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Inhibitory effect of dihydroartemisinin against phorbol ester-induced cyclooxygenase-2 expression in macrophages

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ABSTRACT

Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin isolated from the traditional Chinese herb *Artemisia annua* L., has recently been shown to possess antitumor activity in various cancer cells. However, the effect of anti-inflammatory potentials of DHA in murine macrophage RAW 264.7 cells has not been studied. The present study investigated the effect of COX-2 and molecular mechanisms by DHA in PMA stimulated RAW 264.7 cells. DHA dose-dependently decreased PMA-induced COX-2 expression and PGE_2 production, as well as COX-2 promoter-driven luciferase activity. Additionally, DHA decreased luciferase activity of COX-2 regulation-related transcription factors including NF-κB, AP-1, C/EBP and CREB. DHA also remarkably reduced PMA-induced p65, C/EBPβ, c-jun and CREB nuclear translocation. Furthermore, DHA evidently inhibited PMA-induced phosphorylation of AKT and the MAP Kinases, such as ERK, JNK and p38. Taken together, our data indicated that DHA effectively attenuates COX-2 production via down-regulation of AKT and MAPK pathway, revealing partial molecular basis for the anti-inflammatory properties of DHA.

1. Introduction

Artemisinin, an effective antimalarial drug, is isolated from the traditional Chinese herb *Artemisia annua* L. dihydroartemisinin (DHA), the main active metabolite of artemisinin derivatives, has exhibited the strong anti-cancer effects among the derivatives of artemisinin (Dhingra et al., 2000; Zhou et al., 2005). Many studies have shown that DHA inhibits cell proliferation, induces cell cycle arrest, and promotes apoptosis in human cancer cell lines (Efferth et al., 2001; Lee et al., 2006; Singh and Lai, 2004). Recent hypothesis has been suggesting that artemisinin and its derivatives may be useful as anticancer drugs (Chaturvedi et al., 2010). Previous studies have shown that artemisinin inhibited the production of nitric oxide and the expression of several pro-inflammatory cytokines and matrix metalloproteinases (Hwang et al., 2010; Wang et al., 2009). However, the effect of these anti-inflammatory properties and the signaling pathway of DHA remain unknown.

In experimental practice, RAW 264.7 mouse macrophage cell line stimulated by PMA is widely used as the inflammatory cellular model to study the effect of anti-inflammatory drugs and herbs (Han et al., 2008). Macrophages play an important role in inflammatory disease through the release of cytokines such as TNF-α, IL-1β, IL-6, nitric oxide and other inflammatory mediators (Coussens and Werb, 2002). Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid to prostaglandin E2 in the first step in the biosynthesis of prostanoids. There are two types of COX isoforms: COX-1 and COX-2. COX-1 is constitutively expressed in most tissues, while COX-2 expression is induced by several stimuli, such as mitogens, cytokines, and tumor promoters (Aggarwal et al., 2006). Inducible COX-2 may be responsible for the high prostaglandin levels frequently observed in inflammatory pathology (Jung et al., 2007).

Macrophages stimulated with phorbol 12-myristate 13-acetate (PMA) have been used as a model to overexpress the COX-2 role in the cell differentiation. PMA-induced COX-2 promoter activity requires several enhancer elements including nuclear factor-κB (NF-κB, −223/−214), CCAAT/enhancer-binding protein (C/EBP, −132/−124), and activator protein 1 (AP-1)/cyclic adenosine monophosphate (cAMP)-response element (CRE, −59/−53), which are important for regulating its transcription (Saunders et al., 2001; Schroer et al., 2002). In addition, COX-2 expression also activate mitogen activated protein (MAP) kinases (Han et al., 2008). MAPKs and Akt have been extensively studied relative to their regulation

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of COX-2 **gene** expression (Wang et al., 2009). Upon exposure to PMA transcriptional regulation of COX-2 expression is redundantly modulated by the MAPKs and Akt signaling pathways (Chang et al., 2005).

In the present study, we investigated the effects of DHA on COX-2 expression and its function in mouse macrophages. We found that DHA suppressed PMA-induced COX-2 expression by blocking the MAP kinases (ERK, JNK and p38) and Akt signal transduction cascades, which led to the activation of NF-κB, C/EBP, CRE and AP-1.

2. Materials and methods

2.1. Materials

Chemicals and cell culture materials were obtained from the following sources: phorbol 12-myristate 13-acetate (PMA) from Sigma Co. (St. Louis, MO, USA); prostaglandin E_2 (PGE_2) immunoassay reagents and antibody against COX-2 from Cayman Co. (Ann Arbor, MI, USA); Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin solution from Life Technologies, Inc., (Carlsbad, CA, USA); luciferase assay system from Promega; protein assay kit from Bio-Rad Laboratories, Inc., (Madison, WI, USA); phospho-ERK1/2, phospho-p38 kinase, c-Jun, phospho-JNK, ERK1/2, p38 kinase, JNK, IκB-α, phospho-IκB-α and secondary antibody from Cell Signaling Technology (Danvers, MA, USA); and ECL chemiluminescence system and polyvinylidene difluoride (PVDF) membrane from Amersham Pharmacia Biotech (Uppsala, Sweden). All chemicals were of the highest grade commercially available.

2.2. Cell culture and treatment

RAW 264.7 cells, a mouse macrophage cell line, were obtained from the American Type Culture Collection (Bethesda, MD) and grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine,100 μ/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2/95% air incubator. DHA was dissolved in dimethyl sulfoxide (DMSO), and the stock solutions were added directly to the culture media. Control cells were treated with only DMSO, and the final DMSO concentration was always <0.1%.

2.3. Measurement of cell viability

Cell cytotoxicity was examined using a MTT and a lactate dehydrogenase (LDH) release assay kit by measuring the according to the manufacturer's instruction. Briefly, RAW 264.7 cells (2×10^5 cells/ml) in 10% FBS-DMEM were seeded onto 48-well plates. DHA (1–200 μM) was added to the wells and the plates were incubated at 37 °C for 24 h. The cell supernatant was used for LDH determination at 490 nm using a microplate reader (Varioskan, Thermo Electron Co.). The cells were treated with MTT solution for 1 h, and the dark blue formazan crystals that formed in intact cells were solubilized with dimethyl sulphoxide. The absorbance was measured at 550 nm using a microplate reader.

2.4. PGE_2 production

RAW 264.7 cells were incubated with DHA and/or PMA (100 nM). After incubating for 24 h, the culture medium was collected and the PGE_2 levels therein were measured using a specific enzyme immunoassay, according to the manufacturer's instructions.

2.5. Western blotting

After treatment, the cells were collected and washed with phosphate buffered saline (PBS). The harvested cells were then lysed on ice for 30 min in 100 μL lysis buffer (120 mM NaCl, 40 mM Tris [pH 8], 0.1% NP40 [Nonidet P-40]) and centrifuged at 12,000 rpm for 30 min. The supernatants were collected from the lysates, and protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of total cellular protein (50 μg) were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. After blocking, the membranes were incubated with the target antibody. An HRP-conjugated secondary antibody to IgG was used. Immunoreactive proteins were visualized using the ECL Western blot detection system.

2.6. RNA preparation and gene expression analysis by real-time quantitative PCR

Cells were plated in 6-well plates at a density of 1×10^5 cells/well and incubated with DHA and/or PMA for 16 h. Total RNA from the treated cells was then prepared with RNAiso Reagent (Takara) according to the manufacturer's protocol and stored at −80 °C until use. PCR product formation was continuously monitored during the PCR reaction using Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA, USA). Accumulated PCR products were detected directly by monitoring the increase of the reporter dye (SYBR). The expression levels of COX-2 in the exposed cells were compared to this in control cells at each collection time point using the comparative cycle threshold (Ct)-method (Wadleigh et al., 2000). The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of actin, a housekeeping gene.

2.7. Plasmids

The human COX-2 promoter-luciferase deletion constructs (−1432/+59, −327/+59, −220/+59 and −124/+59) and mutant constructs (mNF-κB, mC/EBP, mAP-1/CRE and mC/EBP-AP-1/CRE) were generous gifts from Dr. Hiroyasu Inoue (Nara Women's University, Nara, Japan) and have been described elsewhere (Han et al., 2008). mNF-κB represents the −327/+59 COX-2 promoter construct in which the NF-κB site was mutagenized; mC/EBP represents the −327/+59 COX-2 promoter construct in which the C/EBP site was mutagenized; mAP-1/CRE represents the −327/+59 COX-2 promoter construct in which the AP-1 and CRE sites were mutagenized; mC/EBP-AP-1/CRE represents the −327/+59 COX-2 promoter construct in which the C/EBP, AP-1 and CRE sites were mutagenized. pNF-κB-Luc, pC/EBP-Luc, pAP-1-Luc and pCRE-Luc were purchased from Stratagene (La Jolla, CA, USA).

2.8. Transfection and β-galactosidase assays

Transient transfection was performed according to a modified method described previously (Han et al., 2008). Cells were incubated in 24-well plates at density of 5×10^4 cells and then transiently co-transfected with test plasmid and pCMV-β-gal using Lipofectamine™ 2000 reagent, according to the manufacturer's instructions. Luciferase activities were normalized to β-galactosidase activities and expressed relative to the luciferase activity of control cells.

2.9. Statistical analysis

All experiments were performed in triplicate. A one-way analysis of variance (ANOVA) was used to determine the significance of