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Caffeine prevents LPS-induced inflammatory responses in RAW264.7 cells and zebrafish

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ABSTRACT

Caffeine is a white crystalline xanthine alkaloid found in the seeds of coffee plants and leaves of the tea bush. In this study, we evaluated whether caffeine exerts anti-inflammatory effects on lipopolysaccharide (LPS)-induced inflammation both *in vitro* and *in vivo*. RAW264.7 cells were treated with various concentrations of caffeine in the presence or absence of LPS. Caffeine decreased the LPS-induced inflammatory mediator, nitric oxide (NO). Caffeine treatment also reduced the expression of pro-inflammatory genes, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin (IL)-3, IL-6 and IL-12, and decreased both IL-6 secretion and phosphorylated p38MAPK expression in LPS-treated RAW264.7 cells. Caffeine inhibited nuclear translocation of nuclear factor κB (NF-κB) via IκBα phosphorylation. In addition, caffeine inhibited LPS-induced NO production in zebrafish. These results suggest that caffeine may suppress LPS-induced inflammatory responses in RAW264.7 cells by regulating NF-κB activation and MAPK phosphorylation.

1. Introduction

There are two types of inflammation, acute and chronic. Acute inflammation is a complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants. Acute inflammatory responses cause pain, heat, itching, swelling, and redness. A dysfunctional acute inflammatory response can cause several serious problems. In addition, chronic inflammation is a cause of cancer, allergies, asthma, and diabetes [1,2]. Therefore, there is an important need for effective methods of regulating inflammation. Inflammatory-mediated key factors, nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK), are important signaling molecules in the Toll-like receptor (TLR) pathway. Regulation of these factors is widely recognized as a good strategy for suppressing various types of inflammation [3–5]. More specifically, NF-κB is an important factor in the transcriptional regulation of pro-inflammatory genes, such as interleukin-6 (IL-6), nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). In addition, activation of the MAPK pathways, including p38, JNK, and ERK, is also required for NF-κB subunit p65 transactivation [6]. Therefore, NF-κB activation is widely implicated in inflammatory diseases and there has been substantial attention on the development of anti-inflammatory drugs targeting NF-κB [7].

Caffeine is one of the phytochemicals found in coffee plants that is known to have biological characteristics, such as antioxidant, antiaging and antiobesity effects [8,9]. The most well-known source of caffeine is the *coffea Arabica* plant seed. Caffeine is a white crystalline xanthine alkaloid extracted from seeds of the coffee plant and leaves of the tea bush. People consume a large quantity of caffeine, a popular natural compound, in their daily lives [10].

Zebrafish (*Danio rerio*) is becoming a popular *in vivo* model for examining the biological mechanisms of compounds. The advantages of zebrafish include its short life cycle, high reproductive rate, and easy care. Recently, zebrafish has emerged as an *in vivo* model system for detection of LPS-induced nitric oxide (NO) production [11]. Therefore, zebrafish is a proper model for assessing drug efficacy, toxicity and safety. A recent study suggested that caffeine

Abbreviations: NF-κB, nuclear factor kappa B; MAPKs, mitogen-activated protein kinases; LPS, lipopolysaccharide; NO, nitric oxide; iNOS, nitric oxide synthase; COX-2, cyclooxygenase-2; IL, interleukin; IKK, IκB kinase; IκB, inhibitor of kappa B; TLR, toll-like receptor; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase.

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suppresses lipopolysaccharide (LPS)-stimulated BV2 microglial cells by inhibiting NF-κB activation and ERK phosphorylation [12]. However, anti-inflammatory effects on LPS-treated RAW264.7 cells and zebrafish have not yet been reported.

The purpose of this study was to assess the anti-inflammatory effects of caffeine and potential signaling pathways in murine macrophage-like RAW264.7 cells. In addition, we investigated whether caffeine inhibits the inflammatory response in LPS-stimulated zebrafish *in vivo*.

2. Materials and methods

2.1. Reagents and antibodies

Caffeine was purchased from Santa Cruz Biotechnology (sc-202514; Santa Cruz, CA, USA). LPS (*Escherichia coli* 0111:B4) was purchased from Sigma (St. Louis, MO, USA). Antibodies against iNOS, COX-2, p-IKKα/β, IKKβ, p-IκBα, IκBα, NF-κB p65, p-p38, p38, p-ERK, ERK, and JNK2 were purchased from Cell Signaling Technology (Boston, MA, USA). p-JNK, β-actin, and Lamin B antibodies were purchased from Santa Cruz Biotechnology.

2.2. Cell culture and viability

RAW264.7 cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were grown at 37 °C in a 5% CO_2/air environment. To evaluate the cell viability, RAW264.7 cells were plated at a concentration of 10,000 cells/well in a 96-well plate, and the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) assay was performed as described by Kim et al. [13]. For the cell viability analysis, RAW264.7 cells were treated with 0, 100, 400, 800, 1000, and 1200 μM of caffeine.

2.3. Nitric oxide assay

RAW264.7 cells were seeded into six-well plates at a density of 1×10^5 cells/well. RAW264.7 cells were treated with caffeine (200 μM) and incubated for 1 h. LPS (1 μg/ml) was added to the culture; then, the cells were incubated for 24 h at 37 °C in a CO_2 incubator. Nitrite release in the culture media was determined using the Griess reaction and presumed to reflect the NO levels. After various treatments, 100-μl cell culture medium was mixed with 100-μl Griess reagent and incubated at room temperature for 15 min. The NO concentration was determined at 540 nm using $NaNO_2$ as a standard.

2.4. Cytokine assays

RAW264.7 cells were pre-treated with caffeine for 1 h and then stimulated with or without LPS (1 μg/ml) for 24 h. Culture media IL-6 levels were quantified using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (BioLegend, San Diego, CA, USA).

2.5. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs were extracted using TRIzol reagent (Invitrogen) and subsequently used to generate cDNA using an RT-PCR system (total RNA, 1 μg). Target gene amplification was performed using specific oligonucleotide primers in a normal PCR system. The primer sequences were as follows: iNOS, forward (5′-CCCTTCCGAAGTTTCTGGCAGCAG-3′) and reverse (5′-GGCTGTCA-GAGCCTCGTGGCTTTG-3′); COX-2, forward (5′-ATGCTCCTGCTT-GAGTATGT-3′) and reverse (5′-CACTACATCCTGACCCACTT-3′); IL-3, forward (5′-GACCCATGGGCCATGAGGAACATT-3′) and reverse (5′-GAAGTGGATCCTGAGGACAGATAC-3′); IL-6, forward (5′-CCATCTCTCCGTCTCTCACC-3′) and reverse (5′-AGACCGCTGCCTGT CTAAAA-3′); IL-12, forward (5′-TTTTGGGGACTCTTCCATC-3′) and reverse (5′-TTCTTTGTTCGAATCCAGCG-3′); GAPDH, forward (5′-AACTTTGGCATTGTGGAAGG-3′) and reverse (5′-ACA-CATTGGGGGTAGGAACA-3′). PCR products were analyzed on 1% agarose gels and bands were visualized using ethidium bromide staining. The expression levels were quantified by scanning with a gel documentation and analysis system (Image J, Bethesda, MD, USA).

2.6. Western blot analysis

RAW264.7 cells were washed in 1 × phosphate-buffered saline (PBS), lysed in lysis buffer (1 M Tris-HCl, 0.5 M EDTA, 0.5 M EGTA, 1.5 M NaCl, 10% NP-40, 10% SDS, 10% sodium deoxycholate, β-mercaptoethanol, 0.1 M PMSF, 0.1 M benzamidine, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 0.25 mg/ml pepstatin, 0.1 M sodium orthovanadate, phosphatase inhibitor cocktail 2, phosphatase inhibitor cocktail 3, 1 M sucrose, sodium fluoride, sodium pyrophosphate, and β-glycerol phosphate), and centrifuged to remove cell debris. Cytosolic and nuclear proteins were extracted using NE-PERTM nuclear and cytoplasmic extraction reagents (Thermo Scientific) according to the manual. The protein content of the supernatant was determined using the Bradford assay. Protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Immune-Blot PVDF membrane, Bio-Rad). Membranes were immunoblotted with primary antibodies specific for iNOS, COX-2, p65, p-IκB-α, IκB-α, p-IKKα/β, IKKβ, p-ERK, ERK, p-p38, p38, p-JNK, JNK, and β-actin at 4 °C overnight. Membranes were then treated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000) for 2 h. Bands were visualized using an enhanced chemiluminescence system (ECL, Thermo Fisher Scientific) and LAS image software (Fuji, New York, NY, USA).

2.7. Nitrite determination in zebrafish

Synchronized zebrafish embryos were collected and rearranged using a pipette (20 embryos/well) in six-well plates containing 2 ml of egg water for 7–9 h post-fertilization (hpf) and incubated with or without the indicated concentrations of caffeine for 1 h. Zebrafish were stimulated with LPS (10 μg/ml) for 24 h at 28.5 °C. Thereafter, zebrafish embryos were transferred into fresh embryo medium. The NO levels in LPS-treated zebrafish were measured using a fluorescent probe dye, diaminofluorophore 4-amino-5-methylanino-2, 7-difluorofluroescein diacetate (DAF-FM DA). Transformation of DAF-FM DA by NO generates highly fluorescent triazole derivatives. LPS-stimulated zebrafish larvae were transferred into 96-well plates and treated with DAF-FM DA solution (5 μM) for 1 h in the dark at 28.5 °C. Following incubation, zebrafish larvae were rinsed in fresh zebrafish embryo medium and anesthetized in tricaine methanesulfonate solution prior to observation. The fluorescence intensity of individual zebrafish larvae was quantified using an ECLIPSE E600 fluorescence microscope (Nikon, Tokyo, Japan).