Prostaglandin Receptors EP2, EP3, and IP Mediate Exudate Formation in Carrageenin-Induced Mouse Pleurisy

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ABSTRACT

The roles of prostaglandins (PGs) as mediators of inflammation have been extensively studied, and production of PGL_2 and PGE_2 at inflammatory sites has been reported. However, it has not yet been clarified which type of PG receptor has a major role in inflammatory exudation. To examine in vivo role of PG receptors in inflammatory exudation, we induced pleurisy in PG receptors (IP, EP1, EP2, EP3, or EP4) knockout mice by intrapleural injection of carrageenin. Pleural exudate accumulation in wild-type (WT) mice at 1 to 5 h, but not at 24 h, was significantly attenuated by the pretreatment with indomethacin, indicating that PGs are responsible for exudate formation at the early phase of pleurisy. Pleural exudation at 1 to 5 h in IP, EP2, or EP3 knockout mice, but not in EP1 and EP4 knockout, was significantly reduced compared with in WT mice. In the exudates, 6-keto-PGF_α_ and PGE_2 were detected as the major PGs, each with its peak concentration at 3 h. In addition, involvement of bradykinin in the phenomenon was suggested by the fact that captopril, a kininase inhibitor, enhanced the exudate formation and increased the amount of 6-keto-PGF_α_ and PGE_2, and that a bradykinin B2-receptor antagonist inhibited the exudate formation. In contrast, leukocyte migration into pleural cavity was not influenced by indomethacin-treatment nor by these receptor deficiencies. These results demonstrate participation of EP2 and EP3 along with IP in pleural exudate formation but not in leukocyte migration in carrageenin-induced mouse pleurisy.

The roles of prostaglandins (PGs) as inflammatory mediators and physiological modulators have been extensively studied (Movat, 1968; Vane, 1976; Rocha e Silva, 1978), and it is well established that nonsteroidal anti-inflammatory agents, such as aspirin and indomethacin, exert their anti-inflammatory action by inhibiting cyclooxygenases in the cascade of PG biosynthesis (Vane and Botting, 1998). Among the various PG species, PGL_2 and PGE_2 have been implicated as the PGs most responsible for exudation at inflammatory sites has been reported. However, it has not yet been clarified which type of PG receptor has a major role in inflammatory exudation. To examine in vivo role of PG receptors in inflammatory exudation, we induced pleurisy in PG receptors (IP, EP1, EP2, EP3, or EP4) knockout mice by intrapleural injection of carrageenin. Pleural exudate accumulation in wild-type (WT) mice at 1 to 5 h, but not at 24 h, was significantly attenuated by the pretreatment with indomethacin, indicating that PGs are responsible for exudate formation at the early phase of pleurisy. Pleural exudation at 1 to 5 h in IP, EP2, or EP3 knockout mice, but not in EP1 and EP4 knockout, was significantly reduced compared with in WT mice. In the exudates, 6-keto-PGF_α_ and PGE_2 were detected as the major PGs, each with its peak concentration at 3 h. In addition, involvement of bradykinin in the phenomenon was suggested by the fact that captopril, a kininase inhibitor, enhanced the exudate formation and increased the amount of 6-keto-PGF_α_ and PGE_2, and that a bradykinin B2-receptor antagonist inhibited the exudate formation. In contrast, leukocyte migration into pleural cavity was not influenced by indomethacin-treatment nor by these receptor deficiencies. These results demonstrate participation of EP2 and EP3 along with IP in pleural exudate formation but not in leukocyte migration in carrageenin-induced mouse pleurisy.

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ABBREVIATIONS: PG, prostaglandin; WT, wild type; IP, PGI_2 receptor; EP, PGE_2 receptor; FR173657, E-3-(6-acetamido-3-pyridyl-N-[N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl)]-oxymethyl]-phenyl][N-methylamino-carbonyl methyl] acrylamide; captopril, S-1-(3-mercaptop-2-methyl-1-oxopropyl)-L-proline; RT-PCR, reverse transcriptase-polymerase chain reaction; KO, knockout.
leads to edema formation or exudate accumulation. Pleurisy, as an excellent model of acute inflammation, has been used as a potent tool to evaluate edema formation, enabling the assessment of both the rate and degree of exudate formation (Vinegar et al., 1973; Oh-ishi et al., 1986). In the present study, we intend to clarify the roles of PGE₂, in addition to LTB₄, in the process of pleurisy production in the pleural cavity of mice under light ether anesthesia as reported previously (Dozen et al., 1989). Immediately after the animals had been killed by exsanguination at the specified time after the carrageenin injection, pleural exudates and saline wash (0.5 ml) were collected in a tube containing 0.05 ml of 3% sodium citrate solution as an anticoagulant. Exudate volume and leukocyte number in the exudates were measured. To assess the exudation rate, we injected mice with a physiological saline solution of Pontamine sky blue (Tokyo Kasei Co., Tokyo, Japan), 50 mg/kg, intravenously 20 min before sacrifice. The dye concentration in the collected exudates and plasma was measured at 620 nm, and the rate of plasma exudation into the pleural cavity was calculated as reported (Imai et al., 1991). Leukocyte classification of a Giemsa-stained smear of the exudates was performed microscopically (Imai et al., 1991).

**Materials and Methods**

**Animals.** IP-, EP1-, EP2-, or EP3-deficient mice and wild-type (WT) mice were prepared and backcrossed over at least six generations to C57BL/6, as reported previously (Murata et al., 1997; Narumiya et al., 1999). EP4-deficient mice had a mixed genetic background of 129sv/ola and C57BL/6 mice, because all the EP4 knockout mice were purchased from Japan SLC (Hamamatsu, Japan). All experiments using animals were carried out under the regulations of the Japanese Pharmacological Society (Guiding Principles for the Care and Use of Laboratory Animals).

**Induction of Pleurisy.** Pleurisy was induced by injection of 0.05 ml of 2% carrageenin saline solution into the pleural cavity of mice under light ether anesthesia as reported previously (Dozen et al., 1989). Immediately after the animals had been killed by exsanguination at the specified time after the carrageenin injection, pleural exudates and saline wash (0.5 ml) were collected in a tube containing 0.05 ml of 3% sodium citrate solution as an anticoagulant. Exudate volume and leukocyte number in the exudates were measured. To assess the exudation rate, we injected mice with a physiological saline solution of Pontamine sky blue (Tokyo Kasei Co., Tokyo, Japan), 50 mg/kg, intravenously 20 min before sacrifice. The dye concentration in the collected exudates and plasma was measured at 620 nm, and the rate of plasma exudation into the pleural cavity was calculated as reported (Imai et al., 1991). Leukocyte classification of a Giemsa-stained smear of the exudates was performed microscopically (Imai et al., 1991).

**Measurement of PGs.** Carrageenin-induced pleurisy was induced in male C57BL/6 mice, and PGs in the exudates collected at 1, 3, 5, and 24 h were extracted and processed as described previously (Matsumoto et al., 1998) and assayed by enzyme-linked immunosorbent assay kits (Cayman Chemical, Ann Arbor, MI). Drugs. Captopril (Sankyo Co., Tokyo, Japan), an angiotensin-converting enzyme and kininase II inhibitor (Erdos, 1979), and FR173657 (Fujisawa Pharmaceutical Co., Tsukuba, Japan), a bradykinin B₂ receptor antagonist (Asano et al., 1997), were used at the dosages reported previously (Dozen et al., 1989; Ueno et al., 2000). Captopril was dissolved in sterile physiological saline, and injected intraperitoneally (10 mg/kg) into mice 10 min before the initiation of the pleurisy. Carrageenin and indomethacin were purchased from Sigma-Aldrich (St. Louis, MO) and bradykinin from Peptide Institute (Minoh, Japan). Indomethacin and FR173657 were suspended in 0.5% sodium carboxymethyl cellulose (Tokyo Kasei Co., Tokyo, Japan) solution in sterile saline and intraperitoneally administered 30 min before the injection of carrageenin (5 and 30 mg/kg, respectively).

**Detection of PG Receptors by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).** Pleura, diaphragm, and lung tissues were used for extraction of RNA to detect mRNAs of the prostaglandin receptors by RT-PCR using the following primers (Ma et al., 2001; Shinomiya et al., 2001): IP receptor primers used were 5'-GGCACGAGAGGTAGAAGTTACC-3' and 5'-GTCAGAGGCACAGCTAATGG-3'; EP1 primers, 5'-ACCCTGACCTCGAGCACAC-TGGCCCTCT-3' and 5'-CGATTGAGGAGACGCAG-3'; EP2 primers, 5'-AGGACTCTGATGCGAGAGAC-3' and 5'-CAGCCCCCTTACACTTCCCAATG-3'; EP3 primers, 5'-GGTATGCGACACCATGAAAC-3' and 5'-CAAGATCTGGTCTCAGGCCAAGCC-3'; and EP4 primers, 5'-TTCGGCTCGTGTCGGAGTTCT-3' and 5'-GGAGTGTTGTCGCTGTGGTCAG-3'. The reverse transcription reaction was performed by using a SuperScript II (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, with 2.5 µg of total RNA from the above-mentioned tissues used as a template. Equal amounts of each RT product were amplifed by PCR with Taq polymerase (Nippon Gene, Tokyo, Japan).

**Statistical Analysis.** Data were expressed as the mean ± S.E.M. Statistical analysis was conducted with Student's t test or one-way analysis of variance followed by Dunnett's t test. Differences with a value of P < 0.05 were considered statistically significant.

**Results**

**Time Course of Carrageenin-Induced Pleurisy in WT Mice and Effect of Indomethacin Treatment.** We first examined the time courses of pleural exudate accumulation, exudation rate, and leukocyte migration in carrageenin-induced pleurisy in WT mice (Fig. 1). The volume of the pleural exudates had already increased by 1 h after the carrageenin injection, peaked at around 3 to 5 h, and then gradually decreased to about one-half of the peak level at 24 h (Fig. 1A). Indomethacin significantly suppressed the exudate accumulation at 1 to 5 h, but not at 16 and 24 h. The exudation rate, assessed by dye leakage into the pleural exudates for 20 min, was the highest at 1 h and decreased with time; thus, the largest plasma leakage occurred at the initiation of the pleurisy (Fig. 1B). Exudation rate at 1 h was measured as the dye exudation during the time from 40 min to 1 h after the carrageenin injection, because 40 min was the earliest time that could be measured reliably in the present experiment. Indomethacin significantly suppressed the exudation rate at 1 and 3 h (Fig. 1B), indicating the involvement of prostanooids in the early phase of the pleurisy.

Total leukocytes in the exudates increased dramatically with time, reached a plateau at 5 h, and remained fairly constant thereafter up to 24 h (Fig. 1C). Eighty to ninety percent of total leukocytes were neutrophils, whereas the resident cells in the pleural cavity consisted of mostly monocytes and mast cells. Indomethacin had no significant effect on the leukocyte number in the pleural exudates throughout the time course (Fig. 1C), suggesting no involvement of prostaglandins in leukocyte migration.

Because indomethacin significantly suppressed the early phase of exudate accumulation, we focused our examination for exudate formation on the time period of 1 to 5 h after the induction of pleurisy in the following experiments.

**Measurement of PGs in the Exudates.** We assessed the production of PGs in the process of carrageenin-induced pleurisy in wild-type C57/BL6 mice. Among PGs measured in the exudates after the carrageenin injection, 6-keto-PGF₁α, a stable metabolite of PGL₂, and PGE₂ were the main PGs. The levels of these PGs had already increased at 1 h, peaked at 3 h, and decreased thereafter, but they remained detectable even at 24 h (Fig. 2). When the animals were pretreated with indomethacin, the levels of both PGs were almost at the lower limit of detection throughout the experimental period. The amounts of other PGs, such as PGD₂ and thromboxane B₂, were less than 0.1 ng/mouse (data not shown).
To assess the involvement of bradykinin in PG synthesis, we examined the effect of captopril, a kininase inhibitor, on the levels of PGs in the exudate. Captopril treatment significantly increased the 6-keto-PGF$_{1\alpha}$ level (from 0.78 $\pm$ 0.13 to 1.43 $\pm$ 0.20 ng/mouse) and PGE$_2$ level (from 0.17 $\pm$ 0.04 to 0.30 $\pm$ 0.07 ng/mouse) at 1 h, suggesting that the prevention of bradykinin degradation increased prostaglandin production.

Exudate Formation in PG Receptor Knockout Mice. Next, we compared the exudate formation induced in IP-, EP1-, EP2-, EP3-, and EP4-knockout (IP-KO, EP1-KO, EP2-KO, EP3-KO, and EP4-KO) and WT mice, because indomethacin significantly attenuated the exudation and because major PGs detected in the exudates were 6-keto-PGF$_{1\alpha}$ and PGE$_2$. The exudate volume in IP-KO, EP2-KO, and EP3-KO mice at 1, 3, and 5 h was significantly less than that in WT mice (Fig. 3, A–C). The exudation rate at 1 and 3 h in IP-KO mice was significantly less than that in WT mice.
(Fig. 3, D and E), whereas those rates in EP2-KO and EP3-KO mice were slightly but not significantly less than that in WT mice. These results suggest that prostanoid receptor IP, EP2 and EP3 participate in the exudate formation.

**Pharmacological Evaluation of Bradykinin Involvement in the Exudate Formation.** We examined whether bradykinin-bradykinin B2 receptor system is involved in the mouse pleurisy; the system was reported to be involved in carrageenin-induced rat pleurisy (Harada et al., 1982; Dozen et al., 1989). The involvement of bradykinin was examined by treatment with a bradykinin B2 receptor antagonist FR173657. In WT mice, the exudate volume at 3 h was significantly suppressed by FR173657 to a similar degree as that by treatment with indomethacin, and no further suppression was found by simultaneous treatment with both inhibitors (Fig. 4). In IP-KO mice, the exudate volume at 3 h was significantly lower compared with that in WT mice, and was significantly suppressed further by indomethacin, indicating that PG other than PGI2, possibly PGE_{2}, participated in the exudate formation. FR173657 significantly decreased the exudate volume to a similar degree with that by indomethacin, and no further suppression was found by simultaneous treatment with both inhibitors (Fig. 4). These results suggest that PG and bradykinin had a common pathway to stimulate the exudate formation.

We also examined the effect of captopril on the exudate volume and rate. Captopril treatment significantly increased the exudate volume in WT and EP3-KO mice at 1 h, whereas the increase in IP-KO and EP2-KO mice was slight (Table 1). For the exudation rate, significant increase was caused by captopril in WT and EP3-KO mice, but only a slight increase was seen in IP-KO and EP2-KO mice.

**Expressions of mRNAs for PG Receptors.** RT-PCR for IP, EP1, EP2, EP3, and EP4 mRNAs was performed in the tissues adjacent to pleural cavity of WT mice. Expression of mRNAs for all receptors except EP1, before and 3 h after carrageenin treatment was detected in the pleura, diaphragm, and lung, suggesting that these receptors could...
mediate signals for PGI2 and PGE2 to induce exudation (Fig. 5). Interestingly, the expression levels of these mRNAs decreased apparently in the pleura after carrageenin injection.

**Discussion**

We first examined the time course of carrageenin-induced pleurisy in WT mice with or without pretreatment of indomethacin. Involvement of PGs in exudate formation was suggested by the significant suppressive effect of indomethacin during the earlier phase, from the initiation up to 5 h after the carrageenin injection (Fig. 1). This feature is similar to the case in carrageenin-induced rat pleurisy, in which involvement of PGs and kinin during an earlier phase of exudation was suggested, and 6-keto-PGF1α and PGE2 were detected in the exudates (Dozen et al., 1989). However, receptor types for PGs mediating exudate formation have not yet been determined. To determine types of PG receptors involved in exudation in the present mouse model, we compared IP- or EP-knockout mice with WT mice. We demonstrated that IP, EP2, and EP3, but not EP1 or EP4, were the receptors participating in exudate formation in the carrageen-induced mouse pleurisy model.

In consideration of the signal transduction caused by stimulation of IP and EP, the activation of either IP or EP2 results in an increased level of cAMP by coupling to Gs (Narumiya et al., 1999). However, the situation is complicated in the case of EP3 because there are several isoform receptors for EP3, all of which have different signaling. For example, among the isoforms of bovine EP3, EP3B, and EP3C increase the cAMP level, but EP3A decreases it (Namba et al., 1993; Narumiya et al., 1999). At least three isoforms for mouse EP3 have been reported, and there may possibly be even more isoforms, as found in rabbit and human (Narumiya et al., 1999). Therefore, identifying the isoform responsible for the exudate formation is much more difficult and must await future investigation.

The expression pattern of PG receptor mRNAs in the tissues around the pleural cavity, such as the pleura, diaphragm, and lungs, is in line with the site of leaking vessels. This site has been shown to be the venules in the pleural tissues, using carbon particles as a marker in the carrageenin-induced rat pleurisy (Majno and Palade, 1961; Tanaka et al., 1980). Although EP4 is known to cause an increase in cAMP, the present study showed no involvement of EP4 in the exudation, suggesting that localization of EP4 differs from that of IP and of EP2. At 3 h of carrageenin injection, there was no apparent change in the expression pattern of these receptors in diaphragm and lung. In the pleura, however, the expression levels of all these mRNA decreased. Although the reason of the decrease was not clear, it may derived from an instability of mRNAs in the pleura, where the severe inflammatory reaction took place. Precise localization of these receptors and identification of the exact leakage sites around the pleural cavity, however, remain to be clarified.

Evidence for involvement of the kinin system in the exudate formation in carrageen-induced mouse pleurisy was obtained in the present study by examining the effects of
bradykinin B2 receptor antagonist and captopril on the exudation formation. We previously reported that carrageenin, a negatively charged polysaccharide, can activate the plasma kallikrein system in human or rat plasma to produce bradykinin through activation of factor XII in the contact phase of the intrinsic blood coagulation cascade (Oh-ishi, 1982). These previous findings suggest that carrageenin injected into the pleural cavity of mice also activates the kallikrein-kinin system in the pleural fluid to produce bradykinin. In accordance with this notion, FR173657 significantly inhibited the pleural exudation, indicating that endogenous bradykinin produced in the pleural cavity enhanced the exudation by acting on the bradykinin B2 receptor. Furthermore, combined treatment with FR173657 and indomethacin did not further suppress the exudation than when treated with each agent alone, suggesting that bradykinin enhanced the exudation by stimulating prostanoid synthesis, as reported previously (Dozen et al., 1989).

From previous reports (Dozen et al., 1989; Erdos and Deddish, 2002), we suspected that treatment with captopril, which is a kininase II inhibitor that prevents the degradation of bradykinin in vivo, could enhance the response to endogenous bradykinin. As expected, the carrageenin-induced pleural exudation was significantly enhanced by captopril in WT mice in a similar manner to that reported in the rat case. In IP- or EP2-KO mice, however, there was no significant enhancement of pleural exudation by captopril, suggesting that IP and EP2 mediate further the signaling of PGE2, respectively, which were increased by captopril. In contrast, pleural exudation was significantly enhanced in EP3-KO mice, indicating that PGE2, which was increased by captopril, could not further stimulate the EP3 signaling, whereas it stimulated EP2. This may suggest that PGE2 produced by carrageenin was able to fully stimulate the EP3 and that the increase in PGE2 level by captopril had little effect on the EP3 no longer. Accordingly, PGE2 has the highest affinity for the EP3 among the EPs (Kiriyama et al., 1997). These results, along with an inability of FR173657 to affect the exudation in mice pretreated with indomethacin and increased levels of 6-keto-PGFα and PGE2, respectively, which were increased by captopril, suggests that IP and EP2 mediate further signaling of PGE2 and PGE2, respectively, which were increased by captopril. In contrast, pleural exudation was significantly enhanced in EP3-KO mice, indicating that PGE2, which was increased by captopril, could not further stimulate the EP3 signaling, whereas it stimulated EP2. This may suggest that PGE2 produced by carrageenin was able to fully stimulate the EP3 and that the increase in PGE2 level by captopril had little effect on the EP3 no longer. 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