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Transduced Tat-Annexin protein suppresses inflammation-associated gene expression in lipopolysaccharide (LPS)-stimulated Raw 264.7 cells

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Annexin-1 (ANX1) is an anti-inflammatory protein as well as an important modulator in inflammation. However, the precise action of ANX1 remains unclear. To elucidate the protective effects of ANX1 on lipopolysaccharide (LPS)-induced murine macrophage Raw 264.7 cells, we constructed a cell-permeable Tat-ANX1 protein. The transduced Tat-ANX1 protein markedly inhibited the expression of cyclooxygenase-2, production of prostaglandin E_2, and generation of pro-inflammatory cytokines in the cells. Furthermore, transduced Tat-ANX1 protein caused a significant reduction in the activation of nuclear factor-kappa B (NF-*k*B) and mitogen-activated protein kinase (MAPK). The results indicate that Tat-ANX1 inhibits the production of inflammatory response cytokines and enzymes by blocking NF-*k*B and MAPK. Therefore, Tat-ANX1 protein may be useful as a therapeutic agent against various inflammatory diseases. [BMB reports 2011; 44(7): 484-489]

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. The overproduction of inflammatory mediators is associated with numerous diseases, such as rheumatoid arthritis, atherosclerosis, chronic hepatitis, pulmonary fibrosis, and the development of cancer (1-3). Prostaglandins (PGs) are potent pro-inflammatory mediators derived from arachidonic acid metabolism by cyclooxygenase (COX) and play an important role in modulating a number of pathophysiological conditions, including inflammatory and allergic immune responses (4). The two isoforms of COX 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 (5, 6).

Nuclear factor-*k*B (NF-*k*B), a nuclear transcription factor, regulates the expression of various genes, including IL-1β, TNF-α, and COX-2, that play critical roles in apoptosis, tumorigenesis, various autoimmune diseases, and inflammation (7, 8). NF-*k*B is currently a target for treating various diseases due to its ubiquitous role in the pathogenesis of inflammatory gene expression (9, 10).

Annexin-1 (ANX1) is a calcium-dependent, phospholipid-binding protein originally identified as a glucocorticoid-inducible 37 kDa protein and lipocortin. ANX1 inhibits phospholipase activity and regulates diverse cellular functions such as cellular proliferation, anti-inflammatory effects, and cell differentiation (11-13). Although the biological function and molecular mechanism of ANX1 protein in inflammation remain unclear, it may be a novel anti-inflammatory therapeutics agent.

The successful delivery of full-length Tat fusion proteins by protein transduction technology has been amply demonstrated previously (14). Several small regions of proteins, called protein transduction domains (PTDs), have been developed to allow the delivery of exogenous proteins into living cells. In a previous study, we used this technology to show that various fusion proteins efficiently protect against cell death *in vitro* and *in vivo* (15-20). In the present study, we examined the inhibitory effects of Tat-ANX1 protein against LPS-induced inflammation-associated gene expression in murin macrophage Raw 264.7 cells.

RESULTS AND DISCUSSION

Construction, purification, and transduction of Tat-ANX1 protein

Annexin-1 (ANX1) has been shown to play anti-inflammatory

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roles. However, the biological function and molecular mechanism of ANX1 protein in inflammation are still unclear. Therefore, to investigate the functional roles and mechanism of this protein, we delivered ANX1 protein into cells using protein transduction technology.

To generate a cell-permeable expression vector, Tat-ANX1, human ANX1 cDNA was subcloned into a pET-15b plasmid that had been reconstructed to contain Tat peptide. The Tat-ANX1 expression vector contained consecutive cDNA sequences encoding human ANX1, a Tat peptide, and six histidine residues at the amino-terminus (Fig. 1A). Following induction of expression, Tat-ANX1 was purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography. SDS-PAGE and Western blot analysis of purified Tat-ANX1 protein were performed. Tat-ANX1 was highly expressed, and purified Tat-ANX1 had an estimated molecular mass of approximately 39 kDa. The fusion proteins were confirmed by Western blot analysis using an anti-rabbit polyhistidine antibody (Fig. 1B, C).

To evaluate the transduction ability of Tat-ANX1, we transduced Tat-ANX1 into Raw 264.7 macrophage cell culture medium either at 0.5 μM for various periods of time (10-60 min) or at various concentrations (0.1-0.5 μM) for 60 min. The levels of transduced Tat-ANX1 were measured by Western blotting. As shown in Fig. 1D, Tat-ANX1 was efficiently transduced into Raw 264.7 cells in a time- and dose-dependent manner. Further, we examined the intracellular stability of transduced Tat-ANX1 in Raw 264.7 cells. Significant levels of transduced Tat-ANX1 persisted in the cells for 36 h (Fig. 1D). To further clarify the cellular localization of transduced proteins in the cells, transduced cells were double-stained with the nucleus-specific marker DAPI. Tat-ANX1 protein was detected in the cytoplasm and nuclei of transduced cells. However, control ANX1 was not transduced into the cells (Fig. 2E). These results indicate that purified Tat-ANX1 was efficiently transduced into Raw 264.7 cells.

Protein delivery into cells is difficult due to their size and biochemical properties. Therefore, application of therapeutic agent proteins is problematic (21). This problem was over-

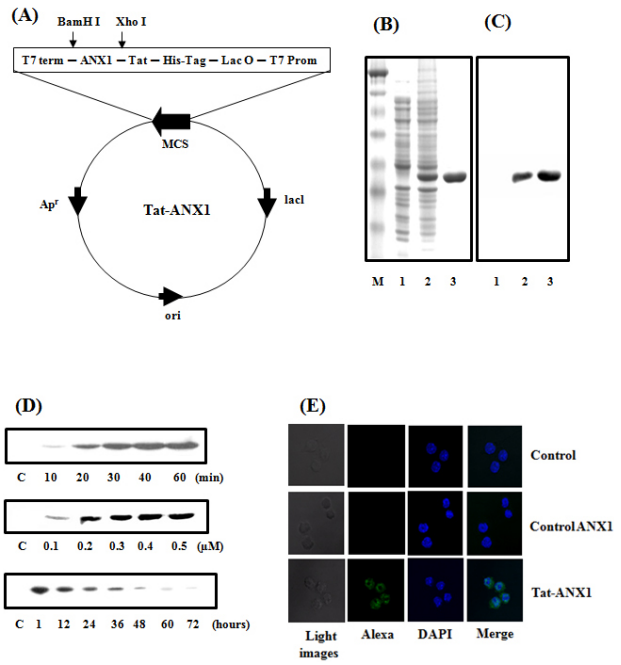

Fig. 1. Construction, purification, and transduction of Tat-ANX1 protein. Construction of pTat-ANX1 expression vector as described in Materials and Methods (A). Protein extracts of cells and purified protein were analyzed by 12% SDS-PAGE (B) and subjected to Western blot analysis with an anti-rabbit polyhistidine antibody (C). Lanes in A and B are as follows: lane 1, non-induced Tat-ANX1; lane 2, induced Tat-ANX1; lane 3, purified Tat-ANX1. Transduction of Tat-ANX1 protein into Raw 264.7 cells (D). Tat-ANX1 protein (0.5 μM) was added to the culture medium for 10-60 min. Tat-ANX1 (0.1-0.5 μM) was added to the culture media for 1 h. Cells pretreated with 0.5 μM Tat-ANX1 were incubated for various times prior to Western blotting. The distribution of transduced with Tat-ANX1 was observed by fluorescence microscopy (E).

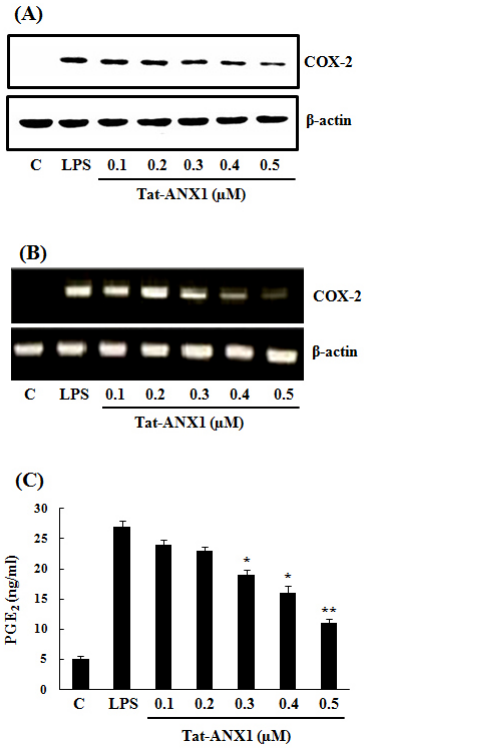

Fig. 2. Inhibitory effect of Tat-ANX1 protein on LPS-induced COX-2 expression and PGE_2 production in Raw 264.7 cells. Raw 264.7 cells were stimulated with LPS (100 ng/ml) for 12 h with or without pretreatment with Tat-ANX1 for 1 h. Cells lysates were prepared and analyzed for COX-2 protein expression by Western blotting (A). Total RNA was extracted. COX-2 mRNA was analyzed by RT-PCR using specific primers (B). Production of PGE_2 in the supernatant was evaluated by ELISA (C).

come using HIV-1 Tat protein transduction domain (PTD) or cell-penetrating peptides (CPP), which are capable of delivering proteins into cells. PTD can deliver exogenous target proteins into cells regardless of protein size and thus has been developed for therapeutic applications (22). Previously, we extensively studied PTD fusion proteins *in vitro* and *in vivo* and showed them to be beneficial in several diseases such as inflammation, Parkinson's disease, diabetes, and ischemia (15-20).

Effect of transduced Tat-ANX1 protein on LPS-induced COX-2 expression, PGE_2 production, and cytokine expression in Raw 264.7 cells

LPS is the main component of bacterial endotoxin and activates macrophages to produce pro-inflammatory factors, which play important roles in the immune response (23, 24).

To examine the effects of Tat-ANX1 on COX-2 expression and PGE_2 production levels upon LPS exposure, Raw 264.7 cells were incubated for 12 h with LPS (100 ng/ml) in the presence or absence of Tat-ANX1. Tat-ANX1 suppressed LPS-induced COX-2 protein and mRNA expression in a dose-dependent manner (Fig. 2A, B). Further, we examined PGE_2 production levels under the same conditions. After treatment with LPS, the medium concentration of PGE_2 was elevated significantly (27 ng/ml). This increase was markedly inhibited by transduced Tat-ANX1 (Fig. 2C).

Hannon *et al.* (2003) previously demonstrated that COX-2 protein and mRNA expression are constitutively increased in ANX1 knockout mice models (25). Further, other studies showed that ANX1 inhibits dexamethason-induced TNF-α and PGE_2 release in human peripheral blood mononuclear cells as well as LPS-induced nitric oxide production in J774 murine macrophages (26-28). These results suggest that ANX1 protein regulates anti-inflammatory functions.

We further examined the effects of Tat-ANX1 on pro-inflammatory cytokine production in LPS-stimulated Raw 264.7 cells. As shown in Fig. 3A and B, cytokine levels increased after treatment with LPS alone. However, transduced Tat- ANX1 significantly reduced production of TNF-α, IL-1β, and IL-6. Tat-ANX1 markedly inhibited LPS-induced mRNA expression of these cytokines in a dose-dependent manner.

It was reported that IL-1β release is increased in ANX1-deficient mice, and most of the anti-inflammatory activities of ANX1 protein are mediated by $ANX1_{2-26}$, which are N-terminus-derived peptides. Further, exogenous treatment with $ANX1_{2-26}$ inhibits nociceptive transmission associated with inflammatory processes in a formalin test, suggesting that ANX1 reduces paw oedema and inflammation (25, 29-32). Several studies have suggested that inhibition of COX-2 and cytokine production is important for alleviating inflammation, since inflammatory cytokines and COX-2 play important roles in the modulation of inflammation (33-35). Our results show that Tat-ANX1 inhibits LPS-induced COX-2, PGE_2, and cytokine expression. Thus, Tat-ANX1 may be an effective new therapeutic agent for the treatment of skin inflammation.

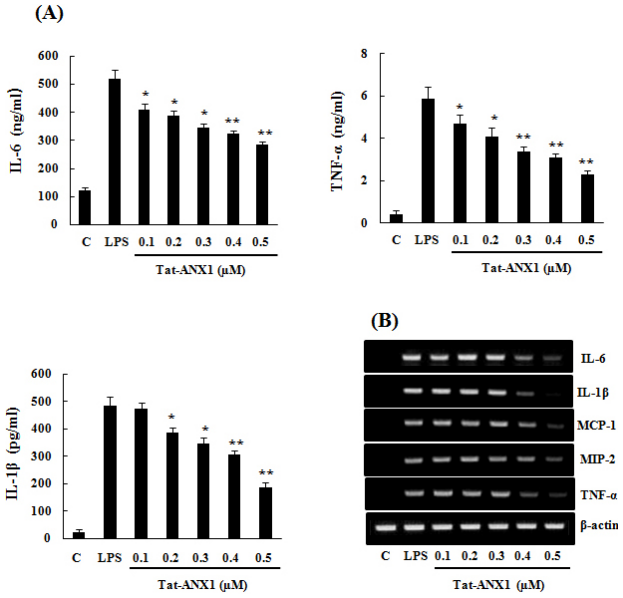

Fig. 3. Inhibitory effect of Tat-ANX1 protein on LPS-induced pro-inflammatory cytokines production in Raw 264.7 cells. Raw 264.7 cells were stimulated with LPS (100 ng/ml) for 12 h with or without pretreatment with Tat-ANX1 for 1 h. The production of pro-inflammatory cytokines in the supernatant was evaluated by ELISA (A). Total RNA was extracted from the cells. IL-1β, IL-6, TNF-α, MCP-1, MIP-2, and β-actin mRNA were analyzed by RT-PCR using specific primers (B).

Effect of Tat-ANX1 on LPS-induced NF-*k*B and MAPK activation in Raw 264.7 cells

NF-*k*B is a transcription factor that controls a number of genes that are important for immunity and inflammation (36, 37). LPS stimulation of macrophages activates NF-*k*B as well as several intracellular signaling pathways, including three MAPK pathways: ERK1/2, p38, and SAPK/JNK. Therefore, NF-*k*B and MAPK are current targets in the treatment of various diseases due to their ubiquitous roles in the pathogenesis of inflammatory gene expression (9, 10).

We therefore examined the regulatory effect of Tat-ANX1 on the LPS-induced signal cascade of NF-*k*B. Tat-ANX1 inhibited LPS-induced IkBα phosphorylation and degradation in cells (Fig. 4A). In addition, we further investigated the regulatory effect of Tat-ANX1 on the activity of MAPKs such as p38, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and extracellular signal-regulated kinase (ERK). To examine the effect of Tat-ANX1 on LPS-induced MAPK activation, Raw 264.7 cells were incubated for 30 min with LPS (100 ng/ml) in the presence or absence of Tat-ANX1 and then analyzed by Western blot analysis using phospho-specific antibodies against the aforementioned MAPK proteins (Fig. 4B). Tat-ANX1 decreased LPS-induced phosphorylation of p38, ERK, and SAPK/JNK in a dose-dependent manner. These results suggest that the NF-*k*B and MAPK pathways could modu-

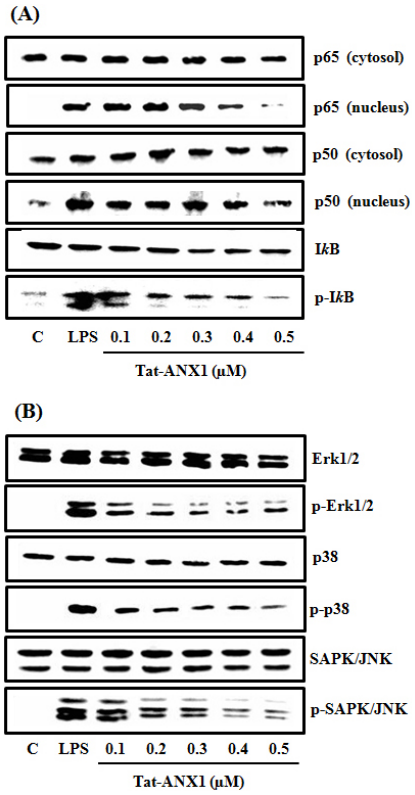

Fig. 4. Inhibitory effect of Tat-ANX1 protein on LPS-induced NF-*k*B and MAPK activation in Raw 264.7 cells. Raw 264.7 cells were stimulated with LPS (100 ng/ml) for 30 min with or without pre-treatment with Tat-ANX1 for 1 h. Phosphorylation and degradation of IkBα were analyzed by Western blotting (A). Extracts from the cells were prepared and analyzed for MAPK protein activation by Western blotting (B).

late the suppression of LPS-induced cytokine expression by Tat-ANX1. Therefore, we suggest that Tat-ANX1 can be used as a new therapeutic strategy for the treatment of inflammation and in the prevention of inflammatory reactions and diseases.

In summary, we demonstrated that human ANX1 fused with Tat peptide (Tat-ANX1) inhibits the production of pro-inflammatory enzymes and cytokines in murine macrophage Raw 264.7 cells. The anti-inflammatory effect of Tat-ANX1 is mediated by the down-regulation of pro-inflammatory gene expression via the suppression of NF-*k*B and MAPK. Our success suggests that transduced Tat-ANX1 may be a potential therapeutic agent for clinical treatment of inflammatory diseases.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), DMEM, and penicillin-streptomycin antibiotics were purchased from Gibco BRL (Grand Island, USA). Antibodies against histidine, COX-2, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies and phospho-specific antibodies against JNK/SAPK, p38, ERK, p65, p50, and IkB were obtained from Cell Signaling Technology (Beverly, MA, USA).

Expression and purification of Tat-ANX1 proteins

An HIV-1 Tat expression vector was prepared in our laboratory as described previously (38). Next, based on the cDNA sequence of human ANX1, two primers were synthesized from custom primers (Gibco BRL, Grand Island, NY, USA). The sense primer 5′-CTCGAGATGGCAATGGTATCAGAA-3′ contains an *Xho*I site, and the antisense primer 5′-GGATCCTTA-GTTTCCTCCACAAAG-3′ contains a *Bam*HI restriction site. Polymerase chain reaction (PCR) was performed, and the PCR product was excised with *Xho*I and *Bam*HI, eluted, ligated into a TA-cloning vector and a pTat vector using T4 DNA ligase (Promega, Madison, WI, USA), and cloned into *Escherichia coli* DH5α. The human ANX1 gene was fused with a nine amino acid Tat peptide in the bacterial expression vector to produce a genetic in-frame Tat-ANX1 protein.

To produce the Tat-ANX1 proteins, the plasmid was transformed into *E. coli* BL21 cells (Novagen). 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 then induced with 0.5 mM of isopropyl-beta-D-thiogalactopyranoside (Duchefa, Budapest, Hungary) at 37°C for 3-4 h. Harvested cells were disrupted by sonication in binding buffer. After centrifugation, the supernatant was immediately loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column. The protein containing the Tat-ANX1 fraction was combined, and the salts were removed using PD-10 column chromatography (Amersham, Piscataway, NJ, USA). The protein concentration was estimated using bovine serum albumin as a standard (39).

Cell culture and transduction of Tat-ANX1 protein

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

For transduction of Tat-ANX1, Raw 264.7 cells were grown to confluence in the wells of a six-well plate. The culture medium was replaced with 1 ml of fresh solution. After the cells were treated with various concentrations of Tat-ANX1 for 1 h, the cells were treated with trypsin-EDTA (Gibco BRL) and washed with phosphate-buffered saline (PBS). The cells were then harvested for the preparation of cell extracts to perform Western blot analysis.

Western blot analysis

Proteins in the cell lysates 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% nonfat dry milk in

PBS. The membrane was probed with the indicated antibodies, and immunoreactive bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

Determination of COX-2 expression levels

Raw 264.7 macrophages were incubated in six-well plates for 12 h to achieve 70% confluence. The cells were then pretreated with Tat-ANX1 protein (0.1-0.5 μM) for 1 h before treatment with LPS (100 ng/ml) for 12 h, after which the culture medium was harvested. The expression levels of COX-2 protein and RNA were determined by Western blotting and reverse transcription (RT)-PCR.

Measurement of cytokines and PGE_2

Raw 264.7 macrophages were incubated in six-well plates for 12 h to achieve 70% confluence. The cells were then pretreated with Tat-ANX1 protein (0.1-0.5 μM) for 1 h before treatment with LPS (100 ng/ml) for 12 h, after which the culture medium was harvested. Levels of TNF-α, IL-1β, IL-6, and PGE_2 in the supernatants were measured using an enzyme-linked immunosorbant assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

RT-PCR analysis

Total RNA was isolated from Raw 264.7 cells using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 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 were performed with the following sense and antisense primers: COX-2 antisense, 5′-TGGACGAGGTTTTTCCACCAG-3′; COX-2 sense, 5′-CAAAGGCCTCCATTGACCAGA-3′; TNF-α antisense, 5′-TGGCACCACTAGTTGGTTGTCTTT-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′-CAAGAAAGACAAAGCCAGAGTCCTT-3′; monocyte chemotactic protein-1 (MCP-1) antisense, 5′-TTCCTTCTTGGGCTTCAGCACAGAC-3′; MCP-1 sense, 5′-ACTGAAGCCAGCTCTCTCTTCCTC-3′; macrophage inflammatory protein-2 (MIP-2) antisense, 5′-AACATAACAACATCTGGGCAA-3′; MIP-2 sense, 5′-GAACAACGGCAAGGCTAACTG-3′; and β-actin antisense, 5′-GGACAGTGAGGCCAGGATGG-3′; β-actin sense, 5′-AGTGTGACGTTGACATCCG TAAAGA-3′. After PCR was performed, PCR products were resolved on 1% agarose gel and visualized with ultraviolet light after ethidium bromide staining.

Statistical analysis

The results are expressed at the mean ± S.E.M. The values were evaluated via one-way ANOVA, followed by Duncan's multiple range tests using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Differences were considered to be significant at $*P < 0.05$ and $**P < 0.01$ compared with LPS alone.

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