CHAPTER 6:
ROLE OF CYCLOOXYGENASE ISOFORMS IN OROFACIAL PAIN
6.1. INVOLVEMENT OF PERIPHERAL PROSTAGLANDINS IN
FORMALIN-INDUCED NOCICEPTIVE BEHAVIORS IN THE
OROFACIAL AREA OF RATS

6.1.1. INTRODUCTION

Painful conditions affecting the trigeminal field of innervation constitute a major public health concern. There are a multitude of painful conditions, with both inflammatory and neuropathic origins, that affect the head and face including odontalgia, migraine, temporomandibular joint pain and trigeminal neuralgia (Eisenberg et al., 1993; Shawn et al., 2001). Orofacial pain can be defined as pain related to the face or mouth. However, the mechanisms underlying orofacial pain of trigeminal origin are still poorly understood. The possibility that these clinical conditions with trigeminal innervation may differ in their response to analgesic drug therapy compared with pain in the spinal system warrants the development of more appropriate tests to investigate these differences. Indeed, there are relatively few animal models that emphasize the mechanisms involved or analgesic responsiveness of the existing analgesics in the orofacial pain (Benoliel et al., 2002; Vos et al., 1994).

The orofacial formalin test represents one of the animal models of nociception mediated by craniofacial sensory afferent neurons and assesses the magnitude of nociceptive sensations elicited by a long-lasting suprathreshold chemical stimulus (Clavelou et al., 1989; 1995).

Peripheral tissue injury or inflammation results in the production and release of inflammatory mediators including PGs, which causes exaggerated pain behavior that includes hyperalgesia, an increased responsiveness to noxious stimuli. PGs are thought to play an important role in inflammatory process, sensitization of nociceptors, generation of pain and nociceptive processing at peripheral sites and in the spinal cord. They are synthesized in tissues by COX, which catalyses conversion of AA to generate PGs (Kulkarni et al., 2000; Simmons et al., 2004).

It has been reported that COX inhibitors significantly attenuate the PGE$_2$ and PGF$_{2\alpha}$-induced hyperalgesia and allodynia (Taiwo and Levine, 1988; Park et al., 2000). Further, the role of both peripherally and centrally administered PGs and the effect of NSAIDs in formalin-induced nociception in the paw have been well established (Malmberg and Yaksh, 1992; Padi et al., 2004). Despite extensive studies
Chapter 6

reported on acute and chronic pain of peripheral origin that relayed sensory information to spinal cord, the role of PGs in the orofacial pain is not fully understood. Although Clavelou et al (1989) used aspirin to characterize and validate the orofacial formalin test, however, the role of PGs is not well established.

Thus, the present study was carried out to examine time course and characteristics of orofacial formalin test and to investigate the effects of systemically or locally administered NSAIDs to address the role of PGs on formalin-induced nociceptive behavior in the orofacial area.

6.1.2. MATERIALS AND METHODS
6.1.2.1. Experimental animals
As per 1.2.1.
Male Wistar rats (bred in Central Animal House of Panjab University, Chandigarh) weighing 160-180 g were randomly divided into various groups consisting of 6 - 8 animals per group.

6.1.2.2. Behavioral test paradigms
6.1.2.2.1. Orofacial formalin test
All animals were acclimatized to laboratory environment for at least 2 h before testing. The formalin was injected in the orofacial region as described previously (Calvelou et al., 1995). Briefly, rats were lightly restrained and a 50 μl subcutaneous injection of formalin diluted in saline was given into one vibrissal pad, whereas control animals received equivalent volume of saline subcutaneously. In preliminary experiments, different groups of rats were injected with different concentrations of formalin (1.5, 2.5, and 5%) in the orofacial area to examine the concentration-dependent nociceptive behavior in both the phases of the assay. Based on these results, 2.5% formalin was used in the subsequent experiments.

6.1.2.3. Behavioral assessment of nociception
For each animal, the cumulative time spent in grooming, rubbing and/or scratching the facial region proximal to the injection site was recorded for 15 successive sequential blocks of 3 min using a stopwatch.

6.1.2.4. Drugs and treatment schedule
Ketorolac tromethamine (Ranbaxy Ltd., India), diclofenac sodium (Panacea Biotec Ltd., India), and formalin (SD Fine Chemicals, India) were used in this study. All the drug solutions were freshly prepared for intraperitoneal and subcutaneous...
administration. Ketorolac tromethamine was dissolved in normal saline whereas diclofenac sodium was suspended in normal saline using two drops of Tween 80 and administered 1 ml/100 g body weight. Because the volume of injection for local administration was low, the drugs for subcutaneous administration (local) were dissolved in a vehicle containing 70% DMSO and 30% normal saline such that all doses were delivered in a total volume of 20 µl.

6.1.2.5. Treatment schedule
For systemic administration, separate groups of animals received vehicle (saline-tween 80 mixture), ketorolac or diclofenac, each 10 and 30 mg/kg intraperitoneally 30 min prior to formalin injection.

To investigate the involvement of peripheral PGs, separate groups of animals received vehicle (70% DMSO), ketorolac or diclofenac, each 100 and 300 µg/lip, locally (subcutaneously) into vibrissal pad 10 min prior to formalin injection.

6.1.2.6. Statistical analysis
As per 3.1.2.5.

6.1.3. RESULTS
6.1.3.1. Characterization of orofacial formalin test
The initial reaction of the rats to the formalin injection was an immediate withdrawal of their head with vocalization. The animals showed continuous face rubbing episodes with vigorous face wash strokes directed to the perinasal area with the ipsilateral paw, sometimes with the hind paw immediately within 15 – 30 sec following formalin injection. However, no such responses were observed in saline injected rats. These formalin-evoked responses could be distinguished from the background level of spontaneous, non-noxious face grooming observed in naïve animals. The nociceptive response presents a typical biphasic time course with early and short lasting periods (0-3 min) of activity followed, after a 3-9 min quiescent period, by a second, prolonged (9-45 min) tonic phase. Further, there was no significant difference between the first phase responses to various concentration of formalin. However, when the formalin concentration was increased from 1.25% to 2.5%, it resulted in increase in the amplitude of the nociceptive behavior, but it slightly decreased at higher concentration (5%) in the second phase (Fig.6.1.1A). Thus, time spent in rubbing and/or scratching behavior during the second phase was considered as a suitable index of nociceptive behavior in this assay. Based on these initial results, we
selected 2.5% of formalin throughout the reminder of the experiments. The mean durations of rubbing activity were $9.67 \pm 1.86$ and $42.83 \pm 6.37$ sec for the first and $58 \pm 8.86$ and $414 \pm 59.71$ for second phase of the formalin test following saline or 2.5% formalin injection, respectively (Fig. 6.1.1B).

6.1.3.2. Effect of systemically administered NSAIDs on formalin-induced orofacial nociceptive behavior

Intraperitoneal administration of ketorolac or diclofenac, each 10 or 30 mg/kg produced a significant decrease in the second phase of the nociceptive response as compared to vehicle (saline-tween 80 mixture) control animals. In contrast, none of the agents at both the doses used failed to decrease the first phase of formalin-induced nociceptive behavior as compared to vehicle pre-treated rats (Fig. 6.1.2).

6.1.3.3. Effect of locally administered NSAIDs on formalin-induced orofacial nociceptive behavior

The subcutaneous administration of ketorolac or diclofenac (100 or 300 µg/lip) into vibrissal pad 10 min prior to formalin injection did not affect the first phase, while it produced a significant decrease in the second phase of the formalin response as compared to vehicle (70% DMSO) control animals (Fig. 6.1.3).

6.1.4. DISCUSSION

The orofacial formalin injection produced characteristic biphasic nociceptive response, with an early followed by a second, prolonged phase. The observed orofacial nociceptive responses to formalin injection were identical to those reported earlier (Clavelou et al., 1989; Eisenberg et al., 1993). It has been well documented that the first phase is a model of acute chemical pain resulting from immediate increase in the activity of slowly conducting C-fibers, whereas the second phase reflects a facilitated state of central sensitization driven by the persistent primary afferent inputs with the release of different pain mediating substances and central sensitization as seen in the paw formalin test (Malmberg and Yaksh, 1992; 1995).

Both, diclofenac and ketorolac, the most commonly used NSAIDs act by inhibiting PGs synthesis. Pretreatment with systemically administered ketorolac or diclofenac significantly attenuated formalin-induced phase-2 responses, but not the phase-1 nociceptive behaviors. These observations not only parallel similar investigation when formalin is injected into paw of rats but also confirm the involvement of PGs in formalin-induced nociception (Malmberg and Yaksh, 1992; Tegeder et al., 2001).
Fig. 6.1.1. (A) Time course of the nociceptive responses evoked by a 50 µl subcutaneous injection of various concentrations of formalin (1.5, 2.5 and 5 %) into the vibrissal pad. (B) Bars represent the total rubbing time induced by formalin or saline (50µl/lip) in rats during the first (0 - 3 min) and the second phase (9 - 45 min). Values are mean ± S.E.M. n = 6-8 per group. *P < 0.05 as compared to corresponding saline-treated group (t-test), * P < as compared to 1.5% formalin-injected control animals (one way ANOVA followed by Dunnett’s test).
Fig. 6.1.2. Effect of ketorolac (Ket; 10 or 30 mg/kg, i.p.) or diclofenac (Dic; 10 or 30 mg/kg, i.p.) on formalin-induced nociceptive behaviors in the orofacial region in rats. Values are mean ± S.E.M. n = 6 - 8 per group. *P < 0.05 as compared to vehicle control; aP as compared to ketorolac 10 mg/kg treated group; bP as compared to diclofenac 10 mg/kg treated group (ANOVA followed by Dunnett’s t-test).

Fig. 6.1.3. Effect of ketorolac (Ket; 100 or 300 µg/lip, s.c.) or diclofenac (Dic; 100 or 300 µg/lip, s.c.) on formalin-induced nociceptive behaviors in the orofacial region in rats. Values are mean ± S.E.M. n = 6 – 8 per group. *P < 0.05 as compared to vehicle control; aP as compared to ketorolac 100 µg treated group (ANOVA followed by Dunnett’s t-test).
Recently, electrophysiological studies have demonstrated that Aδ- and C-fibers, nociceptive specific and convergent (wide dynamic) neurons in the spinal trigeminal nucleus are excited by the injection of formalin in their receptive field (Raboisson et al., 1991; 1995). Accumulating data indicate that injection of formalin into hind paw causes tissue damage and releases pain-producing substances including PGs, SP and glutamate that activate nociceptors on the terminals on the sensory nerve fibers (Malmberg and Yaksh, 1995). Interestingly, formalin injection in the orofacial area caused histological signs of inflammation and tissue injury in the rat lip (Clavelou et al., 1995). Evidences suggest that PGE$_2$ synthesized in the brain may also be involved in the modulation of trigeminal nociception. Stimulation of the intracranial dura mater with PGE$_2$ produces sensitization of brain stem trigeminal neurons (Burstein et al., 1998; Bolton et al., 2001). Further, brain-derived PGE$_2$ induces mechanical hyperalgesia and hypoalgesia of brain stem trigeminal neurons through the E-prostanoid receptors, EP$_3$ and EP$_1$ respectively (Oka et al., 1997). Thus, it is possible that PGs, the arachidonic acid metabolites of COX pathways are critically involved in the formalin-induced orofacial nociception.

The present study demonstrated that diluted formalin injected into vibrissae pad produced tonic nociceptive behavior, which is mediated by the nociceptive mediators released locally at site of injection. Several studies have demonstrated peripheral PGs are involved in cutaneous hyperalgesia. Indeed intradermal and subcutaneous administration of PGE$_2$ plays an important role in cutaneous hyperalgesia by activating polymodal receptors to thermal and mechanical stimulation (Khaser et al., 1993; Hong and Abbott, 1994). Few studies also reported an effect of hypothalamic PGE$_2$ modulation of nociception demonstrate a primarily a hyperalgesic effect in cutaneous tissues such as facial skin or hind paw (Hosoi et al., 1999; Oka et al., 1997). In the present study, ketorolac or diclofenac was also administered subcutaneously into upper lip to address the role of peripheral PGs in the orofacial pain of trigeminal origin. Interestingly, both the NSAIDs showed marked antinociceptive effect in decreasing formalin-induced phase-2 response, but not phase-1 nociceptive behavior. There are electrophysiological and in vivo evidences suggest that PG receptor mediated synaptic transmissions of somatosensory information in the medullary dorsal horn implicating PGs are also one of the mediators at trigeminal nucleus (Burstein et al., 1998; Bolton et al., 2001; Jenkins et
The localization of COX in the axons of primary afferent neurons suggest that PGs may be transported and released in peripheral tissue as well may modulate the activity of peripheral nociceptors. These results indicate the involvement of PGs, particularly peripheral PGs in sensitizing intact peripheral afferents in the orofacial region.

In summary, the present data indicated that tissue injury caused by direct formalin injection in the orofacial area might be as a result of the activation of COX enzymes that produce PGs and further the possible involvement of peripheral PGs in formalin-induced inflammatory pain in the orofacial area has been suggested.
6.2. CYCLOOXYGENASE-1 INHIBITION ATTENUATES FORMALIN-INDUCED NOCICEPTIVE BEHAVIORS IN THE TRIGEMINAL AREA

6.2.1. INTRODUCTION
The orofacial region is innervated by the trigeminal nerve and is one of the most densely innervated areas of the body, which focuses some of the most common acute pains and those accompanying the pathological states of the teeth and related structures (Iwata et al., 2004). It is also site of postherpetic neuralgia, migraine and referred pains. However, the mechanisms underlying these pains are poorly understood, partly due to the general lack of investigations emphasized to the face and the mouth compared to the rest of the body. Most importantly, there are relatively few animal and human models dedicated to the study of nociception in trigeminal region. It is well known that the subcutaneous injection of diluted formalin induces tissue injury, inflammation and generates behavioral and electrophysiological responses that last from few minutes to more than one hour. Recently, Clavelou et al. (1989) developed a new formalin model in the rat to assess nociceptive process in the orofacial region. This is widely validated, reliable, and accepted method for studying mechanisms involved orofacial pain in the trigeminal region (Clavelou et al., 1995; Eisenberg et al., 1993; Shawn et al., 2001).

It has been well demonstrated that PGE$_2$, a principal proinflammatory PG, is released upon various noxious stimuli and inflammatory stimulus insults and plays an important role in nociceptive processing in the spinal cord as well as in the periphery (Willingale et al., 1997; Minami et al., 2001; Turnbach et al., 2002). Recent studies suggest that PGE$_2$ synthesized in the brain may also be involved in the modulation of trigeminal nociception (Jenkins et al., 2001). Further, stimulation of the intracranial duramater with PGE$_2$ sensitizes of brain stem trigeminal neurons (Bolton et al., 2001; Burstein et al., 1998). In one study, brain-derived PGE$_2$ induced mechanical hyperalgesia and hypoalgesia of brain stem trigeminal neurons through the E-prostanoid receptors, EP$_3$ and EP$_1$ respectively (Oka et al., 1997). In addition, Clavelou et al. (1995) reported that formalin injection in the orofacial area caused histological signs of inflammation and tissue injury in the rat lip. It is well known that the analgesic and anti-inflammatory effects of NSAIDs result from suppression of PG synthesis due to COX inhibition. To date three COX isozymes have been identified of

---

257
which physiological roles of two isoforms of COX have been well characterized. Systemic administration of NSAIDs was also reported to inhibit the agitation behavior induced by formalin injection (Clavelou et al., 1989; 7.1. of this chapter) and suggests that PGs, the AA metabolites of COX pathways are critically involved in the formalin-induced orofacial nociception. However, the relative role of COX isoforms in the orofacial pain is not known.

Thus, the present was carried out to investigate the effect of COX inhibitors to address the relative role of COX isozymes in formalin-induced orofacial nociception. The present study also examined the effect of locally administered COX inhibitors to define the role of COX isoforms on peripherally released PGs and their involvement in the orofacial nociception.

7.2.2. MATERIALS AND METHODS
6.2.2.1. Experimental animals
As per 1.2.1.
Male Wistar rats (bred in Central Animal House of Panjab University, Chandigarh) weighing 160-180 g were randomly divided into various groups consisting of 6 - 8 animals per group.

6.2.2.2. Behavioral test paradigms
The rat orofacial formalin test using 2.5% formalin solution was carried out as per 6.1.2.2.1.

6.2.2.3. Behavioral assessment of nociception
As per 6.1.2.3.

6.2.2.4. Drugs and treatment schedule
Resveratrol (Archer Chem, India), naproxen, nimesulide, valdecoxib (Panacea Biotec Ltd., India) and formalin (SD Fine Chemicals, India) were used in the present study. All the drug solutions were freshly prepared for intraperitoneal and subcutaneous administration. All the agents were suspended in normal saline using two drops of Tween 80 and administered 1 ml/100 g body weight. Because the volume of injection for local administration was low, these agents for subcutaneous administration (local) were dissolved in a vehicle containing 70% DMSO and 30% normal saline such that all doses were delivered in a total volume of 20 μl.

6.2.2.5. Treatment schedule
For systemic administration, separate groups of animals received vehicle (saline-tween 80 mixture), resveratrol (3, 10 or 30 mg/kg), naproxen (10, 30 or 100 mg/kg),
nimesulide (3, 10 or 30 mg/kg) or valdecoxib (3, 10 or 30 mg/kg) intraperitoneally 30 min prior to formalin injection.

To investigate the involvement of peripheral PGs, separate groups of animals received vehicle (70% DMSO), resveratrol (30 or 100 µg/lip), naproxen (100 or 300 µg/lip), nimesulide (30 or 100 µg/lip) or valdecoxib (30 or 100 µg/lip), locally (subcutaneously) into vibrissal pad 10 min prior to formalin injection.

6.2.2.6. Statistical analysis
As per 3.1.2.5.

6.2.3. RESULTS
6.2.3.1. Characterization of orofacial formalin test
The nociceptive response after orofacial formalin (2.5%) injection presents a typical biphasic time course with an early and short lasting periods (0-3 min) of activity followed, after a 3-9 min quiescent period, by a second, prolonged (9-45 min) tonic phase (Fig. 6.2.1A). Thus, time spent in rubbing and/or scratching behavior during the first and second phases were significantly higher as compared to orofacial saline injected animals. The mean durations of rubbing activity were 6.67 ± 1.86 and 37.53 ± 4.31 sec for the first and 36.17 ± 3.32 and 365.33 ± 28.14 for second phase of the formalin test following saline or 2.5% formalin injection, respectively (Fig. 6.2.1B).

6.2.3.2. Effect of systemically administered inhibitors on formalin-induced orofacial nociceptive behavior
Intraperitoneal administration of resveratrol (3, 10 or 30 mg/kg) or naproxen (10, 30 or 100 mg/kg) significantly and dose-dependently attenuated the second phase of formalin-induced nociceptive responses as compared to vehicle (saline-tween 80 mixture)-treated control animals. On the contrary, nimesulide (3, 10 or 30 mg/kg) or valdecoxib (3, 10 or 30 mg/kg) had no effect on the phase 2 nociceptive behavior due to orofacial formalin injection (Fig. 6.2.2B). However, none of the agents at all the doses used failed to decrease the first phase of formalin-induced nociceptive behavior as compared to vehicle pre-treated rats (Fig. 6.2.2A).

6.2.3.3. Effect of locally administered COX inhibitors on formalin-induced orofacial nociceptive behavior
The subcutaneous administration of resveratrol (30 or 100 µg/lip) or naproxen (100 or 300 µg/lip) locally (subcutaneously) into vibrissal pad 10 min prior to formalin injection did not affect the first phase, however, these agents significantly suppressed the
second phase nociceptive behavior after formalin injection as compared to vehicle (70% DMSO)-treated control animals (Fig. 6.2.3A and 3B). In contrast, nimesulide (30 or 100 μg/lip) or valdecoxib (30 or 100 μg/lip) did not alter orofacial formalin-induced nociceptive behaviors in any phase (Fig. 6.2.3A and 3B).

Fig. 6.2.1. (A) Time course of the nociceptive responses produced by a 50 μl subcutaneous injection of 2.5 % formalin into the vibrissal pad. (B) Bars represent the total rubbing time induced by formalin or saline (50μl/lip) in rats during the first (0 - 3 min) and the second phase (9 – 45 min). Values are mean ± S.E.M. n = 6-8 per group. *P < 0.05 as compared to corresponding saline-treated group (t-test).
Fig. 6.2.2. Effect of intraperitoneally administered resveratrol (Res; 3, 10 or 30 mg/kg), naproxen (Nap; 10, 30 or 100 mg/kg), nimesulide (Nim; 3, 10 or 30 mg/kg) or valdecoxib (Val; 3, 10 or 30 mg/kg) on formalin-induced (A) phase 1 and (B) phase 2 nociceptive behaviors in the orofacial region in rats. Values are mean ± S.E.M. n = 6 - 8 per group. *P < 0.05 as compared to vehicle control; "p as compared to resveratrol 10 mg/kg treated group; "p as compared to naproxen 30 mg/kg treated group (ANOVA followed by Dunnett’s t-test).
Fig. 6.2.3. Effect of locally administered resveratrol (Res; 30 or 100 µg/lip), naproxen (Nap; 100 or 300 µg/lip), nimesulide (Nim; 30 or 100 µg/lip) or valdecoxib (Val; 30 or 100 µg/lip) into vibrissal on formalin-induced (A) phase 1 and (B) phase 2 nociceptive behaviors in the orofacial region in rats. Values are mean ± S.E.M. n = 6 - 8 per group. *P < 0.05 as compared to vehicle control; *P as compared to resveratrol 100 µg/kg treated group; *P as compared to naproxen 300 µg/kg treated group (ANOVA followed by Dunnett’s t-test).
6.2.4. DISCUSSION

The results of the present study clearly demonstrated that systemically and locally administered preferential COX-2 inhibitor nimesulide or selective COX-2 inhibitor valdecoxib had no effect on the nociceptive behavior induced by formalin injection in the orofacial area. However, resveratrol, a selective COX-1 inhibitor or naproxen, a non-selective COX (COX-1/2) inhibitor attenuated phase 2, but not Phase 1 nociceptive behaviors of the orofacial formalin test indicating that PGs synthesized by COX-1 is involved in nociceptive transmission during the rat orofacial formalin test.

Orofacial formalin test

The subcutaneous injection of diluted formalin produced biphasic nociceptive responses similar to those observed after paw formalin injection. There is direct and indirect evidence that subcutaneous injection of diluted formalin provides a sustained noxious stimulation. The direct evidence comes from the continuous agitation behavior of face rubbing with vigorous face-wash strokes directed to the perinasal area. Further, formaldehyde has property of cross-linking tissue proteins through the formation of one-carbon bridges and provokes injuries, and thus causes tissue inflammation (Clavelou et al., 1989, 1995, Miampamba et al., 1994, Freshwater et al., 2002). It has been well documented that the phase 1 corresponds to the initial elevation of afferent input whereas the phase 2 reflects a state of facilitated central nociceptive processing driven by the moderate ongoing peripheral input (Malmberg and Yaksh, 1992; 1995). Indeed, hystical vibrissae in the rat are densely innervated with centrally projecting trigeminal primary afferents, which extends from the medullary to cervical dorsal horn as far as caudal as cervical C7 (Marfurt and Rajchert, 1991). Thus, both the phases of the formalin test differ in intensity of nociceptive inputs and neurotransmission and depend on extent of tissue damage and inflammation.

Cyclooxygenase isozymes and the formalin test

Previous studies have demonstrated that systemic or spinal administration of NSAIDs produces antinociception in the paw formalin test (Malmberg and Yaksh, 1992, 1995; Dirig et al., 1997). Clavelou et al. (1989) while characterizing and validating the orofacial formalin test assessed and demonstrated the ability of aspirin to attenuate the nociceptive behaviors when formalin was injected into orofacial area. However, the results of the previous study of the first of this chapter as well those in
the present study, demonstrated that inhibition of release of PGs by NSAIDs produces antinociception after orofacial formalin injection.

It has been shown that PGE$_2$ is released from rat duramater encephali following stimulation of the trigeminal ganglion either electrically or chemically with release of serotonin, histamine and bradykinin (Ebersbeger et al., 1999). Further, it has been reported that PGE$_2$ levels are raised in the saliva and jugular venous blood of patients undergoing migraine attacks (Sarchielli et al, 2000). In addition, bradykinin, a known activator of sensory neurons, also induces PGE$_2$ release from cultured trigeminal neurons (Jenkins et al., 2003). A recent report demonstrated that PGE$_2$ could induce the release of CGRP from cultured trigeminal neurons (Jenkins et al., 2001). Moreover, there are evidences for the presence of polymodal C fibers and A$\delta$ fibers in the skin, which are readily sensitized by intradermal injection of PGE$_2$ that results in increased number of action potentials of nociceptors and hyperalgesia (Taiwo and Levine, 1988; Khaseer et al., 1993; Hong and Abbott 1994). This is further evidenced by the electrophysiological studies which demonstrated that A$\delta$, C-fibers and nociceptive specific neurons of the spinal trigeminal nucleus are excited by the formalin injection in their receptive fields (Raboisson 1991, 1995; Puig and Sorkin, 1995). These results suggest that the same mechanism(s) are expected to be involved in the orofacial pain due to formalin injection.

Importantly, the biphasic nociceptive behaviors in the orofacial formalin test resemble the paw formalin test, which has shown to be PGs dependent. Indeed, systemic NSAIDs decreased formalin-induced nociceptive behavior in the paw (Malmberg and Yaksh, 1992; 1995). Most behavioral studies have found that PGE$_2$ produces mechanical hyperalgesia due to sensitization of peripheral nociceptor terminals in animals and humans (Taiwo and Levine, 1988; Ahelgren et al., 1997; Willingale et al., 1997). It is important that systemically administered resveratrol and naproxen inhibited formalin-induced nociception indicating that COX-1 and/or COX-2 might be involved in producing PGs and subsequent nociception. On the contrary, nimesulide and valdecoxib did not alter agitation behavior suggesting that COX-2 is not involved in the orofacial formalin test. Thus, it is plausible that PGs derived preferentially by COX-1 after chemical sensitization and tissue injury are involved in the nociception due to orofacial formalin injection.
In general, the results parallel similar investigation in the paw formalin test in the rat in which selective inhibition of COX-1, but not COX-2 after systemic or intrathecal administration of these agents produces dose-dependent antinociception (Dirig et al., 1997; Ochi et al., 2000; Torres-Lopez et al., 2002). In previously reported studies along with the results of the present study found that systemic and spinal administration of COX-2 inhibitors were ineffective (Dirig et al., 1997; Ochi et al., 2000; Torres-Lopez et al., 2002). However, similar administration of COX-2 inhibitors blocked carrageenan or SP-induced hyperalgesia (Zhang et al., 1997; Dirig et al., 1998; Yaksh et al., 2001). Valdecoxib, nimesulide and resveratrol are non-polar and readily cross lipophilic barriers whereas naproxen is weakly non-polar, thus it may be misinterpreted for its less efficacy in alleviating nociceptive behavior after orofacial formalin injection (Abdel-Halim et al., 1978; Merck and Co Vioxx information, issue 2000; Wang et al., 2002; Boje et al., 2003; Dembo et al., 2005). Further, the doses of these agents employed were in the same range that showed centrally mediated effects following systemic administration (Chan et al., 1999; Josa et al., 2001; Steiner et al., 2001; Wang et al., 2002). Thus, it is implausible to interpret that these agents are poorly distributed to the site of injury and/or CNS. Interestingly, locally administration of the agents with COX- selective or preferential COX-1 inhibiting property also showed antinociceptive effect similar to those observed after systemic administration. These findings suggest that local inhibition of COX-1 at the site of injury prior to formalin injection resulted in antinociceptive effects and further confirm the earlier findings that peripherally derived PGs are also participated in formalin-induced orofacial nociceptive behavior. Therefore, the relative lack of antinociceptive effects of COX-2 inhibitors in the orofacial formalin test is not due to sufficient concentration at the vicinity of trigeminal ganglia and at the site of formalin injection, but it may be due to absence of adequate levels of COX-2 expression or ineffective participation of COX-2 to release PGs. These results suggest that COX-2 isoform is not contributed to the nociception after orofacial formalin injection in the rat.

COX-1 is found in neurons throughout the brain whereas low levels of COX-2 protein and COX-2 mRNA have been detected in fore brain (Yamagata et al., 1993; Breder et al., 1995). However, COX-2 mRNA is induced in brain tissue and cultured glial cells by LPS, IL-1 or TNF-α (Breder and Saper, 1996; Cao et al., 1998).
Moreover, it has been reported that paw formalin-induced biting and licking and/or flinching behavior for 60 min would not be sufficient for activation of COX-2 mRNA and generation of new COX-2 enzyme. In addition, the expression of COX-2 was not detected in trigeminal ganglia of normal rats indicating the absence of constitutive COX-2 in the receptive area of formalin injection (Hill et al., 2001). One possibility is that 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 trigeminal ganglia spinal where sensitization of nociceptors and nociceptive processing occurs, respectively after orofacial formalin injection. Thus, it is likely that COX-2 may not be associated with trigeminal PG synthesis acutely or with facilitated nociception, which occurs within limited time frame of orofacial formalin test.

In summary, inhibition of COX-1, but not COX-2, attenuated the nociceptive behavior induced by orofacial formalin injection. This demonstrates that PGs synthesized by COX-2 are not involved in nociceptive transmission and that COX-1 is the main COX isoform that evoke nociceptive behavior during the orofacial formalin test.