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Down-regulation of MAPK/NF-κB signaling underlies anti-inflammatory response induced by transduced PEP-1-Prx2 proteins in LPS-induced Raw 264.7 and TPA-induced mouse ear edema model

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ABSTRACT

Excessive reactive oxygen species (ROS) production plays a crucial role in causing various diseases, including inflammatory disorders. The activation of mitogen-activated protein kinase (MAPK) and nuclear factor-kappaB (NF-κB) signaling is implicated in stimulating inflammatory response and cytokines. Peroxiredoxin 2 (Prx2) is a 2-cysteine (Cys) peroxiredoxin capable of removing endogenous hydrogen peroxide (H_2O_2). PEP-1 peptide, a protein transduction domain, consists of three domains which are used to transduce exogenous therapeutic proteins into cells. The correlation between effectively transduced PEP-1-Prx2 and ROS-mediated inflammatory response is not clear. In the present study, we investigated the protective effects of cell permeable PEP-1-Prx2 on oxidative stress-induced inflammatory activity in Raw 264.7 cells and in a mouse ear edema model after exposure to lipopolysaccharides (LPS) or 12-O-tetradecanoylphorbol-13-acetate (TPA). Transduced PEP-1-Prx2 suppressed intracellular ROS accumulation and inhibited the activity of MAPKs and NF-κB signaling that led to the suppression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and cytokines in LPS-induced Raw 264.7 cells and TPA-induced mouse ear edema model. Given these results, we propose that PEP-1-Prx2 has therapeutic potential in the prevention of inflammatory disorders.

1. Introduction

Cellular metabolism produces ROS such as hydroxyl radicals, superoxide anion, and hydrogen peroxide, which in turn can cause various diseases, including lipid peroxidation, DNA damage, cell inflammation and death, cancer, Parkinson's disease, and ischemia [1–3]. The use of lipopolysaccharides (LPS) or 12-O-tetradecanoylphorbol-13-acetate (TPA) [4,5], which are usually used to induce inflammation and activate macrophages. Further ROS is produced and pro-inflammatory mediators, including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), interleukin-1beta (IL-1β), and tumor necrosis factor-alpha (TNF-α) are released. Moreover, nuclear factor-kappaB (NF-κB) and the mitogen-activated protein kinase (MAPK) pathways are closely associated with inflammatory responses [6,7].

Peroxiredoxin (Prx) serves as an antioxidant converting diverse peroxides such as H_2O_2 or organic hydroperoxide, which are contained in water and alcohols using electrons donated by thioredoxin [8]. Prxs are widespread in cytoplasm, mitochondria, peroxisome, and subcellular compartments including ER membrane. Prx2 is a typical 2-cysteine (Cys) peroxiredoxin [9,10]. Prx2 is able to regulate the activation of the TNF signaling pathway by controlling ROS production, growth factors and endogenous signaling H_2O_2 [11]. Also Prxs affect cell differentiation such as vascular remodeling and act in various cell models as a molecular chaperone [12–14].

Protein transduction technology is designed to transduce exogenous proteins to mammalian cells using protein transduction domains (PTDs) [15–17]. Among PTDs, PEP-1 peptide exhibits greater efficiency in delivering proteins into cells. PEP-1 peptide is characterized by high stability, a lack of toxicity and a lack of sensitivity to serum [18]. Although the involved mechanism is not clear, PTD fusion proteins successfully deliver

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therapeutic proteins into cells in vitro and vivo, and all transduced proteins prevent cell death [19–26]. In this study, we investigated the effects of transduced PEP-1-Prx2 on oxidative stress and inflammatory responses. PEP-1-Prx2 was directly transduced into Raw 264.7 cells and significantly inhibited LPS-induced oxidative stress and inflammatory responses. Applied locally to mouse ears, PEP-1-Prx2 efficiently suppressed the expression of TPA-induced pro-inflammatory cytokines and enzymes and the activity of NF-κB and MAPKs, leading to suppression of ear edema. Having demonstrated the anti-inflammatory activity of PEP-1-Prx2, we suggest therapeutic potential of PEP-1-Prx2.

2. Material and methods

2.1. Materials

TPA was purchased from Sigma–Aldrich (St.Louis, MO, USA). Ni^{2+}-nitrilotriacetic acid Sepharose Superflow was purchased from Qiagen (Valencia, CA, USA) and Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Duchefa (Haarlem, The Netherlands). Fetal bovine serum (FBS) and antibiotics were purchased from Gibco BRL (Grand Island, NY, USA). Synthetic PEP-1 peptides were synthesized by Peptron (Daejeon, Korea). The indicated primary antibodies and actin were purchased from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other commercial chemicals and reagents were of the highest grade available.

2.2. Expression and purification of PEP-1-Prx2 protein

A PEP-1 expression vector created in a previous experiment was reused for this study [26]. The cDNA sequence for human Prx2 was PCR-amplified using the sense primer 5′-CTCGAGGCCTCCGGTAACG-3′ and the antisense primer 5′-GGATCCCTAATTGTGTTTGGAGAAATA-3′. The resulting PCR products were subcloned in a TA cloning vector and ligated into a PEP-1 vector to produce fully-folded PEP-1-Prx2 protein. Control Prx2 was generated in a similar way without using PEP-1.

The PEP-1-Prx2 plasmid was transformed into *E. coli* BL21 (DE3) cells. PEP-1-Prx2 transformed cells were inoculated with 100 ml of LB media containing 100 μg/ml ampicillin at 37 °C and induced by 0.5 mM IPTG for 6 h. Harvested cells were sonicated, and the extracts were purified according to manufacturer's instruction using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography (Amersham, Braunschweig, Germany). Concentration of purified PEP-1-Prx2 was measured using a Bradford assay and serum albumin as the standard protein [27].

2.3. Transduction of PEP-1-Prx2 protein into Raw 264.7 cells

Murine macrophage Raw 264.7 cells were incubated in Dulbecco's modified Eagle's medium containing 20 mM Hepes/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% FBS, and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) at 37 °C in a 95% air, 5% CO_2 atmosphere.

For transduction of PEP-1-Prx2, macrophages were treated with PEP-1-Prx2 at different concentrations (0.1–0.5 μM) for 1 h and later with trypsin-EDTA and washed with phosphate-buffered saline (PBS). Cells were extracted to perform Western blot analysis.

2.4. Western blot analysis

Proteins were separated from cell lysates using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Sorted proteins were transferred to the nitrocellulose membrane, and the membrane was blocked with Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk. The membrane was immunoblotted using primary antibodies as recommended by the manufacturer. Proteins were detected by chemiluminescence according to the manufacturer's instructions (Amersham, Franklin Lakes, NJ, USA).

2.5. Confocal microscopy analysis

Raw 264.7 cells were plated on glass coverslips and treated with 0.3 μM PEP-1-Prx2. After 1 h of incubation, the cells were washed with PBS twice and fixed with 4% paraformaldehyde at room temperature for 5 min. The cells were permeabilized and blocked with 3% bovine serum albumin and 0.1% Triton X-100 in PBS (PBS-BT) for 40 min before being washed with PBS-BT. The primary antibody (His-probe; Santa Cruz Biotechnology) was diluted 1:2000 and incubated at room temperature for 3 h. The secondary antibody (Alexa Fluor 488; Invitrogen, Carlsbad, CA, USA) was diluted 1:15,000 and incubated at room temperature in the dark for 1 h. Nuclei were stained with 1 μg/ml DAPI (Roche, Mannheim, Germany) for 2 min. The distribution of fluorescence was analyzed using a confocal Model FV-300 microscope (Olympus, Tokyo, Japan).

2.6. Measurement of intracellular ROS levels

Intracellular ROS levels were determined by dichlorofluorescin diacetate (DCF-DA), which is converted into fluorescent dichlorofluorescein by ROS [15,16]. Raw 264.7 cells were incubated for 1 h and treated with 1 μg/ml LPS for 30 min. Cells were washed with PBS twice and incubated with 20 μM DCF-DA for 30 min. The cellular fluorescence intensity was measured using a fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland) with a 485 nm excitation and a 538 nm in emission.

2.7. Real-time RT-PCR analysis

Total RNA was extracted from cells using an Easy Blue RNA extraction kit according to manufacturer's instruction (Intron Biotechnology, Seoul, Korea). RNA was reverse transcribed with reverse transcriptase and an oligo(dT) primer. Real-time RT-PCR was confirmed using a LightCycler Nano real-time PCR system (Roche, Mannheim, Germany). Values of IL-6, IL-1β and TNF-α genes were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels as the endogenous control in each group. Also, relative gene expression was measured according to manufacturer's instructions. For the primer sequence; TNF-α antisense, 5′-TGGCACCACTAGTTGGTTGTCTTT-3′; TNF-α sense, 5′-AAGTTCCCAAATGGCCTCCC-3′ IL-1β antisense, 5′-GTGCTGCCTAATGT CCCCTTGAATC-3′; IL-1β sense, 5′-TGCAGAGTTCCCCAACTGGTACATC-3′; IL-6 antisense, 5′-TGGATGGTCTTGGTCCTTAGCC-3′; IL-6 sense, 5′-CAAGAAAGACAAAGCCAGAGTCCTT-3′; and GAPDH antisense, 5′-AGTG ATGGCATGGACTGTGGTCAT-3′; GAPDH sense, 5′-ACCCCTTCATTGACCT CAACTACA-3′ were used in this study.

2.8. Cytokine measurement by enzyme-linked immunosorbent assay (ELISA)

Raw 264.7 cells were exposed to PEP-1-Prx2 or control Prx2 at different concentrations for 1 h, after which they were treated with LPS (1 μg/ml) and incubated for 24 h. After centrifugation of all culture media at 2000 rpm for 5 min, supernatants were collected. The expression levels of IL-6, IL-1β and TNF-α were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA).

2.9. Measurement of nitric oxide (NO) expression

Raw 264.7 cells were exposed to PEP-1-Prx2 at different concentrations for 1 h, in addition to treatment with LPS (1 μg/ml) and incubation for 48 h. After centrifugation of all culture media at 2000 rpm for 5 min, supernatants were collected. To measure NO expression, the supernatants were blended with Griess reagent (Promega Corp., Madison, WI, USA), according to manufacturer's instructions, and the absorbance was measured at 520 nm using an ELISA plate reader.