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Transduced PEP-1-AMPK inhibits the LPS-induced expression of COX-2 and iNOS in Raw264.7 cells

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AMP-activated protein kinase (AMPK) is a heterotrimeric enzyme that plays a central role in cellular metabolic stress. Modulation of nitric oxide (NO) and cyclooxygenase-2 (COX-2) is considered a promising approach for the treatment of inflammation and neuronal diseases. In this study, the *AMPK* gene was fused in-frame with PEP-1 peptide in a bacterial expression vector to produce a PEP-1-AMPK fusion protein. Expressed and purified PEP-1-AMPK fusion proteins were transduced efficiently into macrophage Raw 264.7 cells in a time- and dose-dependent manner. Furthermore, transduced PEP-1-AMPK fusion protein markedly inhibited LPS-induced iNOS and COX-2 expression. These results suggest that the PEP-1-AMPK fusion protein can be used for the protein therapy of COX-2 and NO-related disorders such as inflammation and neuronal diseases. [BMB reports 2010; 43(1): 40-45]

INTRODUCTION

Inflammation is characterized by the activation of monocytes and macrophages, specifically the secretion of inflammatory mediators such as nitric oxide (NO). Inducible nitric oxide synthase (iNOS) mediate many physiological events, as it is one of only three enzymes that generates NO. Furthermore, overproduction of iNOS is associated with various human diseases including inflammatory and neuronal disorders (1-8).

Prostaglandins (PGs) are potent proinflammatory mediators, synthesized through arachidonic acid metabolism by cyclooxygenase (COXs), that play an important role in modulating a number of pathophysiological conditions, including inflammatory and allergic immune responses (9). The two COX enzyme isoforms have been well studied. COX-1 is constitutively expressed and plays an important role in maintaining normal cell physiology, whereas COX-2 is markdely induced by stimuli such as cytokines during the inflammatory response (10-12).

Lipopolysaccharide (LPS), the main component of endotoxin, is formed by the covalent linkage of phosphoglycolipid to hydrophilic heteropolysaccharide (13). LPS arrests the proliferation of macrophages, activating them to produce pro-inflammatory factors them are important in the immune response (14, 15). Such responses include the induction of pro-inflammatory cytokines and reactive oxygen species (ROS) as well as COX-2 and iNOS expression. Therefore, suppressing the induction of COX-2 and iNOS expression is a new paradigm in the prevention of inflammation (16).

AMP-activated protein kinase (AMPK) is an evolutionary conserved serine/threonine kinase that regulates cellular energy homeostasis (17-19). AMPK is activated in response to changes in cellular energy such as heat shock, hypoxia, ischemia, muscle exercise and other stimuli that compromise cellular ATP levels (20-23). AMPK is a heterotrimeric enzyme consisting of a catalytic subunit (α) and two regulatory subunits (β and γ), each of which has two α, two β and three γ isoforms (24, 25). Several studies have shown that AMPK improves insulin sensitivity, glucose homeostasis and energy metabolism, thus establishing AMPK an attractive and novel target for the treatment of type 2 diabetes, obesity and cardiac hypertrophy (20, 26). Recent studies using 5-aminoimidazole-4-carboxamide ribose (AICAR), the pharmacological activator of AMPK, have confirmed AMPK as an anti-inflammatory target. Specifically, AICAR has been shown to decrease the levels of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and iNOS in macrophages, microglia and astrocytes. However, the anti-inflammatory effects of AICAR are still controversial (19, 27-30). In addition, there is little information concerning the role that AMPK plays in inflammatory processes.

In the present study, we designed a PEP-1-AMPK fusion protein by genetic in-frame transduction. We showed that PEP-1-

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AMPK can be directly transduced into macrophage Raw 264.7 cells, and that it inhibits iNOS and COX-2 mRNA and protein expression in LPS-induced cells. Therefore, we suggest that PEP-1-AMPK fusion protein could be a potential therapeutic agent for the prevention of inflammatory diseases.

RESULTS AND DISCUSSION

Construction and purification of PEP-1-AMPK fusion protein

To generate a cell-permeable expression PEP-1-AMPK vector, human AMPK cDNA was subcloned into a pET-15b plasmid that had been reconstructed with PEP-1 peptide. The newly formed PEP-1-AMPK expression vector contained consecutive cDNA, sequences encoding human AMPK, PEP-1 peptide and six histidine residues at the amino terminus (Fig. 1A). We also constructed an AMPK expression vector for the production of control AMPK protein without PEP-1 transduction peptides (data not shown).

PEP-1-AMPK fusion proteins were purified. The fusion proteins were expressed in *E. coli* and clarified cell extracts were loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column for purification. Fractions containing PEP-1-AMPK fusion proteins were combined and salts were removed PD10 column chromatography. A single major band was obtained by superpose 6 FPLC chromatography. Crude cell extracts obtained from *E. coli* were electrophoresed alongside purified PEP-1-AMPK fusion proteins in 8% SDS-PAGE gel (Fig. 1B). Expressed and purified proteins were further confirmed by Western blot analysis using anti-rabbit polyhistidine antibody and corresponding bands indicating PEP-1-AMPK were detected (Fig. 1C).

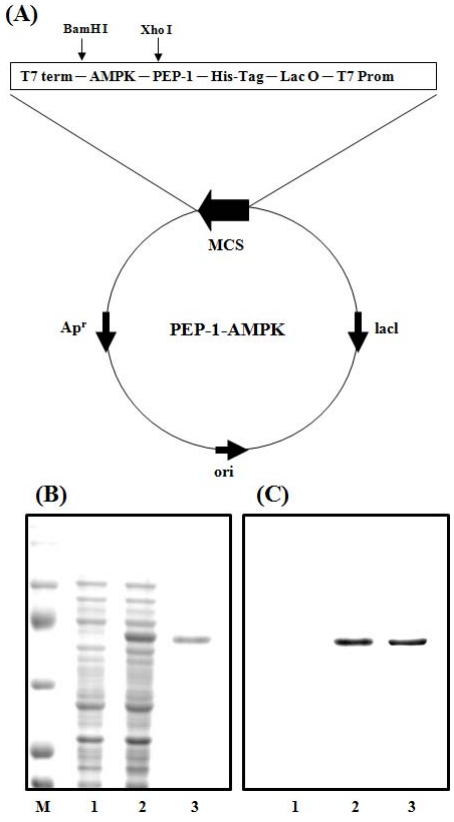

Fig. 1. Expression and purification of PEP-1-AMPK fusion protein. Construction of the PEP-1-AMPK fusion protein expression vector (A). The AMPK coding frame is represented by an open box along with 6His and PEP-1 peptide. Expression was induced by IPTG. Protein extracts and purified fusion proteins were analyzed by 8% SDS-PAGE (B) and subjected to Western blot analysis with anti-rabbit polyhistidine antibody (C). Lanes B and C are as follows; lane 1, non-induced PEP-1-AMPK; lane 2, induced PEP-1-AMPK; lane 3, purified PEP-1-AMPK.

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

We transduced various concentrations (0.5-3 μM) of PEP-1-AMPK fusion protein to Raw 264.7 cells and then analyzed the levels of transduction by Western blotting. As shown in Fig. 2A, the levels of transduced proteins in Raw 264.7 cells increased in a concentration-dependent manner.

The dependency of transduction of PEP-1-AMPK transduction

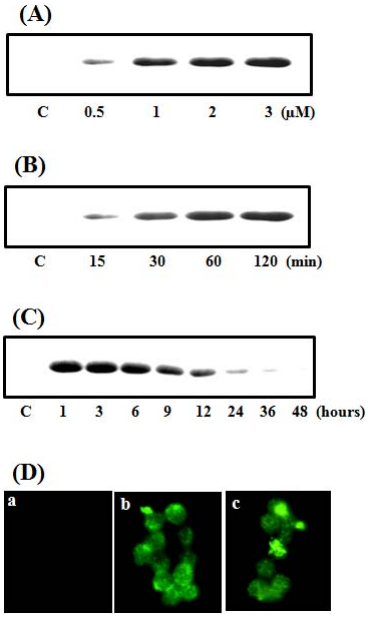

Fig. 2. Transduction of PEP-1-AMPK fusion proteins. PEP-1-AMPK (0.5-3 μM) was added to the Raw 264.7 cells culture media for 60 min (A), 3 μM of PEP-1-AMPK were added to the culture media for 15-120 min (B), cells pre-treated with 3 μM of PEP-1-AMPK were incubated for 1-48 h and analyzed by Western blot analysis (C). FITC-labeled PEP-1-AMPK (3 μM) was transduced into Raw 264.7 cells, which were washed twice with trypsin-EDTA in PBS and immediately observed by fluorescence microscopy. Control cells without PEP-1-AMPK (D-a), non-fixed cells treated with PEP-1-AMPK (D-b), and fixed cells treated with PEP-1-AMPK (D-c).

upon time was further analyzed. Three μM of PEP-1-AMPK proteins were added to the cellular mixture for various time periods (15-120 min) after which the level of transduction was measured by Western blotting. As shown in Fig. 2B, the intracellular concentration of transduced PEP-1-AMPK in cells gradually increased after 60 min. In addition, the intracellular stability of transduced PEP-1-AMPK in cells is shown in Fig. 2C. PEP-1-AMPK fusion proteins were added to the cellular culture media at a concentration of 3 μM for various time periods, and the resulting levels of transduced protein were analyzed by Western blotting. Transduced PEP-1-AMPK fusion protein was initially detected after 1 h, but gradually declined over the observation period. Significant levels of transduced AMPK protein did persist in the cells for 36 h, however.

The intracellular delivery of PEP-1-AMPK fusion protein into Raw 264.7 cells was confirmed by direct fluorescence. To exclude the possibility that cell fixation by paraformaldehyde affects PEP-1-AMPK transduction, we transduced FITC-labeled PEP-1-AMPK fusion protein into non-fixed cells. Immunofluorescence staining using rabbit anti-histidine polyclonal antibody revealed that PEP-1-AMPK fusion protein was transduced into cells, but fluorescence signals were absent in cells not treated with PEP-1-AMPK fusion protein. However, the fluorescence signals of fixed cells were similar to those of non-fixed cells. These results indicate that cell fixation is not required for PEP-1-AMPK fusion protein transduction (Fig. 2D).

Fusion proteins containing protien transduction domains (PTDs) do not transduce across cellular membranes, and therefore any apparent entry is an artifact caused by fixation. Fixation disrupts the cell membrane, internalizing peptiteds and fusion proteins by endocytosis. Thus, fixation is not reliable for studying protein transduction into living cells and is typically avoided (31). However, we were unable to detect any differences in fluorescence distribution of transduced PEP-1-AMPK fusion proteins in non-fixed and fixed cells. Similar observations indicate that artifacts of protein transduction are not induced by paraformaldehyde fixation (32). We have also observed that the transduction of various PTD-fusion proteins into cells was not affected by paraformaldehyde fixation (33, 34).

Effects of PEP-1-AMPK on LPS-induced expression of iNOS and COX-2 in Raw 264.7 cells

LPS, as the main component of endotoxin, arrests macrophage proliferation and activates them to produce pro-inflammatory factors (14, 15). Thus, we examined the effect of PEP-1-AMPK fusion protein transduction on the expression of COX-2 and iNOS under LPS exposure. Raw 264.7 cells were incubated for 12 h with LPS (100 ng/ml) in the absence or presence of PEP-1-AMPK (0.5-3 μM). Our evidence shows that transduction of PEP-1-AMPK fusion proteins suppresses LPS-induced protein expression of COX-2 and iNOS in a dose-dependent manner (Fig. 3). We further examined the effects of PEP-1-AMPK on COX-2 and iNOS mRNA expression levels in LPS-induced cells by RT-PCR. As shown in Fig. 4, PEP-1-AMPK fusion proteins inhibited the LPS-induced expression of COX-2 and iNOS mRNA in a dose-dependent manner. These results suggest that the overall inhibition of COX-2 and NO production was due to the inhibition of mRNA expression by PEP-1-AMPK. COX-2 and iNOS proteins have been reported to be closely associated with cutaneous inflammation, cell proliferation, and skin tumor promotion, all of which are rapidly induced by pro-inflammatory mediators (35-37). Thus, the inhibition of COX-2 and iNOS expression may provide an effective new therapeutic strategy for the treatment of inflammation and the prevention of inflammatory reactions and diseases.

In summary, we demonstrated for the first time that human AMPK fused with PEP-1 peptide (PEP-1-AMPK) can be efficiently transduced into Raw 264.7 cells. In addition, PEP-1-AMPK fusion protein significantly inhibits LPS-induced iNOS and COX-2 expression in Raw 264.7 cells. Although the detailed mechanism remains elucidated, our success in the protein transduction of PEP-1-AMPK may be beneficial in developing protective strategies against inflammatory processes. Furthermore,

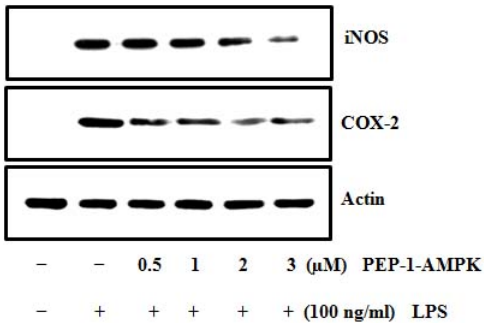

Fig. 3. Inhibitory effect of transduced PEP-1-AMPK fusion proteins on LPS-induced iNOS and COX-2 protein expression in Raw 264.7 cells. Cells were pretreated with PEP-1-AMPK fusion protein for 1 h before incubation with LPS (100 ng/ml) for 12 h. Cells lysates were prepared and protein expression levels of iNOS and COX-2 were analyzed by Western blotting.

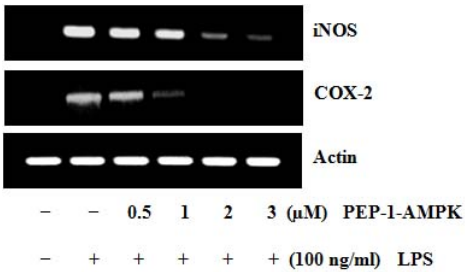

Fig. 4. Inhibitory effect of PEP-1-AMPK fusion protein on LPS-induced iNOS and COX-2 mRNA expression in Raw 264.7 cells. Cells were pretreated with the PEP-1-AMPK fusion protein for 1 h before incubation with LPS (100 ng/ml) for 12 h. After total RNA was extracted, iNOS and COX-2 mRNA were analyzed by RT-PCR using specific primers.

we suggest that PEP-1-AMPK fusion protein can potentially be a clinical drug for inflammation therapy.

MATERIALS AND METHODS

Materials

Ni^{2+}-nitrilotriacetic acid Sepharose superflow was purchased from Qiagen (GmbH, Germany). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Duchefa Co (Haarlen, Netherlands). Fetal bovine serum (FBS), DMEM, and penicillin-streptomycin antibiotics were purchased from Gibco BRL (Grand Island, USA). Primary antibodies against COX-2 and iNOS were purchased from the Santa Cruz Biotechnology company (Santa Cruz, CA, USA). All other chemicals and reagents were of the highest analytical grade available.

Expression and purification of PEP-1-AMPK fusion proteins

Construction of the PEP-1 fusion protein plasmid was performed as previously described (38). AMP-activated protein kinase (AMPK) gene was amplified by PCR using two primers. The sense primer was 5'-CTCGAGGCTGAGAAGCAGAAGC-3' with an *Xho*I restriction site, and the antisense primer was 5'-GGAT CCTCAACGGGCTAAAGTAGTAA-3' with a *Bam*HI restriction site. The PCR products were purified and cloned into a TA cloning vector followed by digestion with *Xho*I and *Bam*HI. The *PEP-1-AMPK* gene was then ligated in-frame into a pET-15b expression vector along with six histidine open reading frames. Host *E. coli* BL21 (DE3) cells were transformed with the PEP-1-AMPK plasmids, and transformants were selected on an LB plate containing ampicillin. Selected colonies were then cultured in LB medium containing ampicillin at 37°C with shaking at 250 rpm. Next, cells were grown to $O.D_{600}$ = 0.5-0.6, after which protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM. Incubation was continued for 3-4 h and harvested cells were lysed by sonication at 4°C in binding buffer (8 M urea, 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). After centrifugation, supernatants containing PEP-1-AMPK were immediately loaded on a 2.0 ml Ni^{2+}-nitrilotriacetic acid Sepharose column. After washing with 10 volumes of binding buffer and 6 volumes of washing buffer (8 M urea, 20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), the fusion protein was eluted with elution buffer (8 M urea, 0.5 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The purified fractions were removed of salt by PD10 column chromatography (Amersham, Braunschweig, Germany). Purified PEP-1-AMPK fusion protein was further purified by Superose 6 FPLC column chromatography. Protein concentrations were estimated by Bradford assay using bovine serum albumin as a standard (39).

Transduction of PEP-1-AMPK into Raw 264.7 cells

Murine macrophage Raw 264.7 cells 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 humidified conditions of 95% air and 5% CO_2. For transduction of PEP-1-AMPK, Raw 264.7 cells were grown to confluence on a 6-well plate and the culture medium was replaced with 1 ml of fresh solution. Cells were treated with various concentrations of PEP-1-AMPK fusion proteins for 1 h, followed by treatment with trypsin-EDTA (Gibco, Grand Island, NY, USA) and washing with phosphate-buffered saline (PBS). Cells were then harvested and cell extracts were used in Western blot analysis.

The intracellular stability of transduced PEP-1-AMPK fusion protein was estimated as follows: after Raw 264.7 cells were treated with 3 μM PEP-1-AMPK for 1 h and washed, fresh culture medium was added to remove whatever PEP-1-AMPK was not transduced. Cells were then further incubated for 48 h after which cell extracts were used for Western blot analysis.

Western blot analysis

Lysates from Raw 264.7 cells were prepared by incubating cells in lysis buffer at 4°C for 30 min. Protein concentration was determined by Bio-Rad protein assay. Proteins (30 μg) were separated on an 8% sodium dodecyl sulfate-polyacrylamide gel, and then 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 1 h, followed by incubation for 1 h at room temperature with anti-COX-2 and iNOS antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1 : 1,000) in TBST. After washing, the membrane was incubated for 1 h with proper secondary antibody conjugated to horseradish peroxidase diluted 1 : 10,000 in TBST. The membrane was incubated with a chemiluminescent substrate and exposed to Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ, USA).

Determination of COX-2 and iNOS protein expression

Raw 264.7 murine macrophage cells were incubated in 24-well plates at 70% confluence for 12 h. After incubation, the cells were pretreated with PEP-1-AMPK fusion protein (0.5-3 μM) for 1 h before treatment with LPS (100 ng/ml) for 12 h. The culture medium was harvested and the protein expression of COX-2 and iNOS were determined by Western blotting using anti-COX-2 and iNOS antibodies, respectively.

RT-PCR analysis

Total RNA was isolated from Raw 264.7 cells using a Trizol reagent kit (Invitrogen, Gaithersburg, MD, USA) according to manufacturer's instructions (40). The RNA (2 μg) was reversibly transcribed with 10,000 U of reverse transcriptase and 0.5 μg/μl of oligo-(dT) primer. PCR amplification of cDNA 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'-AGTGTGACGT-TGACATCCGTAA AGA-3'; iNOS antisense, 5'-CTGTCAGAG-CCTCGTGGCTTT-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 after ethidium bromide treatment.

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