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Emu oil suppresses inflammatory responses of RAW 264 macrophages via inhibition of NF-κB signaling

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Abstract

Emu oil has been shown to alleviate several symptoms of inflammation in animal models, but mechanisms mediating the process are unknown. We investigated the influence of emu oil on the production of inflammatory mediators in the murine macrophage cell line RAW264 to elucidate anti-inflammatory mechanisms. Stimulation with a combination of lipopolysaccharide (LPS) and interferon-γ (IFN-γ) increased production of nitric oxide, interleukin 6 and prostaglandin E_2 in RAW264 cells, and emu oil inhibited the increase in these mediators. Western blot analysis showed that emu oil inhibited phosphorylation of the regulatory protein IκB-α and inhibited NF-κB nuclear import induced by LPS and IFN-γ. These results suggest that emu oil suppresses inflammatory responses of RAW264 by inhibiting NF-κB signaling.

Key Words: emu oil, inflammation, NF-κB

The emu (*Dromaius novaehollandiae*), which is indigenous to Australia, is the second largest extant bird. Its meat and oil are used worldwide, including in Japan. Aboriginal Australians and white settlers in Australia have long used subcutaneous fat of the emu to heal wounds and alleviate joint pain[10, 12)]. Over the past 25 years, a number of investigators, including veterinary scientists, have examined the efficacy of emu oil for the treatment of inflammatory disease. It has been shown that oral administration of emu oil suppressed mucositis-associated inflammatory changes in the intestinal mucosal architecture of rats[16)], and it inhibited several effects of inflammation upon 5-fluorouracil chemotherapy in another rat model[21)]. Additionally, topical application of emu oil reduced inflammatory swelling of rat earplugs treated with croton oil[18)]. Thus, oral and topical administrations of emu oil have significant anti-inflammatory properties, but the mechanisms remain unclear.

A variety of animal and human diseases, including skin diseases, cancer, and neurodegenerative disorders, are closely associated with inflammation. Inflammation is a protective reaction of white blood cells and vascular cells against injury, chemical irritation and infection[1, 24)]. Macrophages play important

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Table 1. Fatty acid composition of emu oil used in the present experiments

Systematic name	Common name	Total (%)
14:0	Myristic acid	0.2
16:0	Palmitic acid	18.8
18:0	Stearic acid	7.5
20.0	Arachidic acid	0.1
16:1 n-7	Palmitoleic acid	3.2
18:1 n-7	Vaccenic acid	2.1
18:1 n-9	Oleic acid	44.2
18:2 n-6	Linoleic acid	13.6
18:3 n-3	α-Linolenic acid	1.0
20:1 n-9	Eicosenoic acid	0.4

roles in inflammation. After recognizing pathogens or toxicants, macrophages produce inflammatory mediators such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and prostaglandins (PGs). These reactions of macrophages lead to inflammatory symptoms such as swelling and heating of tissues and pain[13]. Thus, macrophages are critical targets for the treatment of inflammation. Recently, we demonstrated that the addition of emu oil to the medium of cultured macrophages suppressed the production of NO and TNF-α induced by lipopolysaccharide (LPS)[20]. This result suggested that the anti-inflammatory effects of emu oil are at least partially due to suppression of macrophages. However, the mechanisms by which emu oil affects inflammatory mediators are unknown. In this study, we investigated the influence of emu oil on intracellular signal transduction associated with inflammatory responses of macrophages in order to elucidate the anti-inflammatory properties of emu oil.

The murine macrophage cell line RAW264 was purchased from Riken BioResource Center (Tsukuba, Japan). The cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a CO_2 incubator. Commercially available emu oil was purchased from Tokyo Nodai Bioindustry (Abashiri, Japan). The fatty acid composition of emu oil, as analyzed by gas chromatography, is shown in Table 1.

To investigate the effects of oil, emu oil was mixed with culture medium containing 4% fatty acid-free bovine serum albumin (BSA; Fujifilm Wako Pure Chemical, Osaka, Japan) as an emulsifier[4, 14]. RAW264 cells were seeded in culture dishes or 96-well culture plates at a density of $3 \times 10^4/cm^2$ and cultured overnight. The culture medium was replaced with DMEM containing 4% BSA and emu oil, and the cells were incubated for 24 h. The culture medium was then replaced with DMEM containing 1 μg/ml LPS (Sigma-Aldrich, MO, USA) and 10 ng/ml of murine interferon-γ (IFN-γ; PeproTech, NJ, USA), and the cells were further incubated for the indicated periods. We compared the effects of emu oil with a known inflammatory inhibitor, curcumin[15], which was added to the medium by diluting a stock solution (20 mM curcumin in dimethyl sulfoxide; DMSO) to a final concentration of 20 μM. During the treatments of the cells with emu oil and curcumin, culture mediums for all groups including control and LPS/IFN-γ-stimulation only were supplemented with vehicles (4% BSA and 0.1% DMSO).

We have determined the concentration range of emu oil as 0.001-0.01% in the present study according to the following reasons; 1) In most of in vitro experiments to investigate the oil functions, concentrations of oil less than 0.01% have been adopted[7-9, 25]. Using oil concentrations similar to those used in previous experiments may facilitate discussion comparing the results obtained in this study with previous results. 2) Concentration of oil that can be dissolved in the culture medium containing 4% BSA was up to about 0.01%.

For the measurement of NO, IL-6 and prostaglandin E_2 (PGE_2), cells were seeded in a 96-well plate. After incubation with emu oil, the cells were stimulated with LPS/IFN-γ for 20 h. Then, culture supernatants were collected and stored at -20°C until assayed. The nitrite concentration in the medium was measured as an indicator

of NO production using the Griess reaction, as previously described [20]. The concentrations of IL-6 and PGE_2 in the medium were measured using ELISA kits for IL-6 (R & D Systems, MN, USA) and for PGE_2 (Cayman Chemical, MI, USA). The data were normalized to the amount of cellular protein to account for differences in cell number[20]. The cellular protein content was determined by lysing PBS-washed cells with 0.4% Triton X-100 in PBS and determining the protein concentrations in lysates with a protein assay kit (Pierce BCA protein assay kit; Thermo Fisher Scientific, MA, USA).

For the determination of levels of COX-2 and phosphorylated forms of IκB-α (p-IκB-α), cells were seeded in 6 cm diameter culture dishes. After incubation with emu oil, the cells were stimulated with LPS/IFN-γ for 1 h for p-IκB-α or 6 h for COX-2. The cells were collected and resuspended in 50 μl of lysis buffer and kept on ice for 30 min. After centrifugation at 12000 rpm for 15 min, the supernatant was collected and diluted with SDS buffer. After boiling for 5 min, proteins in the supernatant were separated by SDS-PAGE and then electroblotted onto nitrocellulose membranes. The membranes were blocked for 30 min at room temperature with 5% BSA in TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20). The membranes were then rinsed three times with TBST and incubated overnight at 4°C with anti-COX-2 antibodies (Cell Signaling Technology, MA, USA), anti-p-IκB-α antibodies (Cell Signaling Technology), or anti-actin antibodies (Santa Cruz Biotechnology, CA, USA) diluted 1:1000 in TBST plus 5% BSA. The membranes were washed three times with TBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) diluted 1:1000 in TBST plus 5% BSA. The membranes were washed with TBST, and immunoreactive bands were visualized by chemiluminescence detection. The intensities of the detected bands were analyzed using the Image J v1.38 software package (https://imagej.nih.gov/ij/).

For the detection of nuclear NF-κB, cells were seeded in 10 cm diameter culture dishes. After incubation with emu oil, the cells were stimulated with LPS/IFN-γ for 1 h. Nuclear fractions of RAW264 cells were prepared using an NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The nuclear fraction was then diluted with SDS buffer and boiled for 5 min. Proteins were separated via SDS-PAGE and subjected to immunoblotting as described above using anti-NF-κB p65 antibodies (Cell Signaling Technology) or anti-histone H3 antibodies (Rockland Immunochemicals, PA, USA).

Quantitative results were reported as means ± standard deviation. Differences in mean values were assessed with non-repeated ANOVA, followed by a Student-Newman-Keuls test. Test results were considered significant if the value of P was less than 0.05.

First, we investigated the effects of emu oil on the production of inflammatory mediators from RAW264 macrophages. In a preliminary experiment in which cellular growth was determined with WST-8 reagent (Cell Counting Kit-8; Dojindo, Japan), the viability of RAW264 cells was unaffected by treatment of the cells with 0.001% or 0.01% of emu oil for 24 h (data not shown). Stimulation with LPS/IFN-γ increased the production of NO, IL-6 and PGE_2 (Fig 1). When the cells were pre-treated with emu oil, the LPS/IFN-γ-induced production of these inflammatory mediators was reduced in a dose-dependent manner, although the extent of suppression differed among the substances. The inhibitory effect of emu oil at the highest concentration (0.01%) on production of these mediators was similar to that of curcumin (20 μM). Because PGE_2 production induced by LPS/IFN-γ was suppressed by emu oil, we tested whether emu oil affected the expression of cyclooxygenase-2 (COX-2), a central enzyme in PGE_2 synthesis. Stimulation with LPS/IFN-γ increased COX-2 expression, and this increase was abrogated by treatment with emu oil (Fig. 2A). These results suggest that a decrease in

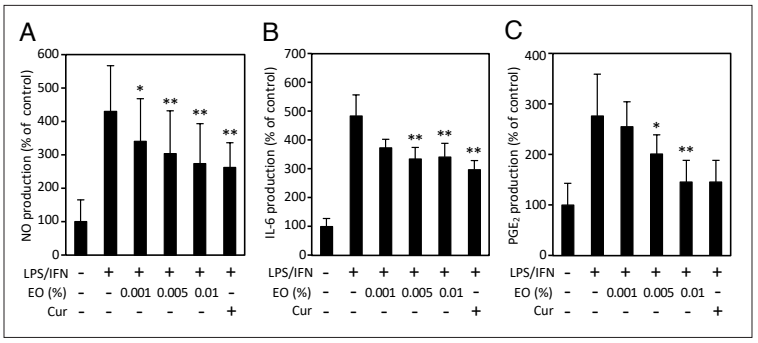

Fig. 1. Effects of emu oil (EO) on the production of NO (A), IL-6 (B) and PGE_2 (C) in RAW264 cells. After pre-incubation with emu oil or curcumin (Cur; 20 μM), cells were stimulated with a combination of 1 μg/ml LPS and 10 ng/ml IFN-γ (LPS/IFN) for 20 h. Concentrations of NO, IL-6 and PGE_2 in the supernatants were normalized to cellular protein contents. Results were expressed as percentages relative to the control value. Values are means ± SD (A: n = 12. B: n = 6, C: n = 4). Asterisks show significant differences from the LPS/IFN group. *, $P < 0.05$; **, $P < 0.01$.

LPS/IFN-γ-induced PGE_2 production by emu oil is due to an inhibition of COX-2 expression.

PGE_2 facilitates the influx of blood cells into tissues, leading to swelling and edema, and it stimulates sensory nerves to increase the pain response[22]. Thus, lowering PGE_2 production is an effective approach for the treatment of inflammatory pain. Accordingly, aboriginal Australians have long used emu oil to treat joint pain, and a recent human clinical study showed that arthralgia in women who had been treated with an aromatase inhibitor for the prevention of breast cancer was favorably improved by massaging with emu oil[6]. These effects of emu oil on pain may be explained by the reduction of COX-2 expression and a subsequent decrease in PGE_2 synthesis.

We next investigated whether NF-κB signaling is involved in the inhibitory effects of emu oil on the production of inflammatory mediators. NF-κB plays a pivotal role in the expression of inflammation-related genes, including *Nos2, Ptgs2* and *Il6*. One of the most common forms of NF-κB is a dimer of the Rel homology domain-containing proteins p65 and p50. NF-κB proteins are sequestered in the cytoplasm in unstimulated cells, and stimulation with several molecules, including LPS, induces the translocation of NF-κB from the cytoplasm to the nucleus, leading to the binding of NF-κB to gene promoters[3]. Figure 2B shows the localization of the p65 subunit of NF-κB to the nuclei of RAW264 cells. NF-κB p65 was weakly detected in the nuclei of control cells, whereas nuclear localization increased after stimulation with LPS/IFN-γ. Pre-treatment of the cells with emu oil reduced the LPS/IFN-γ-induced increase in nuclear p65 localization, suggesting that emu oil inhibited the translocation of NF-κB.

In unstimulated cells, NF-κB is sequestered in the cytoplasm as a complex with the inhibitor protein IκB-α. Stimulation with LPS is known to cause the phosphorylation and degradation of IκB-α and thus the nuclear translocation of NF-κB[3]. Accordingly, we investigated the effect of emu oil on the phosphorylation of IκB-α. As shown in Figure 2C, LPS/IFN-γ increased the phosphorylation of IκB-α, while pre-treatment of the cells with emu oil reduced this increased phosphorylation. These results suggest that emu oil suppresses the activation of NF-κB by inhibiting the phosphorylation of IκB-α.

In the present study, we demonstrated for the first time that emu oil suppresses the

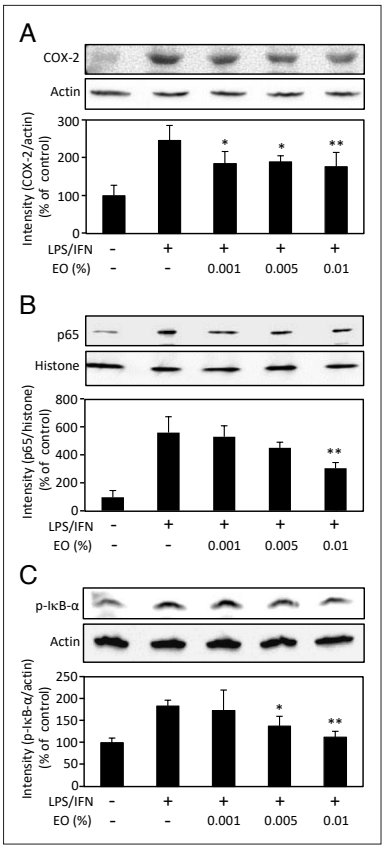

Fig. 2. Effects of emu oil (EO) on (A) the expression of COX-2, (B) nuclear p65 and (C) phosphorylation of IκB-α in RAW264 cells. (A) After pre-incubation with emu oil, cells were stimulated with a combination of 1 μg/ml LPS and 10 ng/ml IFN-γ (LPS/IFN) for 6 h. Upper panels show the representative band patterns of COX-2 and actin. Lower panels show the ratio of band intensities of COX-2 to actin (n = 4). (B) After pre-incubation with emu oil, cells were stimulated with LPS/IFN-γ for 1 h. Upper panels show the representative band patterns of p65 and histone H3. Lower panel shows the ratio of band intensities of p65 to histone H3 (n = 3). (C) After pre-incubation with emu oil, cells were stimulated with LPS/IFN-γ for 1 h. Upper panels show the representative band patterns of p-IκB-α and actin. Lower panel shows the ratio of band intensities of p-IκB-α to actin (n=4). Results are expressed as percentages against the control value. Values are means ± SD. Asterisks show significant difference from the LPS/IFN group. *, $P < 0.05$; **, $P < 0.01$.

inflammatory responses of macrophages by downregulation of pertinent intracellular signaling pathways. Emu oil is rich in unsaturated fatty acids (UFAs) compared with other animal oils[17], and some UFAs are known to have anti-inflammatory properties. Oleic acid and linoleic acid have been reported to inhibit the production of TNF-α and NO by suppressing NF-κB signaling in LPS-stimulated RAW264.7 cells[7]. It has also been shown that α-linolenic acid reduces the production of TNF-α and IL-6 in LPS/IFN-γ-stimulated RAW264.7 cells[25] and inhibits palmitic acid-induced NF-κB activation in C2C12 myotubes[8]. Because oleic acid, linoleic acid and α-linolenic acid are abundant in emu oil, the inhibitory effects of emu oil on inflammatory responses of macrophages shown in the present results might be mediated by these UFAs.

On the other hand, Yoganathan *et al.* compared the healing effects of topical treatments of emu oil, linseed oil and olive oil on the croton oil-induced inflammation of mouse earlobes and demonstrated that emu oil was the most effective, although other oils contain larger proportions of UFAs than does emu oil. These results suggest that the presence of UFAs may not sufficiently explain the anti-inflammatory effects of emu oil[26]. Although the minor constituents in the non-triglyceride fraction of emu oil have been shown to have antioxidant or radical scavenging activities[5], it is unclear whether these constituents contribute to the anti-inflammatory effects of emu oil. Further study is needed to clarify the active components underlying the anti-inflammatory properties of emu oil.

Stimulation with LPS has been shown to enhance phagocytic activity of macrophages[11]. Because activation of phagocytosis is known to induce cytokine productions from macrophages[2], it can be thought that the reduction of cytokine release by emu oil is partly due to an inhibition of phagocytic activity. However, our previous study showed that phagocytic activities were unaffected by emu oil in activated macrophages although the cells were stimulated solely by LPS, not LPS/IFN-γ[20]. Thus it may be unlikely that emu oil reduced cytokine productions from macrophages via the inhibition of phagocytosis. Nevertheless it remains the possibility that emu oil modulates signaling pathways associated with phagocytosis-induced cytokine productions. Further researches

are needed to clarify the effects of emu oil on signal transductions related to phagocytosis.

In the field of clinical practice of veterinary medicine, emu oil has been already used to treat dermatitis of several animals, including dog and cow, with good results (personal communication). However, no scientific case report regarding the effects of emu oil on inflammatory diseases of animal patients has been published. Human clinical trials have revealed that topical treatment with emu oil has beneficial effects in the relief of inflammatory symptoms such as arthralgia and skin swelling[6, 23]. Because skin sensitization testing has supported the safety of emu oil on human skin[19], emu oil will likely have few adverse effects on animals. Thus, emu oil may be a valuable tool for the treatment of inflammatory diseases of animals, and the applicability of the healing effects of emu oil on animal patients in veterinary clinical practice deserves further investigation.

Conflict of interest

The authors declare that they have no conflicts of interest.

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