Role of cyclooxygenase-2 in lipopolysaccharide-induced hyperalgesia in formalin test

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Lipopolysaccharide (LPS)-induced hyperalgesia and the role of cyclooxygenase (COX) isoforms in acute and chronic nociceptive assays have been well established. However, the role of COX isoforms in LPS-induced hyperalgesia in the formalin test is not clear. Thus, the present study was undertaken to characterize the time course of formalin-induced nociceptive response in LPS-pretreated mice and to investigate possible effects of COX inhibitors to address the potential role of COX isoforms in LPS-induced hyperalgesia in the formalin test. All the animals showed typical biphasic response to formalin challenge. At 0 hr (immediately) and 4 hr after LPS pretreatment, animals did not show any alteration in formalin-induced tonic pain. However, 12 and 16 hr after LPS pretreatment, there was a significant increase in the late phase of formalin-induced nocifensive response as compared to control mice. Treatment with intravenously administered ketorolac (a nonselective COX inhibitor) significantly and dose-dependently inhibited the late phase of formalin-induced nociceptive behaviour in saline and LPS-pretreated mice. In contrast, parecoxib (produg of valdecoxib, a selective COX-2 inhibitor) or dexamethasone (COX-2 transcription inhibitor), when administered intravenously or intraperitoneally, respectively, did not show antinociceptive effect in the formalin test in saline-pretreated mice. However, both the agents significantly and dose-dependently decreased the late phase nociceptive behaviour of the formalin test in LPS-pretreated mice to the level of the animals that received saline pretreatment. These results suggest that induction of COX-2 by proinflammatory mediators and subsequent release of prostaglandins could be responsible for LPS enhancement of formalin-induced nocifensive behaviour and supports an important role of COX-2 in LPS-induced hyperalgesia in the formalin test.

Keywords: Cyclooxygenase isoforms, Formalin test, Hyperalgesia, Lipopolysaccharide.

Peripheral tissue injury or inflammation causes exaggerated pain behaviour that includes hyperalgesia, an increased responsiveness to noxious stimuli. Prostaglandins (PGs) specifically in the spinal cord, have long been thought to play a key role in inflammatory process, sensitisation of nociceptors, generation of pain and nociceptive processing. Nonsteroidal anti-inflammatory drugs (NSAIDs) act by inhibiting cyclooxygenase (COX), which is the rate-limiting enzyme, that catalyses 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 for housekeeping functions, while COX-2 is present in low levels in physiological conditions, but is rapidly induced by cytokines, growth factors and inflammatory stimuli. Recently, COX-3 has been identified but its physiological and pathological roles are yet to be characterized.

Lipopolysaccharide (LPS), also known as endotoxin, a major constituent of the outer membrane of the cell wall of gram-negative bacteria, is reported to produce number of pathophysiological changes in the organism. It is also known to release proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumour necrosis factor-alpha (TNF-α) by activated monocytes and macrophages. In addition to proinflammatory cytokines, the subsequent release of nitric oxide (NO) and prostaglandins (PGs) has been found to induce long-lasting hyperalgesia after central, systemic and local administration of LPS. Recently, many studies have been performed using various classes of pharmacological agents including COX inhibitors to characterize and delineate possible underlying mechanisms involved in LPS-induced hyperalgesia in various nociceptive assays.

It is generally agreed that the formalin test reproduces various aspects of acute inflammatory pain analogous to human postoperative pain. Further, the pain intensity in this nociceptive assay is a reproducible and quantifiable behavioural response.
and has been used for the evaluation of the analgesic activity of various pharmacological agents. The diluted formalin when injected into hind paw of mice and rats shows characteristic biphasic licking and biting behaviours to continuous (tonic) noxious stimuli and this complex nociceptive patterns 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 behaviours of the paw. It is generally agreed that the first phase results at least in part from direct activation of primary afferent fibres, both low-threshold mechanoreceptive and nociceptive types, whereas the second phase reflects a facilitated state of central sensitisation 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.

The role of COX isoforms and the effects both peripherally and centrally administered COX inhibitors in formalin-induced nociceptive behaviour have been well established. 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 has been 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 nociceptive behaviour in animals.

Materials and Methods

**Materials**—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 concentration. All the drugs were administered in a dose volume of 1 ml/100 g body weight of mice.

**Experimental animals**—Albino Swiss mice (20-25 g) of either sex (bred in Central Animal House of Panacea Biotec Ltd., Punjab) were housed under standard conditions of light and dark cycle with food and water ad libitum. The protocol was approved by the Institutional Animal Ethics Committee of Panjab University, Chandigarh and carried out in accordance with the guidelines of the Indian National Science Academy. Newly acquired mice were acclimatised to laboratory conditions 2 hr before being used in the experiment. Each animal was used for a single treatment and sacrificed by euthanasia after experimentation. Each group consisted of six animals.

**Formalin-induced tonic pain**—The mouse paw formalin test was carried out according to a previous report. Briefly, mice were injected with 20 μl of 2% formalin solution in normal saline subcutaneously into the plantar surface of the left paw 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.

**Experimental design**—LPS dissolved in normal saline and injected 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 hr (immediately), 4, 12, or 16 hr after LPS administration. Ktorolac (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 hr before saline or LPS pretreatment to mice and another injection of dexamethasone (0.5 mg/kg, ip) was administered 2 hr before formalin challenge. Saline pretreated and LPS pretreated control animals also received equivalent volume of normal saline intravenously 30 min before formalin challenge. In these animals, formalin-induced nociceptive responses were observed 12 hr after saline or LPS pretreatment.

**Statistical analysis**—All the values were expressed as mean ±SE. The mean sum of licking and biting behaviour between two groups was analysed by unpaired Student’s t-test. The mean sum of licking and biting behaviour in both early and late phases of the formalin test was analysed 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.

Results

Effect of lipopolysaccharide pre-treatment on the formalin-induced nociception in mice—All the mice subcutaneously challenged with formalin into hind paw showed characteristic biphasic response with an early and a late phase (Fig. 1). A preliminary study was performed to assess any gross difference in formalin-induced nociceptive behaviours of the LPS-injected mice compared to saline injected mice immediately after the treatment (0 hr). 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 hr, respectively indicating consistency and reproducibility of the measurements among groups (Fig. 1). LPS pretreatment at 0 hr (immediately) and for 4 hr did not show any significant difference in the sum of licking and biting behaviour responses in both early (Fig. 2A) and late phases (Fig. 2B) of the formalin challenge as compared to control mice that received saline pretreatment. In contrast, LPS pretreatment for 12 and 16 hr markedly increased the mean licking and biting responses during the time course of the formalin test resulting in a state of tonic hyperalgesia (Fig. 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 hr or more (Fig. 2). Therefore, in the subsequent experiments, formalin-induced nocifensive behaviour in saline or drug treated animals was observed 12 hr after LPS pretreatment.

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 behaviour as compared to saline treatment in control or LPS-pretreated mice (Fig. 3B). However, it did not alter nociceptive response in early phase of the formalin test in saline or LPS-pretreated mice (Fig. 3A). Both, parecoxib (1, 5 or 10 mg/kg, iv) and dexamethasone (0.5 mg/kg, ip) showed nociceptive responses similar to saline-treated mice following formalin injection (Figs 4 and 5). On the contrary, parecoxib significantly and dose-dependently reduced the enhanced number of licking and biting behaviour 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. 4). Pretreatment with dexamethasone before LPS pretreatment significantly inhibited the enhancement of late phase, but not early phase formalin-induced nocifensive behaviour (Fig. 5).

Discussion

In the present study, systemic administration of parecoxib (Pare) on nociceptive behaviour during (A) early phase (0-10 min) and (B) late phase (10-45 min) of the formalin test in mice pretreated with saline or lipopolysaccharide (LPS) 12 hr before formalin challenge. Parecoxib was intravenously administered 30 min before nociceptive assay. 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). $P <$ as compared to LPS-pretreated control animals (one way ANOVA followed by Dunnett’s test).
LPS enhanced formalin-induced licking and biting behaviours in the late phase, which is indicative of hyperalgesic response. The important aspect in this study is 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 behaviour at 0 hr and after 4 hr, but there was a marked and significant potentiation of nociceptive response at 12 and 16 hr 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. Indeed, the late phase represents facilitated state of central sensitisation, 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.

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. 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 behaviour 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 behaviour 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. 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. It is well known that LPS regulates COX isozymes expression differentially with downregulation of COX-1 and...
upregulation of COX-2 (refs. 25, 26). Previous studies have shown that the dramatic increase in levels of COX-2 isomer in response to proinflammatory cytokines in synovial joints, macrophages, monocytes, and spinal cord4,27,28. Further, various circulating factors increase the expression of COX-2 in the neuronal and nonneuronal elements besides macrophages and fibroblasts within 2 hr and subsides by 4 – 24 hr following LPS administration, but similar changes were also observed after peripheral tissue injury and inflammation14,29-31. In the spinal cord, LPS activates the signalling pathways such as nuclear factor kappaB (NF-κB), AP-1, CREB, mitogen activating protein kinase (MAPK) cascade led to transcriptional activation of COX-2 expression in astrocytes, microglia, endothelial cells, and leptomeningeal cells32,33.

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 hr after IL-1β or systemic LPS pretreatment, respectively28,33. 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 hr after LPS pretreatment. Importantly, the increased expression of COX-2 increases basal and evoked PGs release. These PGs sensitise peripheral nerve endings and facilitate central nociceptive processing in spinal cord resulting in exaggerated pain behaviour (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 hr 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 sensitisation of nociceptors and nociceptive processing occurs, respectively23. 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 vivo10,28,34,35. 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 diabetes36,37. It has also been reported that hyperalgesia in these neuropathy models is dependent on COX-2 and enhanced release of PGs in spinal cord31,39,43. 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 cord39,40. Similarly, selective inhibition of COX-2 reversed cytokine and LPS-induced hyperalgesia in various peripheral and central nociceptive assays8,10,13–15. 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 behaviour 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.

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