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LPS-stimulated RAW264.7 macrophage CAT-2–mediated L-arginine uptake and nitric oxide biosynthesis is inhibited by omega fatty acid lipid emulsion

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ABSTRACT

Background: Omega-3 fatty acid (ω-3 FA) lipid emulsion has been reported to inhibit nitric oxide (NO) production and alter inducible nitric oxide synthase (iNOS) protein expression in lipopolysaccharide (LPS)-stimulated murine macrophages. However, the role of cellular uptake of L-arginine and iNOS transcription in ω-3 FA emulsion-induced inhibition of NO has not been explored. In addition, cationic amino acid transporter-2 (CAT-2) can regulate iNOS activity. The effect of ω-3 FA emulsion on CAT-2 expression is unknown. In the present study, we hypothesized that ω-3 FA emulsion pretreatment would decrease the production of NO in LPS-stimulated macrophages and that this effect would occur through alterations in the cellular uptake of L-arginine and CAT-2 expression, in addition to iNOS expression.

Methods: Confluent immortalized murine macrophages (RAW264.7cells) were incubated with Dulbecco's modified Eagle's medium, ω-3 FA emulsion, or an isoenergetic ω-6 lipid emulsion for 4 h. The cells were washed and then stimulated with LPS (1 μg/mL) or media alone for 12 or 24 h before harvesting. Greiss reagent was used to assess NO production of plate well supernatants. Cellular uptake of L-arginine was assessed through [^{3}H]-L-arginine. The expression of iNOS and CAT-2 mRNA in harvested RAW264.7 was quantified by reverse transcriptase-polymerase chain reaction.

Results: NO production of unstimulated RAW264.7 cells was similar in all groups. After LPS stimulation, ω-3 FA pretreatment at 12 and 24 h produced significantly less NO ($P < 0.05$) compared with ω-6 FA or media only. ω-3 FA pretreatment at 12 and 24 h resulted in less L-arginine uptake. iNOS and CAT-2 mRNA was significantly decreased with ω-3 FA pretreatment compared with ω-6 FA or media-only treatment ($P < 0.05$).

Conclusions: These experiments demonstrated that, in addition to other anti-inflammatory effects, ω-3 FA lipid emulsion also significantly lowers NO production and L-arginine transport through altered expression of iNOS and CAT-2 in LPS-stimulated RAW264.7 macrophage cells.

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1. Introduction

The production and release of nitric oxide (NO) are involved in numerous cellular processes. The induction of inducible nitric oxide (iNOS) and the resultant overproduction of NO have been shown to play an important role in mediating the systemic inflammatory responses and organism injury during sepsis [1,2]. Cationic amino acid transporter-2 (CAT-2) is the main transporter enzyme that mediates cellular uptake of circulating L-arginine (i.e., the sole substrate for iNOS) [3,4]. Kakuda *et al.* [5] reported that CAT-2 mediates L-arginine transport and NO biosynthesis in activated murine macrophages. Using macrophages from CAT-2 knockout mice, Nicholson *et al.* [4] also reported that sustained NO production in macrophages requires CAT-2. These results clearly indicate that CAT-2 plays a crucial role in regulating NO biosynthesis in sepsis.

NF-κB actively participates in the induction of iNOS and CAT-2, and inhibition of NF-κB attenuates NO biosynthesis through inhibition of iNOS and CAT-2 and, in turn, significantly reduces endotoxemia-induced lung injury [6]. Thus, efforts aimed at modulating CAT-2 expression might prove to be a beneficial therapeutic strategy for sepsis.

Previous studies have demonstrated that Omega-3 (ω-3) fatty acids (FAs), specifically, eicosapentaenoic acid (EPA), attenuate cytokine-mediated inflammation through alterations in nuclear transcription factor activation, particularly NF-κB, with resultant inhibition of tumor necrosis factor-α transcription and production [7,8]. ω-3 FA lipid emulsion has been reported to inhibit NO production and alter iNOS protein expression in lipopolysaccharide (LPS)-stimulated murine macrophages [9]. However, the role of cellular uptake of L-arginine in ω-3 FA emulsion-induced inhibition of NO has not been explored. In addition, CAT-2 can regulate iNOS activity and NO production in macrophages [4]. The effect of ω-3 FA emulsion on CAT-2 is unknown. In the present study, we hypothesized that ω-3 FA emulsion pretreatment would decrease the production of NO in LPS-stimulated macrophages and that this effect would occur through alterations in cellular uptake of L-arginine and CAT-2 expression, in addition to iNOS expression.

2. Methods

2.1. Materials

RAW264.7 cells (murine macrophage cell line) were purchased from Chinese Type Tissue Culture Collection (CTCC, Shanghai, China). *Escherichia coli* 0111:B4 LPS was purchased from Sigma Aldrich (St. Louis, MO). A colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) involves the Griess reaction. ω-3 FA lipid emulsion and ω-6 FA lipid emulsion were purchased from Sino-Swed Pharmaceutical (Beijing, China). Endotoxin free serum was purchased from Hyclone (Logan, UT). TRIzol reagent, tissue culture media, and all other cell culture reagents were purchased from Invitrogen (Carlsbad, CA). L-[2.3.4-^{3}H]-arginine hydrochloride was purchased from Perkin Elmer (Waltham, MA) and the Assess reverse transcriptase-polymerase chain reaction (RT-PCR) system from Applied Biosystems (Life Technologies, Carlsbad, CA).

2.2. Cell culture

RAW264.7 cells were suspended in complete medium. Dulbecco's modified Eagle's medium (DMEM) was supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin. The cells were plated in 6-well plates at a density of 2×10^6 cells/well and 24-well plates at a density of 5×10^5 cells/well. All experiments were performed in a humidified atmosphere under 5% carbon dioxide at 37°C.

2.3. Experimental design

The cells were randomly separated into 3 groups: media alone (DMEM), ω-3 FA emulsion, or ω-6 FA emulsion. The cells were allowed to adhere for 2 h and then incubated with each treatment or DMEM alone for 4 h. Equivalent FA concentrations for the ω-3 and ω-6 emulsions were prepared by dilution for experiments as previously reported [7,8]. In brief, the ω-3 FA emulsion containing 2.1 g/100 mL EPA was added in concentrations of 1.44 μL/mL corresponding to 100 μM EPA. Control experiments were performed with a lipid emulsion based on soybean oil (ω-6 FA) at the same dilution to exclude a nonspecific effect of lipid emulsion on the cells. Cell viability assays were also performed and found no increase in toxicity with these emulsions at these concentrations [4]. The cells were then washed twice with DMEM and stimulated with 1 μg/mL LPS for 12 or 24 h.

2.4. Assessment of NO production

The cells were pretreated with media rich in ω-3 FA, ω-6 FA, or media alone for 4 h and then stimulated with LPS for 12 or 24 h. Supernatants were then collected and analyzed for the concentration of stable NO metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), using a colorimetric assay kit (Cayman Chemical) that involves the Griess reaction. Nitrate concentrations in culture media (and nitrate standards) were measured after reducing it into nitrite using the catalyst, with incubation for 1 h at room temperature. The absorbance was read on a microplate reader (Bio-Tek Instruments, Winooski, VT), using a test wavelength of 540 nm.

2.5. Assessment of L-arginine uptake

The cells were cultured for 12 or 24 h in the absence or presence of test substances. Next, the cellular uptake of L-arginine was determined using previously published protocols [10,11]. In brief, macrophages were washed twice with the solution (137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10.0 mM HEPES-Tris [pH 7.4]) and then incubated at 37°C for 2 min in uptake solutions (137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10.0 mM HEPES-Tris [pH 7.4]) supplemented with 0.1 mM L-arginine containing 1.0 μCi/mL L-[3H] arginine. Uptake was stopped with ice-cold stop solution (137 mM NaCl, 14 mM Tris-HCl [pH 7.4]). The cells were then allowed to dry