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(2E,6Z,10E)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10, 14-hexadecatetraen-1-ol (Plaunotol) increases cyclooxygenase-2 expression via nuclear factor κB and cyclic AMP response element in rat gastric epithelial cells

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Abstract

Plaunotol, [(2E,6Z,10E)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14-hexadecatetraen-1-ol], a gastroprotective agent, increases the prostaglandin production in the gastric mucosa and accelerates ulcer healing. The precise mechanisms underlying the gastroprotective actions by plaunotol are not known. On the other hand, cyclooxygenase (COX)-2 is a key enzyme in PGE_2 production and its induction is thought to have an important role in ulcer healing. We investigated the mechanism of plaunotol-mediated COX-2 induction in rat gastric epithelial (RGM1) cells. We used a PGE_2 enzyme-linked immunoassay kit and Western blot analysis to measure PGE_2 production and COX-2 induction with plaunotol treatment in serum-starved RGM1 cells. In addition, gel-shift assay, Western blot analysis and a reporter assay were performed to observe which *Cox-2* promoter was involved in plaunotol-induced *Cox-2* expression. The findings indicated that plaunotol treatment dose-dependently increased COX-2 expression and PGE_2 production. The nuclear factor κB (NF-κB) and cyclic AMP response element (CRE) sites of the COX-2 gene promoter were critical to plaunotol-mediated COX-2 expression.

In conclusion, plaunotol induced COX-2 expression and increased PGE_2 production in serum-starved RGM1 cells via activation of the NF-κB and CRE sites of *Cox-2* gene promoters.

Keywords: COX-2; Plaunotol; Gastric epithelial cells; NF-κB; CRE

1. Introduction

Endogenous prostagladins have an important role in both gastric mucosal defense and ulcer healing (Arakawa et al., 1996; Miller, 1983; Robert et al., 1983). Prostaglandin production is regulated by expression of its catalytic enzyme-cyclooxygenase (COX). There are at least two COX isoforms, known as COX-1 and COX-2. COX-1 is constitutively expressed in most tissue and preserves normal gastric mucosa function. Conversely, COX-2 can be induced by many inflammatory and mitogenic stimuli (Herschman, 1996; Kujubu et al., 1991; Ristimaki et al., 1994). In animal models, there is increasing evidence that COX-2 expression is induced by mucosal injury. Specific COX-2 inhibitors delay healing of erosions and ulcers in mice and rats (Gretzer et al., 1998; Mizuno et al., 1997; Schmassmann et al., 1998; Sun et al., 2000), suggesting an important role for this isozyme in peptic ulcer healing. Promoter regions of the *Cox-2* gene have been cloned and sequenced. Across animal species, these promoter regions contain a canonical TATA box and various putative transcriptional regulatory elements such as CRE, NF-IL6, NF-κB, AP2, and GATA box (Kosaka et al., 1994).

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Plaunotol, [(2E,6Z,10E)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14-hexadecatetraen-1-ol], is an acyclic diterpene alcohol extracted from a Thai medicinal plant, plau-noi, and is widely used in the treatment of peptic ulcers in Japan. As a gastroprotective agent, plaunotol has excellent anti-ulcer activity both in experimental ulcers and in clinical trials (Karita et al., 1995; Kohda et al., 1991). It is also used in combination therapies against *Helicobacter pylori*-associated diseases because of its strong bactericidal effect against *H. pylori* (Karita et al., 1993; Koga et al., 1996; Shirai et al., 1995).

PGE_2 production is increased in gastric tissues with both acute gastric mucosal lesion and gastric ulcer lesion after administration of plaunotol (Ushiyama et al., 1987; Wada et al., 1997). The administration of a prostaglandin synthesis inhibitor abolishes the inhibitory actions of plaunotol on gastric acid secretion, which indicates that PGE_2 has an important role in the gastroprotective effects of plaunotol (Shiratori et al., 1993).

The precise mechanism underlying plaunotol induction of PGE_2 in the gastric mucosa is unclear, and the involvement of COX in this process has not been studied. Here, we investigated PGE_2 production and COX induction after plaunotol was added to cultured rat gastric mucosal (RGM1) cells. The promoter-activation in plaunotol-induced COX-2 expression was confirmed using a reporter gene assay.

2. Materials and methods

2.1. Reagents

The primary antibodies used in this study were COX-1, COX-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), phosphorylated-NF-κBp65 (Ser536), phosphorylated-cyclic AMP response element binding protein (CREB) and phosphorylated-inhibitory κB (IκB)-alpha (Ser32) (Cell Signaling Technology, Inc., Beverly, MA). Anti-goat and anti-rabbit immunoglobulin G antibodies were purchased from DAKO, Glostrup, Denmark and Pierce Biotechnology, Inc., Rockford, IL. Plaunotol was provided by Sankyo Co., Ltd., Tokyo and tumor necrosis factor (TNF)-alpha was purchased from Sigma Chemical Co., St. Louis, MO.

2.2. Cell culture

RGM1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 Ham (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (FBS; Sigma Chemical Co.) and 1% anticiobitics/antimycotic (AA; Invitrogen Corp., Carlsbad, CA) at 37 °C in 5% CO_2 atmosphere.

2.3. PGE_2 measurement

RGM1 cells were seeded at 4×10^5/well in 6-well plates and cultured for 24 h to reach sub-confluence. To silence the *Cox-2* gene expression before plaunotol stimulation, cells were serum-starved for 24 h. Then, the medium was changed and plaunotol was added at different concentrations for up to 6 h. Both the supernatant and the cell lysate were harvested for the following experiments. The supernatant was used to measure the PGE_2 concentrations with a PGE_2 enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions, while cell lysate was used to detect the expressions of COX-1 and COX-2 (also see Western blot analysis).

2.4. Western blot analysis

Cell culture was performed as described above. After serum-starvation for 24 h, the cells were cultured in medium supplemented with plaunotol at different concentrations for up to 15 min (Western blot analysis for phosphorylated-NF-κB, phosphorylated-IκB, or phosphorylated-CREB detection) or 6 h (Western blot analysis for COX-1 or COX-2 detection). The cells were washed three times with phosphate buffered saline (PBS), harvested, lysed in RIPA buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sufate) containing 0.1 mg/ml of phenylmethylsulfonyl fluoride and 51 μg/ml aprotinin as final concentrations, incubated on ice for 1 h, and centrifuged at 4 °C for 20 min at 13 400 × *g*. Protein concentrations of the cell lysates and supernatants were determined by the DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Protein samples (30 μg) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidine difluoride membranes. After blocking with 5% nonfat milk in Tris buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 1 h, the membrane was incubated overnight at 4 °C with the appropriate antibodies. The membrane was washed with TBS-T three times and incubated with anti-goat or anti rabbit immunoglobulin G for 1 h. After the membrane was washed with TBS-T again, the targeted protein was visualized on X-ray film (Hyperfilm; Amersham Pharmacia Biotech Ltd., Buckinghamshire, UK) using the Supersignal Chemiluminescence System (Pierce Biotechnology, Inc.).

2.5. Reporter assay

RGM1 cells were seeded at a density of 7.0×10^4/well in a 24-well plate and cultured for 24 h to reach sub-confluence. Reporter constructs containing different length *Cox-2* promoters, a gift from Dr. H. Inoue (National Cardiovascular Center Research Institute, Osaka, Japan), are shown in Fig. 1. The

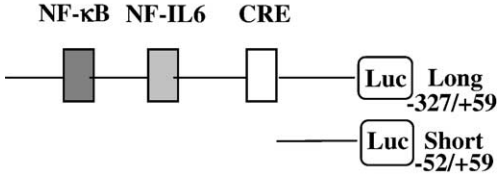

Fig. 1. Schematic representation of reporter vectors containing the 5′-flanking region of the *Cox-2* gene promoter. The construct of long-type of reporter vector containing the 5′-flanking region of the *COX-2* gene promoter contains 3 promoter sites (NF-IL6, NF-κB, and CRE) (−327/+59). No promoter is contained in the short-type of reporter vector containing the 5′-flanking region of the *COX-2* gene promoter (−52/+59). Distances are given as nucleotide positions relative to the transcriptional start site as +1.

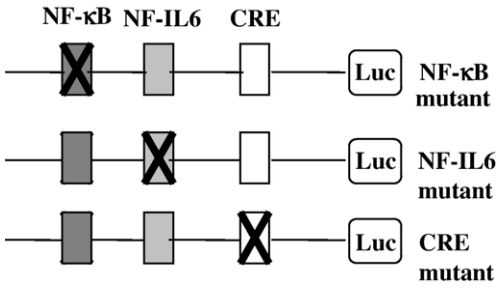

Fig. 2. Schematic representation of reporter vectors containing the 5′-flanking region of the *COX-2* gene promoter with mutations. The luciferase reporter vector driven by the COX-2 promoter region (−327/+59) mutated at the NF-κB, NF-IL6 or CRE site. The mutated sequences are listed as below: NF-κB site (−233/−214): be changed from GGGACTACCC to cacACTACCC; NF-IL6 site (−132/−124): be changed from TTACGCAAT to TTggtaccT; CRE site (−59/−53): be changed from TTCGTCA to TTgagCt.

long-type construct contained the NF-κB, NF-IL-6, and CRE (−327/+59) sites, whereas there was no promoter inserted in the short-type construct (−52/+59). Constructs of *Cox-2* gene reporters with a mutation in the NF-κB, NF-IL-6, or CRE regions of the COX-2 promoter, which were used in this study, are shown in Fig. 2.

A reporter construct (0.2 μg) was mixed with 20 ng/ml of a control vector pRL-TK in 50 μl of OPTI-MEM (Invitrogen Corp., Carlsbad, CA). The solution was mixed with 1 μl of Lipofectamine (Invitrogen Corp.), diluted in 150 μl of OPTI-MEM, and incubated at room temperature for 30 min; two vectors in 200 μl solution were co-transfected into RGM1 cells after the cells were washed twice with OPTI-MEM. The cells were incubated at 37 °C for 24 h in a 5% CO2 atmosphere. The medium was replaced with DMEM/F12 Ham supplemented with 10% FBS, and plaunotol was added to the reaction. After 6 h of culture, cells were harvested and assayed with a PicaGene Dual-Luciferase Assay kit (PG-DUAL SP, Toyo Ink Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions.

2.6. Electrophoretic mobility gel shift assay (EMSA)

EMSA “Gel-Shift” Kit (Panomics Inc., Redwood City, CA) was used to detect the nuclear translocation of NF-κB. RGM1 cells were seeded at 3.8×10^5/well in 6-well plates with DMEM/F12 medium. After cells were serum-starved for 24 h, culture medium containing 10% FBS and 50 μM plaunotol was added for 15 min. The nuclear protein was harvested and NF-κB binding with DNA was detected according to the manufacturer's instructions. The NF-κB sequence consensus oligonucleotide used in this study was 5′-AGTTGAGGGGACTTTCCCAGGC-3′. Instead of plaunotol, 20 ng/ml TNF-alpha was added as the positive control in culture medium.

2.7. Statistical analysis

The results were shown as mean±S.D. A statistical significance was determined by a two-tailed Student's *t*-test. A *P* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of plaunotol on COX-2 expression and PGE2 production in RGM1cells

After plaunotol stimulation, COX-1 and COX-2 expression were detected with their specific antibodies. When plaunotol was added at 50, 100, and 200 μM, COX-2 expression was stronger than that of controls. On the other hand, there was no significant difference in COX-1 expression between groups with or without the addition of plaunotol (Fig. 3A). The reporter assay indicated that the luciferase activity in the long-type construct but not in short-type was significantly increased after plaunotol treatment (Fig. 3B), which was consistent with the COX-2 expression induced by plaunotol. Moreover plaunotol (50, 100, or 200 μM) significantly increased PGE_2 levels in the culture medium compared to the control group (Fig. 4).

3.2. Plaunotol induced COX-2 expression via Cox-2 gene promoter NF-κB and CRE

Using the reporter constructs shown in Fig. 2, we investigated the plaunotol-induced changes in luciferase activity. When the *Cox-2* reporter genes with a mutation in the NF-κB or CRE region was transfected into RGM1 cells, plaunotol (50 μM) did not significantly increase luciferase activity compared with the control and the reporter gene with the mutation in the NF-IL6 region (Fig. 5).

To confirm the plaunotol-induced NF-κB activation, we investigated NF-κB and IκB phosphorylation. NF-κB and IκB were phosphorylated 15 min after addition of 50 μM plaunotol (Fig. 6A). The same level of phosphorylation was also observed when the serum-starved RGM1 cells were stimulated with TNF-

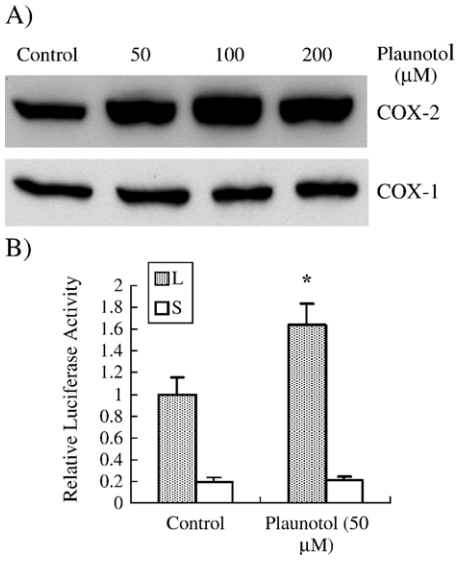

Fig. 3. Effect of plaunotol on the expression on COX-1 and COX-2. (A) RGM1 cells were cultured as described in above. After the treatment with plaunotol, cells were harvested and used for Western blot analysis with COX-1 and COX-2 antibodies. (B) Using reporter assay, *Cox-2* gene transcription was measured after plaunotol stimulation for 6 h (*$P<0.05$ vs. Control, $n=6$).

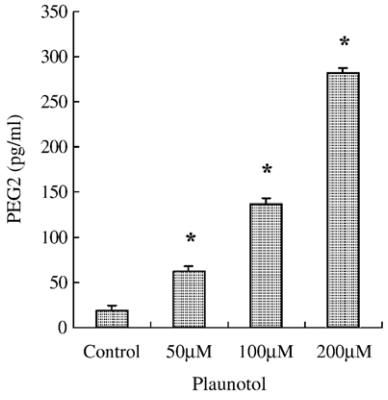

Fig. 4. Effect of Plaunotol on PGE_2 production in RGM1 cells. RGM1 cells were seeded at 4×10^5/well in 6-well plates and cultured for 24 h to reach sub-confluence. After serum-starved for 24 h, plaunotol was added at different concentrations for up to 6 h. Then, the supernatant was collected and PGE_2 was measured with a PGE_2 EIA kit (*$P<0.05$ vs. Control, $n=6$).

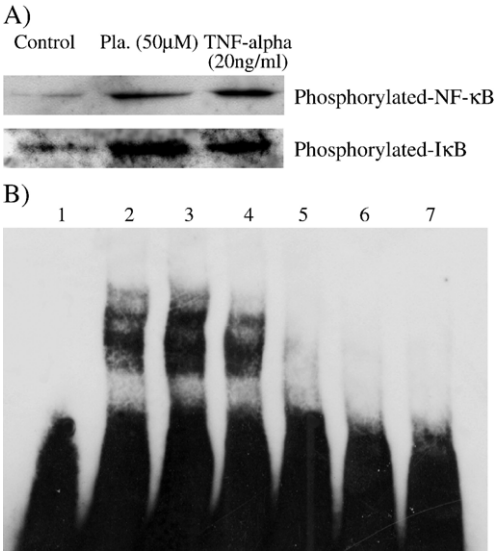

Fig. 6. Effect of Plaunotol on NF-κB and IκB phosphorylation. (A) RGM1 cells were seeded at 3.8×10^5 in 6-well plates. After serum-starved for 24 h, medium containing 10% FBS and 50 μM plaunotol was added for 15 min. Then, cells were harvested and used for Western blot analysis with phosphorylated-NF-κB and phosphorylated-IκB antibodies. TNF-alpha was used as the positive control. (B) Cell culture and treatment with plaunotol was as same as that in (A). The nuclear protein was harvested and the nuclear translocation of NF-κB was detected with EMSA. TNF-alpha was also used as the positive control. Line 1: no probe; line 2: plaunotol 0 μM (hot probe); line 3: plaunotol 50 μM (hot probe); line 4: TNF-alpha 20 ng/ml (hot probe); line 5: plaunotol 0 μM (cold probe); line 6: plaunotol 50 μM (cold probe); line 7: TNF-alpha 20 ng/ml (cold probe).

alpha (20 ng/ml; Fig. 6A). With EMSA gel shift assay, plaunotol-activated NF-κB was bound to DNA, similar to that stimulated by TNF-alpha (Fig. 6B).

Moreover, in the serum-starved RGM1 cells treated with plaunotol for 15 min, the phosphorylation of CRE was also increased compared to that of controls (Fig. 7).

4. Discussion

Gastroprotective agents such as plaunotol [(2E,6Z,10E)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14-hexadecatetraen-1-ol] are widely used in the treatment of peptic ulcers including *H. pylori*-associated and non-steroidal anti-inflammatory drug-induced ulcers in Japan. Plaunotol is an anti-ulcer agent extracted from the medicinal plant plau-noi (Croton sublyratus Kurz, native to Thailand). Plaunotol is effective in patients with gastric ulcer (Ogiso et al., 1985) as well as in animal models of experimental gastric mucosal injury induced by various noxious stimuli (Kobayashi et al., 1982; Okabe et al., 1982). The mechanism underlying the anti-ulcer effects of plaunotol includes the increase of prostaglandin production in gastric mucosa (Ushiyama et al., 1987). On the other hand, Shiratori et al. reported that plaunotol released endogenous secretin, and that secretin is a potential mediator of the anti-ulcer actions of mucosal protective agents. Furthermore, they demonstrated that endogenous prostaglandin have a significant role in the inhibitory action of exogenous and plaunotol-released endogenous secretin in rats (Shiratori et al., 1993). In animal experiments, plaunotol prevents indomethacin-induced gastric mucosal injury by inhibiting neutrophil activation (Murakami et al., 1999). Thus, plaunotol prevents gastric mucosal injury and has gastroprotective actions through various mucosal defensive factors.

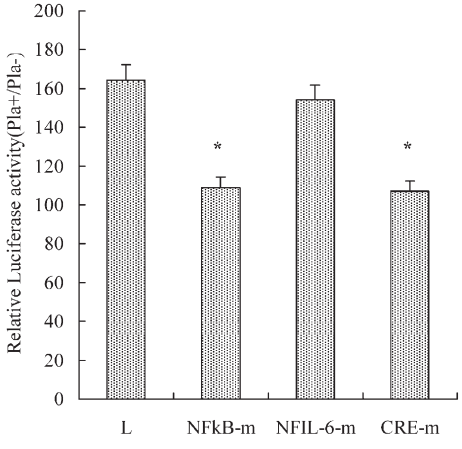

Fig. 5. Effect of plaunotol on the promoter activities. The *Cox-2* gene reporter which has mutation in NL-IL6, NF-κB, or CRE region and the normal one were transfected into RGM1 cells. The luciferase activity was measured after plaunotol stimulation for 6 h (*$P<0.05$ vs. L, $n=6$).

Regarding the mechanism of plaunotol-induced prostaglandin production, Ushiyama et al. reported that plaunotol increased prostaglandin levels by stimulating cellular phospholipase activity in 3T6 fibroblast cells and that plaunotol

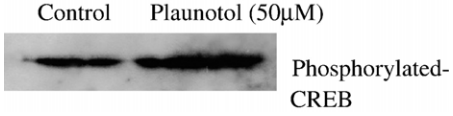

Fig. 7. Effect of plaunotol on CRE activation. RGM1 cells were cultured and treated with plaunotol as described in Fig. 4. Phosphorylated-CREB antibody was used to detect the CRE phosphorylation after plaunotol addition.

stimulated PGE_2 and PGI_2 production when the drug was orally administered to normal rats (Ushiyama et al., 1987).

Thus, prostaglandin has a significant role in the pathogenesis of gastric ulceration and serves to maintain normal gastric mucosal integrity (Kleine et al., 1993; Wada et al., 1997). PGE_2 is one of the major prostaglandins in gastric tissue. The production of PGE_2 is rate-limited by its catalytic enzyme, COX. It has been established that at least 2 forms of COXs exist, termed COX-1 and COX-2. COX-1 is expressed constitutively in most tissues. In contrast, COX-2 expression is usually low under basal conditions. There is increased COX-2 expression under certain conditions (e.g., inflammation, tissue damage, malignant transformation) (Kargman et al., 1996; Vane and Botting, 1995).

The role of COX in plaunotol-induced prostaglandin production, however, is unclear. Here, we focused on PGE_2 production associated with of plaunotol treatment and attempted to clarify the participation of COX in plaunotol-induced PGE_2 production. The results indicate that plaunotol increased PGE_2 production in the RGM1 cell culture supernatant in a dose-dependent manner. Concurrently, COX-2 expression, but not COX-1, increased when 50 μM of plaunotol was added. The *Cox-2* promoter gene assay also confirmed that the addition of plaunotol induced COX-2 expression in RGM1 cells.

The *Cox-2* gene promoter contains a canonical TATA box and various putative transcriptional regulatory elements, such as NF-κB, NF-IL6/C/EBP, PEA3, NFAT, CRE, AP-2, and SP-1 (Kosaka et al., 1994). Depending on the stimulus and the cell type, these transcription factors can modulate *Cox-2* expression.

To investigate the site of promoter involved in plaunotol-induced COX-2 expression, we used a series of reporter vectors for the *Cox-2* gene, and measured their activity (Luciferase activity) after the addition of plaunotol. The mutations at NF-κB or CRE site, but not NF-IL6 site, decreased the response to plaunotol compared with normal controls, which indicated that NF-κB and CRE promoter regions were critical in *Cox-2* gene expression induced by plaunotol.

NF-κB is activated in response to a wide variety of stimuli that promote the dissociation of IκB through phosphorylation followed by ubiquitination and degradation. Moreover, recent studies indicate that phosphorylation of the NF-κB subunit p65 modulates NF-κB transcription activity (Baldwin, 1996; Bhattacharyya et al., 2004). In this study, NF-κB and IκB were phosphorylated after plaunotol addition, similar to when treated with TNF-alpha, an NF-κB signaling activator. Furthermore, using an NF-κB gel-shift assay, the nuclear translocation of NF-κB was detected following treatments with both plaunotol and TNF-alpha. Also, the phosphporylation of CREB, which mediates its nuclear translocation and combination with CRE (Johannessen et al., 2004), increased after the addition of plaunotol.

In conclusion, the results clearly indicate that plaunotol induces COX-2 expression via activating the NF-κB and CRE promoter regions of the *Cox-2* gene and increases PGE_2 production, which might contribute to the gastroprotective functions of plaunotol.

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