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Prostaglandin E_2 Receptors, EP2 and EP4, Differentially Modulate TNF-α and IL-6 Production Induced by Lipopolysaccharide in Mouse Peritoneal Neutrophils

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The expression and function of prostaglandin (PG) E_2 receptors were examined in mouse neutrophils exudated into the peritoneal cavity by casein treatment. Expressions of the EP2 and EP4 receptors were detected in neutrophils by Northern blot, but those of EP1 and EP3 receptors were not detected by RT-PCR. EP2-selective agonist, ONO-AE1-259, and EP4-selective agonist, ONO-AE1-329, stimulated cAMP formation in the cells. PGE_2 affected the TNF-α and IL-6 production in lipopolysaccharide (LPS)-treated neutrophils; it suppressed the TNF-α production and enhanced the IL-6 production. The PGE_2 effects were mimicked by dibutyryl cAMP. This is the first study of the enhancement of IL-6 production by cAMP-elevating reagents in neutrophils. Using neutrophils from EP2- and EP4-deficient mice in combination with EP2- and EP4-selective agonists, it was found that the augmentation of IL-6 was mediated mainly by the EP2 receptor and the suppression of TNF-α by the EP4 receptor and partially by the EP2 receptor. These findings indicate that casein-induced peritoneal neutrophils express Gs-coupled PGE_2 receptors, EP2 and EP4, which might differentially regulate the LPS-induced production of TNF-α and IL-6.

Neutrophils have regulatory roles in the processes of acute inflammatory and immune responses through their functions such as aggregation, chemotaxis, phagocytosis and reactive oxygen release [1]. In addition, neutrophils treated with bacterial lipopolysaccharide (LPS) produce cytokines, such as TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12, G-CSF, and GM-CSF [2].

Prostaglandin (PG) E_2 is a major arachidonate metabolite and is involved in many physiological and pathological processes including inflammation, immune responses, angiogenesis and neoplasia [3]. Recently, it was reported that PGE_2 regulates the functions of neutrophils: for example, exogenously added PGE_2 inhibited chemotaxis [4], superoxide generation [5], and LTB_4 release [6] in human neutrophils, and aggregation in rat neutrophils [7]. However, very little is known about the PGE_2 effect on the cytokine production in neutrophils. The actions of PGE_2 are mediated via its cell surface receptors, which are subdivided into four subtypes on the basis of the distinct genes and signal transduction pathways: the EP1 receptor coupled to stimulation of intracellular Ca^{2+} mobilization [8], the EP2 and EP4 receptor coupled to stimulation of adenylate cyclase [9, 10], and the EP3 receptor coupled to inhibition of this enzyme [11]. However, it remains to be clarified which subtype is responsible for the PGE_2 effects on modulation of neutrophil functions.

The purpose of the present study was to determine the expressed EP receptors and to analyze their regulatory functions on cytokine production in neutrophils. We demonstrate that the EP2 and EP4 receptors differentially modulate the LPS-stimulated production of TNF-α and IL-6 in mouse casein-induced peritoneal neutrophils.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice (8–12 weeks of age) from Japan SLC (Hamamatsu, Japan) were used. EP2-receptor deficient mice, back-crossed 6 generations to C57BL/6 mice, and EP4-receptor deficient mice, in the background of 129/Ola and C57BL/6 F2 progeny, were maintained under specific pathogen-free conditions.

Materials. Lipopolysaccharide (LPS, *Escherichia coli* O55:B5) and PGE_2 were purchased from Funakoshi Pharmaceutical Laboratories (Tokyo, Japan), indomethacin from Nakalai Tesque (Tokyo, Japan), ELISA kits for mouse TNF-α and IL-6 from Pharmingen

Abbreviations used: cAMP, cyclic AMP; cDNA, complementary DNA; COX, cyclooxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; LPS, lipopolysaccharide; mRNA, messenger RNA; PG, prostaglandin; RT-PCR, reverse transcriptase–polymerase chain reaction.

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(San Diego, CA), for cAMP from Amersham Pharmacia Biotech (Uppsala, Sweden). RPMI 1640 from Sigma Chemical Co. (St. Louis, MO) and fetal bovine serum (FBS) from Life Technologies (Rockville, MD; LPS <30 pg/ml). ONO-AE1-259 and ONO-AE1-329 were generous gift from Ono Pharmaceutical Co. (Osaka, Japan). The specificities of the agonists were analyzed by measuring the binding affinity to the respective subtype receptors expressed in CHO cells, which were reported previously [12]. All other chemicals were commercial products of reagent grade.

Preparation of peritoneal neutrophils. Mice were injected intraperitoneally with 2 ml of 5% casein in sterile saline and were killed by cervical dislocation 5–6 h after injection. The lavage fluids were collected in a syringe, and exudated peritoneal cells were precipitated by centrifugation. Neutrophils in peritoneal cells were purified by Percoll stepwise density gradient (1.090 and 1.070 g/ml) centrifugation (600*g* for 20 min at 4°C). The purity of neutrophils was greater than 95% as determined by staining with May–Grunwald–Giemsa. Neutrophils were suspended in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 150 μM 2-mercaptoethanol and 100 μM sodium pyruvate.

RT-PCR and Northern blot analysis. For RNA extraction, neutrophils (3.5–5 $\times$ 10^7 cells/ml) were collected. Total RNA was prepared by acid guanidium thiocyanate–phenol–chloroform method [13]. For RT-PCR analysis, cDNA was amplified using primers specific for EP1, EP2, EP3, EP4 and GAPDH. The primer sequences were as follows: for EP1, 5′-ACCCTGCATCCTGAGCAGCACTGGCCCTCT and 5′-CCCTGCTGGTGTTGGTGGTGTTGGCCATCG; for EP2, 5′-TTCATATTCAAGAAACCAGACCCTGGTGGC, and 5′-TCCCTTCTCCAAAGTAGGTACATCCGTTTC; for EP3, 5′-ATCCTCGTGTACCTGTCACAGCGACGCTGG and 5′-ACGAGTTGGCTGTAGACTAACTTCTAGTAA; for EP4, 5′-GACTGGACCACCAACGTAACGGCCTACGCC and 5′-ACAGGAGGCTGAGAGACTCGTCACGACCC. GAPDH primers were purchased from Life Technologies. For Northern blot analysis, total RNAs (10 μg) were separated by electrophoresis on 1.5% agarose gel, and transferred onto a BIODYNE A membrane (Pall BioSupport Corporation, East Hills, NY). For detection of EP2 mRNA, the *Eco*R1 insert DNA (1.7 kb) of ML202 was used as a hybridization probe. For EP4 mRNA detection, a 970-bp fragment of the mouse EP4 cDNA spanning the putative first to seventh transmembrane domains was used as a probe [14]. Hybridization was carried out under the conditions described previously [11].

Measurement of cAMP formation. The cAMP levels in neutrophils were determined as described previously [15]. Briefly, neutrophils (5×10^6 cells/ml) were preincubated with 100 μM Ro20-1724 for 10 min, and then further incubated with PGE_2 or each agonist for 10 min at 37°C. The reactions were stopped by addition of 10% trichloroacetic acid, and the resulting acid-soluble cAMP was measured by ELISA.

Measurement of cytokine production. Neutrophils (1×10^6 cells/ml) were incubated with or without 100 ng/ml LPS for the indicated time at 37°C in 5% CO_2. After incubation, each culture was centrifuged at 300*g* for 5 min at 4°C to remove the cells. The amounts of TNF-α and IL-6 in the supernatant were assayed using the respective ELISA kits according to the manufacturer's instructions.

RESULTS

Expressions of the EP2 and EP4 Receptors in Mouse Peritoneal Neutrophils

By RT-PCR analysis the amplified products of the EP2 and EP4 receptors were detected in mouse peritoneal neutrophils. No products were observed in the primer sets for the EP1 and EP3 receptors (Fig. 1A).

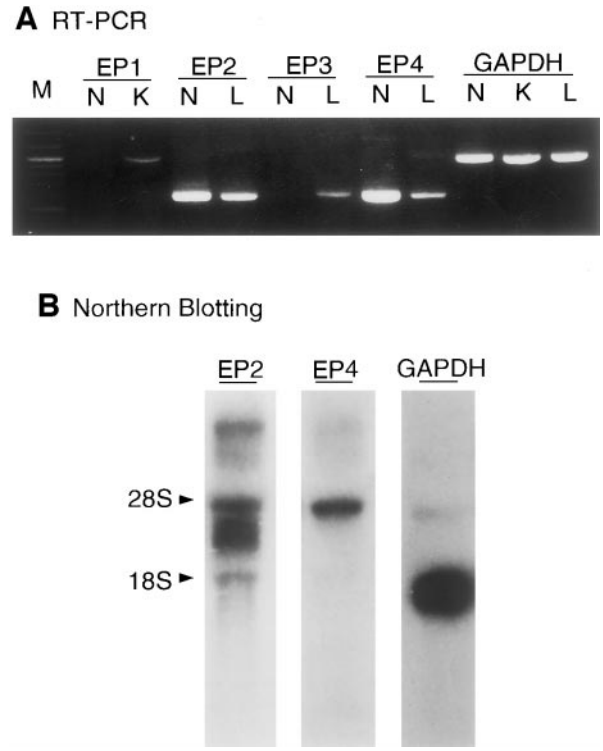

FIG. 1. Expression of EP receptors in peritoneal neutrophils. (A) RT-PCR analysis. Reverse-transcribed products were amplified with the appropriate primers for each EP subtype and GAPDH as described under Materials and Methods. N, neutrophils; K, kidney; L, lung; M, markers. (B) Northern blot analysis. Total RNAs (10 μg/lane) were hybridized with probes specific for EP2, EP4, or GAPDH as described under Materials and Methods.

The expressions of the EP2 and EP4 mRNAs were confirmed by Northern blot analysis (Fig. 1B). The hybridization bands for the EP2 and EP4 mRNAs were detected at the expected sizes of 2.2 and 3.5 kb, respectively [14]. To confirm functional coupling of the EP2 and EP4 receptors to Gs protein, we measured cAMP formation in the cells treated with selective EP agonists, ONO-AE1-259 (AE1-259) for the EP2 receptor and ONO-AE1-329 (AE1-329) for the EP4 receptor. Both agonists as well as PGE_2 increased cAMP formation in the cells (Fig. 2).

Effect of PGE_2 on LPS-Stimulated TNF-α and IL-6 Production in Mouse Peritoneal Neutrophils

LPS treatment (100 ng/ml) induced the production of TNF-α and IL-6 in mouse peritoneal neutrophils (Fig. 3). TNF-α production rapidly increased and then decreased to the basal level by 12 h. The maximum level was obtained at 2 h after LPS stimulation. However, IL-6 production increased gradually and reached a plateau level at 8 h after LPS stimulation. Simultaneous addition of PGE_2 with LPS suppressed the TNF-α production and enhanced the IL-6 production significantly

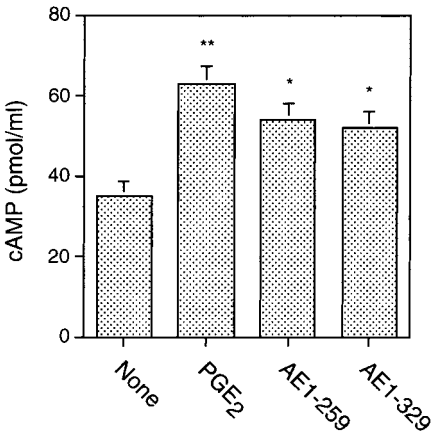

FIG. 2. Effects of PGE_2, ONO-AE1-259, and ONO-AE1-329 on cAMP formation in peritoneal neutrophils. Neutrophils (5×10^6 cells/ml) were stimulated with 1 μM PGE_2, 1 μM ONO-AE1-259 (AE1-259), or 1 μM ONO-AE1-329 (AE1-329) for 10 min at 37°C. cAMP formed was measured by ELISA as described under Materials and Methods. Values represent means ± SEM of three independent experiments. $*P < 0.05$, $**P < 0.01$ by Students' t test.

(Fig. 3). However, the PGE_2 effects in the absence of LPS were relatively small.

Dibutyryl cAMP (1 mM) suppressed the TNF-α production at 2 h and enhanced the IL-6 production at 4 hr after the LPS stimulation in peritoneal neutrophils (Table I).

It was reported that endogenous PG synthesis was induced by LPS treatment through COX-2 induction in human neutrophils [16, 17]. However, in mouse peritoneal neutrophils pretreatment with indomethacin (1 μM), a nonselective COX inhibitor, induced no changes in the LPS-stimulated production of TNF-α and IL-6 (data not shown).

Effects of EP2 and EP4 Selective Agonists on Changes in the LPS-Stimulated TNF-α and IL-6 Production in Peritoneal Neutrophils from EP2- and EP4-Deficient Mice

We recently established EP2- and EP4-deficient mice [18, 19]. From the mutant mice we prepared casein-induced neutrophils and analyzed the effects of selective EP agonists on the LPS-stimulated TNF-α and IL-6 production (Fig. 4). AE1-329 significantly suppressed the TNF-α production in EP2-deficient neutrophils, whereas AE1-259 suppressed it in higher concentrations in EP4-deficient neutrophils. However, AE1-259 significantly enhanced the IL-6 production in EP4-deficient neutrophils, whereas AE1-329 caused weak enhancement even at 1 μM in EP2-deficient neutrophils.

DISCUSSION

Available evidence suggests that PGE_2 is a negative regulator of various cellular activities of neutrophils such as chemotaxis, oxidized metabolite production, LTB_4 production and aggregation [4–7]. In addition, the present study demonstrated that PGE_2 modulates the LPS-stimulated TNF-α and IL-6 production in mouse peritoneal neutrophils; PGE_2 suppressed the LPS-stimulated TNF-α production and enhanced the IL-6 production.

The EP2 and EP4 receptors are essentially coupled to stimulation of adenylate cyclase, which leads to the elevation of intracellular cAMP [9, 10]. Both EP2- and EP4-receptor selective agonists, AE1-259 and AE1-329, respectively, increased cAMP formation, indicating there were functional receptors in peritoneal neutrophils. Dibutyryl cAMP reproduced similar effects with PGE_2 on the TNF-α and IL-6 production, indicating the involvement of cAMP as a second messenger in the PGE_2 effects. However, the signaling pathways after cAMP production leading to both suppression of

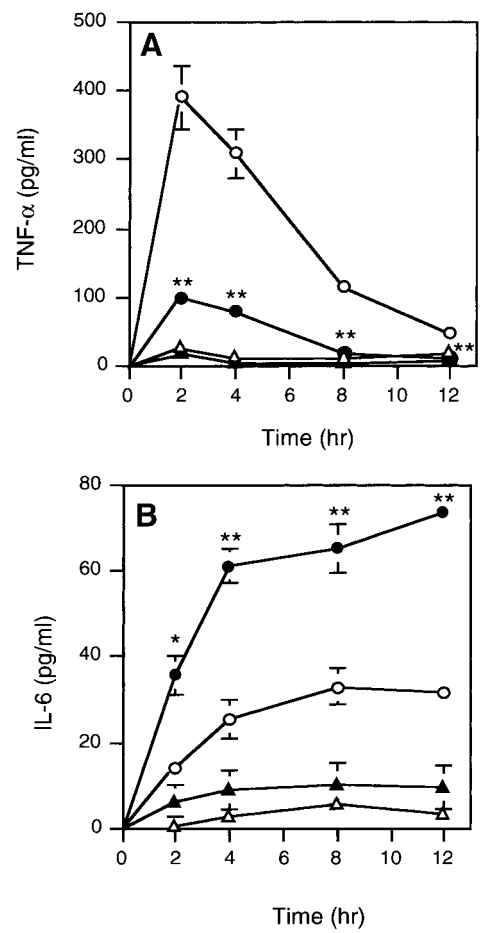

FIG. 3. Effect of PGE_2 on the TNF-α and IL-6 production in peritoneal neutrophils stimulated with LPS. Neutrophils (1×10^6 cells/ml) were incubated at 37°C for the indicated time with solvent (△), 1 μM PGE_2 (▲), 100 ng/ml LPS in the absence (○) or presence (●) of 1 μM PGE_2. Each cytokine released into the culture supernatant was measured by ELISA as described under Materials and Methods. Values represent means ± SEM of three independent experiments. $*P < 0.05$, $**P < 0.01$ by Students' t test.

TABLE I

Effects of Dibutyryl cAMP on Cytokine Productions

Reagents	TNF-α (pg/ml)	IL-6 (pg/ml)
Control	14.6 ± 1.8	3.6 ± 2.2
LPS	165.7 ± 20.4	40.3 ± 0.9
LPS + dbcAMP	2.6 ± 2.1**	86.9 ± 1.3**

Note. Neutrophils (1×10^6/ml) were incubated with the indicated reagents at 37°C (LPS, 100 ng/ml; dibutyryl cAMP, 1 mM). Cell-free supernatants were harvested after 2 h for TNF-α production and after 4 h for IL-6 production, respectively. Each cytokine released into the cell-free supernatant was measured by ELISA as described under Materials and Methods. Values are shown as means ± SEM of three independent experiments. $^{**}P < 0.01$ significantly different from LPS alone.

TNF-α and augmentation of IL-6 production in LPS-stimulated neutrophils remain to be determined. Geng *et al.* reported that LPS-stimulated TNF-α production was regulated by protein kinase C, whereas the IL-6 production was regulated by protein kinase A in human monocytic cells [20].

In LPS-stimulated macrophages it is accepted that PGE_2 and an increased cAMP negatively regulates TNF-α production [21, 22]. However, very little is known about LPS-stimulated neutrophils. In the present experiment, AE1-329 had a potent suppressive effect on TNF-α production in EP2-deficient neutrophils, but AE1-259 had an effect only at higher doses in EP4-deficient neutrophils. These observations indicate that the EP4 receptor dominantly acts as an inhibitory regulator for LPS-stimulated TNF-α production. The EP2 receptor may contribute to this reaction in the presence of higher concentrations of ligands. However, several inconsistent findings have been reported on the effects of PGE_2 and other compounds involved in cAMP formation on the IL-6 production in LPS-stimulated macrophages. Bailly *et al.* reported that PGE_2, dibutyryl cAMP and a phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine, enhanced IL-6 production by LPS-stimulated human monocytes [23]. Oh-ishi *et al.* also demonstrated that PGE_2 augmented LPS-stimulated IL-6 production in rat pleural resident monocytic cells [24]. In contrast, Zhong *et al.* indicated that PGE_2 suppressed the gene activation of IL-6 followed by LPS stimulation, but neither cholera toxin nor 8-bromo-cAMP had such effects [22]. The latter observation was not confirmed by Feng *et al.*, who showed that cholera toxin and 8-bromo-cAMP decreased the LPS-stimulated IL-6 production [25]. In the present study, we demonstrated that the LPS-stimulated IL-6 production in neutrophils was upregulated by PGE_2 and dibutyryl cAMP. Only AE1-259 showed a strong effect on the LPS-stimulated IL-6 production in EP4-deficient neutrophils, and AE1-329 showed very little effect on it.

It is important to clarify how to use two Gs-coupled PGE_2 receptors in regulation of cytokine release from LPS-stimulated neutrophils. Previously, we showed that the EP2 and EP4 receptors differed in their sensitivity to agonist-induced desensitization and to the metabolic inactivation of the agonist [26]. The EP4 receptors underwent short-term agonist-induced desensitization, but no such desensitization was observed for the EP2 receptor. The EP2 receptor has a higher sensitivity to the first metabolite, 15-keto-PGE_2, than the EP4 receptor. These findings indicate the EP4 receptor markedly, whereas the EP2 receptor gradually loses the response to PGE_2. These characters of the EP2 receptor will contribute to the consistent upregulation of LPS-stimulated IL-6 production. However, the EP4 receptor might be helpful in the suppres-

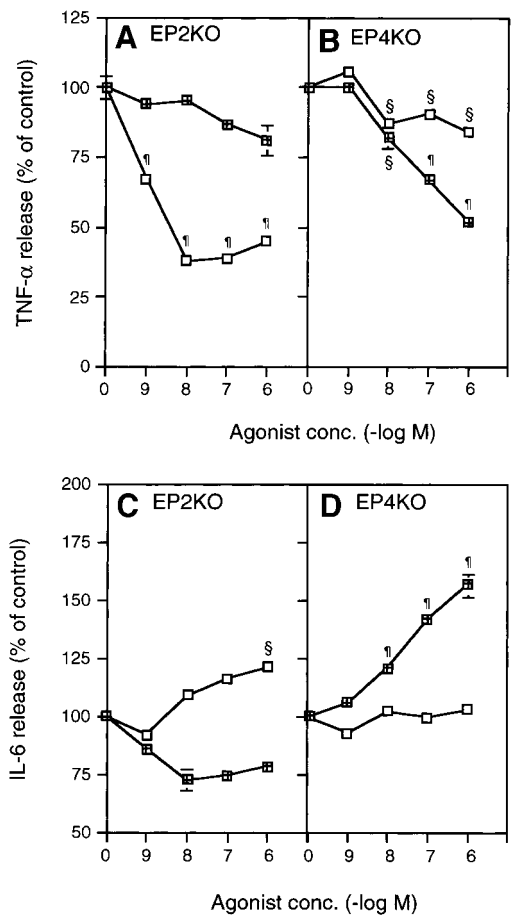

FIG. 4. Effect of ONO-AE1-259 and ONO-AE1-329 on LPS-stimulated TNF-α and IL-6 production in peritoneal neutrophils from EP2- and EP4-deficient mice. Neutrophils (1×10^6 cells/ml) from EP2- and EP4-deficient mice were incubated at 37°C with 100 ng/ml LPS in the presence or absence of ONO-AE1-259 (⊞) or ONO-AE1-329 (□). Reactions were stopped 2 h for TNF-α production or 4 hr for IL-6 production after LPS stimulation. Each cytokine released into the culture supernatant was measured by ELISA as described under Materials and Methods. Values represent means ± SEM of three independent experiments. §$P < 0.005$, ¶$P < 0.001$ by Students' *t* test.

sion of early elevation of TNF-α by LPS stimulation because of the short-term agonist-induced desensitization [26]. These will be helpful for understanding the significance of two Gs-coupled PGE_2 receptor in neutrophils and the diversity of cellular responses to PGE_2.

TNF-α stimulates proinflammatory reactions acting on monocytic cells and functions as a growth factor in fibroblasts [27]. However, IL-6 acts as a stimulator for proliferation and differentiation of B cells and T cells, resulting in enhanced immune responses [28]. Subsequently, the present PGE_2 effects on LPS-stimulated neutrophils may contribute to the shift of immunological environment from local inflammatory responses to systemic immune responses.

In conclusion, LPS-stimulated peritoneal neutrophils might be regulated by PGE_2 via the EP4 and EP2 receptors in the suppression of TNF-α production and via the EP2 receptor in the augmentation of IL-6 production.

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