BMB
Reports

Suppression of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation in mice by transduced Tat-Annexin protein

Sun Hwa Lee[1,#], Dae Won Kim[1,#], Seon Ae Eom[1], Se-Young Jun[1], Meeyoung Park[1], Duk-Soo Kim[2], Hyung Joo Kwon[3], Hyeok Yil Kwon[4], Kyu Hyung Han[1], Jinseu Park[1], Hyun Sook Hwang[1], Won Sik Eum[1,] & Soo Young Choi[1,*]*

[1]Department of Biomedical Science and Research Institute of Bioscience and Biotechnology, Hallym University, Chuncheon 200-702, [2]Department of Anatomy, College of Medicine, Soonchunhyang University, Cheonan 330-090, Departments of [3]Microbiology, [4]Physiology, College of Medicine, Hallym University, Chuncheon 200-702, Korea

We examined that the protective effects of ANX1 on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation in animal models using a Tat-ANX1 protein. Topical application of the Tat-ANX1 protein markedly inhibited TPA-induced ear edema and expression levels of cyclooxygenase-2 (COX-2) as well as pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α). Also, application of Tat-ANX1 protein significantly inhibited nuclear translocation of nuclear factor-kappa B (NF-κB) and phosphorylation of p38 and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) in TPA-treated mice ears. The results indicate that Tat-ANX1 protein inhibits the inflammatory response by blocking NF-κB and MAPK activation in TPA-induced mice ears. Therefore, the Tat-ANX1 protein may be useful as a therapeutic agent against inflammatory skin diseases. [BMB Reports 2012; 45(6): 354-359]

INTRODUCTION

Inflammation is a major contributor to various diseases, such as rheumatoid arthritis, atherosclerosis, chronic hepatitis, pulmonary fibrosis, and the development of cancer. Inflammatory mediators, such as interlukin-1 beta (IL-1β), interlukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and cyclooxygenase-2 (COX-2) play important roles in inflammatory diseases (1-4). 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 (5, 6). Of the two isoforms of COX enzymes, 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 (7-9).

Nuclear factor-kappa B (NF-κB) is a nuclear transcription factor that plays a central role in the onset of inflammation. In its inactive state, NF-κB is bound to IκB in the cytosol. The activation of NF-κB, which in turn phosphorylates IκB and leads to degradation of NF-κB and translocation to the nucleus, induces the expression of various inflammatory genes including IL-1β, TNF-α, and COX-2 which leads to the activation of mitogen-activate protein kinases (MAPKs) such as p38, extracellular signal-regulated kinase (ERK1/2) and c-Jun N-terminal kinase (JNK) (10). Others studies have suggested that NF-κB may be a target for treating various diseases because of its ubiquitous role in the pathogenesis of inflammatory gene expression (11).

Annexin-1 (ANX1), a member of a superfamily of annexin proteins, was originally identified as a glucocorticoid inducible 37 kDa protein also known as lipocortin. ANX1 is highly expressed in the cytoplasm and has various functions including the inhibition of cytosolic phospholipase A2 (cPLA2), cellular proliferation as well as anti-inflammatory effects (12-14). Although ANX1 has been implicated as an anti-inflammatory agent, its protective mechanism remains poorly understood.

Protein transduction technology has been used to deliver a wide range of proteins into living mammalian cells. Since cell-permeable peptides, called protein transduction domains (PTDs) or cell penetrating peptides (CPPs), were identified two decades ago, these peptides have numerous therapeutic applications and are used to successfully deliver a range of molecules into cells. Among the cell-permeable peptides, Tat peptide is well known for its ability to deliver exogenous proteins

*Corresponding author. Soo Young Choi, Tel: +82-33-248-2112; Fax: +82-33-248-3201; E-mail: sychoi@hallym.ac.kr; Won Sik Eum, Tel: +82-33-248-2112; Fax: +82-33-248-3201; E-mail: wseum@hallym.ac.kr
#These authors equally contributed to this work.
http://dx.doi.org/10.5483/BMBRep.2012.45.6.036

Received 19 February 2012, Revised 6 March 2012, Accepted 19 March 2012

Keyword: Cytokine, Inflammation, MAPK, Tat-ANX1, TPA

into cells (15). In previous studies, we have shown that a number of transduced fusion proteins including ANX1 efficiently protected against cell death *in vitro* and *in vivo* (16-20).

In this study, we examined the protective effects of Tat-ANX1 protein on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation in animal models. The topical application of Tat-ANX1 to mice ears significantly inhibited TPA-induced ear edema, the expression of pro-inflammatory cytokines, enzymes as well as TPA-induced activation of NF-κB and mitogen-activated protein kinases (MAPKs). Therefore, Tat-ANX1 protein could be useful as a potential topically applied therapeutic agent for the treatment of skin inflammation.

RESULT AND DISCUSSION

Purification and transduction of Tat-ANX1 protein

Construction, purification and transduction of the Tat-ANX1 protein was previously described (20). In this study, we investigated how transduced Tat-ANX1 protein inhibited TPA-induced skin inflammation. Tat-ANX1 protein was purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography. The purified Tat-ANX1 and control ANX1, without a Tat protein transduction domain, are shown in Fig. 1A. The purified proteins were further confirmed by Western blot analysis using an anti-rabbit polyhistidine antibody. Tat-ANX1 proteins were detected at the corresponding bands in Fig. 1B.

To determine whether the Tat-ANX1 protein transduced across the skin barrier, we performed immunohistochemistry on ear sections of control ANX1-treated, Tat-ANX1-treated and sham control mice. In the control ANX1-treated mice, ANX1 protein level was slightly detected. However, ANX1 protein levels were significantly increased throughout the ear of Tat-ANX1-treated mice (Fig. 1C). These results indicate that Tat-ANX1 protein efficiently transduced beyond the skin barrier.

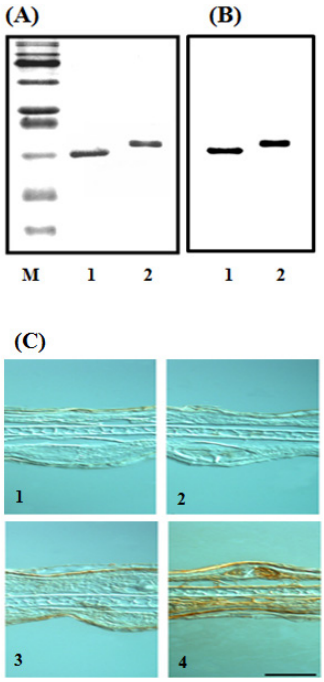

Fig. 1. Purification and histochemical analysis of animal skin transduced with Tat-ANX1 protein. Purified control ANX1 and Tat-ANX1 protein were analyzed by SDS-PAGE (A) and subjected to Western blot analysis (B) with an anti-rabbit polyhistidine antibody. Lanes are as follows: lane 1, control ANX1; lane 2, Tat-ANX1. Control ANX1 and Tat-ANX1 proteins (50 μg) were topically applied to the mice ear for 1 h. Frozen sections of ears were immunostained with rabbit anti-histidine IgG (1:400) and stained with biotinylated goat-anti-rabbit IgG (1:200). The sections were visualized with 3,3′-diaminobenzidine and observed under an Axioscope microscope (C). Panels are as follows: 1, control; 2, PBS treated; 3, control ANX1 treated; 4, Tat-ANX1 treated. Bar = 50 μm.

Effect of Tat-ANX1 on TPA-induced ear edema in mice

Mouse ear edema induced by TPA has been used as a model for inflammation studies (21). To investigate the anti-inflammatory effects of Tat-ANX1 protein, we tested Tat-ANX1 protein on TPA-induced inflammatory cell infiltration and in-

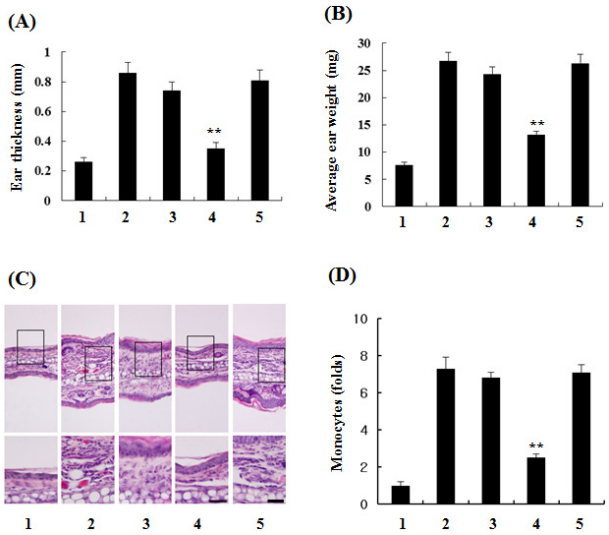

Fig. 2. Inhibitory effect of Tat-ANX1 on TPA-induced ear edema. The ears of mice (five mice in each group) were treated with TPA (1 μg/ear) once a day for 3 days. Tat-ANX1 protein (3 μM) was topically applied to mice ears 1 h after TPA treatment for 3 days. Inhibition of TPA-induced ear edema by topical application of Tat-ANX1 was analyzed by measuring changes in ear thickness (A) and ear weight (B). For histological analysis, ear skin sections were prepared and then stained with hematoxylin and eosin (C) and monocyte infiltration folds (D). Lanes are as follows: lane 1, normal control; lane 2, TPA treated mice; lane 3, ANX1 treated mice; lane 4, Tat-ANX1 treated mice; lane 5, Tat-GFP treated mice. Bar = 50 μm and 25 μm. $^{**}P < 0.01$ compared with mice treated with TPA along.

creased ear thickness as well as ear weight. Topical application of Tat-ANX1 protein to mice ears 1 h after TPA application once a day for 3 days afforded suppression of TPA-induced edema as determined by examining ear thickness and weight (Fig. 2A and B). In addition, Tat-ANX1 protein markedly inhibited infiltration of inflammatory cells, while control ANX1 had a minimal effect. To examine the effect of Tat itself on TPA-induced inflammation, we treated mice ears with Tat-GFP protein. However, Tat-GFP protein had no effect on TPA-induced ear edema (Fig. 2C and D). Topically applied Tat-ANX1 protein significantly inhibited the infiltration of monocytes into the skin, a step that is one of the early events in skin inflammation. Although the detailed mechanism remains to be further elucidated, Tat-ANX1 protein appears to have protective effects on TPA-induced skin inflammation.

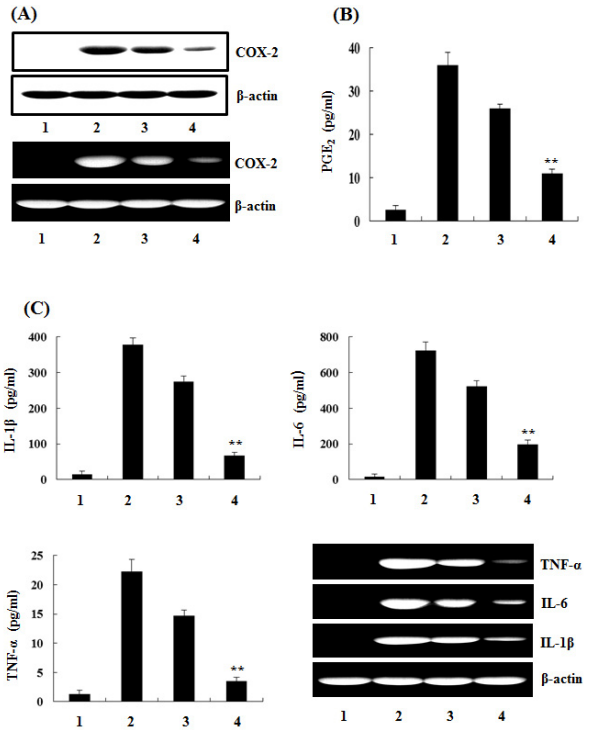

Fig. 3. Inhibitory effect of Tat-ANX1 protein on TPA-induced COX-2 expression, PGE_2 production, and pro-inflammatory cytokines in mice ears. Mice ears (five mice in each group) were treated with TPA (1 μg/ear) once a day for 3 days. Tat-ANX1 protein (3 μM) was topically applied to mice ears 1 h after TPA treatment for 3 days. Mice ear extracts were prepared and analyzed for COX-2 protein expression. Total RNA was extracted. COX-2 mRNA was analyzed by RT-PCR using specific primers (A). The production of PGE_2 in the supernatant was evaluated by ELISA (B). Supernatant fractions of homogenates from ear biopsies were examined for cytokine production using ELISA and total RNA was extracted from ear biopsies. IL-1β, IL-6, TNF-α, and β-actin mRNA were analyzed by RT-PCR using specific primers (C). Lanes are as follows: lane 1, normal control; lane 2, TPA treated mice; lane 3, ANX1 treated mice; lane 4, Tat-ANX1 treated mice. $^{**}P < 0.01$ compared with mice treated with TPA along.

Effects of Tat-ANX1 on TPA-induced COX-2 expression and PGE_2 production

To examine whether Tat-ANX1 protein can inhibit COX-2 expression and PGE_2 production in mice, Tat-ANX1 protein was topically applied to TPA-treated mice ears, and COX-2 expression and PGE_2 production were analyzed in the ear biopsies. The topical application of Tat-ANX1 significantly inhibited COX-2 mRNA and protein expression levels as determined by RT-PCR and Western blot analysis (Fig. 3A). Also, the topical application of TPA alone resulted in an increase in PGE_2 production. Tat-ANX1 significantly inhibited TPA-induced PGE_2 production (Fig. 3B). However, control ANX1 had a minimal effect. These results indicate that Tat-ANX1 can efficiently inhibit COX-2 and PGE_2 generation in TPA-induced in mice ears.

Effects of Tat-ANX1 on TPA-induced pro-inflammatory cytokines

It is well known that pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 might be involved in skin inflammation (22-24). Thus, we examined the effects of Tat-ANX1 protein on pro-inflammatory cytokine production in TPA-induced mice ears. The cytokine levels increased after treatment with TPA alone. Tat-ANX1 protein significantly reduced the production of TNF-α, IL-1β, and IL-6 (Fig. 3C). The effects of Tat-ANX1 on pro-inflammatory gene expression were further examined under the same conditions by RT-PCR. Tat-ANX1 markedly inhibited TPA-induced mRNA expression of these cytokines. In animal models of acute and chronic inflammation, recombinants ANX1 (renamed lipocortin 1) plays an anti-inflammatory action of glucocorticoid. Glucocorticoid inhibits prostaglandin release via a different mechanism (25). Also, ANX1 is known to act on the lipoxin A4 receptor (ALXR). In many inflammation animal models, ALXR has shown anti-inflammatory effects (26, 27). In this study, transduced Tat-ANX1 protein demonstrated more protective effects against inflammation.

Effects of Tat-ANX1 on TPA-induced on NF-κB and MAPK activation

Since NF-κB and MAPK activation are generally considered to play a role in many immune responses such the regulation of pro-inflammatory cytokine response (28), we examined the regulatory effect of Tat-ANX1 on TPA-induced signal cascades of NF-κB activation such as p65 translocation. Topically applied Tat-ANX1 inhibited TPA-induced p65 translocation from cytosol to nucleus (Fig. 4A).

Next, we investigated the regulatory effects of Tat-ANX1 on the activity of MAPKs such as p38 and ERK protein kinase. Tat-ANX1 was topically applied to TPA-treated mice ears after which MAPK activation was analyzed by Western blot analysis on cellular extracts from ear biopsies using phosphorylation

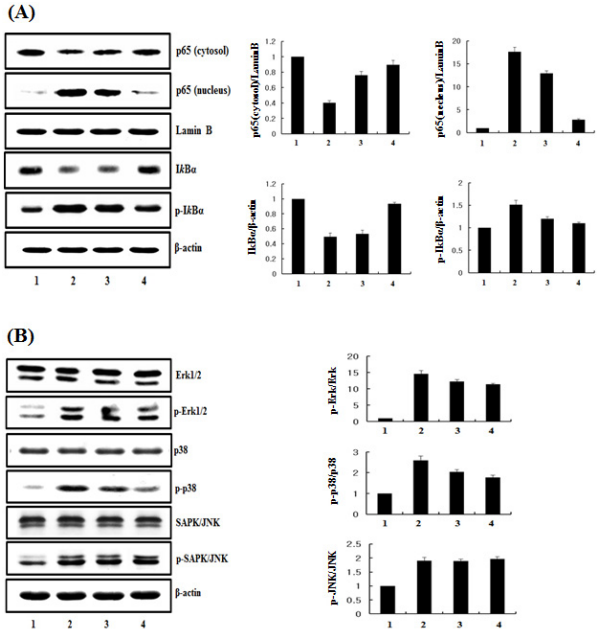

Fig. 4. Inhibitory effect of TPA-induced NF-κB and MAP kinase activation by Tat-ANX1 protein in mice ears. Mice ears (five mice in each group) were treated with TPA (1 μg/ear) once a day for 3 days. Tat-ANX1 protein (3 μM) was topically applied o mice ears 1 h after TPA treatment for 3 days. Degradation and phosphorylation of IkBα was analyzed by Western blotting. The level of proteins detected by Western blot was quantified by densitometer (A). Extracts from ear biopsies were prepared and analyzed for MAP kinase protein activation by Western blotting. The level of proteins detected by Western blot was quantified by densitometer (B). Lanes are as follows: lane 1, normal control; lane 2, TPA treated mice; lane 3, ANX1 treated mice; lane 4, Tat-ANX1 treated mice.

specific antibodies against MAPK proteins. Tat-ANX1 inhibited activation of TPA-induced phosphorylation of ERK and p38 MAPK. However, Tat-ANX1 did not affect SAPK/JNK activation (Fig. 4B). Further studies are needed to examine whether the expression of various inflammation factors is modulated by Tat-ANX1 protein. However, the present study revealed that Tat-ANX1 protein has anti-inflammatory effects by inhibiting the expression of COX-2, cytokines, NF-κB and MAPKs in TPA-treated mice. Therefore, we suggest that Tat-ANX1 can be used as a new therapeutic strategy for the treatment of inflammation and the prevention of inflammatory reactions and diseases.

In summary, we demonstrate that Tat-ANX1 protein inhibits the production of pro-inflammatory enzymes and cytokines via the suppression of NF-κB and MAPKs such as ERK and p38, suggesting that Tat-ANX1 protein may be relevant for the topical clinical treatment of inflammatory skin diseases.

MATERIALS AND METHODS

Materials

TPA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against COX-2 were purchased from Santa Cruz Biotechnology (Santacruz, CA, USA). The antibodies against p65, IkB, ERK, p38 and phsopho-specific antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kits including TNF-α, IL-1β, IL-6, and PGE_2 were purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals and reagents were the highest analytical grade available.

Expression and purification of Tat-ANX1 proteins

A Tat-ANX1 expression vector was constructed to express the Tat peptide as a fusion with human ANX1. Expression and purification of the ANX1 fusion proteins were carried out as described previously (20). To produce the Tat-ANX1 proteins, the plasmid was transformed into *E. coli* BL21 cells (Novagen, Sandiego, CA, USA). 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 of isopropyl-beta-D-thiogalactopyranoside (Duchefa, Budapest, Hungary) at 37°C for 3-4 h. Harvested cells were disrupted by sonication in a binding buffer. After centrifugation, the supernatant was immediately loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column. The fusion 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 (29).

TPA-induced skin inflammation

Male 6-8-week-old ICR mice were purchased from the Experimental Animal Center, at Hallym University, Chunchon, South Korea. The animals were housed at constant temperature (23ºC) and relative humidity (60%) with a fixed 12 h light:12 h dark cycle and free access to food and water. All experimental procedures involving animals and their care conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research & Quarantine Service of Korea and were approved by the Hallym Medical Center Institutional Animal Care and Use Committee.

Skin inflammation was induced in the right ear of each mouse by topical application of TPA as previously described (16). Five mice were used in each group. TPA (1.0 μg) dissolved in 20 μl of acetone was applied to the inner and outer surfaces of the mice ears every day for 3 days for 1 h. Tat-ANX1 protein was topically applied to the ears of mice every day for 3 days 1 h after TPA treatment to the same area. After 3 days, mice were sacrificed and 5 mm-diameter ear biopsies were obtained with a punch (Kai Industries, Gifu, Japan) and ear thickness and weight were measured. For histological analysis, ear biopsies were fixed in 4% paraformaldehyde, em-

bedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxyline and eosin. Stained tissue sections were examined to analyze the infiltration of inflammatory cells using standard bright-field optics with an AXIOIMAGER M1 apparatus (Carl Zeiss, Jena, Germany).

Western blot analysis

Ear biopsies were homogenized vigorously in tissue protein extraction reagent buffer with protease inhibitor cocktail and the samples were resolved by 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 phosphate buffered saline (PBS). The membrane was probed with the indicated antibodies, and the immunoreactive bands were visulalized by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Millipore, Billerica, MA, USA). The densities of the bands were quantitated with a densitometer (Image J software, USA).

RT-PCR

Total RNA from the ear biopsy samples was isolated using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR was performed with the following specific primers: COX-2 antisense, 5'-TGGACG AGGTTTTTCCACCAG-3'; COX-2 sense, 5'-CAAAGGCCTCC ATTGACCAGA-3'; TNF-α antisense, 5′-TGGCACCACTAGT TGGTTGTCTTT-3′; TNF-α sense, 5′-AAGTTCCCAAATGGCC TCCC-3′; IL-1β antisense, 5′-GTGCTGCCTAATGTCCCCTTG AATC-3′; IL-1β sense, 5′-TGCAGAGTTCCCCAACTGGTACA TC-3′; IL-6 antisense, 5′-TGGATGGTCTTGGTCCTTAGCC-3′; IL-6 sense, 5′-CAAGAAAGACAAAGCCAGAGTCCTT-3′; and β-actin antisense, 5'-GGACAGTGAGGCCAGGATGG-3'; β-actin sense, 5'-AGTGTGACGTTGACATCCGTAAAGA-3'. PCR products were resolved on a 1% agarose gel and visualized with ultraviolet light after ethidium bromide staining.

Measurement of cytokines and prostaglandin E_2 (PGE_2)

The ear biopsies described above were homogenized vigorously in tissue protein extraction reagent buffer with a protease inhibitor cocktail (Sigma-Aldrich). For measurement of PGE_2, the biopsies were homogenized in PBS containing 10 mM indomethacin and a protease inhibitor cocktail. The homogenate was centrifuged at 10,000 × g for 10 min. After the centrifugation, TNF-α, IL-1β, IL-6, and PGE_2 in the supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA), according to the manufacturer's instructions.

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 $^{**}P<0.01$ compared with TPA alone.

Acknowledgements

This work was supported by the Next Generation Growth Engine Program Grant (2010K001266) and in part by the Priority Research Centers Program grant (2009-0093812) through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology.

REFERENCES

1. Bertolini, A., Ottani, A. and Sandrini, M. (2002) Selective COX-2 inhibitors and dual acting anti-inflammatory drugs: critical remarks. *Curr. Med. Chem.* **9**, 1033-1043.
2. Firestein, G. S. (2003) Evolving concepts of rheumatoid arthritis. *Nature* **423**, 356-361.
3. Korhonen, R., Lathi, A., Kankaanranta, H. and Moilanen, E. (2005) Nitric oxide production and signaling in inflammation. *Curr. Drug Targets Inflamm. Allergy* **4**, 471-479.
4. Libby, P., Ridker, P. M. and Maseri, A. (2000) Inflammation and atherosclerosis. *Circulation* **105**, 1135-1143.
5. Cho, H. J., Seon, M. R., Lee, Y. M., Kim, J., Kim, J. K., Kim, S. G. and Park, J. H. (2008) 3,3′-Diindolylmethane suppresses the inflammatory response to lipopolysaccharide in murine macrophage. *J. Nutr.* **138**, 17-23.
6. Tilley, S. L., Coffman, T. M. and Koller, B. A. (2001) Mixed message gene: modulation of inflammation and immune responses by prostaglandins and thromboxanes. *J. Clin. Invest.* **107**, 191-195.
7. Carey, M. A., Germolec, D. R., Langenbach, R. and Zeldin, D. C. (2003) Cyclooxygenase enzymes in allergic inflammation and asthma. *Prostaglandins Leukot Essent Fatty Acids* **69**, 157-162.
8. Lee, E., Choi, M. K., Lee, Y. J., Ku, J. L., Kim, K. H., Choi, J. S. and Lim, S. J. (2006) Alpha-tocopheryl succinate, in contrast to alpha-tocopherol and alpha-tocopheryl acetate, inhibits prostaglandin E_2 production in human lung epithelial cells. *Carcinogen* **27,** 2308-2315.
9. Vancheri, C., Mastruzzo, C., Sortino, M. A. and Crimi, N. (2004) The lung as a privileged site for the beneficial actions of PEG_2. *Trends Immunol.* **25**, 40-46.
10. Buchanan, M. M., Hutchinson, M., Watkins, L. R. and Yin, H. (2010) Toll-like receptor 4 in CNS pathologies. *J. Neurochem.* **114**, 13-27.
11. Mararov, S. S. (2000) NF-κB as a therapeutic target in chronic inflammation: recent advances. *Mol. Med. Today* **6**, 441-448.
12. Babbin, B., Laukoetter, M. G., Nava, P., Koch, S., Lee, W. Y., Capaldo, C. T., Peatman, E., Severson, E. A., Flower, R. J., Perretti, M., Parkos, C. A. and Nusrat, A. (2008) Annexin A1 regulates intestinal mucosal injury, inflammation, and repair. *J. Immunol.* **181**, 5035-5044.
13. Kamal, A. M., Flower, R. J. and Perretti, M. (2005) An overview of the effects of annexin 1 on cells involved in the inflammatory process. *Mem. Inst. Oswaldo Cruz Rio de Janeiro* **100(Suppl 1)**, 39-48.

14. Perretti, M. and D'Acquisto, F. (2009) Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat. Rew. Immunol.* **9**, 62-70.
15. Wadia, J. and Dowdy, S. F. (2002) Protein transduction technology. *Curr. Opin. Biotechnol.* **13**, 52-56.
16. Ahn, E. H., Kim, D. W., Kang, H. W., Shin, M. J., Won, M. H., Kim, J., Kim, D. J., Kwon, O. S., Kang, T. C., Han, K. H., Park, J., Eum, W. S. and Choi, S. Y. (2010) Transduced PEP-1-ribosomal protein S3 (rpS3) ameliorates 12-O-tetradecanoylphorbol-13-acetate-induced inflammation in mice. *Toxicol.* **276**, 192-197.
17. An, J. J., Lee, Y. P., Kim, S. Y., Lee, S. H., Lee, M. J., Jeong, M. S., Kim, D. W., Jang, S. H., Yoo, K. Y., Won, M. H., Kang, T. C., Kwon, O. S., Cho, S. W., Lee, K. S., Park, J., Eum, W. S. and Choi, S. Y. (2008) Transduced human PEP-1-heat shock protein 27 efficiently protects against brain ischemic insult. *FEBS J.* **275**, 1296-1308.
18. Kim, D. W., Jeong, H. J., Kang, H. W., Shin, M. J., Sohn, E. J., Kim, M. J., Ahn, E. H., An, J. J., Jang, S. H., Yoo, K. Y., Won, M. H., Kang, T. C., Hwang, I. K., Kwon, O. S., Cho, S. W., Park, J., Eum, W. S. and Choi, S. Y. (2009) Transduced PEP-1-catalase fusion protein attenuates ischemic neuronal damage. *Free Radic. Biol. Med.* **47**, 941-952.
19. Lee, S. H., Jeong, H. J., Kim, D. W., Sohn, E. J., Kim, M. J., Kim, D. S., Kang, T. C., Lim, S. S., Kang, I. J., Cho, S. W., Lee, K. S., Park, J., Eum, W. S. and Choi, S. Y. (2010) Enhancement of HIV-1 Tat fusion protein transduction efficiency by bog blueberry anthocyanins. *BMB Rep.* **43**, 561-566.
20. Lee, S. H., Kim, D. W., Back, S. S., Hwang, H. S., Park, E. Y., Kang, T. C., Kwon, O. S., Park, J. H., Cho, S. W., Han, K. H., Park, J., Eum, W. S. and Choi, S. Y. (2011) Transduced Tat-annexin protein suppresses lipopolysaccharide (LPS)-stimulated inflammation gene expression in Raw 264.7 cells. *BMB Rep.* **44**, 484-489.
21. Stanley, P. L., Steiner, S., Havens, M. and Tramposch, K. M. (1991) Mouse skin inflammation induced by multiple topical applications of 12-O-tetradecanoylphorbol-13-acetate. *Skin Pharmacol.* **4**, 262-271.
22. DeVry, C. G., Valdez, M., Lazarov, M., Muhr, E., Buelow, R., Fong, T. and Iyer, S. (2005) Topical application of a novel immunomodulatory peptide, RDP58, reduces skin inflammation in the phorbol ester-induced dermatitis model. *J. Invest. Dermatol.* **125**, 473-481.
23. Ha, H. Y., Kim, Y., Ryoo, Z. Y. and Kim, T. Y. (2006) Inhibition of the TPA-induced cutaneous inflammation and hyperplasia by EC-SOD. *Biochem. Biophys. Res. Commun.* **348**, 450-458.
24. Murakawa, M., Yamaoka, K., Tanaka, Y. and Fukuda, Y. (2006) Involvement of tumor necrosis factor (TNF)-α in phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin edema in mice. *Biochem. Pharmacol.* **71**, 1331-1336.
25. Flower, R. J. (1988) Lipocortin and the mechanism of action of the glucocorticoid. *Br. J. Pharmacol.* **94**, 987-1015.
26. Chiang, N., Fierro, I. M., Gronert, K. and Serhan, C. N. (2000) Activation of lipoxin A4 receptors by aspirin-triggered lipoxins and select peptides evokes ligands-specific responses in inflammation. *J. Exp. Med.* **191**, 1197-1208.
27. Kieran, N. E., Doran, P. P., Connolly, S. B., Greenan, M. C., Higgins, D. F., Leonard, M., Godson, C., Taylor, C. T., Henger, A., Kretzler, M., Burne, M. J., Rabb, H. and Brady, H. R. (2003) Modification of the transcriptomic response to renal ischemia/reperfusion injury by lipoxin analog. *Kidney Int.* **64**, 480-492.
28. Gayathri, B., Manjula, N., Vinaykumar, K. S., Lakshmi, B. S. and Balakrishnan, A. (2007) Pure compound from Boswellia serrata extract exhibits anti-inflammatory property in human PBMCs and mouse macrophages through inhibition of TNF-α, IL-1β, NO and MAP kinases. *Int. Immunopharmacol.* **7**, 473-482.
29. Bradford, M. A. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* **72**, 248-254.