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Inhibition of LPS-induced cyclooxygenase 2 and nitric oxide production by transduced PEP-1-PTEN fusion protein in Raw 264.7 macrophage cells

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Abbreviations: COX-2, cyclooxygenase-2; NO, nitric oxide; PTD, protein transduction domain; PTEN, phosphatase and tensin homologue deleted on chromosome 10

Abstract

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a tumor suppressor. Although it is well known to have various physiological roles in cancer, its inhibitory effect on inflammation remains poorly understood. In the present study, a human PTEN gene was fused with PEP-1 peptide in a bacterial expression vector to produce a genetic in-frame PEP-1-PTEN fusion protein. The expressed and purified PEP-1-PTEN fusion protein were transduced efficiently into macrophage Raw 264.7 cells in a time- and dose- dependent manner when added exogenously in culture media. Once inside the cells, the transduced PEP-1-PTEN protein was stable for 24 h. Transduced PEP-1-PTEN fusion protein inhibited the LPS-induced cyclooxygenase 2 (COX-2) and iNOS expression levels in a dose-dependent manner. Furthermore, transduced PEP-1-PTEN fusion protein inhibited the activation of NF-κB induced by LPS. These results suggest that the PEP-1-PTEN fusion protein can be used in protein therapy for inflammatory disorders.

Keywords: cyclooxygenase 2; inflammation; lipopolysaccharides; nitric oxide; PTEN phosphohydrolase

Introduction

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN), also named MMAC-1 (mutated multiple advanced cancers) or TEP-1 (TGF-β-regulated and epithelial cell-enriched phosphatase) was identified as a new tumor suppressor gene involved in a wide variety of human cancers located at 10q23 (Li *et al*., 1997; Steck *et al*., 1997). PTEN is well known to play a key role in suppressing cancer, cell migration, survival and apoptosis (Yamada and Araki, 2001). PTEN is a major negative regulator of the phosphatidylinositol 3-kinase (PI3K) and serine/theronine protein kinase (Akt) signaling pathway by catalyzing degradation of the phosphatidylinositol-3,4,5-triphosphate (PIP3) to PI-4,5-diphosphate (Vazquez and Sellers, 2000).

Prostaglandins (PGs) are potent proinflammatory mediators derived form arachidonic acid metabolism by cyclooxygenase (COXs), and play an important role in modulating a number of pathophysiological conditions, including inflammatory and allergic immune response (Tilly *et al*., 2001). The two isoforms of COX enzymes have been well studied. COX-1 is constitutively expressed and plays an important role in maintaining the normal physiological function of cells. COX-2 is markedly induced by a number of stimuli including cytokines during the inflammatory response (Smith and Dewitt, 1990; Carey *et al*., 2003; Vancheri *et al*., 2004).

LPS is the main component of endotoxin and is formed by a phosphoglycolipid that is covalently linked to a hydrophilic heteropolysaccharide (Rietschel *et al*., 1994). LPS arrests macrophage

proliferation and activates them to produce pro-inflammatory factors, which play important roles in the immune response (Adams and Hamilton, 1984; Morrison and Ryan, 1987). Eosinophils act as effectors in the inflammatory reactions of allergic diseases such as asthma, allergic rhinitis, and atopic dermatitis (AD), as well as in the chronic development of allergic inflammation (Fugihara *et al*., 2002; Gleich, 2000; Wong *et al*., 2002). Kwak *et al*. (2003) demonstrated that administration of an adenovirus gene transfer vector expressing a PTEN cDNA inhibitors reduced the inflammation and airway hyper-responsiveness in a murine model of allergic asthma. Although there has been increased interest in the role of PTEN in cellular function particularly in inflammations, the underlying mechanisms still need to be established.

Several small regions of proteins, called protein transduction domains (PTDs), have been developed to allow the delivery of exogenous protein into living cells. Up to the present, many researchers have demonstrated the successful delivery of full-length Tat fusion proteins by protein transduction technology (Wadia and Dowdy, 2002). We successfully transduced Tat-SOD directly into insulin-producing RINm-5F and pancreatic islet cells and transduced Tat-SOD by increased radical scavenger activity in the pancreas (Eum *et al*., 2002, 2004a). Recently, we showed that Tat-pyridoxal kinase (Tat-PK) and Tat-pyridoxal oxidase (Tat-PO) fusion protein were efficiently transduced into PC12 cells and catalytically active in the cells (Kim *et al*., 2005, 2006). Also, we transduced PEP-1-SOD and PEP-1-CCS into neuronal cells and across the blood-brain barrier which efficiently protected against ischemic insults. Also, Tat-α-synuclein fusion protein protects against oxidative stress *in vitro* and *in vivo* (Eum *et al*., 2004b; Choi *et al*., 2005, 2006a,b).

In the present study, we designed the PEP-1-PTEN fusion protein by genetic in-frame transduction and showed that the PEP-1-PTEN fusion protein can be directly transduced into macrophage Raw 264.7 cells, as well as inhibit levels of iNOS and COX-2 mRNA and protein in LPS-induced cells. Therefore, we suggest that PEP-1-PTEN fusion protein could be useful as a potential therapeutic agent for inflammatory diseases.

Materials and Methods

Materials

Restriction endonuclease and T4 DNA ligase were purchased from Promega Co. (Madison, WI). Oligonucleotides were synthesized from Gibco BRL custom primers (Grand Island, NY). Ni^{2+}-nitrilotriacetic acid sepharose superflow was purchased from Qiagen (Valencia, CA). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Duchefa Co. (Haarlem, Netherlands). Plasmid pET-15b and *Escherichia coli* strain BL21 (DE3) were obtained from Novagen (Hilden, Germany). A human PTEN cDNA fragment was isolated using the PCR technique. Primary antibodies against histidine, COX-2, actin (Santa Cruz, CA) and phosphor-IκBα, total IκBα (Cell signaling Technology, Beverly, MA) were obtained commercially. All other chemicals and reagents were of the highest analytical grade available.

Cloning and purification of PEP-1-PTEN fusion proteins

The PEP-1 expression vector was constructed to express the PEP-1 peptides (KETWWETWWTEW-SQPKKKRKV) as a fusion with human PTEN. First, two oligonucleotides (top strand) 5'-TATGAA-AGAAACCTGGTGGGAAACCTGGTGGA CCGAATGGTCTCAGCCGAAAAAAAAACGTAAA GTGC-3' and (bottom strand) 5'-TCGABCACT-TTACGTTTTTTTTTCGGCTGAGACCATTCGGTC CACCAGGTTTCCCACCAGGTTTCTTTCC-3' were synthesized and annealed to generate a double-stranded oligonucleotide encoding the PEP-1 peptides. The double-stranded oligonucleotide was directly ligated into a *Nde*I-*Xho*I digested pET-15b vector. Second, on the basis of the cDNA sequence of human PTEN, two primers were synthesized. The sense primer, 5'-CTCGAGATGGCAGCCATC-ATCAAAGAGATC-3' contains an *Xho*I site, and the antisense primer, 5'-GGATCCTCAGACTTTTGTA-ATTTGTGTATG-3', contains a *Bam*HI restriction site. The resulting PCR products were digested with *Xho*I and *Bam*HI, eluted (Invitek, Berlin, Germany), ligated into a TA-cloning vector (Promega, Madison, WI) and a pPEP-1 vector using T4 DNA ligase (Takaka, Otsu, Shiga, Japan), and cloned in *E. coli* BL21 (DE3) cells.

To produce the PEP-1-PTEN fusion protein, the plasmid was transformed into *E. coli* BL21 cells. The transformed bacterial cells were grown in 100 ml of LB media at 37°C to a D_{600} value of 0.5-1.0 and induced with 0.5 mM IPTG at 37°C for 3-4 h. Harvested cells were lysed by sonication at 4°C in a binding buffer containing 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9). After centrifugation, the supernatant was immediately loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column (Qiagen, Valencia, CA). After the column was washed with 10 volumes of a binding buffer

and 6 volumes of a washing buffer (35 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9), the fusion protein was eluted with an eluting buffer (0.5 M imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). The fusion proteins were combined and the salts were removed using PD10 column chromatography (Amersham, Braunschweig, Germany). Then, the purified PEP-1-PTEN fusion proteins were further purified by chromatography on Superose 6 FPLC column. The protein concentration was estimated by the Bradford procedure using BSA as a standard (Bradford, 1976).

Transduction of PEP-1-PTEN into macrophage Raw 264.7 cell

The murine macrophage Raw 264.7 cells were cultured in DMEM containing 20 mM HEPES/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% 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-PTEN, macrophage Raw 264.7 cells were grown to confluence on a 6-well plate. The culture medium was then replaced with fresh solution. After Raw 264.7 cells were treated with various concentrations of PEP-1-PTEN for 1 h, the cells were then treated with trypsin-EDTA and washed with PBS. Thereafter Raw 264.7 cells were harvested for the preparation of cell extracts to perform Western blot analysis.

The intracellular stability of transduced PEP-1-PTEN fusion protein was estimated by treating: after Raw 264.7 cells were treated with 3 μM native PEP-1-PTEN for 1 h, after which cells were washed and changed with a fresh culture medium to remove the PEP-1-PTEN that was not transduced. Cells were then further incubated for 48 h, followed by preparations of cell extracts for Western blot analysis.

Western blot analysis

The transduced PEP-1-PTEN proteins on the polyacrylamide gel were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk in Tris-buffered saline (TBS; 20 mM Tris, 0.2 M NaCl, pH 7.5) containing 0.05% Tween-20 (TBST) for 2 h and was then incubated for 1 h at room temperature with an anti-histidine antibody (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:400) in TBST. After washing, the membrane was incubated for 1 h with a proper secondary antibody conjugated to HRP diluted 1:10,000 in TBST. The membrane was incubated with a chemiluminescent substrate and exposed to Hyperfilm ECL.

Determination of COX-2 protein expression

The macrophage Raw 264.7 cells were cultured in 6-well plates. The cells were washed with fresh medium and stimulated with 1 μg/ml LPS for 24 h. Then, the expression of COX-2 protein levels were determined by Western blotting using anti-COX-2 antibody.

Determination of NO production

NO was measured as its stable oxidative metabolite, nitrite, as described in (Misko *et al*., 1993). 100 μl of the culture medium was mixed with an equal volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). The absorbance at 550 nm was measured, and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

RT-PCR analysis

Total RNA was isolated from Raw 264.7 cells using a Trizol reagent kit (Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions (Zhang *et al*., 2005). The RNA (2 μg) was reversibly transcribed with 10,000 U of reverse transcriptase and 0.5 μg/μl oligo-(dT) primer. PCR amplification of cDAN aliquots was performed with the following sense and antisense primers: COX-2 antisense, 5'-TGGACGAGGTTTTTCCACCAG-3'; sense, 5'-CAAAGGCCTCCATTGACCAGA-3'; beta-actin antisense, 5'-GGACAGTGAGGCCAGGATGG-3'; sense, 5'-AGTGTGACG TTGACATCCGTAAAGA-3'; iNOS antisense, 5'-CTGTCAGAGCCTCGTGGCTTT-3'; sense, 5'-ATGGCTCGGGATGTGGCTAC-3'. PCR was performed in 50 μl of 10 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$, 10 mM dNTP, 100 U of *Taq* DNA polymerase, and 0.1 μM of each primer and was terminated by heating at 70°C for 15 min. PCR products were resolved on a 1% agarose gel and visualized with UV light in ethidium bromide.

Electrophoretic mobility shift assay (EMSA)

Raw 264.7 cells were treated with PEP-1-PTEN for 1 h, then nuclear extracts of the cells were prepared and analyzed for NF-κB binding activity by EMSA as described in (Song *et al*., 2007). An NF-κB consensus oligonucleotide was used in the EMSA. The complementary oligonucleotide was annealed and end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. EMSA was performed in

a total volume of 20 μl at 4°C. Five micrograms of nuclear extracts were equilibrated for 15 min in a binding buffer (10 mM Tris-HCl, pH 8.0, 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.25 mM DTT) and 1 μg of poly dI/Dc. ^{32}P-labeled oligonucleotide probe (20,000 cpm) was then added and the reaction was incubated on ice for an additional 20 min. Bound and free DNA were then resolved by electrophoresis in a 6% native polyacrylamide gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA).

Results

Construction and purification of PEP-1-PTEN fusion protein

To generate a cell-permeable expression vector, PEP-1-PTEN, a human PTEN cDNA was subcloned into the pET-15b plasmid that had been reconstructed to contain the PEP-1 peptide. The PEP-1-PTEN expression vector thus formed contained consecutive cDNA sequences encoding human PTEN, PEP-1 peptide (21 amino acids) and six histidine residues at the amino-terminus (Figure 1A). We also constructed the PTEN expression vector to produce control PTEN protein without PEP-1 transduction peptides.

Following the induction of expression, the PEP-1-PTEN fusion proteins were purified. The fusion proteins were expressed in *E. coli* and the clarified cell extracts were loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column under native conditions. A fusion protein containing fractions was combined and salts were removed using a PD10 column. A major single band was obtained by superpose 6 FPLC chromatography. The crude cell extracts obtained from *E. coli* and purified PEP-1-PTEN fusion proteins were electrophoresed in 10% SDS-PAGE (Figure 1B and C). The purified proteins were further confirmed by Western blot analysis using an anti-rabbit polyhistidine antibody. PEP-1-PTEN was detected at the corresponding bands in Figure 1C and D.

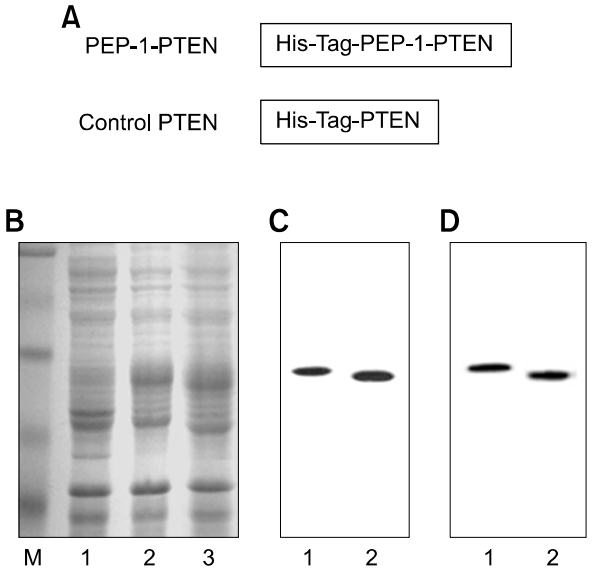

Figure 1. Expression and purification of PEP-1-PTEN fusion protein. A schematic representation of the PEP-1-PTEN fusion protein containing 6His, PEP-1, and PTEN coding sequence (A). Protein extracts from cells and purified fusion proteins were analyzed by 10% SDS-PAGE (B, C) and subjected to Western blot analysis with an anti-rabbit polyhistidine antibody (D). Lanes in B are as follows: lane 1, non-induced; lane 2, induced PEP-1-PTEN; lane 3, induced PTEN. Lanes in C and D are as follows: lane 1, purified PEP-1-PTEN; lane 2, purified PTEN.

Transduction of PEP-1-PTEN into macrophage Raw 264.7 cells

To evaluate the transduction ability of PEP-1-PTEN fusion protein, we analyzed the transduction of PEP-1-PTEN proteins by adding them to Raw

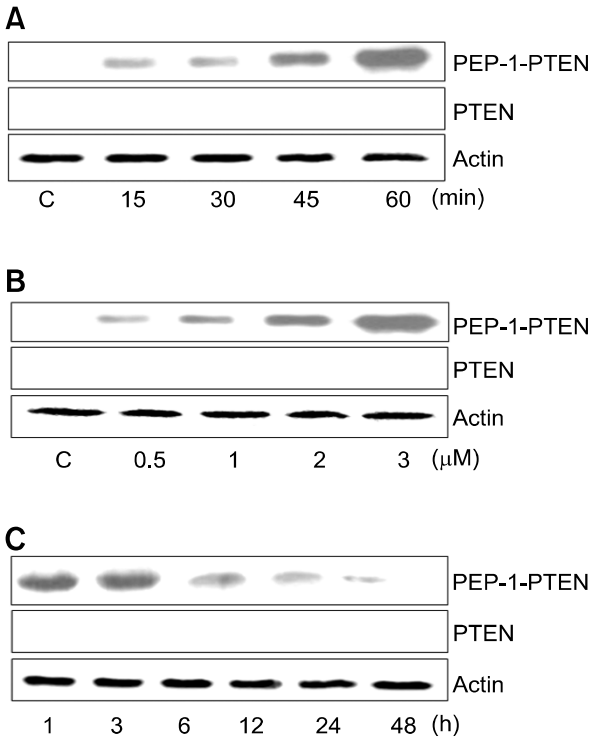

Figure 2. Transduction of PEP-1-PTEN fusion proteins. (A) 3 μM PEP-1-PTEN were added to the culture media of Raw 264.7 cells for 15-60 min, (B) 0.5-3 μM of PEP-1-PTEN were added to the culture media for 1 h, (C) cells were pre-treated with 3 μM PEP-1-PTEN incubated for 1-48 h, and analyzed by Western blot analysis. PTEN and β-actin were used as a control.

264.7 cells culture media at 3 μM for various periods (15-60 min), and then analyzed the transduced protein levels by Western blotting. The intracellular concentration of transduced PEP-1-PTEN in cells was seen to gradually increase at 60 min (Figure 2A).

The dose-dependency of the transduction of PEP-1-PTEN fusion proteins was further analyzed. Various concentrations (0.5-3 μM) of PEP-1-PTEN proteins were added to Raw 264.7 cells in culture for 60 min, and the levels of transduced proteins were measured by Western blotting. As shown in Figure 2B, the levels of transduced proteins in Raw 264.7 cells increased in a concentration-dependent manner with the amount of fusion protein in the culture media. These results indicate that PEP-1-PTEN fusion proteins are transduced into Raw 264.7 cells in a dose- and time-dependent manner. However, control PTEN was not transduced into the cells.

The intracellular stability of transduced PEP-1-PTEN into Raw 264.7 cells is shown in Figure 2C. The PEP-1-PTEN fusion protein was added to the culture media of Raw 264.7 cells at a concentration of 3 μM for various time periods and the resulting levels of transduced protein were analyzed by Western blotting. The intracellular level of transduced PEP-1-PTEN fusion protein into cells was initially detected after 1 h, and then declined gradually over the period of observation. However, significant levels of transduced PTEN protein persisted in the cells for 24 h.

Effect of PEP-1-PTEN fusion protein on LPS-induced COX-2 expression and NO production

Macrophage plays crucial roles in the initiation and maintenance of inflammation. Since the level of COX-2 and NO is important in addressing the extent of inflammation, the effect of PEP-1-PTEN fusion protein on the inhibition of COX-2 expression and NO production was investigated. Raw 264.7 cells were incubated for 24 h with LPS (1 μg/ml) in the presence or absence of various concentrations (0.5-3 μM) of PEP-1-PTEN fusion protein. PEP-1-PTEN fusion protein suppressed LPS-induced COX-2 expression levels in Raw 264.7 cells in a dose-dependent manner (Figure 3).

The effect of PEP-1-PTEN fusion protein on NO production was examined in Raw 264.7 cells. Cells were stimulated with LPS (1 μg/ml) for 24 h in the presence or absence of various concentrations (0.5-3 μM) of PEP-1-PTEN fusion protein. After which cell culture media were collected and nitrite levels were determined. The exogenous PEP-1-PTEN fusion protein reduced NO production in a dose- dependent manner (Figure 4). However, the control PTEN protein did not suppress LPS-induced COX-2 expression levels in the same conditions and no significant cytotoxicity of PEP-1-PTEN fusion protein was determined in the cells (data not shown).

We further examined the effects of PEP-1-PTEN on COX-2 and iNOS mRNA expression in LPS-induced cells by RT-PCR. As shown in Figure 5, post-treatment with PEP-1-PTEN fusion protein markedly inhibited LPS-induced mRNA expression of COX-2 and iNOS. These results suggest that

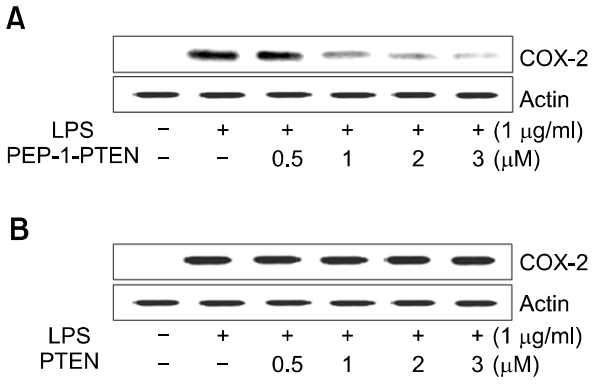

Figure 3. Effect of transduced PEP-1-PTEN on LPS-induced COX-2 protein expression. Raw 264.7 cells were pretreated with the PEP-1-PTEN for 1 h before incubation with LPS (1 μg/ml) for 24 h. The cells were lysed and the lysates were analyzed by immunoblotting using an anti-COX-2 antibody.

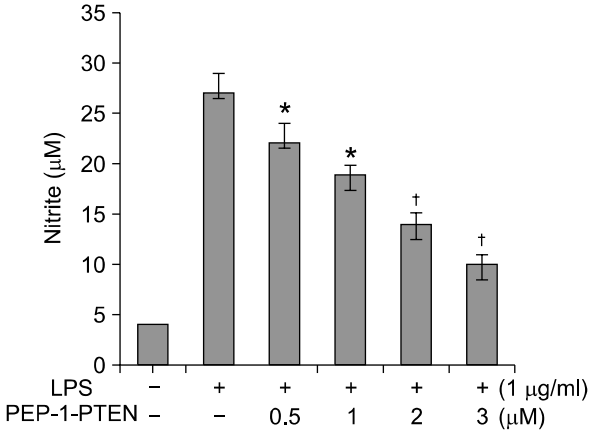

Figure 4. Effect of transduced PEP-1-PTEN on LPS-induced NO production. Raw 264.7 cells were pretreated with the PEP-1-PTEN for 1 h before incubation with LPS (1 μg/ml) for 24 h. Nitrite levels were measured in the culture media of LPS-stimulated cells by the Griess reaction. Each bar represents the mean ± SEM obtained from five experiments. $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ versus LPS alone. The statistical analysis was evaluated by Student's *t*-test.

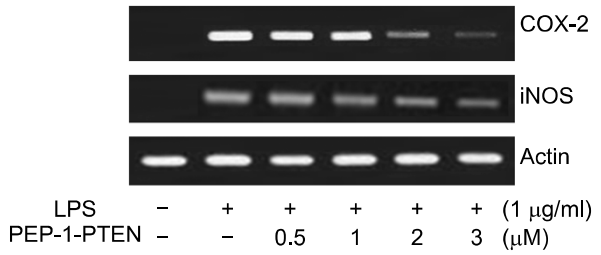

Figure 5. Inhibitory effect of PEP-1-PTEN on LPS-induced iNOS and COX-2 mRNA levels in Raw 264.7 cells. Raw 264.7 cells were pretreated with the PEP-1-PTEN for 1 h before incubation with LPS (1 μg/ml) for 12 h. Total RNA was extracted. iNOS and COX-2 mRNA was analyzed by RT-PCR using specific primers.

the inhibition of COX-2 and iNOS mRNA expression by transduced PEP-1-PTEN fusion protein were responsible for the inhibition of COX-2 and NO production.

Effect of PEP-1-PTEN on LPS-induced NF-κB activation

As NF-κB plays critical role in the LPS-induced expression of iNOS and COX-2 in Raw 264.7 cells, we attempted to determine the effect of transduced PEP-1-PTEN on LPS-induced activation of NF-κB. Nuclear extracts from LPS-induced cells were analyzed using EMSA. As shown in Figure 6A, transduced PEP-1-PTEN fusion protein decreased in the LPS-induced DNA binding activity of NF-κB in a dose-dependent manner. Next, we examined the regulatory effects of PEP-1-PTEN fusion protein on the LPS-induced signal cascade of NF-κB activation such as IκBα phosphorylation and IκBα degradation. As shown in Figure 6B, PEP-1-PTEN fusion protein inhibited LPS-induced IκBα phosphyrylation and degradation in the cells.

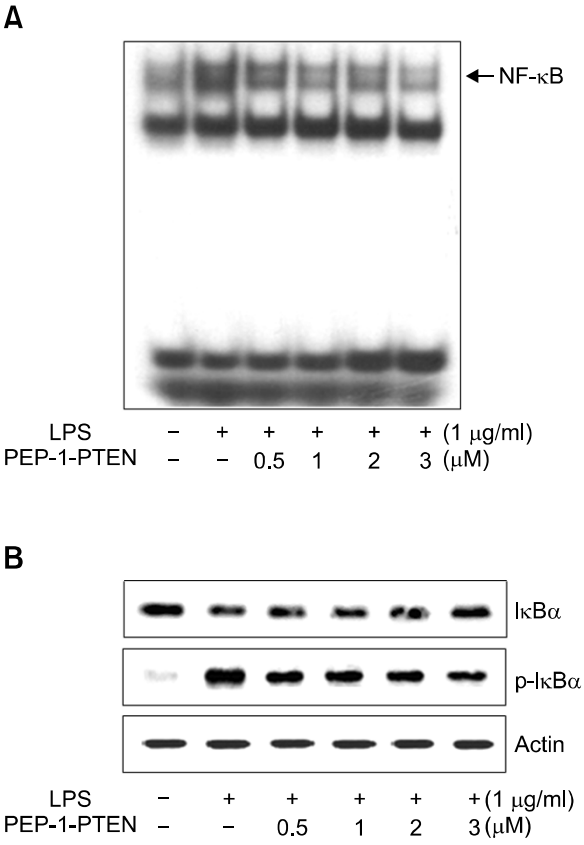

Figure 6. Effect of transduced PEP-1-PTEN on LPS-induced activation of NF-κB in Raw 264.7 cells. Raw 264.7 cells were pretreated with the PEP-1-PTEN for 1 h before incubation with LPS (1 μg/ml) for 1 h and analyzed for NF-κB binding by EMSA (A). Phosphorylation and degradation of IκB-α were analyzed by Western blot analysis (B).

Discussion

It is well known that PTEN is a tumor suppressor gene that suppresses cell growth, inhibits cell migration, and induces apoptosis. It has been implicated in regulating cell survival signaling through the PI3K/Akt pathway (Maehama and Dixon, 1998; Myers *et al*., 1998; Cantley and Neel, 1999; Lu *et al*., 1999; Yamada and Araki, 2001).

Many inflammatory mediators attract and activate eosinophils via signal transduction pathways involving the enzyme PI3K (Dunzendorfer *et al*., 1998; Palframan *et al*., 1998; Tigani *et al*., 2001). Asthma is a chronic inflammatory disorder of the airways in which many cell types play a role. Eosinophil response appears to be a critical feature in asthma (Frigas and Gleich, 1986; Bousquet *et al*., 2000). It was reported that the PTEN protein expression and activity were decreased in OVA-induced asthma. They demonstrated that the administration of an adenovirus gene transfer vector expressing a PTEN cDNA or PI3K inhibitor reduced inflammation and airway hyperresponsiveness in a murine model of allergic asthma, and the inhibition of PI3K may be a good therapeutic strategy (Kwak *et al*., 2003). Several studies reported that the role of PTEN in immunity has been shown using PTEN-deficient mice. They have generated T cell-specific PTEN-deficient ($tPten^{flox/-}$) mice in which the T cells exhibit autoreactivity, enhanced proliferation, increased levels of Th1 and Th2 cytokines, and inhibition of apoptosis. Similar phenomena are observed in the B cells derived from B cell-specific PTEN-deficient ($bPten^{flox/flox}$) mice (Suzuki *et al*., 2001, 2003a). They suggest that PTEN negatively regulates most cellular functions in the immune

system. Although PTEN has been considered as a potential therapeutic protein against inflammatory diseases, its inability to enter cells hinders its use for this purpose. Therefore, in an effort to deliver PTEN protein to cells, we investigated the possibility of a protein transduction. In previous studies, we have shown that PEP-1-SOD fusion protein can be efficiently transduced into cells and skin tissue. Moreover, transduced PEP-1-SOD proved enzymatically and biologically active, and efficiently protected against neuronal cell death caused by transient forebrain ischemia (Eum *et al*., 2004b). Also, we reported that transduced PEP-1-Grb7 fusion protein markedly increased cell viability in macrophage Raw 264.7 cells treated with LPS by inhibition of the COX-2 expression level (An *et al*., 2007). Recently, we showed that transduced PEP-1-PLP phosphatase (PLPP) fusion protein significantly decreased PLP concentration in PC12 cells pretreated with the vitamin B_6 precursors (Lee *et al*., 2008).

The PEP-1-PTEN fusion protein was expressed, purified and it was found to be nearly homogeneous and greater than 95% pure, as determined by a SDS-PAGE analysis. The expressed and purified PEP-1-PTEN fusion proteins were confirmed by Western blot using an anti-rabbit polyhistidine antibody. Purified PEP-1-PTEN fusion proteins were efficiently transduced into Raw 264.7 cells in a time- and dose-dependent manner. The intracellular stability of transduced PEP-1-PTEN persisted in the cells for 24 h. Although the mechanism of transduction is unclear, protein transduction domain (PTD) fusion protein transduction is a major development in protein therapeutics. Morris *et al*. (2001) showed that PEP-1 peptide/GFP (green fluorescent protein, 30 kDa) or β-Gal (β-galactosidase, 119 kDa) mixtures transduce into a human fibroblast cell line (HS-68) and into Cos-7 cells by incubating with a PEP-1 peptide carrier and proteins (GFP or β-gal) for 30 min at 37°C. These differences in the time courses of transduction may depend on whether the target protein is fused with the PEP-1 vector or mixed with the PEP-1 peptide. As a result of fusion with the PEP-1 vector, the conformation, polarity, and the molecular shape of a target protein might be altered, which would improve the transduction of fusion proteins into cells.

It is well known that COX-2 and NO produced in macrophages play critical roles in inflammatory diseases (Romanovsky *et al*., 2006; Lee *et al*., 2007; Kim *et al*., 2007). Thus, the inhibition of COX-2 and NO expressions may constitute an effective new therapeutic strategy for the treatment of inflammation and the prevention of inflammatory diseases. To determine whether transduced PEP-1-PTEN can play a biological role in cells, we tested the effects of transduced PEP-1-PTEN on COX-2 expression levels and NO production under LPS exposure. COX-2 expression is induced by a number of stimuli, including cytokines, during the inflammatory response (Carey *et al*., 2003). Nonsteroidal anti-inflammatory drugs inhibit COX, leading to a marked decrease in prostaglandin synthesis and inflammation (Simon, 1996). The transduced PEP-1-PTEN fusion protein significantly suppressed LPS-induced COX-2 expression levels and NO production in Raw 264.7 cells in a dose- dependent manner.

NF-κB is a transcription factor that controls a number of genes, such as, iNOS and COX-2, TNF-α, and IL-6, which are important for immunity and inflammation (Barnes *et al*., 1997; Yun *et al*., 2008). Upon stimulation with LPS, NF-κB is translocated in the cytoplasm as an inactive complex bound to IκB-α, which is phosphorylated and subsequently degraded, and then dissociates to produce activated NF-κB. Therefore, we examined the effect of transduced PEP-1-PTEN on the LPS-induced activation of NF-κB. We found that the translocation of NF-κB was inhibited by transduced PEP-1-PTEN fusion protein in a dose-dependent manner, as well as the phosphorylation and degradation of IκB-α in the cells. In addition, we performed experiments to explore the effect of PEP-1-PTEN fusion protein on the LPS-induced expression levels of iNOS and COX-2 mRNA. The transduced PEP-1-PTEN fusion protein markedly inhibited iNOS and COX-2 mRNA expression levels. Thus, inhibition of these mediators may constitute an effective therapeutic strategy for the prevention of inflammatory reactions and diseases.

Suzuki *et al*. (2003b) indicates that *k5Pten*$^{flox/flox}$ mice, which a keratinocyte-specific null mutation of *Pten*, exhibit noticeably wrinkled skin, and more than 90% of the mice died within 3 weeks of birth. Skin is continuously exposed to many hazardous environmental agents. PTEN is an essential regulator of normal development and oncogenesis in the skin. Thus, the cell permeable PTEN fusion proteins used in this study may have therapeutic potential against skin inflammation disorders when applied topically. PEP-1 peptide carriers present several advantages for protein therapy, which include the translocation of native proteins, high stability, a lack of toxicity, and a lack of sensitivity to serum. In particular, no toxicity to PEP-1 peptide was observed in several cell lines at up to 1 mM. Although the exact mechanisms of protein transduction are unclear, transduction of the PTEN fused with PEP-1 vector offers more attractive

advantages for protein therapy.

In summary, we demonstrated for the first time that human PTEN fused with PEP-1 peptide (PEP-1-PTEN) can be efficiently transduced into Raw 264.7 cells. In addition, transduced PEP-1-PTEN fusion protein significantly suppressed LPS-induced COX-2 expression, NO production. Moreover, the inhibitory effects of PEP-1-PTEN fusion protein were found to be associated with NF-κB inactivation via the blockade of IκB-α phosphorylation. Although the detailed mechanism needs to be further elucidated, our success in the protein transduction of PEP-1-PTEN may be beneficial in developing topical application against inflammatory skin disorders.

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