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Oleamide suppresses inflammatory responses in LPS-induced RAW264.7 murine macrophages and alleviates paw edema in a carrageenan-induced inflammatory rat model

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ABSTRACT

Oleamide compounds purified from green algae have been used for the prevention and treatment of atherosclerosis, thrombosis, arthritis, and cancer. They function through their metabolic conversion into prostaglandins, thromboxanes, and leukotrienes. However, the actual mechanism of action has not been well characterized. To investigate the underlying anti-inflammatory activity and associated mechanisms, oleamide purified from *Codium fragile* was studied using RAW264.7 murine macrophages and a carrageenan-induced inflammatory rat model. Our results indicate that pre-treatment of RAW264.7 cells with oleamide significantly suppressed LPS-induced nitrite production and PGE_2 secretion. Oleamide inhibited LPS-induced iNOS and COX-2 mRNA and protein expression. It also inhibited the LPS-induced production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6. In addition, oleamide prevented the nuclear translocation of NF-κB by suppressing the phosphorylation of the inhibitor of kappa B (IκB)-α. Oleamide also suppressed the phosphorylation of mitogen-activated protein kinases such as ERK1/2 and JNK. Furthermore, inhibition of paw swelling (%) was suppressed 2 h after the intraperitoneal injection of oleamide (20 mg/kg, body weight) in a carrageen-induced rat model. Therefore, our results suggest that oleamide can be used as a single ingredient treatment for inflammatory diseases.

1. Introduction

Marine biological products have proven to be valuable raw materials for the treatment of a variety of inflammatory diseases, without causing adverse effects [1]. *Codium fragile* is an edible alga from the family Codiaceae, and is widely distributed on the coasts of East Asia, Oceania, and Northern Europe. In Korea, *Codium fragile* is one of the green algae used as a culinary ingredient and has been used in traditional medicine for the treatment of enterobiasis, dropsy, and dysuria [2]. Several studies have reported that various extracts of *Codium fragile* have anti-inflammatory effects [3,4]. Oleamide is a naturally occurring oleic acid metabolite which is to be involved as a neuropeptide related to sleep. It may also be found in a traditional Chinese medicine involved in sedation [5]. Oleamide is reported to perform a variety of other functions, including inhibition of gap junction functions and apoptosis, hypothermia, anti-nociception, and modulation of receptor and neurotransmitter systems [6–8]. However, few studies have reported the anti-inflammatory effects of oleamide.

Inflammation is a defense response that is triggered by many different stimulating factors, including physical damage, precursor chemicals, microbial invasion, and immune responses in the body [9]. It is essential for healing and recovery when humans cope with an infection or are injured. Unfortunately, the defense responses can sometimes lead to other inflammatory diseases [10]. The inflammatory process is usually characterized by the recruitment of leukocytes and macrophages. Lipopolysaccharide (LPS) rapidly activates macrophages and stimulates the secretion of pro-inflammatory cytokines as well as inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2), via inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, respectively [11,12].

The expression of these inflammatory mediators is induced by the nuclear factor-kappa B (NF-κB) p65 subunit and mitogen-activated

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protein kinases (MAPKs), which also control important molecular mechanisms that drive anti-inflammatory effects [13,14]. Under naïve cellular conditions, NF-κB is localized in the cytoplasm as an inactive transcription factor that is associated with the inhibitor of kappa B (IκB)-α [15]. Phosphorylation of NF-κB p65, which is induced by pro-inflammatory cytokines, causes NF-κB to dissociate from IκB and translocate to the nucleus as an active transcription factor to regulate the inflammatory response [16]. In addition, phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, C-Jun N terminal kinase (JNK), and p38 is involved in catabolic metabolism and inflammatory responses [17]. Recently, Oh et al. reported that oleamide suppresses the LPS-induced inflammatory response-related expression of iNOS and COX-2 through inhibition of NF-κB activation in BV2 murine microglial cells [18]. The anti-inflammatory effect of oleamide in BV2 microglia suggests that it may have inflammation-inhibitory effects in macrophages.

In the present study, we confirmed the anti-inflammatory effect and molecular mechanism of oleamide, both in vitro and in vivo. Our results suggest that oleamide reduces the expression of inflammatory mediators such as iNOS, COX-2, and pro-inflammatory cytokines by inhibiting ERK1/2 and JNK phosphorylation and NF-κB activity in LPS-induced RAW264.7 macrophages. Oleamide also suppresses paw edema thickness following its intraperitoneal injection in a carrageenan-induced rat paw edema model.

2. Materials and methods

2.1. Reagents

Oleamide ((*Z*)-octadec-9-enamide) was 95% pure according to lot-specific CoA and was purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin solution were purchased from WelGene (Deagu, Republic of Korea). Fetal bovine serum (FBS) was purchased from Corning (Corning, NY, USA).

2.2. Purification of oleamide purified from Codium fragile by preparative HPLC

Powdered aqueous extract from *Codium fragile* (1.00 g) was extracted ten times with 10 ml of methanol by sonicating for 10 min. After centrifugation at 3000 rpm for 10 min, the supernatants were combined in a 10 ml volumetric flask and adjusted to the final volume with methanol. The sample solutions were separated using a Shim-pack Prep-ODS column (20 mm i.d. × 250 mm L.; Shimadzu) protected by a Security Guard SemiPrep C18 column (10 mm i.d. × 10 mm L.; Phenomenex, Torrance, CA, USA) at 40 °C in a Prominence preparative HPLC system equipped with a photo diode array (PDA) (Shimadzu, Kyoto, Japan). Mobile phases included acetonitrile (solvent A) and water (solvent B). A gradient elution program was used for the preparative separation as follows: 50% A (0.1 min), 50–100% A (20 min), then 100% A (30 min). The flow rate was 25 ml/min while the detected wavelength was 203 nm. The injection volume was 2 ml. The preparative HPLC equipment was controlled by LabSolution ver. 5.89 Chromatography Data Software (Shimadzu, Kyoto, Japan).

2.3. Identification of oleamide

Identification of oleamide was further analyzed by using a liquid chromatography-electrospray ionization-mass spectroscopy (LCMS-IT-TOF, Shimadzu, Kyoto, Japan). Gradient programming was performed with the mobile phase, combining solvent A (acetonitrile) and solvent B (water) as follows: 70% A (0.1 min), 70–100% A (7 min), then 100% A (20 min). The flow rate was 0.2 ml/min. The injection volume was 5 μl, and the column temperature was maintained at 40 °C. The signal was monitored at 203 nm with the diode array detector (DAD). The MS was operated with a probe voltage of 4.50 kV by ESI in positive mode, CDL temperature of 200 °C, block heater temperature of 200 °C, nebulizer gas flow of 1.5 L/min, ion accumulation time of 10 ms, and MS range of *m/z* 100 to 1000. All data were recorded and analyzed using Shimadzu software, LCMS solution ver. 3.60 and Formula Predictor Ver. 1.2 for Accurate Mass Calculator (Shimadzu, Kyoto, Japan).

2.4. Cell culture and viability assay

Murine RAW264.7 macrophages were obtained from the Korea Research Institute of Bioscience & Biotechnology (KRIBB, Daejeon, Republic of Korea). Cells were cultured at 37 °C in a 5% CO_2-humidified incubator in DMEM containing 10% FBS and 1% penicillin/streptomycin. RAW264.7 cells were seeded at 5×10^5 cells/ml in 12-well plates. They were pre-treated for 1 h with various concentrations of oleamide and subsequently cultured with LPS (0.1 μg/ml) for 24 h. A total of 50 μl of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Sigma-Aldrich) was added to each well. After 4 h of incubation at 37 °C, the supernatant was aspirated, and formazan crystals were dissolved in 1 ml of DMSO at room temperature for 15 min under gentle agitation. The absorbance per well was measured at 595 nm using a microplate Reader (Epoch BioTek Instruments, Inc., headquartered in Winooski, VT, USA).

2.5. Measurement of NO and PGE_2

Cells were seeded at 1×10^6 cells/ml in 6-well plates. They were pretreated for 1 h with various concentrations of oleamide and subsequently cultured with LPS (0.1 μg/ml) for 24 h. Nitrite accumulation in the culture medium was measured as an indicator of nitrite production, based on the Griess reaction. In brief, 100 μl of each supernatant was mixed with an equal volume of Griess reagent (1% [w/v] sulfanilamide in 5% [v/v] phosphoric acid and 0.1% [w/v] naphthylethylenediamine) in a dark room for 10 min. The absorbance was then measured at 540 nm using a microplate Reader (Epoch BioTek Instruments, Inc., headquartered in Winooski, VT, USA). The nitrite concentration was determined by comparison to a standard curve for sodium nitrite. The production of PGE_2 was measured using a Parameter Prostaglandin E_2 Assay Kit (R&D Systems, Minneapolis, MN, USA).

2.6. Isolation of total RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells, using the TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using 1 μg of total RNA and the PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio Inc., Otsu, Japan). cDNA was amplified by polymerase chain reaction (PCR) using the following primers: mouse iNOS (732 bp) sense 5′-TTG TGCATCGACCTAGGCTGGAA-3′ and antisense 5′-GACCTTTCGCATTA GCATGGAAGC-3′, mouse COX-2 (395 bp) sense 5′-GTACTGGC TCATG CTGGACGA-3′ and antisense 5′-CACCATACACTGCCAGGTCAGCAA-3′, and GAPDH (382 bp) sense 5′-GGACTGTGGTCATGAGCCCTTCCA-3′ and antisense 5′-ACTCACGG CAAATTCAACGGCAC-3′. The PCR products were visualized via agarose gel electrophoresis. Standard deviations represent data from three independent experiments. GAPDH was used as an internal control to evaluate the relative expression of iNOS and COX-2.

2.7. Extraction of nuclear and cytoplasmic protein

The nucleic and cytoplasmic extraction was prepared using an NE-PER Nuclear Cytoplasmic Extraction Reagent kit (Pierce, Rockford, IL, USA) according to the manufacturer's instruction. Briefly, cells were washed twice with PBS and centrifuged at 500*g* for 5 min. The cell pellet was suspended in 200 ml of cytoplasmic extraction reagent I (CER I) by vigorously vortex. The suspension was incubated on ice for 10 min