Effects of a Novel Selective Agonist for Prostaglandin Receptor Subtype EP$_4$ on Hyperalgesia and Inflammation in Monoarthritic Model

Keiichi Omote, M.D.,* Tomoyuki Kawamata, M.D.,† Yoshito Nakayama, M.D.,‡ Hiroki Yamamoto, M.D.,‡ Mikito Kawamata, M.D.,§ Akiyoshi Namiki, M.D.‖

Background: Cytokines have crucial role in the development and maintenance of inflammation and pain in arthritis. Activation of prostaglandin receptor subtype EP$_4$ suppresses cytokine production in immune cells. The purpose of this study was to evaluate whether a novel EP$_4$ agonist would be able to suppress thermal and mechanical hyperalgesia and paw swelling in acute and chronic phases in rat monoarthritic model.

Methods: Monoarthritis was induced by an injection of complete Freund's adjuvant (CFA) intracapsularly into the tibiotarsal joint of the rats. Withdrawal latencies to thermal stimulation on the hind paw, withdrawal thresholds to mechanical stimulation, paw volume, and ankle diameter were measured 24 h and 4 weeks after the CFA injection. A novel selective EP$_4$ receptor agonist, ONO-AE1–329 (10, 25, or 50 μg) or saline was administered intracapsularly into the joint.

Results: Withdrawal latencies and withdrawal thresholds were significantly ($P < 0.05$) shortened and decreased, respectively, on the arthritic side but not on the contralateral side 24 h and 4 weeks after the CFA injection. In addition, significant ($P < 0.05$) increases in paw volume and ankle diameter on the arthritic side were observed. Intracapsularly administered ONO-AE1–329 showed significant ($P < 0.05$) inhibition of thermal and mechanical hyperalgesia and significant ($P < 0.05$) decrease in paw volume and ankle diameter in a dose-dependent manner at 24 h and 4 weeks after CFA.

Conclusion: Intracapsular administration of EP$_4$ receptor agonist effectively inhibited mechanical and thermal hyperalgesia and inflammatory reactions in acute and chronic monoarthritis. An EP$_4$ agonist would be a potential strategy for inflammatory pain in arthritis.

ARTHRITIS is a disorder of synovial joint characterized by joint pain, which is the major subjective symptom. Synovitis is the primary triggering mechanism of joint pain and is typically associated with the production of chemical mediators that serve to stimulate quiescent articular afferent nerves.

Cytokines have been implicated as mediators of inflammation in the pathophysiology of various types of arthri-

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tis. The cytokines stimulate chondrocytes, osteoclasts, osteoblasts, fibroblasts, and synoviocytes and are thought to contribute to the excessive growth of the synovium and proliferation of fibroblasts; overproduction of connective tissue–degrading enzymes by synoviocytes, fibroblasts, and chondrocytes; overproduction of prostaglandins by fibroblasts; and excessive reabsorption of calcium by bone cells.[1] Tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), IL-6, and IL-8 possess a pivotal role in the development of inflammatory hyperalgesia in arthritis. Among them, TNF-α is able to stimulate the release of several other cytokines implicated with nociception.[2-4] Cytokines are also able to trigger the further release of chemical mediators such as neuropeptides, kinins, and leukotrienes in joints previously exposed to inflammation, inducing intense and long-lasting incapacitating nociception.[5] Cytokines thus have a crucial role in the development and maintenance of arthritis-induced hyperalgesia.

Several lines of studies showed that prostaglandin E$_2$ suppressed the production of cytokines in lipopolysaccharide-stimulated neutrophils and macrophages,[6-11] and this suppression was mediated by the activation of prostaglandin E$_2$ receptor subtype EP$_4$.[9-11] In addition, the increased expression of EP$_4$ mRNA in mouse macrophage-like cells was observed by the addition of lipopolysaccharide in a dose-dependent manner.[10] In a recent study, Kurihara *et al.*[12] reported that EP$_4$ receptor expression was observed in inflamed synovial tissue. They also reported that selective EP$_4$ receptor agonist ONO-AE1–329 inhibited IL-6 production. Thus, there is a possibility that the activation of EP$_4$ receptors in the inflammatory joint cavity would be able to suppress the pain, hyperalgesia, and inflammatory responses in arthritis.

Recently, a novel selective EP$_4$ receptor agonist, 16-(m-methoxymethyl)phenyl derivative (ONO-AE1–329), has been produced.[13] It is reported that this compound inhibits ^{3}H-prostaglandin E$_2$ binding to crude membrane preparation of EP$_4$ subtype, expressing cells with Ki value of 9.7 nM, and exhibited excellent selectivity for the EP$_4$ receptor (KiEP$_1$/KiEP$_4$ > 1,000, KiEP$_2$/KiEP$_4$ = 210, KiEP$_3$/KiEP$_4$ = 120). ONO-AE1–329 increased adenosine 3',5'-cyclic monophosphate (cAMP) concentration in these cells, with a 50% effective concentration value of 3.1 nM.[13] In addition, this compound inhibited the production of TNF-α stimulated by lipopolysaccharide in mouse neutrophils exudated into the peritoneal cavity.[9]

In the present study, we examined the effects of an intracapsularly administered ONO-AE1–329 on the mechanical and thermal hyperalgesia and on the inflammatory reaction in the rat monoarthritic model.

Materials and Methods

Experiments were conducted according to a protocol approved by the Sapporo Medical University Animal Care and Use Committee. The animals used in this study were male Sprague-Dawley rats (weighing 250–260 g, Japan SLC, Hamamatsu, Japan), which were housed individually in a temperature-controlled (21 ± 1°C) room with a 12-h light–dark cycle and given free access to food and water.

Animal Preparation

Monoarthritis was induced by the intracapsular injection of 50 μl of complete Freund's adjuvant (CFA) into the right tibiotarsal joint. This procedure was performed with brief isoflurane-oxygen anesthesia, as described previously.[14,15] In brief, the tarsal area of the hind paw was grasped and the fossa distal and medial to the lateral malleolus of the fibula was palpated. A 26-gauge needle was introduced into the capsule of the tibiotarsal joint percutaneously by directing it cephalad, mesiad, and superiorly from the midpoint of the inframalleolar fossa until a distinct loss of resistance was felt—approximately 4 mm—and CFA or saline was injected. With a true intracapsular injection, a firm resistance to injection was characteristically felt after the injection of 50 μl of fluid.

The acute monoarthritis consisted of animals 24 h after the CFA injection, and the chronic monoarthritis consisted of animals 4 weeks after the CFA injection.

Evaluation of Pain Behavior

In order to evaluate the degree of hyperalgesia, withdrawal latencies to thermal stimulation and withdrawal thresholds to mechanical stimulation were assessed.

For evaluation of thermal hyperalgesia, thermal nociceptive testing was conducted, using an analgesimeter (Plantar test 7370; Ugo Basile, Comerio-Varese, Italy). Radiant heat was applied on the plantar surface of hind paws on the CFA-injected side and on the contralateral side. The thermal nociceptive threshold was determined as paw withdrawal latency from the heat source. Bulb intensity was adjusted so that the control latency was 12–14 s.

To evaluate the mechanical hyperalgesia, withdrawal threshold to mechanical stimulation was determined using calibrated von Frey filaments (0.0045–75.8580 g bending force), which were applied from underneath the cage through openings (12 × 12 mm) in the wire mesh floor to the plantar of the hind paw on the CFA-injected side and to the same area on the contralateral side. Each filament was applied once starting with 0.0045 g and continuing until a withdrawal response occurred. A withdrawal response was considered to be complete lifting of the hind paw off the surface of the cage or flinching. The test was repeated three times in each time point. The lowest force producing a response was considered the withdrawal threshold.

Evaluation of Inflammation

The magnitudes of inflammatory response were evaluated by measuring the volume of the hind paw and the ankle diameter. The paw volume was determined using plethysmometry (Plethysmometer 7140; Ugo Basile). The ankle diameter was measured with a vernier micrometer.

Drugs

Complete Freund's adjuvant was purchased from Sigma Chemical Co. (St. Louis, MO). The adjuvant (1 ml) contains 1 mg of mycobacterium tuberculosis (H37 Ra, ATCC 25177) that was heat killed and dried, 0.85 ml paraffin oil, and 0.15 ml mannide monooleate. The novel selective EP$_4$ agonist, 16-(m-methoxymethyl)phenyl derivative (ONO-AE1–329) was supplied by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Recombinant rat TNF-α was purchased from Pepro Tech EC Ltd. (London, England). These compounds were dissolved in saline.

Experimental Procedure

Before the injection of CFA or saline, withdrawal latency to thermal stimulation, withdrawal threshold to mechanical stimulation, volume of the hind paw, and diameter of ankle were measured as control value on each side of hind paw. Fifty microliters of CFA or saline was injected intracapsularly into the right tibiotarsal joint. Twenty-four hours after the injection (acute monoarthritis), the baseline values of the latency, threshold, volume, and diameter were determined. ONO-AE1–329 (10, 25, or 50 μg) or saline in a volume of 50 μl was administered intracapsularly into the tibiotarsal joint on the ipsilateral side to the arthritis. The latencies, thresholds, or inflammatory responses were assessed for up to 60 min. Four weeks after the CFA injection (chronic monoarthritis), the baseline of latencies, thresholds, or inflammatory responses was again determined, and ONO-AE1–329 (10, 25, or 50 μg) or saline in a volume of 50 μl was administered intracapsularly on the ipsilateral side to the arthritis.

In another experiment, the inhibitory effect of ONO-AE1–329 on the intracapsular TNF-α–induced hyperalgesia was examined. TNF-α at the dose of 100 ng in a volume of 50 μl was injected intracapsularly into the right tibiotarsal joint. In a treatment group, 50 μg of ONO-AE1–329 was concomitantly injected with 100 ng of TNF-α. Withdrawal latencies and withdrawal thresholds to thermal and mechanical stimuli, respectively,
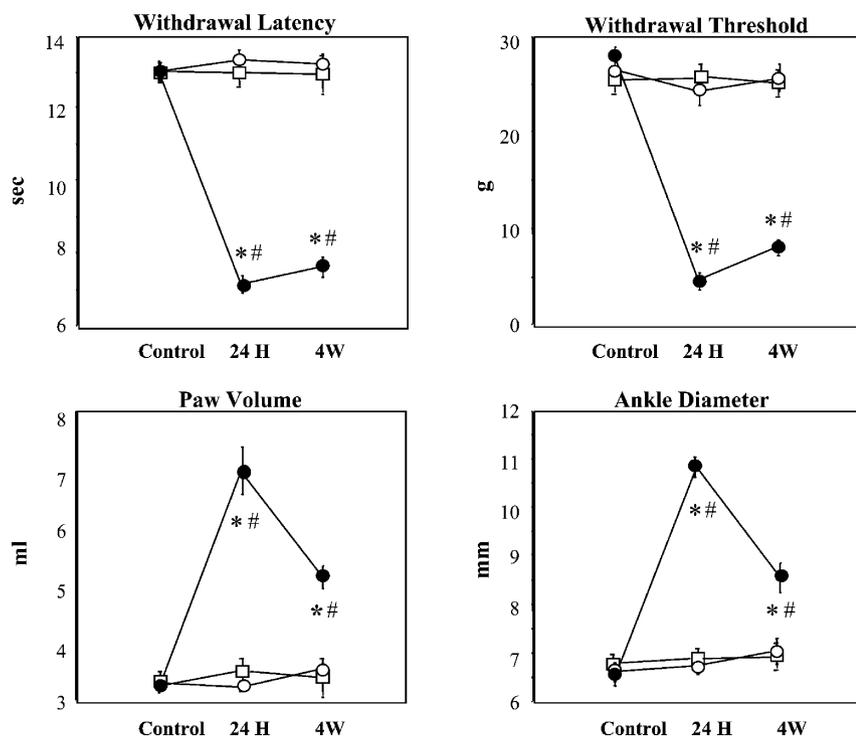
Fig. 1. Withdrawal latencies to thermal stimulation (*top left*), withdrawal thresholds to mechanical stimulation (*top right*), paw volumes (*bottom left*), and ankle diameters (*bottom right*) before and 24 h and 4 weeks after intracapsular injection of complete Freund's adjuvant (circles) or saline (squares). Closed circles are arthritic side and open circles are contralateral side. Data are mean ± SD. N = 6 to 7 in each group. *$P < 0.05$ compared to contralateral side, #$P < 0.05$ compared to saline.

were determined before and after the administration of TNF-α or TNF-α combined with ONO-AE1–329.

Statistical Analysis

The withdrawal latencies, withdrawal thresholds, paw volumes, and ankle diameters were represented as mean ± SD. Baseline values (post-CFA) were compared to control values (pre-CFA) using a paired Student *t* test. To assess the effects of ONO-AE1–329 on the CFA-induced changes, the values from the baseline (post-CFA) were compared within group and between groups, using a two-way analysis of variance for repeated measurements followed by Scheffé F test. To assess the effect of ONO-AE1–329 on TNF-α–induced hyperalgesia, a two-way analysis of variance for repeated measurements followed by Scheffé F test was used. A *P* value < 0.05 was considered to be statistically significant.

Results

Time Courses of Hyperalgesia and Inflammation

Figure 1 shows the time courses of changes in withdrawal latency to thermal stimulation, withdrawal threshold to mechanical stimulation, paw volume, and ankle diameter. Twenty-four hours after intracapsular injection of CFA into the tibiotarsal joint, withdrawal latency and withdrawal threshold were significantly (*P* < 0.05) shortened and decreased, respectively. Paw volume and ankle diameter were significantly (*P* < 0.05) increased. These changes were also observed 4 weeks after CFA, but the intensities of paw swelling were likely less than those seen at the time point of 24 h after the

injection. On the contralateral side to the arthritis, no significant changes of latency, threshold, paw volume, and ankle diameter were observed 24 h and 4 weeks after CFA injection. Intracapsular saline did not show any significant changes.

Effects of ONO-AE1–329 on Withdrawal Latency and Threshold

Twenty-four hours after the CFA injection, the intracapsular administration of ONO-AE1–329, but not saline, significantly (*P* < 0.05) prolonged the withdrawal latency in a dose- and time-dependent manner (fig. 2, top). Four weeks after the CFA injection, the baseline latencies were comparable to those observed 24 h after the injection; thus, thermal hyperalgesia was persistent. ONO-AE1–329, but not saline, significantly (*P* < 0.05) prolonged the withdrawal latency at the doses of 25 and 50 μg (fig. 2, bottom). Peak antihyperalgesic effects were observed 30 min after the administration. The duration of the effect following administration of 50 μg of ONO-AE1–329 was 45 min. Withdrawal latencies to thermal stimulation on the contralateral hind paw did not show any changes following ONO-AE1–329 administration at all doses at the time points of 24 h and 4 weeks after CFA injection (data not shown).

Figure 3 (top and bottom) shows the effects of ONO-AE1–329 on the withdrawal thresholds to mechanical stimulation at the time points of 24 h and 4 weeks after the CFA injection. Similar to the thermal withdrawal latencies, ONO-AE1–329, but not saline, significantly (*P* < 0.05) increased the mechanical withdrawal threshold in a dose- and time-dependent manner 24 h after the
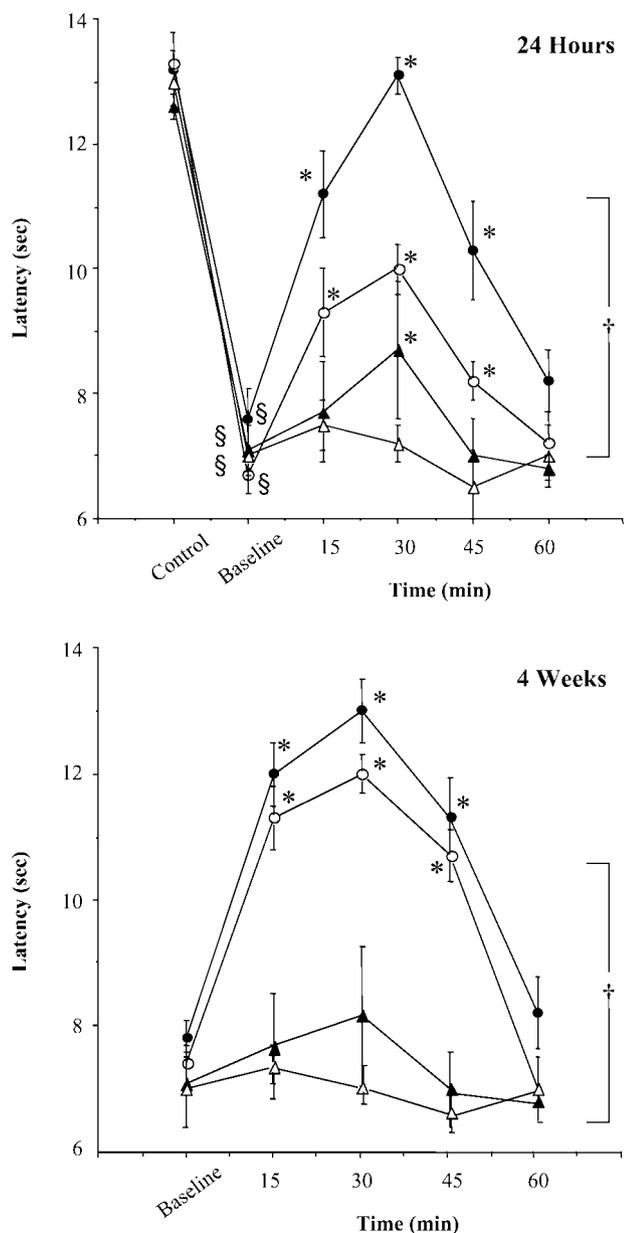
Fig. 2. Effects of ONO-AE1–329 on withdrawal latency to thermal stimulation on the arthritic side at the time points of 24 h (*top*) and 4 weeks (*bottom*) after CFA injection. Closed circles, 50 μg; open circles, 25 μg; closed triangles, 10 μg; and open triangles, saline. Data are mean ± SD. N = 6 to 7 in each group. §$P < 0.05$ compared to control, *$P < 0.05$ compared to baseline, †$P < 0.05$ compared among groups.

Effects of ONO-AE1-329 on Paw Volume and Ankle Diameter

In the rats injected with CFA 24 h before, intracapsular injection of ONO-AE1–329 significantly ($P < 0.05$) decreased the increased volumes of the paw on the arthritic side, but not on the contralateral side, 30 min after the administration in a dose-dependent manner (fig. 4, top). Four weeks after CFA injection, ONO-AE1–329 at the doses of 25 and 50 μg significantly ($P < 0.05$)
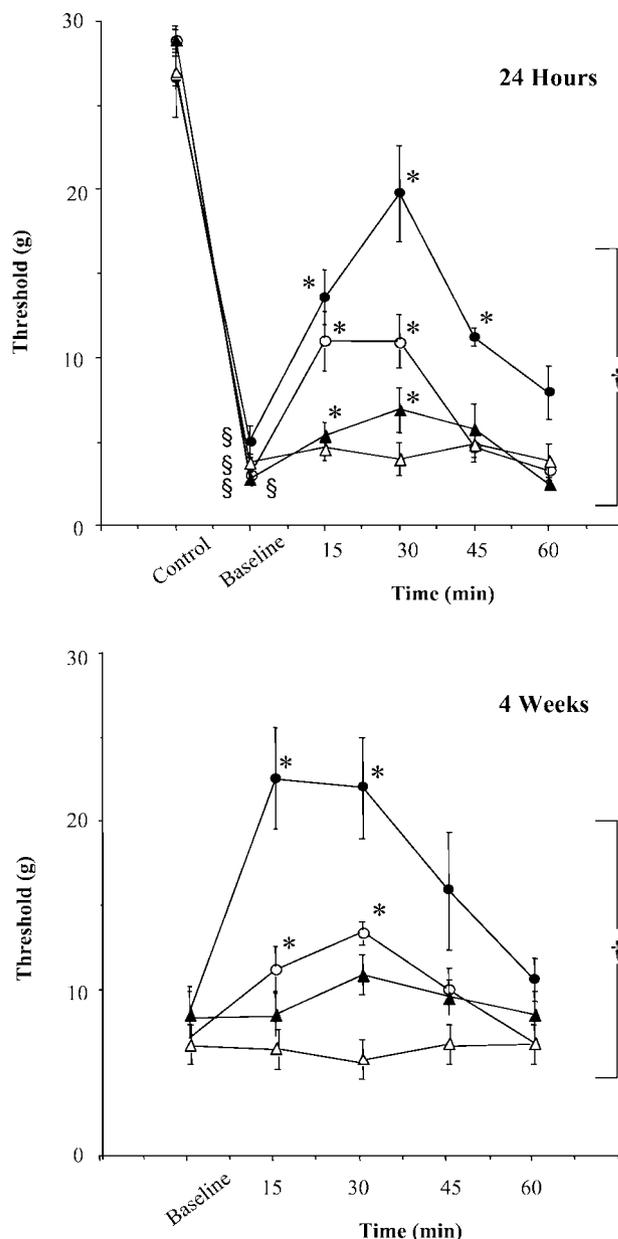

Fig. 3. Effects of ONO-AE1–329 on withdrawal threshold to mechanical stimulation on the arthritic side at the time points of 24 h (*top*) and 4 weeks (*bottom*) after injection of complete Freund's adjuvant. Closed circles, 50 μg; open circles, 25 μg; closed triangles, 10 μg; and open triangles, saline. Data are mean ± SD. N = 6 to 7 in each group. §$P < 0.05$ compared to control, *$P < 0.05$ compared to baseline, †$P < 0.05$ compared among groups.

CFA injection (fig. 3, top). Peak effect was observed 30 min after the administration. In the rats that received CFA 4 weeks before, ONO-AE1–329, but not saline, significantly ($P < 0.05$) increased the withdrawal threshold at the doses of 25 and 50 μg (fig. 3, bottom). ONO-AE1–329 at all doses did not show any changes in withdrawal threshold on the contralateral side 24 h and 4 weeks after CFA administration (data not shown).

In the rats that had received intracapsular injection of saline, ONO-AE1–329 did not show any changes in latencies and thresholds (data not shown).
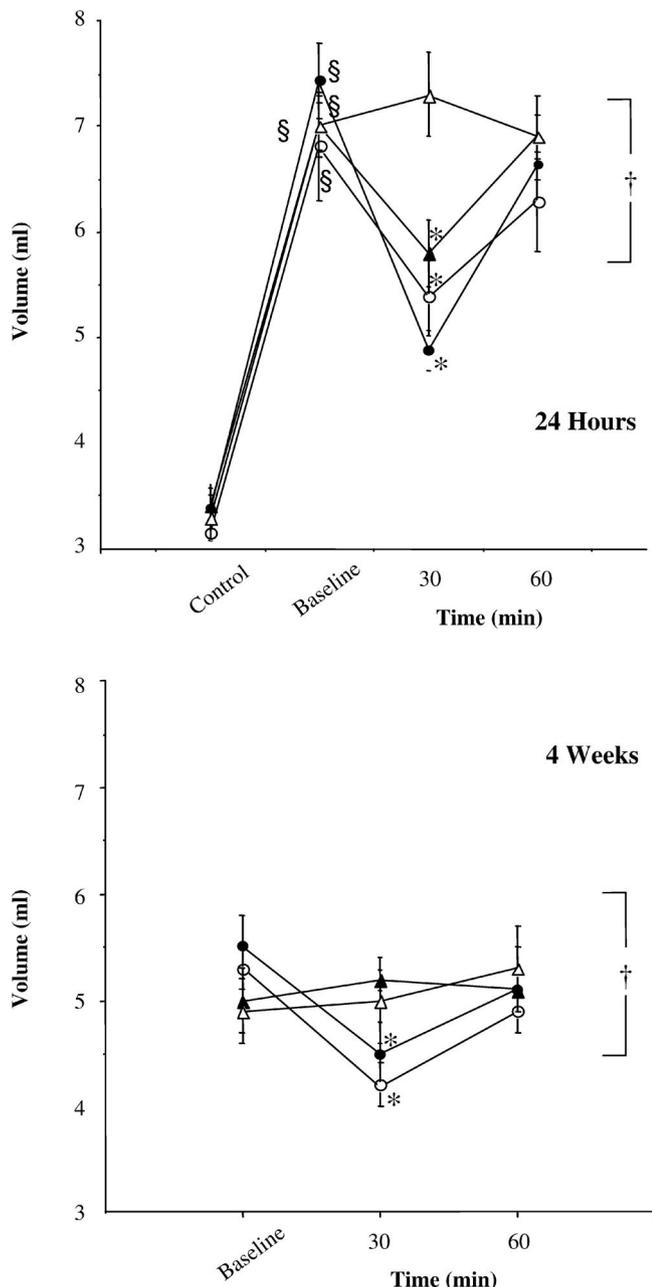
Fig. 4. Effects of ONO-AE1–329 on paw volume on the arthritic side at the time points of 24 h (*top*) and 4 weeks (*bottom*) after injection of complete Freund's adjuvant. Closed circles, 50 μg; open circles, 25 μg; closed triangles, 10 μg; and open triangles, saline. Data are mean $\pm$ SD. N = 6 to 7 in each group. §$P < 0.05$ compared to control, *$P < 0.05$ compared to baseline, †$P < 0.05$ compared among groups.

decreased the volume 30 min after the administration (fig. 4, bottom).

Similar to the changes in paw volume, ONO-AE1–329 significantly ($P < 0.05$) decreased the increased diameter of the ankle on the arthritic side, but not on the contralateral side, in a dose-dependent manner 24 h after the CFA injection (data not shown). Twenty-five and 50 μg of ONO-AE1–329 were significantly ($P < 0.05$)

effective 4 weeks after the CFA injection (data not shown).

Effects of ONO-AE1-329 on Tumor Necrosis Factor α–induced Hyperalgesia

Figure 5 shows the effect of ONO-AE1–329 on intracapsular TNF-α–induced thermal and mechanical hyperalgesia. Intracapsular injection of TNF-α 100 ng alone significantly ($P < 0.05$) shortened and decreased withdrawal latency and withdrawal threshold, respectively. The durations of the thermal and mechanical hyperalgesia observed were 60 min. Concomitantly administered ONO-AE1–329 50 μg with TNF-α 100 ng did not show any inhibition of TNF-α–induced thermal and mechanical hyperalgesia.

Discussion

The present study demonstrated that intracapsular injection of CFA into tibiotarsal joint induced mechanical and thermal hyperalgesia and paw swelling on the ipsilateral side, but not on the contralateral side, 24 h and 4 weeks after the injection. At both time points, the intracapsular injection of a novel selective EP$_4$ agonist ONO-AE1–329 inhibited the mechanical and thermal hyperalgesia and reduced the paw swelling in a dose-dependent manner.

In this study, we injected CFA intracapsularly into the tibiotarsal joint, as described previously.[14,15] Many researchers have turned to unilateral subcutaneous plantar injection of CFA[16-18]; however, the process evolved to produce widespread polyarthritis. CFA-induced polyarthritis is a severe widespread systemic disease, and it is not possible with this model to compare directly the effects of stimuli and analgesics on an inflamed side and on a normal contralateral side. The model we used in the present study showed a reliable monoarthritis.[14,15] The paw on the inflamed side, but not on the normal side, showed mechanical and thermal hyperalgesia and swelling 24 h after CFA injection and for over 4 weeks. This model therefore enables evaluation of whether a compound would suppress the hyperalgesia and inflammatory reaction in acute and chronic arthritic phases.

Proinflammatory cytokines are found in synovial fluid from patients with rheumatoid arthritis and have been implicated as a major mediator of the joint pathology associated with arthritic disease.[1] TNF-α, IL-1β, IL-6, and IL-8 contribute to the perpetuating joint inflammation in rheumatoid arthritis because of its ability to induce proliferation of synoviocytes and to stimulate the production of other putative mediators such as kinins, prostaglandins, and cytokines.[3,5,18,19] This action is sustained by the formation of positive feedback loops resulting in continuous formation of high amounts of cytokine and other mediators. This will lead to the conception that

Fig. 5. Effects of ONO-AE1–329 on intracapsular tumor necrosis factor α (TNF-α)–induced thermal (*left*) and mechanical (*right*) hyperalgesia. Open circles are TNF-α injection group and closed circles are TNF-α and ONO-AE1–329 injection group. Data are mean ± SD. N = 5 in each group. *$P < 0.05$ compared to control.
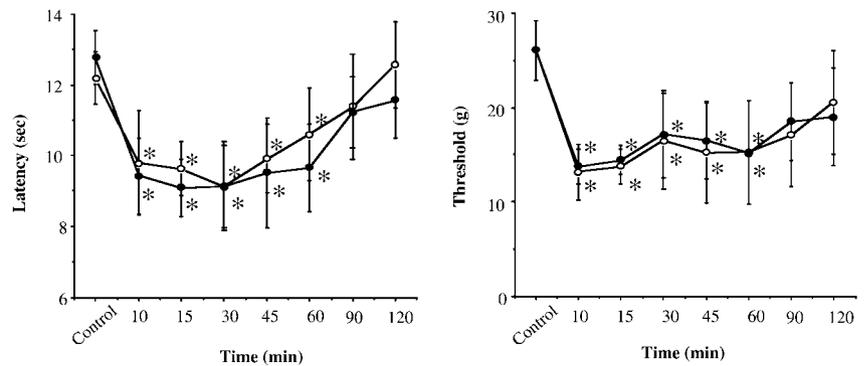
cytokine such as TNF-α is a therapeutic target in the management of acute and chronic arthritis. Many drugs are capable of cytokine modulation *in vivo* and *in vitro*,[20] and promising results have been obtained with selective antagonism of cytokine in rheumatoid arthritis. Actually, antiserum neutralizing endogenous TNF-α and anti–TNF-α antibodies abolished the symptoms of arthritis[20,21]; however, the likely adverse effects and the cost involvement limit their use in long-term therapy.

The receptors for prostaglandin E$_2$ are subdivided into four subtypes (EP$_1$, EP$_2$, EP$_3$, and EP$_4$) on the basis of the distinct genes and signal transduction pathways. The EP$_4$ receptors are essentially coupled to stimulation of adenylate cyclase, which leads to the elevation of intracellular cAMP.[22,23] The EP$_4$ receptor activation inhibits the productions of TNF-α, IL-6, and IL-12 in neutrophils and macrophages.[9–11,24] This led to the hypothesis that EP$_4$ agonist may inhibit cytokine production in arthritis, resulting in suppression of inflammation and resulting in effective analgesia. In the present study, a very selective EP$_4$ agonist, ONO-AE1–329, suppressed mechanical and thermal hyperalgesia in CFA-induced monoarthritis in both acute and chronic phases. In addition, paw swelling due to arthritis was also effectively suppressed after intracapsular selective EP$_4$ agonist in both acute and chronic phases of arthritis. These results will be consistent with the inhibitory effects of EP$_4$ receptor activation on cytokine production. However, the signaling pathways by which EP$_4$-induced cAMP production leads to the suppression of cytokine production remain to be determined.

The effects of EP$_4$ receptor activation on increasing intracellular cAMP might induce possible excitable effects on the peripheral nervous system, since the agents that elevate cAMP in the peripheral nerves induce changes in ion channel permeability, resulting in afferent hyperexcitability.[25,26] However, EP$_4$ receptor subtype is a minor prostanoid receptor expressed in DRG neurons. Only 20% of total DRG neurons, including small and large neurons, expressed EP$_4$ receptor.[27] This may indicate that EP$_4$ receptor on the peripheral neuron does not have a main contribution to pain transmission. Actually, as shown in this study, intracapsular administration of

EP$_4$ agonist in the rats that had saline injected intracapsularly did not show any changes in withdrawal latency to thermal stimulation and withdrawal threshold to mechanical stimulation.

As part of the inflammatory process in the synovium, cellular humoral mediators are produced that maintain the synovitis. All of these mediators, such as prostaglandins, cytokines, and kinins, are integrated into a network where they modulate the production and effects of each other, thereby contributing directly or indirectly to the effect on afferent terminals, leading to the generation of arthritic pain.[3] From these points of view, it seems that ONO-AE1–329 inhibits cytokine production through EP$_4$ receptor activation, resulting in blocking the complicated network of mediators. In the present study, we also examined the effect of EP$_4$ agonist on intracapsular TNF-α–induced hyperalgesia and found that the intracapsular ONO-AE1–329 at the dose that inhibited CFA-induced hyperalgesia showed no effects on the TNF-α–induced thermal and mechanical hyperalgesia. This means that ONO-AE1–329 does not inhibit the hyperalgesic effect of TNF-α that had been released in the inflamed joint. A recent study has shown that ONO-AE1–329 inhibited IL-6 production by synovial cells of arthritic rats.[12] Although this result suggests the possible inhibitory effect of EP$_4$ agonist on cytokine production in adjuvant-induced arthritic model, further study needs to examine the effect of EP$_4$ agonist on the production of other pivotal cytokines such as TNF-α, IL-1β, and IL-8 in synovial fluid in arthritic model.

Cytokines could excite nociceptors and thus induce the release of neuropeptides such as CGRP from the peripheral nerve terminals,[28] resulting in reactions of vasodilation and plasma extravasation. The interference of the neuropeptide production by inhibition of cytokine production by EP$_4$ agonist may change the inflammatory reaction. In addition, the expression of EP$_4$ mRNA was up-regulated in endothelial cells in small vessels of inflamed synovia.[12] Thus, the activation of EP$_4$ receptors might have changed the vasotonia, capillary permeability, and blood flow, resulting in the changing in paw swelling. Intracapsular ONO-AE1–329 would contribute to reducing paw volume and ankle diameter;

however, the reasons and mechanisms of the rapid, transient, and reversible changes observed in this study are unclear.

For ONO-AE1–329 to be useful clinically, it is important to know about the long-term efficacy of this agent. However, in the present study, we applied ONO-AE1–329 with single administration. Therefore, further study is needed to examine whether the effect of this agent is persistent and whether repeated administration can reproduce the effect of a single administration.

In summary, we demonstrated that intracapsularly administered EP_4 agonist ONO-AE1–329 effectively inhibited the mechanical and thermal hyperalgesia and paw swelling in acute and chronic phases in the CFA-induced monoarthritis model. Consequently, an EP_4 receptor agonist would be a potential analgesic for acute and chronic arthritic pain.

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