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Anti-inflammatory effect of transduced PEP-1-Cyclophilin A in Raw 264.7 cells and 12-O-tetradecanoylphorbol-13-acetate-induced mice

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ABSTRACT

Aims: Cyclophilin A (CypA) is an immunophilin that acts as a receptor for the immunosuppressant drug cyclosporine A (CsA). CypA has emerged as a potential drug target for several inflammatory diseases, although the details of its mechanism are unclear. We examined the protective effects of CypA on inflammation in Raw 264.7 cells and animal models.

Main methods: A human CypA gene was fused with a protein transduction domain, PEP-1 peptide, to construct a cell permeable PEP-1-CypA protein. The protein expression level of cyclooxygenase-2 (COX-2) and cytokines was detected by Western blot, ELISA and mRNA level of COX-2 and cytokines were measured by RT-PCR. The nuclear factor-kappa B (NF-*k*B) and mitogen-activated protein kinase (MAPK) activation were analyzed by Western blot and electrophoretic mobility shift assay. Skin inflammation was detected with immunohistochemistry.

Key findings: Transduced PEP-1-CypA protein markedly inhibited lipopolysaccharide- and 12-O-tetradecanoyl phorbol-13-acetate-induced expression levels of COX-2 as well as pro-inflammatory cytokine levels *in vitro* and *in vivo*. Furthermore, transduced PEP-1-CypA protein resulted in a significant reduction in the activation of NF-*k*B and MAPK.

Significance: The results indicate that PEP-1-CypA inhibits inflammatory response cytokines and enzymes by blocking NF-*k*B and MAPK activation upon stimulation of inflammation *in vitro* and *in vivo*. PEP-1-CypA protein may potentially be used as a therapeutic agent against skin diseases-related inflammation.

Introduction

Macrophages play an important role in inflammatory diseases by producing nitric oxide (NO), prostaglandins, cytokines such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and other inflammatory mediators (Lowenstein et al., 1996). Inflammatory mediators are associated with numerous diseases including rheumatoid arthritis and the development of cancer (Bertolini et al., 2002; Isomaki and Punnone, 1997; Korhonen et al., 2005).

It is well-known that nuclear factor-*k*B (NF-*k*B), a nuclear transcription factor, regulates the expression of various genes, including IL-1β, TNF-α, and cyclooxygenase-2 (COX-2), that play critical roles in apoptosis, tumorigenesis, various autoimmune diseases, and inflammation (Lawrence et al., 2001; Riehemann et al., 1999). NF-*k*B is present in most cells as homodimeric or heterodimeric complexes of p50 and p65 subunits. Various inflammatory stimuli induce NF-*k*B activation by increasing nuclear p65 protein associated with decreased cytosolic I*k*B protein. Its ubiquitous role in the pathogenesis of inflammatory gene expression has made NF-*k*B a target for treating various diseases (Mararov, 2000; Renard and Raes, 1999). Furthermore, inflammatory stimuli induce mitogen-activated protein kinase (MAPK) activation. Three MAPKs (extracellular signal-regulated protein kinase (ERK), c-Jun terminal kinase (JNK), and p38 MAPK) have been studied in the transcriptional regulation of COX-2 expression (Ho et al., 2007; Song et al., 2008).

Cyclophilin A (CypA) belongs to the immunophilin family and is recognized as the receptor for the immunosuppressive drug cyclosporine A (CsA) (Fruman et al., 1992). CypA demonstrates cis-trans peptidylprolyl isomerase (PPIase) activity, which plays an important role in protein folding, and also acts as a potent chemoattractant to

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human monocytes, neutrophils, and T cells (Arora et al., 2005; Ou et al., 2001). CypA is secreted from smooth muscle cells and macrophages in response to inflammatory stimuli such as oxidative stress and lipopolysaccharide (LPS) (Jin et al., 2000; Sheery et al., 1992). CypA levels are also increased in patients with sepsis and rheumatoid arthritis (RA) (Billich et al., 1997; Tegeder et al., 1997). Although the pro-inflammatory roles of CypA in RA are well known, the biological function and molecular mechanism of the CypA protein in inflammation are not yet clear.

Many studies have demonstrated the successful delivery of full-length fusion proteins by protein transduction technology (An et al., 2008; Choi et al., 2006a, 2006b; Eum et al., 2004; Kim et al., 2010; Morris et al., 2001; Wadia and Dowdy, 2002). Several small regions of proteins, called protein transduction domains (PTDs), have been developed to allow the delivery of exogenous protein into living cells. In the present study, a PEP-1-CypA fusion protein was genetically designed in-frame for transduction directly *in vitro* and *in vivo*. The PEP-1-CypA could be directly transduced into Raw 264.7 cells, where it potently protected against LPS-induced cell damage. Topical application of PEP-1-CypA to mice ears significantly inhibited 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced ear edema as well as TPA-induced expression of pro-inflammatory cytokines and enzymes in mice. Furthermore, PEP-1-CypA inhibited LPS- or TPA-induced activation of NF-*k*B and MAPKs. These results indicate that PEP-1-CypA could be useful as a potential topically applied therapeutic agent for the treatment of skin inflammation.

Materials and methods

Materials

Male 6–8-week-old ICR mice were purchased from the Experimental Animal Center, Hallym University. TPA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ni^{2+}-nitrilotri-acetic acid Sepharose superflow was purchased from Qiagen (Valencia, CA, USA). Plasmid pET-15b and *Escherichia coli* strain BL21 (DE3) were obtained from Novagen (Hilden, Germany). A primary antibody against histidine was purchased from Santa Cruz, Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents were of the highest analytical grade available.

Expression and purification of PEP-1-CypA fusion protein

A PEP-1 expression vector was prepared as described previously in our laboratory (Eum et al., 2004). On the basis of the cDNA sequence of human CypA, two primers were synthesized and amplified by PCR using the sense primer, 5′-CTCGAGCTCGAGATGGTCAACCC CACCGTGTTCTTC-3′ and the antisense primer, 5′-GGATCCTTAATC-GAGTTGTCCACAGTCAGC-3′. The PCR product was excised, eluted, and ligated into a TA-cloning vector. The purified TA vector containing human CypA cDNA was ligated into the expression vector, PEP-1, to produce a genetic in-frame PEP-1-CypA fusion protein. In a similar fashion, a control CypA was constructed that expressed the CypA fusion protein without the PEP-1.

To produce the PEP-1-CypA and control CypA fusion proteins, the plasmid was transformed into *E. coli* BL21 cells (Novagen) and grown in 100 ml of LB media at 37 °C to an optimal density 600 nm value of 0.5–1.0, and was induced with 0.5 mM of isopropyl-β-D-thiogalactoside (Duchefa, Haarlem, Netherlands) at 37 °C for 3–4 h. Harvested cells were lysed by sonication and purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography according to the manufacturer's instruction. The protein concentration was estimated by the Bradford procedure using bovine serum albumin as a standard (Bradford, 1976).

Cell culture and transduction of PEP-1-CypA fusion protein

The murine macrophage cells, Raw 264.7, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM HEPES/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% fetal bovine serum (FBS) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37 °C under a humidified condition of 95% air and 5% CO_2.

For the transduction of PEP-1-CypA, Raw 264.7 cells were grown to confluence in wells of a 6-well plate. After the cells were treated with various concentrations of PEP-1-CypA for 1 h, they were treated with trypsin-EDTA (Gibco, Carlsbad, CA, USA) and washed with phosphate-buffered saline (PBS). The cells were harvested for the preparation of cell extracts to perform Western blot analysis.

Western blot analysis

Cell lysate proteins were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrotransferred to a nitrocellulose membrane, which was then blocked with 5% non-fat dry milk in PBS. The membrane was probed with a rabbit antihistidine polyclonal antibody (1:1000; Santa Cruz Biotechnology), followed by incubation with goat antirabbit immunoglobulins (dilution 1:10,000; Sigma-Aldrich). The bound antibodies were then visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Piscataway, NJ, USA).

Fluorescence analysis

For direct detection of fluorescein-labeled protein, purified PEP-1-CypA and control CypA were labeled using an EZ-Label FITC protein labeling kit (Pierce, Rockford, IL, USA). FITC labeling was carried out according to the manufacturer's instructions. Cultured cells were grown on glass coverslips and treated with 0.3 μM PEP-1-CypA and control CypA fusion proteins. Following incubation for 1 h at 37 °C, the cells were washed twice with PBS and trypsin-EDTA. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature. The distribution of fluorescence was analyzed on a fluorescence microscope (Carl Zeiss, Jena, Germany).

Determination of COX-2 expression levels

Raw 264.7 cells were incubated in wells of 6-well plates for 12 h, to achieve 70% confluence. After incubation, the cells were pretreated with PEP-1-CypA (0.1–0.5 μM) for 1 h before treatment with LPS (100 ng/ml) for 12 h, and the culture medium was harvested. The expression of COX-2 protein as well as RNA levels was determined by Western blotting and RT-PCR.

RT-PCR analysis

Total RNA was isolated from Raw 264.7 cells using a Trizol reagent kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA (2 μg) was reversibly transcribed with reverse transcriptase and oligo-(dT) primer. PCR amplification of cDNA aliquots was performed with the following sense and antisense primers: COX-2 antisense, 5′-TGGACGAGGTTTTTCCACCAG-3′; COX-2 sense, 5′-CAAAGGCCTCCATTGACCAGA-3′; TNF-α antisense, 5′-TGGCACCAC-TAGTTGG TTGTCTTT-3′; TNF-α sense, 5′-AAGTTCCCAAATGGCCTCCC-3′; IL-1β antisense, 5′-GTGCTGCCTAATGTCCCCTTGAATC-3′; IL-1β sense, 5′-TGCAGAGTTCCCCAACTGGTACATC-3′; IL-6 antisense, 5′-TGGATGGTCTTGGTCCTTAGCC-3′; IL-6 sense, 5′-CAAGAAAGACAAA-GCCAGAGTCCTT-3′; and β-actin antisense, 5′-GGACAGTGAGGCCAG-GATGG-3′; β-actin sense, 5′-AGTGTGACGTTGACATCCGTAAAGA-3′. PCR was performed using a PCR Premix kit (Intron Biotechnology, Seoul, Korea) and each primer was terminated by heating to 72 °C