CHAPTER 2:

LIPOPOLYSACCHARIDE-INDUCED HYPERSENSITIVITY:
ROLE OF CYCLOOXYGENASE-2
2.1. SELECTIVE INHIBITION OF CYCLOOXYGENASE-2 REVERSES LIPOPOLYSACCHARIDE-INDUCED HYPERALGESIA

2.1.1. INTRODUCTION

Prostaglandins produced in both in the periphery and in the CNS have been well demonstrated to play key roles in inflammatory process, sensitization of nociceptors, and nociceptive transmission. Therefore, the COX enzyme, which participate in the synthesis of PGs have been the target for the action of NSAIDs (Garavito and DeWitt, 1999; Kulkarni et al., 2000). The effectiveness and sensitivity of animal models differ depending on the participation of different COX isoforms. Various studies have demonstrated the induction of COX-2 enzyme in chronic pain and inflammatory conditions and produced PGs mainly at sites of inflammation without affecting expression of COX-1 enzyme (Beiche et al., 1996; Zhang et al., 1997; Ebersberger et al., 1999; Tonoi et al., 1999).

Lipopolysaccharide, also known as endotoxin, a major constituent of the outer membrane of the cell wall of gram-negative bacteria, is reported to release pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α by activated monocytes and macrophages, which further induce the expression of inducible nitric oxide synthase (iNOS) and COX-2 leading to the release of eicosanoids, neuropeptides, and reactive oxygen species (Watkins et al., 1994; O’Neill and Lewis, 1998; Eriksson et al., 2000). Moreover, systemic and local administration of LPS demonstrated marked hyperalgesia in various animal models of peripheral and central nociceptive assays (Kanaan et al., 1996; Matsumoto et al., 1998; Jain et al., 2001b and 2002). Previous studies have demonstrated the modulatory role of nitric oxide and COX enzyme pathway, cytokines and growth factors as the possible mediators of LPS-induced hyperalgesia (Kanaan et al., 1996, 1997; Talhouk et al., 2000; Jain et al., 2001b, 2002). Despite the fact that LPS induces hyperalgesia in various nociceptive assays the role of COX-2 in LPS-induced hyperalgesia has not been fully understood.

The discovery of COX-2 inhibitors with potentially low risk of gastrointestinal side effects has generated lot of interest in the possible role of COX-2 in various pathophysiological conditions. Therefore, the present study was designed to investigate and characterize the role of COX-2 by examining the effects of selective inhibition of COX-2 on LPS-induced hyperalgesia using peripheral and central nociceptive models in mice and rats, respectively.
2.1.2. MATERIALS AND METHODS

2.1.2.1. Experimental animals
As per 1.2.1.

2.1.2.2. Induction of lipopolysaccharide-induced hypersensitivity
All animals were acclimatized to laboratory environment for at least 2 h before testing. Hyperalgesia was induced by intraperitoneal or subcutaneous administration of LPS 50 µg/mouse or 25 µg/left hind paw of rat, respectively. Control mice received equivalent volume of saline intraperitoneally whereas rats received saline subcutaneously into left hind paw. Hyperalgesic response was assessed 8 h after LPS injection by tail flick test and acetic acid-induced writhes in mice and by thermal and mechanical nociception in rats. A dose-response curve was established using five to six animals per group. In all these assays, normal saline was used as vehicle.

2.1.2.3. Behavioral assays of hyperalgesia

2.1.2.3.1. Acetic acid-induced writhing assay in mice
As per 1.2.2.1.

The effect of LPS treatment was considered hyperalgesic if the mean writhes was significantly increased compared to that of saline-injected animals measured at the same time after injection.

2.1.2.3.2. Tail immersion assay in mice
This assay was used to assess central hyperalgesia in LPS-pretreated mice and carried out as described in a previous study (Larson et al., 2000). In brief, animals were manually restrained and the tail was submerged to a distance of 1.0 cm of the base of the tail (mouse) in a water bath maintained at 53°C. The withdrawal latency was defined as the time for the animal to withdraw its tail from water. A cut off time of 15 sec was used to prevent any damage to the tail. The effect of LPS treatment was considered hyperalgesic if the average latency of tail flick response was significantly decreased compared to that of saline-injected animals measured at the same time after injection.

2.1.2.3.3. Lipopolysaccharide-induced thermal and mechanical hyperalgesia in rat
As per 1.2.2.3a and b.

The mean paw withdrawal latency of the LPS or saline-injected paw when dipped in water bath maintained at 47 ± 0.5°C was measured and expressed in seconds as per 1.2.2.3a. The withdrawal threshold was evaluated using an analgesymeter (Ugo
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Basile, Italy) by applying noxious pressure to hind paw and expressed in grams as per 1.2.2.3b.

The mean withdrawal latency of LPS or saline injected paw was observed at 30, 60, and 120 min after drug administration. The effect of LPS treatment was considered hyperalgesic if the average withdrawal latency or threshold was significantly decreased compared to that of saline-injected animals measured at the same time after injection.

2.1.2.4. Drugs and treatment schedule

Parecoxib sodium (Panacea Biotec Ltd., India), dexamethasone (Unichem Labs, India), lipopolysaccharide from Salmonella typhimurium (Sigma, USA), and acetic acid (SD Fine Chemicals, India) were used in this study. Parecoxib sodium was freshly prepared by dissolving in normal saline and administered intravenously in a dose volume of 10 ml/kg body weight of mice and 2 ml/kg body weight of rats at the time as mentioned above.

In all the assays, parecoxib (1, 2, 5, 10 or 20 mg/kg) was administered intravenously 30 min before assessing hyperalgesia. Dexamethasone (0.5 or 2 mg/kg) was administered intraperitoneally 1 or 2 h before LPS pre-treatment to rats and mice, respectively and another injection of dexamethasone (0.5 mg/kg, i.p.) was administered 1 h before behavioral assay to rats and mice. Similarly, the effect of parecoxib and dexamethasone on acetic acid-induced writhes and tail immersion test was assessed in mice that had not received LPS pre-treatment. Saline and LPS injected animals received equivalent volume of vehicle intravenously.

2.1.2.5. Statistical analysis

All the values were expressed as mean ± S.E.M. in dose-response curves. The significance of the difference in the responses between saline and LPS pre-treated control animals was analyzed by unpaired Student’s t-test and that of various groups of drug-treated animals in comparison to LPS-pretreated control animals was analyzed by one-way analysis of variance with a Dunnett’s test for multiple comparisons. \( P < 0.05 \) was considered as statistically significant.

2.1.3. RESULTS

2.1.3.1. Acetic acid-induced writhing in mice

Intraperitoneal administration of acetic acid resulted in characteristic writhing responses in control animals. LPS pre-treatment (50 \( \mu \)g/mouse, i.p., 8 h before)
significantly enhanced the number of writhes for 20 min in comparison to control mice that received saline only (without LPS pre-treatment). Parecoxib (1 – 20 mg/kg, i.v.) produced significant reduction in the enhanced number of writhes dose dependently in LPS-pretreated mice to the level of the animals that had not received LPS but did not reduce the number of writhes in saline pre-treated mice (without LPS pre-treatment) (Fig. 2.1.1). Dexamethasone (0.5 mg/kg, i.p.) pretreatment also significantly attenuated enhancement of writhing responses in LPS-injected mice. However, it had no effect on the number of writhes due to acetic acid injection in saline-pretreated mice (Fig. 2.1.1).

2.1.3.2. Tail immersion assay in mice
LPS pre-treatment (50 μg/mouse, i.p., 8 h before) resulted significant decrease in mean tail withdrawal latency (hyperalgesic response) as compared to saline control animals. Parecoxib (1 – 20 mg/kg, i.v.) dose-dependently increased the mean tail withdrawal latency in LPS-pretreated mice as compared to LPS control mice. Similar effect was also observed with dexamethasone (0.5 mg/kg, i.p.) pretreatment. None of the agents at the same dose used alone had a significant effect in increasing the mean withdrawal latency in saline pre-treated mice (without LPS pre-treatment) (Fig. 2.1.2).

2.1.3.3. Lipopolysaccharide-induced thermal and mechanical hyperalgesia in rats
LPS (25 μg/paw, s.c., 8 h before) administration into left hind (ipsilateral) paw produced marked hyperalgesia as shown by significant decrease in paw withdrawal latency to thermal stimulus and paw withdrawal threshold to mechanical pressure as compared to basal levels. The observed hyperalgesia in each LPS-pretreated animal was relatively stable during the experimentation period (10 h) and showed no significant variations. In control group, the withdrawal latency and withdrawal threshold on both paws (saline injected and non-injected) were comparable. Our results indicate that marked hyperalgesia was observed only in the ipsilateral paw of LPS-pretreated animals as compared to the ipsilateral paw of the saline control animals while the contralateral paw showed withdrawal latency and withdrawal threshold comparable to the saline control (Fig. 2.1.3B and 2.1.4B). Dexamethasone (0.5 mg/kg, i.p.) and parecoxib (1 – 20 mg/kg, i.v.) dose-dependently attenuated thermal and mechanical hyperalgesia in the ipsilateral paw of LPS-pretreated animals (Fig. 2.1.3A and 2.1.4A). However, both dexamethasone and parecoxib did not alter the withdrawal latency and withdrawal threshold of the contralateral paw of LPS-pretreated animals as compared to LPS-pretreated control rats that had received saline (Fig. 2.1.3 and 2.1.4).
Fig. 2.1.1. Effect of parecoxib (Pare; 1 - 20 mg/kg i.v.) and dexamethasone (Dex; 0.5 mg/kg i.p.) on acetic acid-induced writhes in saline and lipopolysaccharide (LPS) pre-treated animals (50 μg per mouse). Values are mean ± S.E.M. *P < 0.05 vs saline control (without LPS pre-treatment) (Student’s t-test) and aP < 0.05 vs LPS pre-treated control animals (one-way ANOVA followed by Dunnett’s test).

Fig. 2.1.2. Effect of parecoxib (Pare; 1 - 20 mg/kg i.v.) and dexamethasone (Dex; 0.5 mg/kg i.p.) on tail withdrawal latencies in saline and lipopolysaccharide (LPS) pre-treated animals (50 μg per mouse). Values are mean ± S.E.M. *P < 0.05 vs saline control (without LPS pre-treatment) (Student’s t-test) and aP < 0.05 vs LPS pre-treated control animals (one-way ANOVA followed by Dunnett’s test).
Fig. 2.1.3. Effect of parecoxib (Pare; 1 - 20 mg/kg i.v.) and dexamethasone (Dex; 0.5 mg/kg i.p.) on (A) ipsilateral (LPS-injected) and (B) contralateral (LPS-noninjected) paw withdrawal latencies in lipopolysaccharide (LPS) pre-treated animals. Values are mean ± S.E.M. *p < 0.05 vs saline control (without LPS pre-treatment) (Student’s t-test) and *p < 0.05 vs LPS pre-treated control animals (one-way ANOVA followed by Dunnett’s test).
Fig. 2.1.4. Effect of parecoxib (Pare; 1 - 20 mg/kg i.v.) and dexamethasone (Dex; 0.5 mg/kg i.p.) on (A) ipsilateral (LPS-injected) and (B) contralateral (LPS-noninjected) paw withdrawal threshold in lipopolysaccharide (LPS) pre-treated animals. Values are mean ± S.E.M. *P < 0.05 vs saline control (without LPS pre-treatment) (Student’s t-test) and aP < 0.05 vs LPS pre-treated control animals (one-way ANOVA followed by Dunnett’s test).
2.1.4. DISCUSSION

The present study demonstrated that COX-2 derived products of arachidonic acid metabolism contribute to the development and maintenance of LPS-induced hyperalgesia in animals. Further, inhibition of COX-2 or its expression attenuated the hyperalgesic effects of LPS. The effect of COX-2 inhibition was seen irrespective of systemic or local administration of LPS.

Administration of LPS produced marked hyperalgesic behavior as observed by a significant enhancement of the number of acetic acid-induced writhes and decrease in the tail withdrawal latency in mice. This alteration in the threshold caused could be due to inducible COX-2 activity since parecoxib, a selective COX-2 inhibitor and dexamethasone, an inhibitor of COX-2 expression significantly attenuated the hyperalgesic response. However, both the agents attenuated only the enhancement of writhes to the level of that observed in saline control mice. These results emphasize the involvement of COX-2 and are in accordance with those of a previous study where induction of COX-2 caused an enhancement of writhing response (Matsumoto et al., 1998).

The locally administered LPS into plantar region of the hind paw produced a marked thermal and mechanical hyperalgesia that was significantly reversed by administration of dexamethasone and parecoxib suggests that LPS-induced hyperalgesia could be due to inducible COX-2. In contrast, no hyperalgesia was observed in the contralateral (non-injected) paw indicating the localized expression of COX-2 activity. Furthermore, parecoxib treatment did not alter the basal nociceptive response of the right paw thereby supporting the fact that COX-2 inhibitors are effective in hyperalgesic conditions that are due to COX-2 expression. It is well reported that COX inhibitors per se had no effect in increasing nociceptive threshold (Dubinsky et al., 1987; Dirig et al., 1998). The present findings indicate that there is no involvement of COX-2 in these nociceptive assays under physiological conditions but suggests the possible role of inducible COX-2 in mediating LPS-induced hyperalgesic response.

It is well known that LPS regulates COX isozyme expression differentially with downregulation of COX-1 and upregulation of COX-2 (Liu et al., 1996; Matsumoto et al., 1997). Further, various circulating factors increase the expression of COX-2 in the neuronal and non-neuronal elements, such as astrocytes and microglia, besides macrophages and fibroblasts within 2 h and subsides by 4 – 24 h following LPS.
administration, but similar changes were also observed after peripheral tissue injury and inflammation (Beiche et al., 1996; Dirig et al., 1997; Ebersberger et al., 1999; Tonoi et al., 1999; Samad et al., 2001). In the spinal cord, LPS activates the signaling pathways such as nuclear factor kappaB (NF-κB), activator protein (AP)-1, cyclic AMP response element binding (CREB) Protein, MAPK cascade led to transcriptional activation of COX-2 expression in non-neuronal cells, such as astrocytes and microglia, endothelial and leptomeningeal cells (Hwang et al., 1997; Tonoi et al., 1999; Molina-Holgado et al., 2000; Samad et al., 2001). Importantly, the increased expression of COX-2 increases in basal and evoked PG release. The released PGs sensitize peripheral nerve endings and facilitate central nociceptive processing in spinal cord results in exaggerated pain behavior (hyperalgesia). These data indicate that the elevated COX-2 can be a major contributor to hypersensitivity after LPS administration. In the present study, the effect of selective inhibition of COX-2 prior to LPS pre-treatment was not evaluated because marked hyperalgesia was observed after 8 h of LPS administration. Moreover, selective COX-2 inhibitors have no role in the activation of COX-2 mRNA and generation of new enzyme. Although, COX-2 expression was not measured but the administration of dexamethasone, an inhibitor of COX-2 expression prior to LPS injection significantly prevented hyperalgesia in the present study. Furthermore, clear inhibition of COX-2 expression by dexamethasone has been demonstrated in both in vitro and in vivo (Hempel et al., 1994; Zhang et al., 1997; Matsumoto et al., 1998). This provides the support for the proposal that inducible COX-2 is mainly responsible for PG release and subsequent hyperalgesia following LPS administration.

In a recently reported study, Ballou et al. (2000) has observed that the number of writhing responses induced by diluted acetic acid injection in the COX-1 knockout mice, but not in the COX-2 knockout mice, was less than that observed in the wild-type mice accentuating the potential role of PGs derived from COX-1 rather than from COX-2 in acetic acid-induced writhing. Moreover, selective inhibition of COX-2 at dose(s) tested did not affect basal nociceptive response in acetic acid-induced writhing and tail immersion assay in saline pre-treated mice and also withdrawal response to thermal and mechanical stimuli on the contralateral (LPS non-injected) paw. The results of the present study in accordance with previously reported studies where selective COX-2 inhibitors have failed to show efficacy in acute nociceptive
tests (acetic acid-induced writhing and formalin-induced tonic pain) due to the absence of functional COX-2 in the periphery and in the spinal cord where sensitization of nociceptors and nociceptive processing occurs, respectively (Dirig et al., 1997; Matsumoto et al., 1998; Jain et al., 2001a). These results implicate that PGs derived from COX-1 but not by COX-2 play a role in acute nociceptive tests where COX-1 inhibition is involved in decreasing nociceptive inputs and supporting the lack of efficacy of parecoxib in these tests.

Selective inhibition of COX-2 produced significant antihyperalgesic effect against chronic pain in experimental animals in which there is a marked increase in COX-2 mRNA and PGs in spinal cord (Beiche et al., 1996; Ebersberger et al., 1999). Moreover, acetic acid-induced writhes for 20 min and tail withdrawal to thermal stimulus response would not be sufficient for activation of COX-2 mRNA and generation of COX-2. It is likely that COX-2 may not be associated with spinal prostanoid synthesis acutely or with facilitated nociception, which occurs within limited time frame of acute analgesic tests. Consistent with previous reports, the results of the present study well support the role of COX-2 derived PGs for facilitation of nociceptive processing that occurs in inflammatory late phase and chronic pain conditions. Together, these results indicate that peripheral and central PGs released by inducible COX-2 involve in central nociceptive processing that results in hyperalgesic behavior in LPS-pretreated animals.

In conclusion, the present study demonstrated that inducible COX-2 is required for induction of hyperalgesia by LPS and selective inhibition of inducible COX-2 results in attenuation of established LPS-induced hyperalgesia.
Chapter 2

2.2. LIPOPOLYSACCHARIDE ENHANCES FORMALIN-INDUCED NOCICEPTION: ROLE OF CYCLOOXYGENASE-2

2.2.1. INTRODUCTION

Peripheral tissue injury or inflammation causes exaggerated pain behavior that includes hyperalgesia, an increased responsiveness to noxious stimuli. PGs specifically in the spinal cord have long been thought to play a key role in inflammatory process, sensitization of nociceptors, generation of pain and nociceptive processing (Malmberg and Yaksh, 1992; Willingale et al., 1997). NSAIDs act by inhibiting COX, which is the rate-limiting enzyme, that catalyses conversion of AA to generate PGs.

It is generally agreed that subcutaneous injection of diluted formalin produces various aspects of acute inflammatory pain analogous to human postoperative pain. Further, the pain intensity in this nociceptive assay is a reproducible and quantifiable behavioral response and has been used for the evaluation of the analgesic activity of various pharmacological agents (Abbott and Franklin, 1986; Murray et al., 1988). The diluted formalin when injected into hind paw of mice and rats shows characteristic biphasic licking and biting behavior to continuous (tonic) noxious stimuli and this complex nociceptive pattern last for approximately 60 min. The first or acute phase lasts for about 5 min which is followed by a longer-lasting, more persistent phase (about 40 min) that is characterized by shaking or licking and biting behavior of the paw (Murray et al., 1988). It is generally agreed that the first phase results at least in part from direct activation of primary afferent fibers, both low-threshold mechanoreceptive and nociceptive types whereas the second phase reflects a facilitated state of central sensitization driven by the persistent primary afferent inputs and this ongoing activity releases excitatory amino acids and neuropeptides that are necessary for the development of the second phase (Malmberg and Yaksh, 1992, 1995; Abbadie et al., 1997; Scheuren et al., 1997).

The role of COX isoforms and the effects both peripherally and centrally administered COX inhibitors in formalin-induced nocifensive behavior have been well established (Malmberg and Yaksh, 1992, 1995; Dirig et al., 1997) Despite extensive studies reported on LPS-induced hyperalgesia, the role of COX isoforms in LPS-induced hyperalgesia in the formalin test is not known. Thus, the present study was carried out to examine time course and characteristics of LPS-induced
hyperalgesia in the formalin test and to investigate the effects of COX inhibitors to address the role of COX isoforms on LPS enhanced formalin-induced nocifensive behavior in animals.

2.2.2. MATERIALS AND METHODS

2.2.2.1. Experimental animals

As per 1.2.1.

Albino Swiss mice (20 – 25 g) of either sex bred in Central Animal House of Panjab University, Chandigarh were used in the present study.

2.2.2.2. Induction and characterization of lipopolysaccharide-induced hypersensitivity

LPS dissolved in normal saline and 50 μg/0.1 ml/mouse was administered intraperitoneally at different time intervals. Control animals received normal saline 0.1 ml/mouse. The LPS pretreated and control animals were subjected to formalin-induced tonic pain at 0 h (immediately), 4, 12, or 16 h after LPS administration.

2.2.2.3. Formalin-induced tonic pain

Formalin induced tonic pain in the paw of mice was assessed according to the method reported earlier (Minami et al., 2001). Briefly, mice were injected with 20 μl of 2% formalin solution in normal saline subcutaneously (s.c.) into the plantar surface of the left paws with a 26-gauge needle fitted to a microsyringe. Pain response was quantified by counting the time spent in licking and biting of the injected paw for 5 min periods from 0–45 min. Two phases of spontaneous licking was observed after formalin injection. The interval from 0 – 10 min was defined as early phase and the interval 10 – 45 min as late phase, respectively.

2.2.2.4. Drugs and treatment schedule

Parecoxib sodium (Panacea Biotec Ltd., India) and ketorolac tromethamine (Ketanov® 15 mg/ml) intravenous injection (Ranbaxy Ltd., India), dexamethasone (Unichem Labs, India), lipopolysaccharide from Salmonella typhimurium (Sigma, USA), and formalin (37% formaldehyde) (SD Fine Chemicals, India) were used in this study. Parecoxib sodium, 2% formalin and lipopolysaccharide were freshly prepared by dissolving in normal saline to suitable concentrations. All the drugs were administered in a constant volume of 1 ml/100 g body weight of mouse.

Ketorolac (nonselective COX inhibitor) or parecoxib (selective COX-2 inhibitor) (1, 5 or 10 mg/kg) was administered intravenously 30 min before assessing
formalin-induced tonic pain in saline or LPS-pretreated mice. A single injection of dexamethasone (0.5 mg/kg) was administered intraperitoneally 2 h before saline or LPS pre-treatment to mice and another injection of dexamethasone (0.5 mg/kg, i.p.) was administered 2 h before formalin challenge. Saline pretreated and LPS pretreated control animals also received equivalent volume of normal saline intravenously 30 min before formalin challenge. In all these animals, formalin-induced nociceptive responses were observed 12 h after saline or LPS pretreatment.

2.2.2.5. Statistical analysis
All the values were expressed as mean ± S.E.M. The mean sum of licking and biting behavior between two groups was analyzed by unpaired Student’s t-test. The mean sum of licking and biting behavior in both early and late phases of the formalin test was analyzed by one-way analysis of variance with Dunnett’s t-test for multiple comparisons between different groups. A value of \( P < 0.05 \) was considered as statistically significant.

2.2.3. RESULTS

2.2.3.1. Effect of lipopolysaccharide pre-treatment on the formalin-induced nociception in mice
All the mice injected with formalin (2% solution, 20μl/paw) into the left hind paw showed characteristic biphasic responses, an early and a late phase response (Fig. 2.2.1A and 2.2.1B). A preliminary study was performed to assess any gross difference in formalin-induced nociceptive behaviors of the LPS-injected mice compared to saline injected mice immediately after the treatment (0 h). No significant difference between in time course of formalin-induced nociceptive response was observed in various control groups of animals tested at various time intervals after saline injection i.e., at 0, 4, 12, and 16 h, respectively indicating consistency and reproducibility of the measurements among groups (Fig. 2.2.1A and 2.2.1B). LPS pretreatment at 0 h (immediately) and for 4 h did not show any significant difference in the sum of licking and biting behavior responses in both early (Fig. 2.2.2A) and late phases (Fig. 2.2.2B) of the formalin challenge as compared to control mice that received saline pretreatment. In contrast, LPS pretreatment for 12 and 16 h markedly increased the mean licking and biting responses during the time course of the formalin test resulting in a state of tonic hyperalgesia (Fig. 2.2.1B). A significant enhancement of formalin-induced nociceptive response as compared to saline pretreatment group was observed.
during the late phase (10 – 45 min) but not in the early phase (0 – 10 min) in mice with LPS pretreatment for 12 h or more (Fig. 2.2.2A and 2.2.2B). Therefore, in the subsequent experiments, formalin-induced nocifensive behavior in saline or drug treated animals was observed 12 h after LPS pretreatment.

2.2.3.2. Effect of cyclooxygenase inhibitors in lipopolysaccharide enhanced formalin-induced nociception in mice

Intravenous administration of ketorolac (1, 5, or 10 mg/kg) significantly and dose-dependently decreased formalin-induced nociceptive behavior as compared to saline treatment in control or LPS-pretreated mice (Fig. 2.2.3B). However, it did not alter nociceptive response in early phase of the formalin test in saline or LPS-pretreated mice (Fig. 2.2.3A). Both, parecoxib (1, 5 or 10 mg/kg, i.v.) and dexamethasone (0.5 mg/kg, i.p.) showed nociceptive responses similar to saline-treated mice following formalin injection (Fig. 2.2.4 and 2.2.5). On the contrary, parecoxib significantly and dose-dependently reduced the enhanced number of licking and biting behavior in late phase of the formalin test in LPS-pretreated mice to the level of the animals that received only saline, however it had no effect on early phase of the formalin test (Fig. 2.2.4). Pretreatment with dexamethasone before LPS pretreatment significantly inhibited the enhancement of late phase, but not early phase formalin-induced nocifensive behavior (Fig. 2.2.5).

2.2.4. DISCUSSION

In the present study, systemic administration of LPS enhanced formalin-induced licking and biting behaviors in the late phase, which is an indicative of hyperalgesic response. The important observation was the time course of hyperalgesia that was most evident and consistent in all the LPS-pretreated animals. Although there was no significant difference in the tonic pain behavior at 0 hand after 4 h, but there was a marked and significant potentiation of nociceptive response at 12 and 16 h after LPS pretreatment in mice challenged with formalin resulting in a state of hyperalgesia. Although dose-dependent LPS potentiation of formalin-induced tonic pain was not studied, the same concentration of LPS was employed which was previously reported to cause hyperalgesia in mice (Kanaan et al., 1996; Jain et al., 2001). Indeed, the late phase represents facilitated state of central sensitization, however, marked enhancement of nociceptive response was observed in late phase following LPS pretreatment suggesting that increased release of nociceptive mediators and/or release
of certain mediators other than those normally involved in formalin nociceptive responses are specifically induced by LPS pretreatment.

Fig. 2.2.1. Time course of formalin response in mice pretreated with lipopolysaccharide (LPS) or saline (A) 0 and 4 h and (B) 12 and 16 h before the nociceptive test. Points represent the mean ± S.E.M. of formalin-induced licking and biting responses in seconds during 5 min intervals observed for 45 min.
Fig. 2.2.2. Duration of nociceptive behavior during (A) the early phase (0 - 10 min) and (B) the late phase (10 - 45 min) of the formalin test in mice pretreated with lipopolysaccharide (LPS) or saline 0, 4, 12, and 16 h before formalin challenge. The data represent the mean ± S.E.M. of sum of formalin-induced licking and biting responses in seconds during the early and late phase. * P < 0.05 as compared to corresponding saline pretreated group (t-test).
Fig. 2.2.3. Effect of ketorolac (Ket) on nociceptive behavior during (A) the early phase (0 - 10 min) and (B) the late phase (10 - 45 min) of the formalin test in mice pretreated with saline or lipopolysaccharide (LPS) 12 h before formalin challenge. The data represent the mean ± S.E.M. of sum of formalin-induced licking and biting responses in seconds during the early and late phase. * P < 0.05 as compared to corresponding saline pretreated group (t-test). * P < as compared to LPS-pretreated control animals (one way ANOVA followed by Dunnett’s test).
Fig. 2.2.4. Effect of parecoxib (Pare) on nociceptive behavior during (A) the early phase (0 - 10 min) and (B) the late phase (10 – 45 min) of the formalin test in mice pretreated with saline or lipopolysaccharide (LPS) 12 h before formalin challenge. The data represent the mean ± S.E.M. of sum of formalin-induced licking and biting responses in seconds during the early and late phase. * $P < 0.05$ as compared to corresponding saline pretreated group ($t$-test). $a$ $P <$ as compared to LPS-pretreated control animals (one way ANOVA followed by Dunnett's test).
Fig. 2.2.5. Effect of dexamethasone (Dex) on nociceptive behavior during (A) the early phase (0 - 10 min) and (B) the late phase (10 - 45 min) of the formalin test in mice pretreated with saline or lipopolysaccharide (LPS) 12 h before formalin challenge. The data represent the mean ± S.E. of sum of formalin-induced licking and biting responses in seconds during the early and late phase. * $P < 0.05$ as compared to corresponding saline pretreated group (t-test). $^a$ $P < 0.05$ as compared to LPS-pretreated control animals (one way ANOVA followed by Dunnett’s test).
Systemic administration of ketorolac, a nonselective COX inhibitor, but not parecoxib (a prodrug of valdecoxib, a selective COX-2 inhibitor) or dexamethasone (COX-2 transcription inhibitor) showed antinociceptive effect in the formalin test in saline-pretreated mice. Selective COX-1 and nonselective COX inhibitors, but not selective COX-2 inhibitors reduced formalin-induced nociceptive responses (Malmberg and Yaksh, 1992; Dirig et al., 1997; Ochi et al., 2000). Consistent with previous reports, the results of the present study well support the role of COX-1 derived PGs for facilitation of nociceptive processing that occurs in inflammatory late phase of the formalin test. Importantly, ketorolac markedly decreased the late phase nociceptive behavior of the formalin test in LPS-pretreated mice. Further, both parecoxib and dexamethasone also reduced LPS-induced hyperalgesia. However, both these agents reduced only the enhancement of nociceptive behavior by LPS to the level of the animals that receive only saline treatment. These results implicate that PGs derived from COX-1 but not by COX-2 play a role in this acute nociceptive test in normal animals whereas PGs derived specifically from COX-2 may be involved in LPS potentiation of formalin-induced nociceptive responses.

In the recent past, a number of studies attempted to unravel the mechanisms underlying in LPS-induced hyperalgesia (Safieh-Garabedian et al., 1997; Matsumoto et al., 1998; Jain et al., 2001; Anjaneyulu et al., 2003). LPS, when administered intraperitoneally does not cross blood-brain barrier, however, it stimulates the expression and release of various immunological factors, cytokines such as IL-1, IL-6, and TNF-α by activated monocytes and macrophages and proinflammatory mediators in the periphery and in the central nervous system. In addition, these cytokines increase the expression of iNOS, and COX-2, and also induce the expression and release of various proinflammatory mediators including PGs and neuropeptides (O'Neill et al., 1989; Matsumoto et al., 1998; Eriksson et al., 2000; Samad et al., 2001; Anjaneyulu et al., 2003). It is well known that LPS regulates COX isozymes expression differentially with downregulation of COX-1 and upregulation of COX-2 (Liu et al., 1996; Matsumoto et al., 1997). Previous studies have shown that the dramatic increase in levels of COX-2 isoform in response to proinflammatory cytokines in synovial joints, macrophages, monocytes, and spinal cord (Marziniak and Sommer, 2000; Samad et al., 2001; Stichtenoth et al., 2001). Further, various circulating factors increase the expression of COX-2 in the neuronal and non-neuronal
elements besides macrophages and fibroblasts within 2 h and subsides by 4 – 24 h following LPS administration, but similar changes were also observed after peripheral tissue injury and inflammation (Beiche et al., 1996; Ebersberger et al., 1999; Samad et al., 2001; Ma and Eisenach, 2003). In the spinal cord, administration of causes hypersensitivity (Reeve et al., 2000) and activates the signaling pathways such as NF-κB, AP-1, CREB, MAPK cascade led to transcriptional activation of COX-2 expression in astrocytes, microglia, endothelial cells, and leptomeningeal cells (Hwang, 1997; Samad et al., 2001).

Increased expression of COX-2 that catalyses arachidonic acid into PGH₂ and PGE synthase that produces PGE₂ from PGH₂ in skin, dorsal root ganglia, white and gray matter of spinal cord or synoviocytes was observed 4 – 24 h after IL-1β or systemic LPS pretreatment, respectively (Stichtenoth et al., 2001; Schuligoi et al., 2003). The results of the present study are coherent to time course of increased expression of COX-2 where time-dependent hyperalgesia was observed 12 – 16 h after LPS pretreatment. Importantly, the increased expression of COX-2 increases basal and evoked PGs release. These PGs sensitize peripheral nerve endings and facilitate central nociceptive processing in spinal cord resulting in exaggerated pain behavior (hyperalgesia). These data indicate that the elevated COX-2 could be a major contributor to hypersensitivity after LPS administration.

In the present study, the effect of selective inhibition of COX-2 prior to LPS pre-treatment was not evaluated because marked hyperalgesia was observed 12 h after LPS administration. Moreover, formalin-induced biting and licking for 60 min would not be sufficient for activation of COX-2 mRNA and generation of COX-2. In addition, COX-1 is present constitutively and readily releases PGs in response to tissue injury and noxious stimuli whereas COX-2 is inducible and not present constitutively in the periphery and in spinal cord where sensitization of nociceptors and nociceptive processing occurs, respectively (Willingale et al., 1997; Vane et al., 1998; Simmons et al., 2004). Indeed, selective COX-2 inhibitors have no role in the activation of COX-2 mRNA and generation of new enzyme. It is likely that COX-2 may not be associated with spinal prostanoid synthesis acutely or with facilitated nociception, which occurs within limited time frame of acute analgesic tests.

Although, COX-2 expression was not measured, the administration of dexamethasone, an inhibitor of COX-2 expression prior to LPS injection significantly
prevented hyperalgesia in the present study. Further, clear inhibition of COX-2 expression by dexamethasone has been demonstrated in both *in vitro* and *in vivo* (Hempe et al., 1994; Zhang et al., 1997; Matsumoto et al., 1998; Stichtenoth et al., 2001). In various studies, enhancement of the late phase of formalin nociceptive responses have also been reported in rats that were already in a state of neuropathy-induced hyperalgesia due to spinal nerve ligation and diabetes (Courteix et al., 1993; Calcutt et al., 1996; LaBuda et al., 2001). It has also been reported that hyperalgesia in these neuropathy models is dependent on COX-2 and enhanced release of PGs in spinal cord (Ma and Eisenach, 2002, 2003; Pop-Busui et al., 2002). Further, selective inhibition of COX-2 produced significant antihyperalgesic effect against chronic pain in experimental animals in which there is a marked increase in COX-2 mRNA and PGs in spinal cord (Beiche et al., 1996; Ebersberger et al., 1999). Similarly, selective inhibition of COX-2 reversed cytokine and LPS-induced hyperalgesia in various peripheral and central nociceptive assays (Jain et al., 2001b, 2002; Samad et al., 2001). Taken together, the results provide support to the concept that the PGs released by COX-2 involve in central nociceptive processing that results in hyperalgesic behavior in LPS-pretreated animals.

In conclusion, the above results indicate that LPS potentiated formalin-induced nociceptive response with marked hyperalgesia produced in the late phase. Further, LPS-mediated induction of COX-2 contributes to the development of inflammatory pain hypersensitivity in the formalin test.