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Induction of cyclooxygenase-2 causes an enhancement of writhing response in mice

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Abstract

Pretreatment of mice with lipopolysaccharide for 16 h enhanced the number of acetic acid-induced writhing reactions by 2 to 3-fold. In the peritoneal exudates at 10 min after acetic acid injection, 6-keto-prostaglandin $F_{1\alpha}$ was detected as a major prostanoid, and this level increased by several-fold by the pretreatment with lipopolysaccharide. The writhing reaction and the prostaglandin formation were almost completely suppressed by indomethacin. However, the lipopolysaccharide-induced enhancement of writhing reaction and an increment of 6-keto-prostaglandin $F_{1\alpha}$ level were diminished by the administration of cyclooxygenase-2-selective inhibitors, such as NS-398, nimesulide, or L-745337, to a level similar to the mice that did not receive lipopolysaccharide. Cyclooxygenase-2 protein in the exudates became detectable at 5–48 h after the lipopolysaccharide-pretreatment. These results suggest that the increased prostaglandin production by cyclooxygenase-2 could be responsible for enhancement of the acetic acid-induced writhing reaction by lipopolysaccharide pretreatment.

Keywords: Writhing reaction; 6-Keto-prostaglandin $F_{1\alpha}$; Cyclooxygenase-2; Lipopolysaccharide; NS-398; Nimesulide

1. Introduction

The writhing reaction, a stretching response in mice, has been used for evaluation of the analgesic activity of non-steroidal anti-inflammatory drugs (Vinegar et al., 1979). Stretches produced by acetic acid (Koster et al., 1959) have been frequently used, as well as those induced by other algogenic substances, such as phenylquinone (Siegmund et al., 1957), acetylcholine (Collier et al., 1968) or kaolin (Fujiyoshi et al., 1990). A review of the analgesic action of inhibitors of cyclooxygenase indicated that such inhibitors could block cyclooxygenase product-mediated sensitization of polymodal pain receptors around various inflammatory tissues (Moncada et al., 1975; Higgs, 1980; Nuki, 1990; Kumazawa, 1996) and that prostaglandin I_2 and prostaglandin E_2 are the most potent agonists for sensitization of peripheral pain receptors among various cyclooxygenase products (Moncada et al., 1975). However, there have been only a few reports describing the molecular species of prostaglandins involved in nociception of the writhing reaction, i.e., Berkenkopf and Weichman (1988) indicated that prostaglandin I_2 may be a candidate for nociceptive mediator, since 6-keto-prostaglandin $F_{1\alpha}$ was found as a major prostanoid in the peritoneal cavity during the writhing reaction of mice induced by acetic acid or phenylbenzoquinone. Recently, inducible cyclooxygenase-2 has been reported as the enzyme responsible for inflammatory exudation and pain (Seibert et al., 1994), and cyclooxygenase-2 expression has been demonstrated in inflammatory cells and numerous tissues (DeWitt and Smith, 1995; Vane, 1994), including in lipopolysaccharide-stimulated macrophages (Hempel et al., 1994; Matsumoto et al., 1997). However, direct evidence for its biological role in vivo, especially in inflammatory reactions is still lacking. In this communication we demonstrate that acetic acid-induced writhing reaction was enhanced by pretreatment with lipopolysaccharide and that inducible cyclooxygenase-2 may be involved in this enhancement.

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2. Materials and methods

2.1. Materials

Lipopolysaccharide (*Escherichia coli* O111:B4, indomethacin, dexamethasone, and nimesulide (*N*-[4-nitro-2-phenoxyphenyl]-methanesulfonamide) were purchased from Sigma (St. Louis, MO, USA). NS-398 (Futai et al., 1994) and L-745337 (Chan et al., 1995) were generous gifts from Taisho Pharmaceutical (Saitama, Japan) and Merck Frosst Canada (Quebec, Canada), respectively. For administration to animals, indomethacin, 1 mg/ml; nimesulide, 0.3 mg/ml; NS-398, 0.3 mg/ml; L-745337, 0.3 mg/ml and dexamethasone, 0.5 mg/ml were suspended in 1% sodium carboxymethylcellulose solution containing 0.9% sodium chloride. Rabbit antiserum to mouse cyclooxgenase-1 was a kind gift from Dr. W.L. Smith (Michigan State University, East Lansing, MI, USA). Rabbit antiserum to mouse cyclooxygenase-2 (Cayman Chemical, Ann Arbor, MI, USA) and peroxidase-conjugated goat anti-rabbit IgG (Zymed Labs., CA, USA) were purchased.

2.2. Induction of writhing reaction and administration of anti-inflammatory drugs

Writhing reaction was induced by an intraperitoneal injection of 0.9% acetic acid solution into 5–6-week old male ICR mice (SLC Japan, Hamamatsu, Japan). Some animals received lipopolysaccharide, 25 μg/0.1 ml per mouse given intraperitoneally at selected times before the induction of the writhing reaction. Indomethacin, 10 mg/kg, was intraperitoneally injected 30 min before the induction of the writhing. Nimesulide, 3 mg/kg; NS-398, 3 mg/kg; and L-745337, 3 mg/kg were administered orally 90 min before the induction of writhing. Dexamethasone, 0.5 mg/kg, was intraperitoneally injected 2 h before the lipopolysaccharide-treatment.

2.3. Determination of prostaglandins in the peritoneal exudates

Prostaglandins were extracted from the peritoneal exudates of mice that had been injected with 0.9% acetic acid. Mice were exsanguinated at 5, 10, and 15 min after the acetic injection: and their peritoneal cavity was washed 3 times with 2 ml of Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution containing 10 mM indomethacin. The pooled washings were adjusted to pH 3.0 with *N*-HCl and passed through a Sep-Pak C18 cartridge (Waters, Milford, MA, USA), and the retained prostaglandins were eluted with 8 ml of methanol as described previously (Yamaki and Oh-ishi, 1992). The solvent of the samples was evaporated, and then the prostaglandins were dissolved in an aliquot of buffer (200 ml) and assayed with commercial enzyme immunoassay kits for each prostaglandin (Cayman Chemical).

2.4. SDS-PAGE / immunoblotting

Pooled peritoneal washings were centrifuged at $900 \times g$ for 10 min, and the obtained cells were washed once with Ca^{2+}, Mg^{2+}-free Hanks, balanced salt solution and suspended at a concentration of 1×10^7 cells/ml in 50 mM Tris–HCl buffer containing 0.1% sodium dodecylsulfate, 0.5% Nonident-P40, 5 μM Na_3VO_4, 50 μg/ml leupeptin, 1.5 μM pepstatin A and 1 mM phenylmethylsulfonyl fluoride. Then a half volume of sample buffer (188 mM Tris–HCl, pH 6.8, containing 6% sodium dodecylsulfate, 30% glycerol, 15% 2-mercaptoethanol, 0.3% bromophenylblue) was added and boiled for 10 min. A 10-μl of aliquot of each sample solution was applied to a sodium dodecylsulfate-polyacrylamide gel, and electrophoresed under reducing conditions for immunoblotting with antibodies specific for cyclooxygenase-1 and cyclooxygenase-2. Immunoblots were performed in the same manner as previously reported (Matsumoto et al., 1997). In this experiment antibodies against mouse cyclooxygenase-1 (1:10 000 dilution), cyclooxygenase-2 (1:5000 dilution), and the horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:7000 dilution) were used.

2.5. Statistical analysis

Data were expressed as the mean with S.E.M. of more than three independent experiments. Statistical analysis was conducted with Student's *t*-test or one-way analysis of variance followed by Dunnet's *t*-test.

3. Results

3.1. Effect of lipopolysaccharide pretreatment on the acetic acid-induced writhing reaction of mice

Intraperitoneal injection of acetic acid caused writhing (stretching) responses in the mice, and the number of responses that occurred during each sequential 5-min periods for 30 min was counted, as shown in Fig. 1. Numbers of the response in 5 min peaked at the 5–10-min period after the injection of 0.9% acetic acid, and the reaction ceased after 30 min. As expressed by closed columns in Fig. 1, lipopolysaccharide pretreatment (25 μg/mouse, i.p., 16 h beforehand) significantly increased the number of the writhings per 5 min, especially at 5–10 min, in comparison with those in the mice without the pretreatment (open columns). Since significant enhancement was observed over the time lapse of 0–15 min, the accumulated number of writhings during the initial 15 min was used for evaluation, and compared between the mice pretreated with lipopolysaccharide and those of vehicle-pretreated animals. Numbers of writhing responses were significantly increased in the mice that received lipopolysaccharide at 5, 12, 16, 24 and 48 h before the acetic acid injection over

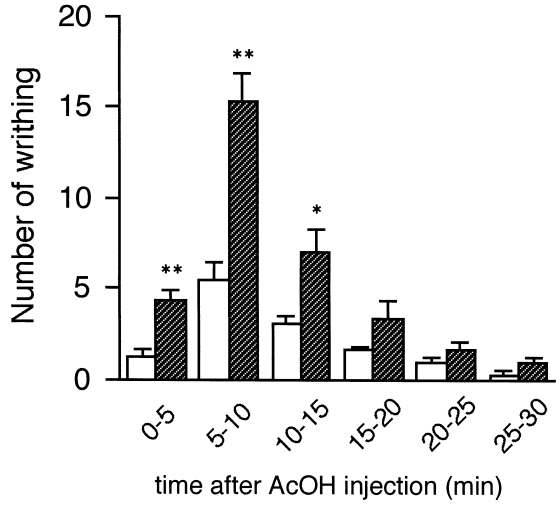

Fig. 1. Writhing reaction induced by intraperitoneal injection of acetic acid and effect of pretreatment with lipopolysaccharide. Frequency of the stretch response (writhing) was counted in each of six sequential 5-min periods after the intraperitoneal injection of 0.9% acetic acid solution (5 ml/kg) into male ICR mice (open columns). Another group of mice was pretreated with lipopolysaccharide (lipopolysaccharide, 25 μg/mouse, intraperitoneally) 16 h prior to the acetic acid injection (hatched columns). Each value is the mean with S.E.M. of eight mice. $*$ and $**$ Indicate statistically significant difference from corresponding values of non-pretreated groups by Student's *t*-test at $P < 0.05$ and $P < 0.01$, respectively.

the response of mice without the lipopolysaccharide pretreatment. This enhancement peaked around 12–16 h after lipopolysaccharide treatment, as shown in Fig. 2. Therefore, in subsequent experiments, the pretreatment with lipopolysaccharide for 16 h prior to the acetic acid injection was used.

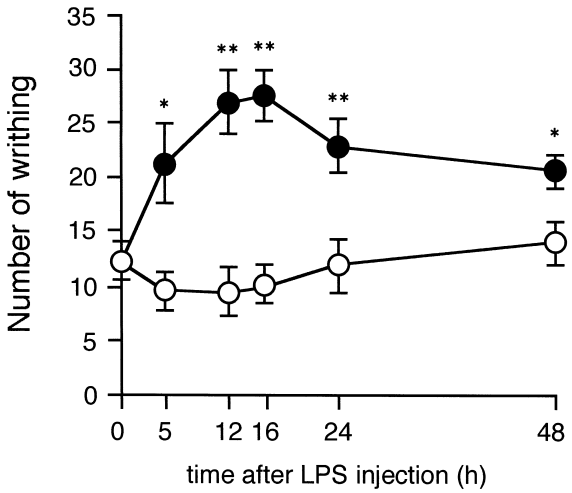

Fig. 2. Time-course of enhancement of the number of the acetic acid-induced writhing responses by pretreatment with lipopolysaccharide. Accumulated number of stretch responses for 15 min after intraperitoneal injection of 0.9% acetic acid solution (5 ml/kg) was determined. Mice received lipopolysaccharide (closed circles) or physiological saline as a vehicle (open circles) at the indicated hours before the injection of acetic acid. Each point indicates the mean with S.E.M. of 10 to 11 mice. $*$ and $**$ Indicate statistically significant difference from vehicle-pretreated groups by Student's *t*-test at $P < 0.05$ and $P < 0.01$, respectively.

3.2. Effects of several anti-inflammatory agents on the acetic acid-induced writhing reaction in mice with or without lipopolysaccharide pretreatment

In order to know which cyclooxygenase, constitutive or inducible, is responsible for the basal response and the enhanced response of the acetic acid-induced writhing reaction by lipopolysaccharide, we examined the effects of indomethacin (10 mg/kg) and several cyclooxygenase-2-selective anti-inflammatory agents. The doses of the latter agents were selected because these doses had been reported to suppress cyclooxygenase-2 induced production of prostaglandin E_2 in vivo (Chan et al., 1995; Harada et al., 1996). As illustrated in Fig. 3, cyclooxygenase-2-selective inhibitors, i.e., nimesulide (3 mg/kg), NS-398 (3 mg/kg), and L-745337 (3 mg/kg), reduced the enhanced number of writhings in lipopolysaccharide-pretreated mice to the level of the animals that had not received lipopolysaccharide, but did not reduce the acetic acid-induced writhing number of normal mice. Whereas indomethacin significantly suppressed both the lipopolysaccharide-induced enhanced response and the response of non-pretreated mice. Dexamethasone, when injected before the

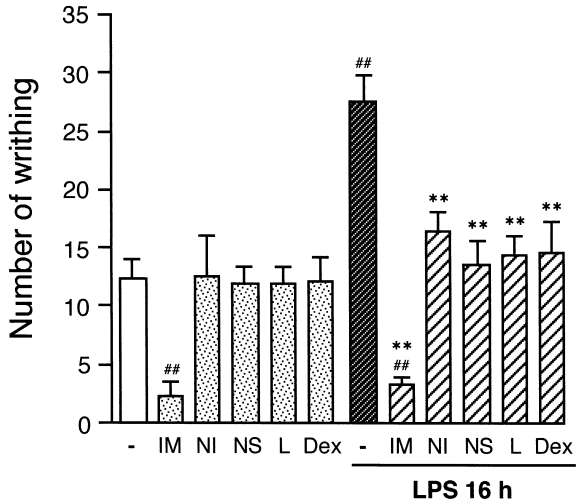

Fig. 3. Effects of anti-inflammatory drugs on the number of acetic acid-induced writhing responses in lipopolysaccharide-pretreated and non-pretreated mice. Effects of indomethacin (IM, 10 mg/kg), nimesulide (NI, 3 mg/kg), NS-398 (NS, 3 mg/kg), L-745337 (L, 3 mg/kg), and dexamethasone (Dex, 0.5 mg/kg) on the number of acetic acid-induced stretch (writhing) responses in lipopolysaccharide- or physiological saline-pretreated mice were examined. Lipopolysaccharide (hatched columns) or physiological saline (open and dotted columns) was injected intraperitoneally 16 h before the injection of acetic acid. Dexamethasone or indomethacin was injected intraperitoneally 2 h or 30 min, respectively, before the acetic acid injection; and other drugs were administered orally 90 min beforehand. Each column indicates the mean with S.E.M. of eight mice. ## Indicates significant difference vs. the control group injected with acetic acid solution only (open column) at $P < 0.01$. $*$ and $**$ Indicate statistically significant difference from the lipopolysaccharide-pretreated, anti-inflammatory drug non-pretreated group (a finely hatched column) at $P < 0.05$ and $P < 0.01$, respectively. One-way analysis of variance followed by Dunnet's *t*-test was performed.

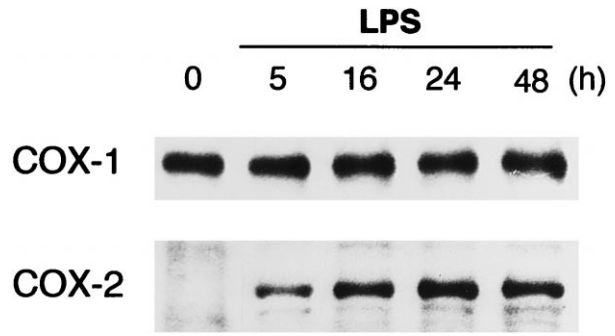

Fig. 4. Western blot analysis of cyclooxygenase species in the peritoneal cells of mice pretreated with lipopolysaccharide for various lengths of time. A representative immunoblot pattern from the peritoneal exudated cells collected at 0, 5, 16, 24 and 48 h after the injection of lipopolysaccharide is shown. Each lane contained lysates of approximately 6.7×10^4 cells.

lipopolysaccharide treatment, also inhibited only the enhancement of writhing frequency, but did not affect the number of responses by the mice that had not received the lipopolysaccharide. These results suggest that enhancement of acetic acid-induced writhing response by lipopolysaccharide treatment could be associated with induction of cyclooxygenase-2, which may, in turn, produce increased amounts of prostaglandins.

3.3. Western blot analysis of cyclooxygenases

To confirm the expression of cyclooxygenase enzymes, we conducted Western blot analysis of the cell lysates of peritoneal cells obtained 0, 5, 16, 24, and 48 h after the injection of lipopolysaccharide (Fig. 4). Cyclooxygenase-2 protein was not detectable in the cells without lipopolysaccharide treatment, but it was found in the cells from mice subjected to lipopolysaccharide treatment for 5 to 48 h. However, constitutive cyclooxygenase-1 expression in these cells was not significantly changed by lipopolysaccharide treatment.

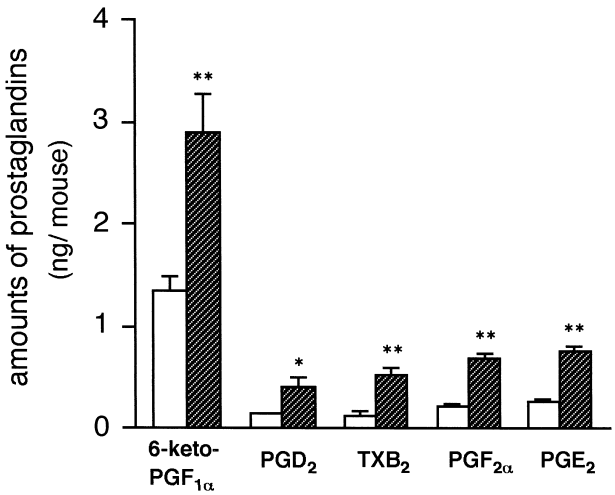

Fig. 5. Production of prostaglandins in the peritoneal exudates during the acetic acid-induced writhing reaction in non-pretreated mice and in those pretreated with lipopolysaccharide for 16 h. Peritoneal exudates were collected from the mice at 10 min after acetic acid injection, and prostaglandins were extracted; and assayed with each enzyme immunoassay kit. Open columns indicate prostaglandin levels in the peritoneal exudates from mice without lipopolysaccharide pretreatment; and hatched ones, those in the exudates from mice that received lipopolysaccharide 16 h prior to the acetic acid injection. Values are expressed as means with S.E.M. of 16 mice for 6-keto-prostaglandin $F_{1\alpha}$ and of eight mice for the other prostaglandins.

3.4. Prostaglandin levels in the peritoneal exudates of mice during the writhing reaction and effects of NS-398 and indomethacin on these levels

Prostaglandin levels were measured in the peritoneal exudates of mice obtained at 10 min after acetic acid injection with or without lipopolysaccharide pretreatment for 16 h (Fig. 5). 6-Keto-prostaglandin $F_{1\alpha}$, prostaglandin D_2, thromboxane B_2, prostaglandin $F_{2\alpha}$, and prostaglandin E_2 were detected in the exudates taken 10 min after acetic acid injection in non-pretreated mice. The levels of these prostaglandins increased significantly after pretreatment with lipopolysaccharide 16 h prior to the acetic acid injection. 6-Keto-prostaglandin $F_{1\alpha}$ showed the highest level among the prostanoids, with a peak at 10 min after the acetic acid injection, especially in the lipopolysaccha-

Table 1
Effect of NS-398 and indomethacin on the level of 6-keto-prostaglandin $F_{1\alpha}$ in the peritoneal exudates during acetic acid-induced writhing reaction of mice

Treatment	*n*	6-Keto-prostaglandin $F_{1\alpha}$
Wash[a]	8	0.27 + 0.04
Without lipopolysaccharide[b]		
Vehicle	8	1.14 + 0.25
NS-398	8	1.28 + 0.16
Indomethacin	8	0.42 + 0.04[d]
Lipopolysaccharide-treated[c]		
Vehicle	16	2.90 + 0.38
NS-398	6	0.77 + 0.09[e]
Indomethacin	6	0.43 + 0.01[f]

Level of 6-keto-prostaglandin $F_{1\alpha}$ (ng/mouse) was measured in the exudates obtained 10 min after the intraperitoneal injection of 0.9% acetic acid, and expressed as the mean ± S.E.M. of the indicated number (*n*) of mice.

[a] Peritoneal wash sample was collected from non-treated mice.

[b] Sample was collected from mice without lipopolysaccharide pretreatment.

[c] Sample was collected from the mice that had been injected with lipopolysaccharide 16 h before the acetic acid injection. Indomethacin (10 mg/kg), NS-398 (3 mg/kg), or vehicle was administered in the same way as described in the legend of Fig. 2.

[e] and [f] Indicate statistically significant difference from the data of each vehicle group, the animals in which were injected with acetic acid but with no anti-inflammatory drugs, at $P < 0.05$ and $P < 0.01$, respectively. One-way analysis of variance followed by Dunnet's *t*-test was performed.

ride pretreated mice; and this peak well corresponded to the peak of writhing responses shown in Fig. 1. Then, to know if inducible cyclooxygenase plays a role in this increase in the level of 6-keto-prostaglandin $F_{1\alpha}$, we examined the effect of cyclooxygenase inhibitors. As seen in Table 1, NS-398, at a dose that suppressed the enhanced writhing reaction, significantly reduced the increment of 6-keto-prostaglandin $F_{1\alpha}$ level in the peritoneal exudates from the mice pretreated with lipopolysaccharide 16 h beforehand. However, NS-398 did not affect the 6-keto-prostaglandin $F_{2\alpha}$ level of the mice not pretreated with lipopolysaccharide. Indomethacin reduced the prostaglandin level in both mice with and without lipopolysaccharide pretreatment more strongly than NS-398.

4. Discussion

There is a common consensus that mediators involved in the nociception of the writhing reaction are cyclooxygenase products, and this reaction has been used for preclinical evaluation of the analgesic potency of newly developed non-steroidal anti-inflammatory drugs or cyclooxygenase inhibitors. However, only a few reports have been published to provide direct evidence for the involvement of prostaglandins in this pain model for visceral algesia. Furthermore, there has been no previous report on the involvement of inducible cyclooxygenase in the writhing reaction.

We considered the writhing reaction to be a model for inflammatory hyperalgesia and sought to assess the possible involvement of inducible cyclooxygenase (cyclooxygenase-2) in this pain model. For this purpose we pretreated mice with lipopolysaccharide of *E. coli*, since there are reports describing that such treatment increases prostaglandin production (Masferrer et al., 1990) and that this lipopolysaccharide induces cyclooxygenase-2 in various cells including rat peritoneal macrophages (Hempel et al., 1994; Matsumoto et al., 1997). We found enhancement of the number of acetic acid-induced writhings in lipopolysaccharide-pretreated mice. This enhancement may be caused by inducible cyclooxygenase-2 activity, since cyclooxygenase-2-selective inhibitors attenuated only the enhancement, and cyclooxygenase-2 protein expression increased during the time period of enhancement. These data could well support the role of cyclooxygenase-2 in inflammatory late-phase pain (Seibert et al., 1994; Masferrer et al., 1994).

As for the species of prostaglandins involved, we conclude that prostaglandin I_2 could be a main mediator for nociception of writhing in normal mice, and which conclusion is consistent with the report of Berkenkopf and Weichman (1988), and our previous paper (Murata et al., 1997). In the former, they measured 6-keto- prostaglandin $F_{1\alpha}$ as a major prostaglandin in the peritoneal cavity during writhing. In our previous paper, we demonstrated that IP-receptor could be involved in the nociception of acetic acid induced writhing, since the receptor disruption in mice reduced the response significantly (Murata et al., 1997).

To identify the mediators responsible for the enhancement of the writhing response by the pretreatment with lipopolysaccharide, we measured several prostanoids, and found that prostaglandin I_2 produced by cyclooxygenase-2 could be a major one, but also found that the levels of several other prostanoids were increased significantly, though to a lesser extent than the level of 6-keto-prostaglandin $F_{1\alpha}$ in the exudates of lipopolysaccharide-pretreated, acetic acid-injected mice. Therefore, we cannot completely exclude the participation of other prostanoids in this enhancement. These results indicate that prostaglandin I_2 in the mice without lipopolysaccharide pretreatment is produced by constitutive cyclooxygenase-1 and that prostaglandin I_2 in the lipopolysaccharide-pretreated mice is produced by both produced by cyclooxygenase-2.

In conclusion, the above results taken together suggest that the increase in frequency of the writhing response in lipopolysaccharide-treated animals is associated with an increased level of prostaglandins produced by cyclooxygenase-2. Furthermore, the results indicate that prostaglandin I_2 could be the main nociceptive mediator for the acetic acid-induced writhing reaction in mice and that it may also have a role in the late-phase response, in which case nociception would be enhanced by inducible cyclooxygenase-2, acting to produce more prostanoids in response to an irritant such as acetic acid.

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