

Increased Tumor Necrosis Factor- α and Prostaglandin E₂ Concentrations in the Cerebrospinal Fluid of Rats with Inflammatory Hyperalgesia: The Effects of Analgesic Drugs

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BACKGROUND: We examined the changes in cerebrospinal fluid (CSF) concentrations of prostaglandin E₂ (PGE₂) and tumor necrosis factor- α (TNF- α) after intraplantar administration of complete Freund's adjuvant (CFA) in rats. In addition, we investigated whether different analgesic drugs orally administered at antihyperalgesic doses were able to prevent the changes in PGE₂ and TNF- α spinal levels associated with hindpaw inflammation.

METHODS: The Randall-Selitto paw-withdrawal test was used to measure inflammatory hyperalgesia. Tramadol (7.5 mg/kg), paracetamol (65 mg/kg), tramadol plus paracetamol and nimesulide (5 mg/kg) were administered orally twice a day, starting from the first day after the CFA injection. PGE₂ in the CSF was measured by enzyme immunoassay, and TNF- α by ELISA. Behavioral and biochemical parameters were measured on Day 7 after intraplantar injection of CFA or saline. **RESULTS:** Withdrawal thresholds to mechanical stimuli decreased markedly in the CFA-treated paw. In these animals the quantification of proinflammatory mediators in the CSF revealed a significant increase in both PGE₂ and TNF- α concentrations. All the pharmacological treatments prevented the development of the hyperalgesia as well as the PGE₂ increase in the CSF. Conversely, a prevention of the increase in TNF- α levels was observed only in rats treated with nimesulide or tramadol and paracetamol in combination.

CONCLUSIONS: Our results demonstrate that peripheral inflammatory hyperalgesia is associated with significant changes of proinflammatory mediators in the CSF. It is important to note, however, that spinal PGE₂ and TNF- α proved to be differently affected by pharmacological treatments able to fully abolish the hyperalgesia.

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Peripheral tissue injury and inflammation induce central sensitization that results in the development of hyperalgesia to noxious stimuli (1-3). The enhanced response to noxious stimuli (hyperalgesia) is the main characteristic of pathological pain. In many clinical pain syndromes, painful sensations are greatly amplified (4,5). Experimental models of hyperalgesia, therefore, seem particularly suitable in order to study and differentiate analgesic drugs in preclinical conditions (1,6,7).

During the last decade, spinally produced prostaglandins (PGs), with special reference to PGE₂, and proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) have been recognized as active participants in the initiation and maintenance of pain

induced by inflammation and damage to peripheral tissue (8-10).

It has been demonstrated that PGE₂ depolarizes spinal neurons and that PGE₂ produced in the spinal cord after peripheral inflammation can induce central sensitization and inflammatory pain hypersensitivity (11,12).

There is growing evidence that TNF- α is an important mediator of persistent pain states, and that it can act at the spinal level. In the rat, intrathecally administered TNF- α enhances dorsal horn neuronal responses to nociceptive stimuli (13). Consistent with these data, the authors of an electrophysiological study showed that the application of TNF- α to the nerve root induces a significant increase in the spontaneous discharge of wide dynamic range and nociceptive-specific spinal neurons, as well as enhanced responses of wide dynamic range neurons to noxious stimulation (14). Milligan et al. (15) demonstrated that intrathecal administration of a TNF- α antagonist can prevent the hyperalgesia produced by intrathecal injection of the human immunodeficiency virus-1 envelope, glycoprotein gp120. Recently, it has been reported that pain

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behavior induced by the intrathecal injection of TNF is reduced by intrathecal pretreatment with cholera toxin (16). Thus, intrathecal administration of TNF- α can enhance nociceptive behaviors by activating specific receptors, whereas intrathecal administration of a TNF antagonist can block and/or reverse hyperalgesia. All these findings support a role for spinal TNF- α in the development and maintenance of pain facilitation. Although the involvement of both spinal PGE₂ and TNF- α in pain transmission has been demonstrated, their release into cerebrospinal fluid (CSF) of animals with peripheral inflammatory hyperalgesia has been poorly investigated.

The purpose of the present study, therefore, was to measure the concentrations of PGE₂ and TNF- α in the CSF of rats after intraplantar administration of complete Freund's adjuvant (CFA) in the hindpaw. In addition, we investigated whether different analgesic drugs could prevent the biochemical changes at the spinal level associated with peripheral inflammation. We focused our attention on three drugs frequently used for the treatment of pain. We investigated the effects produced by paracetamol (acetaminophen), a centrally acting non-opioid analgesic, tramadol, a drug that is thought to relieve pain through monoaminergic and opioid mechanisms of action and widely used for the treatment of several kinds of pain, and nimesulide, a nonsteroidal anti-inflammatory drug with good analgesic activity (7,17–19). Moreover, as a fixed dose oral combination of tramadol plus paracetamol has become available for treating different painful conditions (18,20), we considered it of interest to evaluate the effects of these two drugs when administered together.

METHODS

All procedures were approved by the Department of Pharmacology of the University of Milan Animal Care and Use Committee and followed the ethical guidelines for the treatment of animals of the International Association for the Study of Pain (21). All efforts were made to minimize the number of animals and their suffering.

Male Sprague Dawley albino rats (Charles River, Calco, Italy) weighing between 200 and 250 g were used for these studies. The animals were housed four to a cage, at 22°C \pm 2°C with a light-dark cycle of 12/12-h and free access to water and food. The rats were allowed to habituate to the housing facilities for 1 wk before the experiments began. Behavioral studies were performed in a quiet room between 10.00 and 12.00. Eight rats were used in each experimental group.

Peripheral inflammation was induced by the injection of a suspension of 0.1 mg/0.1 mL CFA containing heat-killed and dried mycobacterium tuberculosis (H37Ra, ATCC 25177) in 85% paraffin oil and 15% mannide monooleate into the plantar surface of the left hindpaw. Control animals were injected with 0.1

mL of saline in the left hindpaw. Behavioral and biochemical variables were measured on Day 7 after intraplantar injection of CFA or saline. This timepoint was chosen because previous data indicate that at 1 wk postinjection, hyperalgesia is particularly evident (4,22). The Randall-Selitto paw-withdrawal test, which uses mechanical force as nociceptive stimulus, was used to measure inflammatory hyperalgesia. The stimulus was applied with an analgesymeter (Basile, Comerio, Italy) which generates a linearly increasing mechanical force, applied by a conical piece of plastic with a dome-shaped tip on the dorsal surface of the rat's hindpaw. The animals were gently held and incremental pressure (maximum 250 g) was applied onto the dorsal surface of the hindpaw. The thresholds represent the pressure (expressed in grams) at which the animal withdrew its hindpaw. The paw pressure thresholds were determined at 60 min after the administration of the last dose of each drug. To avoid tissue damage, only one trial was performed at this time point. The observer was blind to treatment allocation of the animals.

The following drugs were used: tramadol hydrochloride (Prodotti Formenti, Milano, Italy), paracetamol (Acetaminophen, Sigma, Milano, Italy), and nimesulide (Helsinn Healthcare, Lugano, Switzerland). All drugs were dissolved in 0.5% methylcellulose in 0.9% NaCl. Tramadol (7.5 mg/kg), paracetamol (65 mg/kg), tramadol plus paracetamol and nimesulide (5 mg/kg) were administered orally twice a day, starting from the first day after the CFA injection. These doses were chosen on the basis of results obtained in the experiments we had previously conducted with these drugs (4,6,7). Control animals were orally treated with an equal volume of vehicle (0.5 mL/100 g body weight).

Under barbiturate anesthesia (Nembutal 50 mg/kg i.p.) CSF was collected into ice-cold Eppendorf microvials by cisternal puncture performed with a 26G needle attached to a PE 10 cannula and then immediately frozen and stored at -20°C until analysis. All samples (200 μ L) were collected immediately (within 30 min) after the measurement of behavioral variables. PGE₂ and TNF- α measurements were performed in the same sample of CSF.

Quantitative determination of PGE₂ in the CSF was performed by the enzyme immunoassay (EIA) using a commercially available EIA kit (Amersham, Cologno Monzese, Italy). The sensitivity of the PGE₂ EIA kit was 10 pg/mL. The levels of TNF- α in CSF were measured by means of an enzyme-linked immunosorbent assay (ELISA) kit specific for rat TNF- α (Bender Medsystem, Prodotti Gianni, Milano, Italy). The sensitivity of the TNF- α assay was 1.0 pg/100 μ L.

All values are expressed as means \pm SEM. Comparisons among groups were performed using one way analysis of variance (ANOVA) followed by Bonferroni's *t*-test for multiple comparisons. An effect was determined to be significant if the *P* value was <0.05.

Figure 1. Effects of the treatment with nimesulide (nime, 5 mg/kg p.o.), paracetamol (para, 65 mg/kg p.o.), tramadol (tra, 7.5 mg/kg p.o.), and tramadol plus paracetamol (tra + para) on mechanical hyperalgesia produced by complete Freund's adjuvant (CFA) injection into the hindpaw of rats. Control animals were treated intraplantarly with saline and orally with vehicle. Paw withdrawal latencies from mechanical stimulation were measured by the Randall-Selitto test 7 days after CFA injection. Values are grams, means \pm SEM of eight rats. * = $P < 0.05$ vs. Controls (ANOVA + Bonferroni's *t*-test).

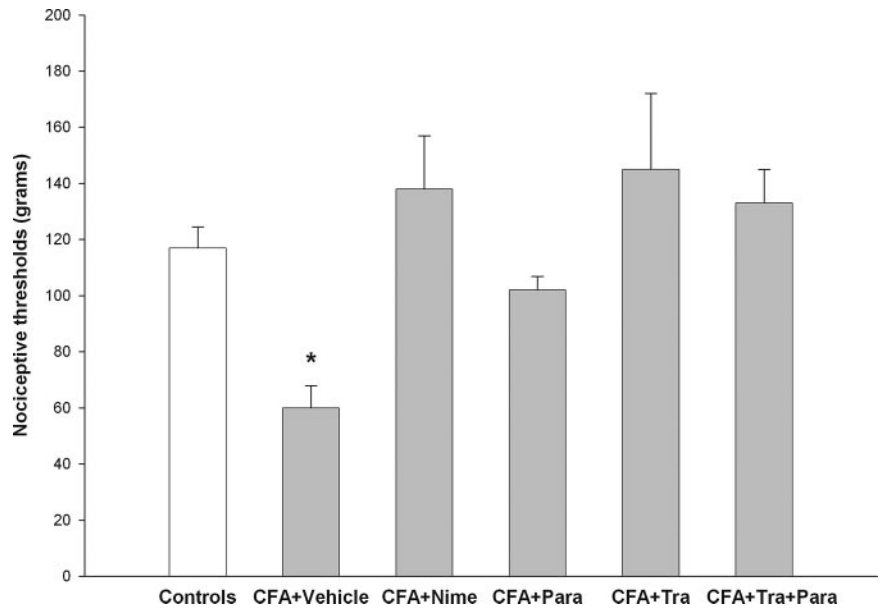
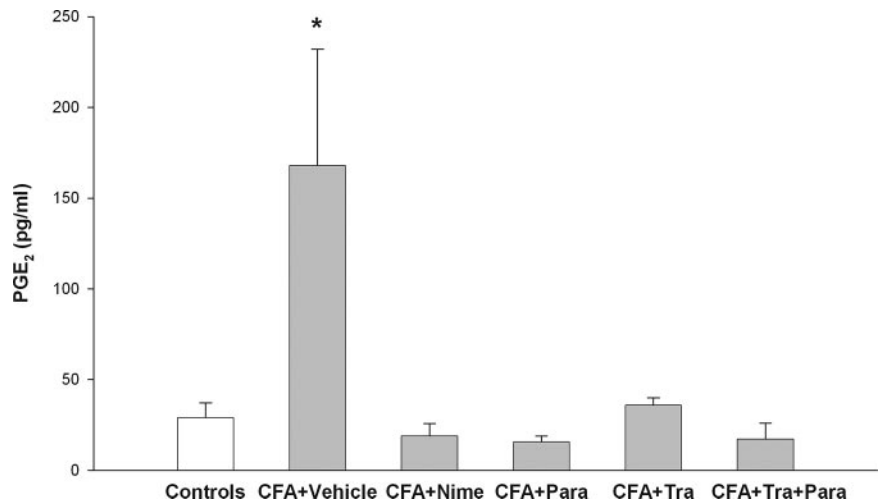


Figure 2. Effects of the treatment with nimesulide (nime, 5 mg/kg p.o.), paracetamol (para, 65 mg/kg p.o.), tramadol (tra, 7.5 mg/kg p.o.), and tramadol plus paracetamol (tra + para) on the increase of prostaglandin E₂ (PGE₂) concentrations in the cerebrospinal fluid produced by complete Freund's adjuvant (CFA) injection into the hindpaw of rats. Control animals were treated intraplantarly with saline and orally with vehicle. The measurements were performed 7 days after CFA injection. Values are pg/mL, means \pm SEM of eight rats. * = $P < 0.05$ vs. Controls (ANOVA + Bonferroni's *t*-test).



RESULTS

As expected, the administration of CFA into the hindpaw caused a significant decrease in paw withdrawal latency (in grams) to noxious mechanical stimuli. Withdrawal latency was significantly different between the CFA- and saline-treated (controls) rats (Fig. 1). CFA-induced mechanical hyperalgesia was completely prevented by oral treatment with nimesulide, paracetamol, tramadol, and paracetamol plus tramadol. Therefore it was impossible to find evidence of a potential additive effect of tramadol and paracetamol when administered in combination. All drug treatments produced a comparable antihyperalgesic effect (Fig. 1).

After the evaluation of the nociceptive thresholds to mechanical stimuli, the CSF was collected for PGE₂ and TNF- α measurement. A possible effect of the Randall-Selitto testing on PGE₂ and TNF- α release in inflamed animals was excluded as we demonstrated that the values of PGE₂ concentrations measured in rats that underwent the Randall-Selitto paw-withdrawal test

were not different from those measured in a group of rats that was killed 7 days after CFA injection without performing the test (data not shown).

The injection of CFA produced a significant increase in PGE₂ concentrations in the CSF, compared with the control group (Fig. 2).

Oral administration of all drugs tested in these experiments completely prevented CFA-induced increase of spinal PGE₂ (Fig. 2). Indeed, ANOVA comparing the values of PGE₂ concentrations in the different groups revealed a significant difference between vehicle- and drug-treated inflamed animals: $F(5,47) = 4.93$, $P = 0.001$; CFA+Vehicle versus CFA+Nimesulide, $P = 0.005$; CFA+Vehicle versus CFA+Paracetamol, $P = 0.004$; CFA+Vehicle versus CFA+Tramadol, $P = 0.018$; CFA+Vehicle versus CFA+Tramadol+Paracetamol, $P = 0.004$. No difference was detected between drug-treated animals and controls (uninflamed, vehicle-treated rats).

Intraplantar administration of CFA increased the CSF levels of TNF- α measured 7 days after the induction of hindpaw inflammation (Fig. 3). Unlike PGE₂,

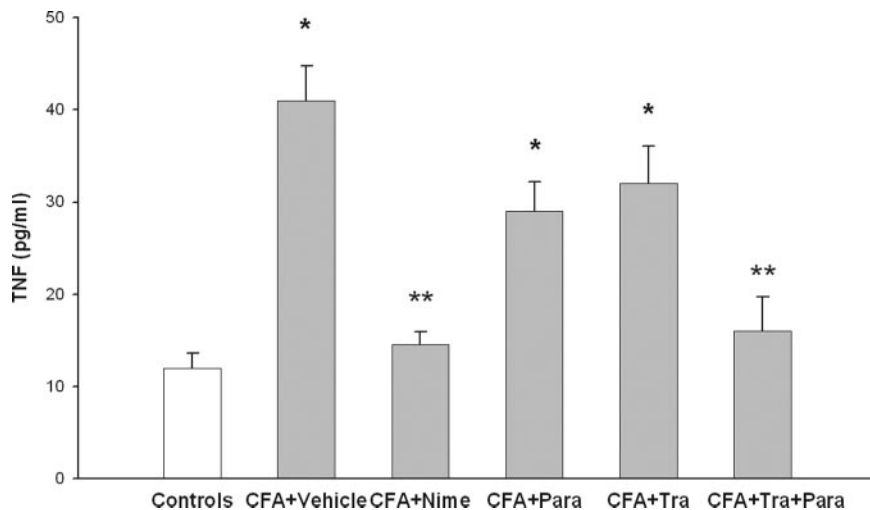


Figure 3. Effects of the treatment with nimesulide (nime, 5 mg/kg p.o.), paracetamol (para, 65 mg/kg p.o.), tramadol (tra, 7.5 mg/kg p.o.), and tramadol plus paracetamol (tra + para) on the increase of tumor necrosis factor- α (TNF- α) concentrations in the cerebrospinal fluid produced by complete Freund's adjuvant (CFA) injection into the hindpaw of rats. Control animals were treated intraplantarly with saline and orally with vehicle. The measurements were performed 7 days after CFA injection. Values are pg/mL, means \pm SEM of eight rats. * = $P < 0.05$ vs. Controls; ** = $P < 0.05$ vs. CFA+Vehicle (ANOVA + Bonferroni's *t*-test).

the increase in TNF- α concentrations was prevented by treatment with nimesulide or paracetamol and tramadol in combination, but not by paracetamol and tramadol alone (Fig. 3). In fact, ANOVA comparing the values of TNF- α concentrations in the different groups revealed a significant difference between vehicle-treated animals and the groups treated with nimesulide or paracetamol and tramadol in combination: $F(5,47) = 13.61$, $P < 0.001$; CFA+Vehicle versus CFA+Nimesulide, $P < 0.001$; CFA+Vehicle versus CFA+Tramadol+Paracetamol, $P < 0.001$. In contrast, no difference was detected between the inflamed animals orally treated with vehicle and those treated with paracetamol and tramadol alone. The values measured in these two latter groups of animals were significantly different from those measured in controls ($P = 0.007$ and $P < 0.001$, respectively).

DISCUSSION

The results of the present study show that the injection of CFA into the hindpaw of rats produces a condition of hyperalgesia to mechanical stimuli, which is associated with an increase in the levels of PGE₂ and TNF- α in the CSF. Our findings regarding the increase in PGE₂ concentration are consistent with data provided by a number of other authors demonstrating that peripheral inflammation causes an increase in spinal levels of PGs (23–25). In particular, it has been demonstrated that intraplantar injection of CFA produced mechanical hyperalgesia as well as a marked increase in the level of PGE₂ in the lumbar spinal cord (24). These data indicate that spinal PGE₂ is involved in pain initiated by acute peripheral inflammation. In other experiments, it has been demonstrated that PGE₂ increase, as well as the associated behavior, can be prevented by the administration of analgesic drugs such as paracetamol and flurbiprofen (26,27).

In our present experiments, the increase in spinal PGE₂ induced by CFA injection was completely inhibited by the treatment with analgesic drugs administered at antihyperalgesic doses. Thus, the prevention

of hyperalgesia was associated with the prevention of the changes in PGE₂ concentration in the CSF. The effects on PGE₂ concentrations by a nonsteroidal anti-inflammatory drug such as nimesulide, and by paracetamol, could be explained by their ability to inhibit cyclooxygenase enzymes (17,19). On the other hand, tramadol has been shown to exert its action without directly inhibiting cyclooxygenase activity (28). Several actions, both at the central and peripheral level, could be the basis of the effects of tramadol. Tramadol, in fact, exerts a dual mechanism of action: binding to the μ opioid receptor and enhancement of the monoaminergic system (29,30). Peripheral opioid receptors have been described as mediating several effects of opioid agonists; interestingly, such effects are particularly prominent in inflammatory conditions (31). The activation of opioid receptors by tramadol in the periphery may contribute, in addition to its central action, to the effects of this drug. There is also evidence that monoamines play an important role in inflammation and pain modulation. It can be hypothesized, therefore, that the enhancement of serotonergic and noradrenergic tone that follows tramadol administration could have contributed to the effects exerted by this analgesic drug in our experimental conditions (32,33).

Our current findings show that the injection of CFA into the hindpaw also produced a significant increase in TNF- α levels in the CSF. Up-regulation of central proinflammatory cytokines after peripheral inflammation has been documented (34). An enhanced expression of TNF- α and other cytokines in the spinal cord throughout several phases of CFA-induced inflammation in rats has been demonstrated (3). To our knowledge, however, there are few data concerning the changes in TNF- α levels in the CSF in animals with inflammatory hyperalgesia (15,35). Ours is the first evidence of a marked increase in the CSF concentration of TNF- α in a rodent model of peripheral inflammation.

In the present study we collected the CSF by cisterna magna, and therefore the changes that we

observed after the induction of peripheral inflammation may not have reflected only what was happening in the lumbar area, where the primary afferents innervating the inflamed hindpaw terminate, but could have been representative of more general functional and biochemical changes in the central nervous system.

With regard to the effects of pharmacological treatments, it is interesting that nimesulide and paracetamol plus tramadol could prevent both hyperalgesia and the TNF- α increase in the CSF. Conversely, when administered at a dose shown to prevent the development of hyperalgesia, paracetamol or tramadol alone do not prevent CFA-induced changes in spinal levels of TNF- α . Thus, prevention of hyperalgesia is not necessarily associated with the prevention of an increase in TNF- α concentration in the CSF.

Our present data do not enable us to establish a clear relationship between the ability of an analgesic drug to fully prevent spinal changes produced by peripheral inflammation and a specific mechanism of action. As it has been demonstrated that nimesulide, paracetamol, and tramadol are able to reduce peripheral inflammation (4,6,33), it is conceivable that the attenuation of spinal PGE₂ and TNF- α release is, at least in part, related to reduced input from the primary afferents. All the drugs tested in this study may act on the central nervous system. Thus, both central and peripheral actions may contribute to the effects produced by the drugs in our present study.

It has been shown that, in addition to PGE₂, TNF- α also plays a fundamental role in changing the response of central pain signaling neurons leading to persistent pain states (9). Pain facilitation is traditionally viewed as being mediated solely by sensory neurons. However, it is now clear that activated glial cells in the spinal cord play a major role in mediating enhanced pain states by releasing proinflammatory cytokines and other substances thought to facilitate pain transmission (36). TNF- α released by activated glial cells was established as an important mediator of pain and hyperalgesia in the central nervous system (10,37).

Our present data indicate that the increase in TNF- α concentrations in the CSF and hyperalgesia represent two phenomena that can be dissociated, at least from a pharmacological point of view. Therefore, we believe that pharmacological treatments that can provide symptomatic relief (i.e., the prevention of hyperalgesia) as well as complete normalization of spinal changes produced by persistent peripheral inflammation deserve special attention for possible use in clinical conditions of inflammatory pain.

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