Ozonides of olive oil and methyl oleate inhibit the expression of cyclooxygenase-2 through the suppression of IκB/NFκB-dependent pathway in lipopolysaccharide-stimulated macrophage-like THP-1 cells

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Abstract

To elucidate the anti-inflammatory action mechanism of ozonized olive oil, we investigated the effects of ozonized olive oil and methyl oleate ozonides on the generation of PGE_2 and the expression of Cyclooxygenase-2 (COX-2) in Lipopolysaccharide (LPS)-stimulated macrophage-like THP-1 cells. Olive oil, methyl oleate and their ozonides did not affect cell viability and morphology within a concentration used. LPS-induced COX-2 expression and PGE_2 generation in macrophage-like THP-1 cells were not affected by olive oil itself. However, ozonized olive oil inhibited both reactions in a concentration-dependent manner. Methyl oleate ozonides also inhibited COX-2 expression and PGE_2 generation whereas oleic acid and methyl oleate did not show such inhibitory effects. In addition, ozonized olive oil and methyl oleate ozonide inhibited IκB phosphorylation which is required for Nuclear factor-kappa B (NFκB) activation and subsequent COX-2 gene transcription in LPS-stimulated macrophages. These results demonstrate that ozonized olive oil and methyl oleate ozonides suppress LPS-induced PGE_2 generation in macrophage-like THP-1 cells through inhibition of COX-2 gene transcription by suppressing the IκBα/NFκB pathway.

Introduction

Ozonized olive oil has been reported to accelerate the wound healing and exert anti-inflammatory effect [1]. However, the details of chemical structure of ozonized olive oil and its action mechanism have long been unclear. We have recently revealed that the major component of the ozonized olive oil is triozonide of triolein (Fig. 1) [2]. This finding enables us to perform the structure-based studies on the pharmacological action mechanism of ozonized olive oil.

Two isoforms of cyclooxygenase (COX), COX-1 and COX-2, have been identified [3]. COX-1 is constitutively expressed in most tissues and plays physiologically important roles through synthesis of prostaglandins. In contrast, COX-2 is merely detectable in most tissues under normal condition but is induced by many inflammatory stimuli such as bacterial lypopolysaccharide (LPS) and pro-inflammatory gene products including IL-1 and TNF-α,leading to overproduction of prostaglandin E_2 (PGE_2) [4].

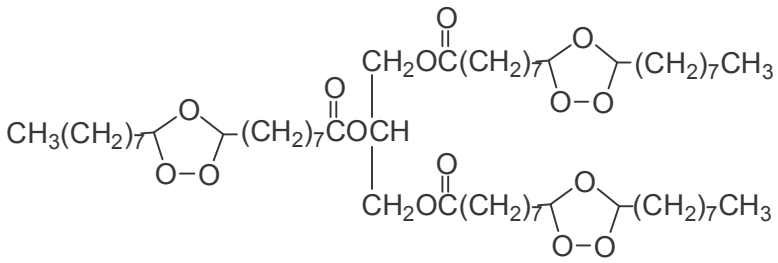

Fig.1 Chemical structure of Triolein triozonide

High amount of PGE_2 contributes to sustain more favorable environment for progression and continuation of inflammation at lesion by stimulating vasodilation, and enhancing vascular permeability and the action of kinin. Therefore, the suppression of PGE_2 overproduction by inhibiting the expression or activity of COX-2 has been an important goal to treat inflammatory diseases. Therefore, in the present study, we examined whether and how anti-inflammatory effect of ozonized olive oil is associated with LPS/COX-2/PGE_2 pathway in macrophage-like THP-1 cells.

Materials and methods

Materials. Olive oil was purchased from Yakuhan Pharm. (Sapporo, Japan). Methyl oleate was obtained from Nu-Chek Prep (Elisyan, MN). Oleic acid was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Ozonide of olive oil was prepared by bubbling O_3/O_2 mixed gas (10 μg/ml of O_3) into olive oil at 30°C. After the ozonization, yellow olive oil turned to white vaseline. Ozonide of methyl oleate was prepared by the similar manner except that O_3/O_2 mixed gas containing 80 μg/ml of O_3 was used. Methyl oleate ozonide thus obtained was slightly solidified. These ozonides were dissolved in dimethyl sulfoxide at a concentration of 30 mM and used after dilution with appropriate buffer.

Cells. Human leukemia cells, THP-1 (Riken Gene Bank, Tsukuba, Japan), were cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 0.03% D-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of fungison. Cells were differentiated into macrophage-like cells by incubation with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) for 48 h at 37°C and then used for experiments. Cell viability was estimated by Trypan Blue exclusion test. Cell morphology was observed under the phase-contrast microscope.

PGE_2 measurement. Cells were incubated with 10 or 30 μM of each ozonide, olive oil or methyl oleate at 37°C for 24 h, subsequently with 1 μg/ml of LPS at 37°C for 8 h. After incubation, the amounts of PGE_2 accumulated in medium were determined by enzyme immunoassay using STAT-prostaglandin E_2 EIA kit (Cayman, Ann Arbor, MI).

Western blot analysis of COX-2 expression. Cells were incubated with 1, 3, 10, 30 μM of ozonide, olive oil or methyl oleate at 37°C for 24 h subsequently with 1 μg/ml of LPS at 37°C for 6 h. After incubation, cells were collected, dissolved in lysis buffer (50 mM Tris-HCl buffer, pH 7.8, containing 10 mM EDTA, 0.5% Nonidet P-40, 0.5% deoxycholic acid, 0.01% SDS, 50 mM NaCl, 10 μg/ml aprotinin,

10 μg/ml pepstatin, 10 μg/ml leupeptin and 2 mM diisopropyl fluorophosphates) and centrifuged at 12,000g for 30 min. Fifty μg of protein in the supernatant were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were transferred to nitrocellulose membrane and then COX-2 was detected by reacting with rabbit anti-COX-2 antibody (previously prepared in this laboratory) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG antibody. Antibody-bound COX-2 on the membrane was visualized by ECL using ECL chemiluminescence kit (Perkin-Elmer) and hyperfilm (Amersham Japan, Tokyo). The density of COX-2 bands on the film was quantified by Image master (Pharmacia).

Analysis of phosphorylation of IκBα, p38 MAP kinase and JNK. Cells were incubated with ozonide, olive oil or methyl oleate at 37°C for 30 min subsequently with 1 μg/ml of LPS at 37°C for 60 min. Cells were lysed with 20 mM HEPES buffer, pH 7.5, containing 1% Nonidet P-40, 150 mM NaCl, 10 mM $Na_4P_2O_7$, 2mM EDTA, 150 mM NaF, 20 mM β-glycerophosphate, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 mM Na_3VO_4. After separation of cellular proteins (40 μg) by SDS-PAGE, phosphorylated proteins were analyzed by Western blot using following antibodies as primary antibodies followed by ECL; anti-active p38 antibody for detection of phosphorylated p38, anti-active JNK antibody for phosphorylated JNK and ani-phospho IκBα for phosphorylated IκBα.

Results and discussion

We have first examined whether ozonides act as cytotoxic agents on macrophage-like THP-1 cells. Cells were incubated with ozonides of olive oil and methyl oleate for 24 h and then analyzed cell viability and cell morphology. Cells were also incubated with dimethyl sulfoxide, olive oil, methyl oleate or oleic acid as controls. As shown in Table 1, all agents used did not show any effect on cell viability within a concentration used. In addition, cell morphology was not affected by these agents as well (not shown). These results demonstrate that ozonides of olive oil and methyl oleate, olive oil, methyl oleate and oleic acid of the concentrations used in this study are not cytotoxic against macrophage-like THP-1 cells.

Table 1. Effects of ozonides of olive oil and methyl oleate on cell viability.

Agents	Concentration	Cell viability (%)
vehicle (DMSO)	0.03 %	98.67 ± 0.33
	0.1%	97.67 ± 0.66
olive oil	10 uM	98.00 ± 1.15
	30 uM	97.00 ± 0.58
methyl oleate	10 uM	97.67 ± 1.45
	30 uM	96.67 ± 0.66
oleic acid	10 uM	97.00 ± 0.57
	30 uM	98.33 ± 0.67
ozonized olive oil	10 uM	98.33 ± 0.88
	30 uM	97.67 ± 1.20
ozonized methyl oleate	10 uM	99.33 ± 0.33
	30 uM	99.00 ± 0.57

PGE_2 is a potent chemical mediator in inflammatory disease. To examine effects of ozonides on PGE_2 generation in macrophages, we used macrophage-like THP-1 cells since the cells generate enhanced amount of PGE_2 upon stimulation with LPS. This response occurred time-dependently and the maximal response was attained with 12 h-stimulation. When cells were cultured for 24 h with olive oil subsequently for 9 h with an addition of LPS, no effect of olive oil on the enhanced PGE_2 generation was observed. In contrast, ozonized olive oil exhibited significant inhibitory effect on LPS-induced PGE_2 generation and its effect was concentration-dependent (Fig. 2). Interestingly, methyl oleate ozonide also inhibited PGE_2 generation, suggesting that triolein triozonide structure is not necessarily required for and a single ozonide structure may be enough for anti-inflammatory and analgesic action.

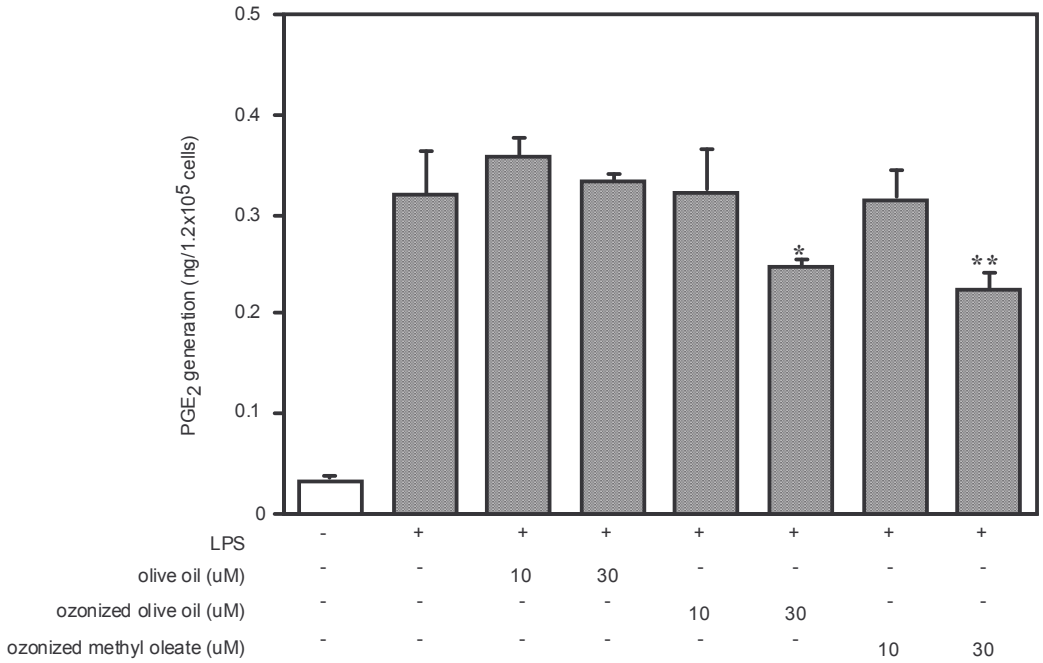

*Fig. 2. Effects of ozonides of olive oil and methyl oleate on LPS-induced PGE_2 generation. Cells were preincubated for 24 h with various concentrations of ozonized olive oil, ozonized methyl oleate and olive oil, and then incubated with LPS for further 9 h. The amounts of PGE_2 generated were analyzed by ELISA. The results are shown as means ± S.E. (n=3). (** : p < 0.01, * : p< 0.05)*

Figure 3 shows the effects of ozonized olive oil and ozonized methyl oleate on the expression of COX-2 protein in stimulated macrophage-like THP-1 cells. Both the ozonides significantly suppressed the COX-2 expression, indicating that the ozonides inhibit the LPS-induced PGE_2 overproduction by suppression of COX-2 expression.

The transcription of COX-2 gene is regulated through nuclear factor-kappa B (NFκB). NFκB can rapidly activate various inflammatory cytokines. It is therefore a central mediator of the immune and inflammatory response [5]. NFκB dimmers are sequestered in an inactive cytoplamic complex by binding to its inhibitor, IκBα, and other IκB proteins. Many extracellular stimuli including LPS activate NFκB by phosphorylation of IκB. We therefore examined the effect of ozonides on the phosphorylation of IκBα. As shown in Fig. 4, ozonized olive oil and ozonized methyl oleate significantly inhibit the phosphorylation of IκBα in LPS-stimulated macrophage-like THP-1 cells whereas no significant effect was observed with olive oil, methyl oleate and oleic acid.

Besides NFκB, various other transcription factors are involved in the regulation of the inflammatory response. The activation protein-1 (AP-1) also mediates activation of COX-2 transcription [6]. However, ozonized olive oil and ozonized methyl oleate showed no effect on the phosphorylations of p38 and JNK, which are involved in the activation of AP-1 (data not shown).

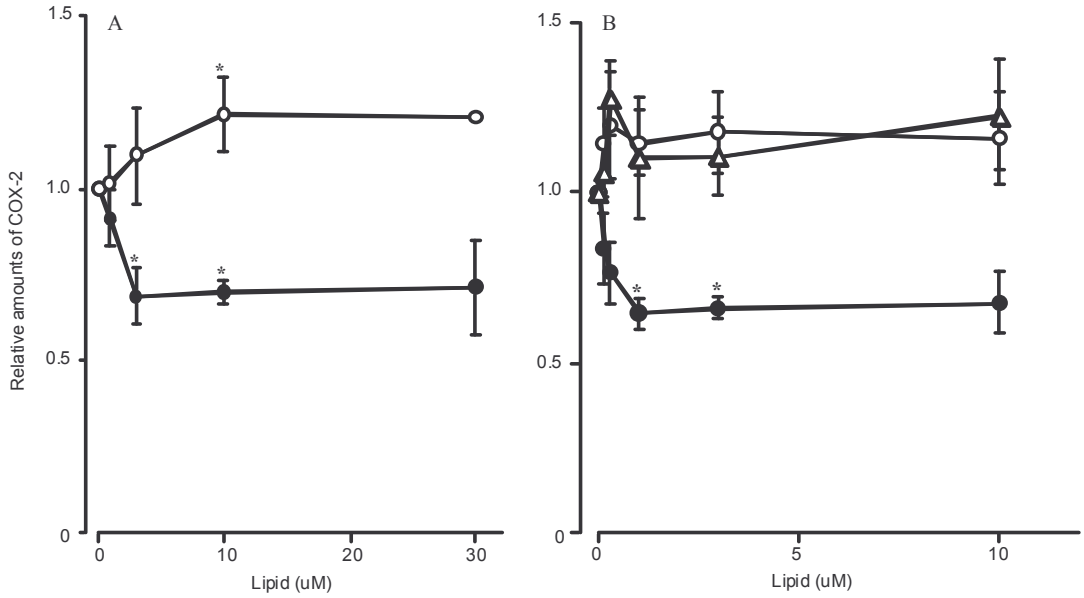

Fig. 3. Effect of ozonized olive oil on the LPS-induced COX-2 expression in macrophage-like THP-1 cells. The cells were preincubated for 24 h with various concentrations of (A) ozonized olive oil (closed circle) and olive oil (open circle), (B) ozonized methyl oleate (closed circle), methyl oleate (open circle) and oleic acid (open triangle), and then incubated with LPS for 6 h. COX-2 expression was analyzed by Western blot. The results are shown as means ± S.E. (n=3). (: p < 0.05)*

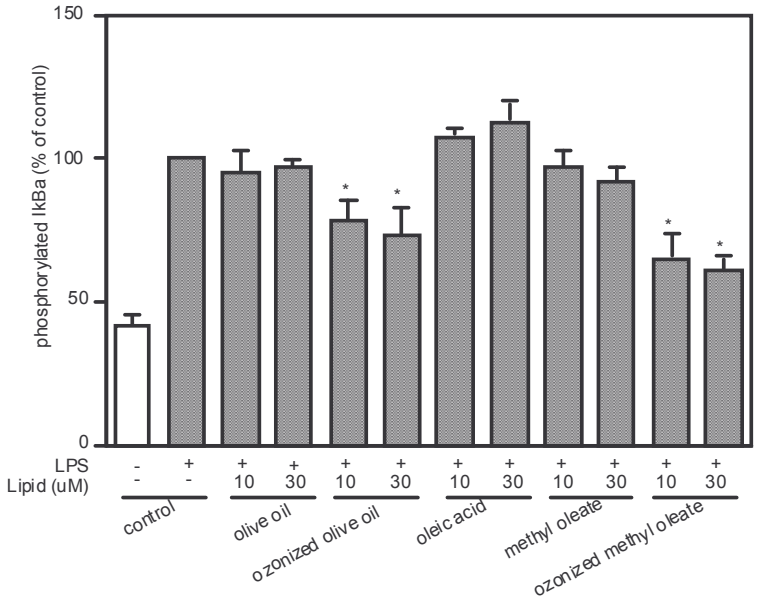

Fig. 4. Effects of ozonized olive oil and ozonized methyl oleate on the phosphorylation of IκBα. The cells were pretreated for 30 min with various concentrations of ozonized olive oil and ozonized methyl oleate, and then incubated with LPS (1 μg/ml) for 60 min. The phosphorylation of IκBα was analyzed by Westrn blot. The results are shown as means ± S.E. (n=3). (: p < 0.05)*

Conclusion

Ozonized olive oil is known to exert anti-inflammatory and analgesic effects, and has been applied topically to a variety of cutaneous diseases for disinfecting the lesions and promoting their healing though its action mechanism has remained uncertain. In this paper, we first demonstrated that ozonized olive oil significantly inhibits the overproduction of PGE_2 as well as COX-2 expression in LPS-stimulated macrophage-like THP-1 cells. The effect of ozonized olive oil on COX-2/ PGE_2 pathway was shown to be the consequence of inactivation of NFκB by inhibiting phosphorylation of IκBα as shown in Fig.5. The same effect was also observed with ozonized methyl oleate, suggesting that any type of ozonide exerts anti-inflammatory and analgesic effects by the same mechanism. In addition, ozonized olive oil may suppress LPS-induced generation of pro-inflammatory cytokines such as TNF-α and IL-1β.

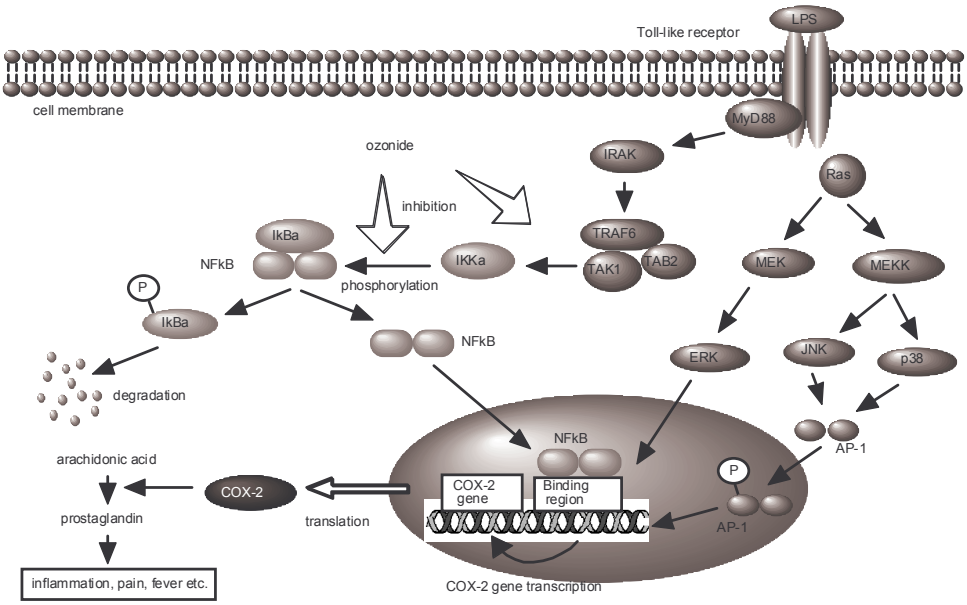

Fig. 5. A possible mechanism for inhibition of COX-2 gene transcription by ozonides in LPS-stimulated macrophage-like THP-1 cells

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