhibitors on day 14 and for 7 days (from day 7 to day 14 following nerve injury) had no effect on the contralateral paw withdrawal responses in these tests as
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Fig. 3.2.1. Effect of intraperitoneally administered resveratrol (Res; 3, 10 or 30 mg/kg) on day 14 following nerve injury on ipsilateral paw withdrawal responses to (A) cold and (B) mechanical stimulation in neuropathic rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group. n = 6-8 in each group.
Fig. 3.2.2. Effect of intraperitoneally administered naproxen (Nap; 3, 10 or 30 mg/kg) on day 14 following nerve injury on ipsilateral paw withdrawal responses to (A) cold and (B) mechanical stimulation in neuropathic rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group. aP < 0.05 as compared to saline/tween mixture-treated group. n = 6-8 in each group.
Fig. 3.2.3. Effect of intraperitoneally administered ibuprofen (Ibu; 3, 10 or 30 mg/kg) on day 14 following nerve injury on ipsilateral paw withdrawal responses to (A) cold and (B) mechanical stimulation in neuropathic rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group. a P < 0.05 as compared to saline/tween mixture-treated group. n = 6-8 in each group.
Fig. 3.2.4. Effect of intraperitoneally administered rofecoxib (Rof; 1, 3 or 10 mg/kg) on day 14 following nerve injury on ipsilateral paw withdrawal responses to (A) cold and (B) mechanical stimulation in neuropathic rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group. a P < 0.05 as compared to saline/tween mixture-treated group. n = 6-8 in each group.
Fig. 3.2.5. Effect of intraperitoneally administered valdecoxib (Val; 1, 3 or 10 mg/kg) on day 14 following nerve injury on ipsilateral paw withdrawal responses to (A) cold and (B) mechanical stimulation in neuropathic rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group. a P < 0.05 as compared to saline/tween mixture-treated group. n = 6-8 in each group.
Fig. 3.2.6. Effect of intraperitoneally administered resveratrol (Res; 3, 10 or 30 mg/kg), naproxen (Nap; 3, 10, or 30 mg/kg) or ibuprofen (Ibu; 3, 10, or 30 mg/kg) on day 14 following nerve injury on contralateral paw withdrawal responses to (A) cold and (B) mechanical stimulation in neuropathic rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *$P<0.05$ vs sham-operated group. $n = 6-8$ in each group.
Fig. 3.2.7. Effect of intraperitoneally administered rofecoxib (Rof; 1, 3 or 10 mg/kg) or valdecoxib (Val; 1, 3, or 10 mg/kg) on day 14 following nerve injury on contralateral paw withdrawal responses to (A) cold and (B) mechanical stimulation in neuropathic rats. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group. n = 6-8 in each group.
Fig. 3.2.8. Effect of intraperitoneally administered resveratrol (Res; 3 or 10 mg/kg) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Treatment was initiated after behavioral assessment on day 7 and continued once daily till day 14 following nerve injury. Responses on day 0 represent baseline paw withdrawal responses. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and **P < 0.05 vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.9. Effect of intraperitoneally administered naproxen (Nap; 3 or 10 mg/kg) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Treatment was initiated after behavioral assessment on day 7 and continued once daily till day 14 following nerve injury. Responses on day 0 represent baseline paw withdrawal responses. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and P < 0.05 vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.10. Effect of intraperitoneally administered ibuprofen (Ibu; 3 or 10 mg/kg) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Treatment was initiated after behavioral assessment on day 7 and continued once daily till day 14 following nerve injury. Responses on day 0 represent baseline paw withdrawal responses. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and †P < 0.05 vs saline-tween mixture treated group, n = 6-8 in each group.
Fig. 3.2.11. Effect of intraperitoneally administered rofecoxib (Rof; 1 or 3 mg/kg) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Treatment was initiated after behavioral assessment on day 7 and continued once daily till day 14 following nerve injury. Responses on day 0 represent baseline paw withdrawal responses. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and †P < 0.05 vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.12. Effect of intraperitoneally administered valdecoxib (Val; 1 or 3 mg/kg) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Treatment was initiated after behavioral assessment on day 7 and continued once daily till day 14 following nerve injury. Responses on day 0 represent baseline paw withdrawal responses. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and \( ^{a}P < 0.05 \) vs saline-tween mixture treated group, n = 6-8 in each group.
Fig. 3.2.13. Effect of intraperitoneally administered resveratrol (Res; 3 or 10 mg/kg), naproxen (Nap; 3 or 10 mg/kg) or ibuprofen (Ibu; 3 or 10 mg/kg) on contralateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Treatment was initiated after behavioral assessment on day 7 and continued once daily till day 14 following nerve injury. Responses on day 0 represent baseline paw withdrawal responses. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group.
Fig. 3.2.14. Effect of intraperitoneally administered rofecoxib (Rof; 1 or 3 mg/kg) or valdecoxib (Val; 1 or 3 mg/kg) on contralateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Treatment was initiated after behavioral assessment on day 7 and continued once daily till day 14 following nerve injury. Responses on day 0 represent baseline paw withdrawal responses. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group.
Fig. 3.2.15. Effect of intrathecally administered resveratrol (Res; 100 or 300 µg/rat) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and *P < 0.05 vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.16. Effect of intrathecally administered naproxen (Nap; 100 or 300 μg/rat) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *$P < 0.05$ vs sham-operated group and $^aP < 0.05$ vs saline-tween mixture treated group. $n = 6-8$ in each group.
Fig. 3.2.17. Effect of intrathecally administered ibuprofen (Ibu; 100 or 300 μg/rat) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and **P < 0.05 vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.18. Effect of intrathecally administered rofecoxib (Rof, 30 or 100 μg/rat) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *$P < 0.05$ vs sham-operated group and $^aP < 0.05$ vs saline-tween mixture treated group. $n = 6-8$ in each group.
Fig. 3.2.19. Effect of intrathecally administered valdecoxib (Val; 30 or 100 µg/rat) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and aP < 0.05 vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.20. Effect of intrathecally administered resveratrol (Res; 100 or 300 µg/rat), naproxen (Nap; 100 or 300 µg/rat) or ibuprofen (Ibu; 100 or 300 µg/rat) on contralateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and **P < 0.05 vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.21. Effect of intrathecally administered rofecoxib (Rof; 30 or 100 μg/rat) or valdecoxib (Val; 30 or 100 μg/rat) on contralateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and ^P < 0.05 vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.22. Effect of perineurally administered resveratrol (Res; 3 or 10 mg/kg) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and aP < 0.05 vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.23. Effect of perineurally administered naproxen (Nap; 3 or 10 mg/kg) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and ^aP < 0.05 vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.24. Effect of perineurally administered ibuprofen (Ibu; 3 or 10 mg/kg) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and \( aP < 0.05 \) vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.25. Effect of perineurally administered rofecoxib (Rof; 1 or 3 mg/kg) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and aP < 0.05 vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.26. Effect of perineurally administered valdecoxib (Val; 1 or 3 mg/kg) on ipsilateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group and "P < 0.05 vs saline-tween mixture treated group. n = 6-8 in each group.
Fig. 3.2.27. Effect of perineurally administered resveratrol (Res; 3 or 10 mg/kg), naproxen (Nap; 3 or 10 mg/kg) or ibuprofen (Ibu; 3 or 10 mg/kg) on contralateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group. n = 6-8 in each group.
Fig. 3.2.28. Effect of perineurally administered rofecoxib (Rof; 1 or 3 mg/kg) or valdecoxib (Val; 1 or 3 mg/kg) on contralateral paw withdrawal responses to (A) thermal and (B) mechanical stimulation in nerve injured rats. Responses on day 0 represent baseline paw withdrawal responses. Arrow indicates the time of drug administration. Values are mean ± S.E.M. *P < 0.05 vs sham-operated group. n = 6-8 in each group.
3.2.4. DISCUSSION

The results of the present study indicated that acute systemic administration of selective COX-2 inhibitor rofecoxib or valdecoxib, but not a selective COX-1 inhibitor resveratrol, or nonselective COX inhibitor naproxen or ibuprofen, at the time when hypersensitivity is established, effectively reversed the behavioral manifestation of neuropathic pain. However, such effect was observed on the day of experimentation only, but did not persist longer. Previous studies have shown that administration of COX inhibitors attenuated behavioral and neurochemical indices of tactile allodynia following spinal strychnine or bicuculline, which has been reported as PGs dependent (Hall et al., 1999; Zhang et al., 2001). This is consistent with the electrophysiological properties of the PGs indicating that it plays a role in the modulation of neuronal excitation (Baba et al., 2001; Ahmadi et al., 2002). Because COX, the rate-limiting enzyme that existed in two isoforms, COX-1 and COX-2, it seems likely that the reversal of hypersensitivity could be due to selective inhibition of COX-2.

It has been suggested that the development and maintenance of behavioral manifestations in neuropathic pain are due, at least in part, to spontaneous ectopic discharges of primary afferent neurons and subsequent sensitization of dorsal horn neurons by PGs (Syriatowicz et al., 1999; Hefferan et al., 2003). Further, direct administration of PGE\(_2\) and PGF\(_{2\alpha}\) into spinal cord results in spontaneous agitation, hyperalgesia and tactile allodynia, which were significantly attenuated by COX inhibitors in mice and rats (Taiwo and Levine, 1988; Minami et al., 1994; Park et al., 2000; Tumbach et al., 2002). The PGs, specifically PGE\(_2\), are released in peripheral sensory neurons of the DRG and in spinal cord, not only decreases nociceptive threshold but also increases response to noxious stimulation (Malmberg et al., 1995; Vasquez et al., 2001). The enhanced activity presents as increased neuronal responses to stimuli and also spontaneous discharges that may originate from the site of injury, the DRG, and also from remaining intact fibers and therefore contributing to hypersensitivity.

An interesting observation is that chronic administration of rofecoxib or valdecoxib significantly attenuated the maintenance of hypersensitivity in nerve-injured rats suggesting that there is persistent generation of PGs at both spinal and peripheral sites. However, the highest dose of naproxen or ibuprofen showed weak
antiallodynic and antihyperalgesic effects in neuropathic rats. On the contrary, resveratrol, a selective COX-1 inhibitor failed to alter hypersensitivity. It has been previously reported that continuous administration of systemic or epidural NSAIDs relieved neuropathic pain in cancer patients indicating that PGs are continuously produced for longer periods in neuropathic pain (Ripamonti et al., 1996; Lauretti et al., 1998). Moreover, daily intraplantar injections of PGE₂ in rats for 14 days cause the development of persistent mechanical nociceptor hypersensitivity state that lasting for more than 30 days was significantly attenuated by chronic treatment with indomethacin (Ferreira et al., 1990). Recent evidence suggested that COX-2 expression is not static, but changes in a time-dependent manner after peripheral nerve injury (Ma and Eisenach, 2002, 2003a, b). Together, the present findings suggest that neuropathic pain is PGs dependent and a gradual and persistent increase in the production of PGs by COX-2 might be expected to last for several weeks in the periphery, injured sciatic nerve and spinal cord, and constantly contribute to the hypersensitivity. Our findings are in general agreement with this, as chronic treatment with rofecoxib or valdecoxib, which selectively inhibits COX-2, significantly attenuated the maintenance of neuropathic state following nerve injury.

It is well known that spinal and peripheral PGs are involved in sensitization of central and peripheral afferent fibers, respectively, that contribute to the hypersensitivity (Ferreira et al., 1990; Taiwo and Levine, 1990; Willingale et al., 1997; Vasquez et al., 2001). Therefore, in the present study, the drugs were also administered via intrathecal and perineural route to nerve injured rats to distinguish the potential spinal and peripheral mechanisms in attenuating the hypersensitivity since systemically administered drug is distributed to both spinal and peripheral sites. Interestingly, intrathecally or perineurally administered ibuprofen, a nonselective COX inhibitor that possess some selectivity for COX-2 inhibition and selective COX-2 inhibitors attenuated hypersensitivity on the day of experimentation indicating that PGs produced by both spinal as well as peripheral COX-2 might be involved in the hypersensitivity following nerve injury. It has been reported that systemic and subcutaneous injection of EP₁ receptor antagonist blocked hypersensitivity and suggested a PG dependent mechanisms in the receptive field of injured neurons (Syriatowicz et al., 1999; Kawahara et al., 2001). Although, we did not measure PGs, the results agree with the observation that peripheral nerve injury results in an increase in the PGs at the site of injury and in the spinal cord (Syriatowicz et al.,
These data along with the present results clearly demonstrate the antinociceptive activity of spinally, perineurally or systemically administered COX-2 inhibitors in alleviating thermal and mechanical hypersensitivities after CCI to sciatic nerve.

The results are in contrast to previous reports in which systemically administered COX inhibitor did not reverse hypersensitivity and the doses employed for systemic and spinal administration of drugs are the same (Syriatowicz et al., 1999; Hefferan et al., 2003). Rofecoxib and valdecoxib are highly non-polar and readily cross lipophilic barriers whereas naproxen is weakly non-polar, thus it may be misinterpreted for its less efficacy in alleviating hypersensitivity to nerve injury (Abdel-Halim et al., 1978; Merck and Co. Vioxx information, issue 2000; Dembo et al., 2005). However, the doses of naproxen employed were in the same range that showed antipyretic and analgesic effects following systemic administration (Jain et al., 1999; Josa et al., 2001). Furthermore, naproxen and ibuprofen are relatively more potent inhibitors of COX-1 than COX-2. So the relatively less efficacy of these drugs is not due to sufficient drug concentration at the vicinity of spinal cord, but it may be due to absence of adequate levels of COX-1 expression to release PGs or ineffective participation of COX-1. It is further supported by the absence of effects of resveratrol, a selective COX-1 inhibitor in attenuating neuropathic pain. Resveratrol is a potent inhibitor of both cyclooxygenase and peroxidase reactions of COX-1 and causes a mechanism-based inhibition of COX-1, however, it only weakly inhibited the peroxidase activity of COX-2 (Jang et al., 1997; Brzozowski et al., 2001; Sylvia et al., 2001; Szewczuk et al., 2004). Moreover, resveratrol cross the blood brain barrier and produced marked neuroprotection following systemic administration (Wang et al., 2002). These data supports the important findings that there is no role of COX-1 in maintenance of hypersensitivity following nerve injury. Further, the effects of rofecoxib or valdecoxib do not reflect hypoalgesic activity because contralateral paw withdrawal responses were not affected by systemic, intrathecal, and perineural administration. It has been reported that COX-2 expression is increased without any change in COX-1 expression in the spinal nerve, at nerve injury site and adjacent region from 2 weeks onwards after nerve injury indicating the COX-2 participation during the maintenance of neuropathic pain (Ma and Eisenach, 2002, 2003a, b). Recently, various studies demonstrated antiallodynic and antihyperalgesic effects of systemically administered selective COX-2 inhibitors in a model of post-operative
pain, cancer neuropathic pain in rats and electrically evoked hyperalgesia in humans (Yamamoto et al., 2000; Fox et al., 2004; Koppert et al., 2004). Thus it is plausible that COX-2 rather than COX-1 are involved in generating PGs and contributed to the hypersensitivity following nerve injury.

It has been well reported that the central terminals of Aβ-mechanoreceptive afferents, which normally terminate in deep lamina III and IV of the dorsal horn, have been shown to sprout into the superficial lamina I and II of the dorsal horn, where afferent Aδ- and C-fibers terminate and form a novel physiological synapse with transmission neurons after peripheral nerve injury (Woolf et al., 1992). This presynaptic interaction between low threshold mechanoreceptors and C fibers through these second order neurons, which normally code for nociceptive input now appears to receive non-noxious input. Moreover, high densities of binding sites for PGE$_2$ are co-localized with high-threshold, thin unmyelinated primary afferent C-fibers in lumbar spinal horn lamina I and II (Willingale et al., 1997; Beiche et al., 1998; Narumiya et al., 1999). Recently, it has been shown that COX-2 immunoreactive cells with glial morphology in the superficial laminae of the ipsilateral spinal dorsal horn L4-L6 of spinal cord and PGE$_2$ receptors in the injured nerve are increased 2 weeks after spinal nerve ligation (Ma and Eisenach, 2003a, b, c). Further, the time course of the activation and upregulation of COX-2 in those studies parallels the development and maintenance of hypersensitivity in the present study. Indeed, the neuroanatomical sites for the increased COX-2 expression, receptors for PGE$_2$, termination of nociceptive afferent Aδ- and C-fibers, and second order neurons due to nerve sprouting are in superficial laminae I and II, which further supports that PGs produced by COX-2, but not COX-1 play an important role in the maintenance of hypersensitivity following peripheral nerve injury.

In summary, the results of the present study not only demonstrate the effectiveness of the selective COX-2 inhibition on attenuating peripheral neuropathic pain but also show a significant role for COX-2 in nerve injury model of neuropathic pain. Further, the fact that rofecoxib or valdecoxib attenuated behavioral hypersensitivity due to nerve injury is likely associated with its ability to inhibit both spinal as well as peripheral COX-2. Thus, chronic treatment with COX-2 inhibitors could prevent hypersensitivity to peripheral nerve injury.