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Protocatechuic acid inhibits Toll-like receptor-4-dependent activation of NF-κB by suppressing activation of the Akt, mTOR, JNK and p38-MAPK

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ABSTRACT

Protocatechuic acid has demonstrated to have antioxidant and anti-inflammatory effects. We assessed whether protocatechuic acid may reduce the inflammatory mediator production, which is regulated by the Toll-like receptor-4-dependent Akt, mTOR and NF-κB pathway, and JNK and p38-MAPK in HaCaT cells and primary keratinocytes. Protocatechuic acid, Akt inhibitor, Bay 11-7085 and *N*-acetylcysteine reduced the lipopolysaccharide-caused production of cytokines and chemokines, expression of cyclooxygenase, increase in the levels and activities of Toll-like receptor-4, p-Akt and mTOR, activation of NF-κB, phosphorylation of the JNK and p38-MAPK, and production of reactive oxygen species in keratinocytes. Inhibitors of the c-JNK (SP600125) and p38-MAPK (SB203580) reduced lipopolysaccharide-caused production of inflammatory mediators, activation of the JNK and p38-MAPK, and production of reactive oxygen species in keratinocytes. These results show that protocatechuic acid may inhibit the lipopolysaccharide-stimulated inflammatory mediator production in keratinocytes by reducing the Toll-like receptor-4-dependent activation of Akt, mTOR and NF-κB pathways, and activation of JNK and p38-MAPK. The suppressive effect of protocatechuic acid appears to be associated with inhibition of the reactive oxygen species production. Protocatechuic acid appears to reduce the microbial product-caused inflammatory skin diseases.

1. Introduction

Microbial products, including lipopolysaccharide, cause the production of proinflammatory cytokines and chemokines through the activation of Toll-like receptors (TLRs) [1–3]. Lipopolysaccharide binding to keratinocyte CD14 and subsequent activation of TLR-4 causes the activation of nuclear factor (NF)-κB, leading to the production of cytokines and chemokines [3,4]. Lipopolysaccharide triggers the activation of the phosphatidylinositol (PI) 3-kinase/Akt/mammalian target of rapamycin (mTOR) signaling pathways, which is followed by activation of transcription factors, including NF-κB [5,6]. NF-κB regulates genes responsible for the innate and adaptive immune responses as well as inflammation [7]. The mitogen-activated protein (MAP) kinases play a crucial role in maintenance of intracellular functions, inflammation, innate immunity [8–10]. MAPK subfamilies include ERKs, c-Jun N-terminal kinases (JNKs), and p38-MAPKs [9,10]. Lipopolysaccharide has been shown to exhibit a proinflammatory effect on various tissues through activation of the NF-κB and the JNK, p38-MAPK, Akt pathways [11–13].

Protocatechuic acid (PCA), a major metabolite of antioxidant polyphenols found in green tea, has been shown to have antioxidant and anti-inflammatory effects [14–16]. PCA suppresses production of IL-6 and IL-8, and activation of NF-κB in lipopolysaccharide-stimulated human gingival fibroblasts [15]. PCA inhibits lipopolysaccharide-caused production of TNF-α, IL-6, IL-1β, and PGE2, and expression of TLR-4 by inhibiting NF-κB and MAPKs signaling pathways in BV2 microglia [17]. PCA suppresses ovalbumin-induced airway inflammation by the inhibition of the extracellular signal-regulated protein kinase (ERK), p38-MAPK phosphorylation and the NF-κB activation in a mouse allergic asthma model [18]. PCA attenuates the carrageenan-induced paw edema, cotton pellet-induced granuloma and Freund's adjuvant-induced arthritis in rats [14]. PCA inhibits the leukocyte number, the levels of TNF-α, PGE_2, cyclooxygenase (COX)-2 expression, and NF-κB activation in the exudates of the air pouch in carrageenan-treated BALB/c mice [19]. In contrast to these reports, PCA potentiates inflammatory leukocyte-derived oxidative stress in mouse skin via a tyrosinase-bioactivation pathway [20].

PCA has demonstrated to have antioxidant and anti-inflammatory effects. Nevertheless, in keratinocytes the PCA effect on the lipopolysaccharide-stimulated inflammatory mediator production has not been

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studied. Furthermore, it is unclear whether the preventive effect of PCA on the lipopolysaccharide-stimulated inflammatory mediator production is achieved by reducing activation of the Akt, mTOR and NF-κB and the activation of the JNK and p38-MAPK. In this respect, we assessed the PCA effect on lipopolysaccharide-stimulated inflammatory mediator production in human keratinocytes.

2. Materials and methods

2.1. Materials

Protocatechuic acid, Bay 11-7085 (An inhibitor of NFκB activation and phosphorylation of IκBα, (2E)-3-[[4-(1,1-dimethylethyl)phenyl] sulfonyl]-2-propenenitrile), the Akt inhibitor (type II, SH-5), rapamycin (a mTOR inhibitor), SB203580 (a selective inhibitor of p38-MAPK) and horseradish peroxidase-conjugated anti-mouse IgG were obtained from EMD-Calbiochem. Co. (La Jolla, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for human CXCL1/IL1β, human IL6, human TARC/CCL17, human CTACK/CCL27, human cyclooxygenase (COX)-2 and human/mouse/rat phosphorylated-Akt (Pan) were obtained from R&D systems, Inc. (Minneapolis, MN, USA). Antibodies (Toll-like receptor (TLR)-4, NF-κB p65, NF-κB p50, phosphorylated-IκB-α, Akt1, phosphorylated-Akt1, mTOR, JNK, phosphorylated-JNK, p38, phosphorylated-p38, and β-actin) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The TransAM™ NF-κB p65 to DNA binding assay kit was obtained from Active Motif® (Carlsbad, CA, USA). Lipopolysaccharide (from *Escherichia coli*), SP600125 (a selective inhibitor of c-JNK) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). MitoSOX Red mitochondrial superoxide indicator for live-cell imaging (M36008) was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA).

2.2. Keratinocyte culture

HaCaT cells, a spontaneously transformed aneuploid immortal keratinocyte cell line, were provide from Dr. Seo (Chung-Ang University Hospital, Seoul, South Korea) and cultured in DMEM medium (high glucose) supplemented with 2 mM L-glutamine and 10% fetal bovine serum.

Normal human keratinocytes were provided from the Department of Urology, Chung-Ang University Hospital (Seoul, Korea). The cells were prepared from neonatal foreskin discarded after circumcision [21] in accordance with the ethical guidelines. Neonatal foreskin was chopped and split overnight in a sucrose-trypsin-EDTA solution (0.1% sucrose, 0.25% trypsin and 1 mM EDTA) at 4 °C. Keratinocyte suspension was cultured in the growth factor-supplemented EpiLife® medium (Cascade Biologics™, Portland, OR, USA).

Cell numbers used in the ELISA were based on manufacturers' protocols and adjusted to measure the values under suitable experimental conditions.

Changes in cell morphology were visualized under phase-contrast illumination with an Olympus IX71 inverted microscope (Tokyo, Japan).

2.3. Immunoassays for IL-1β, IL-6, COX-2, CCL17 and CCL27

HaCaT cells (1×10^6 cells/ml of medium for the cytokine and COX assay, and 5×10^6 cells/ml of medium for the chemokine assay) were grown in 24-well plate and treated with 1 μg/ml lipopolysaccharide for 24 h. After centrifugation at 412 × *g* for 10 min, the amounts of IL-1β, IL-6, COX-2, CCL17 and CCL27 in the culture medium supernatants were measured using ELISA kits, according to the manufacturer's instructions. Absorbance of mixture was measured at 450 nm using a microplate reader (Magellan, TECAN, Salzburg, Austria).

2.4. Preparation of keratinocyte cytosolic and nuclear extracts

HaCaT cells (5×10^6 cells/ml of medium) were treated with lipopolysaccharide for 30 min at 37 °C. The levels of TLR-4 in keratinocytes were measured after a 24 h incubation. The cytosolic and nuclear extracts of keratinocytes were prepared, as a previously described method [22]. Keratinocytes collected by centrifugation at 1000 × *g* for 5 min were washed twice with phosphate buffered saline (PBS). Keratinocytes were suspended in lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 100 μM EDTA, 100 μM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 10 mM HEPES-KOH, pH 7.8) and were made to swell on ice for 15 min. Next, a 10% Nonidet NP-40 solution (final concentration of approximately 0.6%) was added, and the mixtures were vigorously vortexed for 10 s. The homogenates were centrifuged at 12000 × *g* for 10 min at 4 °C. The supernatants were stored as cytoplasmic extracts and kept at − 70 °C. The nuclear pellets were re-suspended in an ice-cold hypertonic solution containing 5% glycerol and 0.4 M NaCl. The tubes were located on ice for 30 min and then centrifuged at 12000 × *g* for 10 min at 4 °C. The supernatants as the nuclear extracts were stored at − 70 °C. Protein concentrations were measured, according to the Bio-Rad's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Western blot assay

The cytosolic and nuclear extracts for Western blot were treated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and then boiled. Samples (45 μg protein/well) were loaded onto each lane of a 10–12% sodium dodecyl sulfate-polyacrylamide gel, electrophoresis was done and the proteins were transferred onto polyvinylidene difluoride membranes (GE Healthcare Chalfont St. Giles, Buckinghamshire, UK). The membranes were blocked for 2 h in TBS (50 mM Tris-HCl, pH 7.5 and 150 mM NaCl) containing 0.1% Tween 20. The membranes were labeled with the appropriate antibody (TLR-4, NF-κB p65, NF-κB p50, phosphorylated-IκB-α, Akt1, phosphorylated-Akt1, mTOR, JNK, phosphorylated-JNK, p38, phosphorylated-p38 and β-actin) overnight at 4 °C with gentle agitation. After 4 washes in TBST (TBS containing 0.1% Tween 20), the membranes were treated with horseradish peroxidase-conjugated anti-mouse IgG for 2 h at room temperature. The membranes were then treated with SuperSignal® West Pico chemiluminescence substrate and protein bands have the enhanced chemiluminescence were visualized using an image analyzer (ImageQuant™ LAS4000, GE Healthcare Bio-Sciences AB, Björkgatan 30, 751 84 Uppsala Sweden).

The densities of protein bands were determined using TINA 2.10 g software licensed for Seoul National University (SNU and SNUMD, Seoul, South Korea) and expressed as a fold increase compared to the control density.

2.6. Assay for DNA binding activity of NF-κB p65

The DNA binding activity of NF-κB p65 was measured, according to the TransAM™ NF-κB kit user's manual. Keratinocytes (2×10^6 cells/ml of medium) were treated with lipopolysaccharide for 30 min. Nuclear extracts were prepared, according to the procedure described in the Active Motif® protocol, and added to a 96-well plate to which oligonucleotides containing an NF-κB p65 consensus binding site (5′-GGGA CTTTCC-3′) were immobilized. The amount of nuclear extract in the sample was determined to be 5 μg/20 μl. The NF-κB p65 bound to DNA was treated with a primary antibody specific for NF-κB p65 and then with anti-rabbit horseradish peroxidase-conjugated IgG. The color developer and stop solutions were sequentially added to the well plate. The absorbance of mixture was measured at 450 nm with a reference wavelength of 655 nm in a microplate reader.