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Topical transduction of superoxide dismutase mediated by HIV-1 Tat protein transduction domain ameliorates 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice

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ABSTRACT

A domain (RKKRRQRRR) derived from HIV-1 Tat is one of the most efficient protein transduction domains (PTD) for delivering macromolecules including proteins into cells and tissues. Antioxidant enzymes such as superoxide dismutase (SOD) and catalase are major cellular defenses against oxidative stress which results in various diseases including skin inflammation. In this study, we examined the effect of SOD fused with HIV-1 Tat PTD (Tat-SOD) on TPA-induced skin inflammation in mice. Topical application of Tat-SOD to mice ears 1 h after TPA application once a day for 3 days dose-dependently inhibited TPA-induced ear edema in mice. Topical application on mice ears of Tat-SOD also suppressed TPA-induced expression of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 as well as cyclooxygenase-2 (COX-2) and production of PGE_2. Furthermore, topical application of Tat-SOD resulted in significant reduction in activation of NF-κB and mitogen-activated protein kinases (MAPK) in the mice ears treated with TPA. These data demonstrates that Tat-SOD inhibits TPA-induced inflammation in mice by reducing the levels of expression of proinflammatory cytokines and enzymes regulated by the NF-kappaB and MAPK and can be used as a therapeutic agent against skin inflammation related to oxidative damage.

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Abbreviations: SOD, superoxide dismutase; PTD, protein transduction domain; TPA, 12-O-tetradecanoylphorbol-13-acetate; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase.

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1. Introduction

Exposure of the skin to a variety of stimuli such as cytokines (interferon-γ, TNF-α), UV radiation and TPA leads to the excessive generation of reactive oxygen species (ROS) directly or indirectly [1]. Considerable evidences suggest that oxidative stress resulting from overproduction of ROS plays a critical role in the development of various inflammatory skin diseases such as atopic dermatitis [2]. Increased levels of ROS in the skin can induce cellular damage that leads to various pathophysiological conditions including inflammatory responses [3].

ROS such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) can also act as second messengers in the intracellular signaling cascades to activate the mitogen-activated protein kinases (MAPKs) and redox-sensitive transcriptional factors such as NF-κB and AP-1 [4,5]. These signaling pathways play a critical role in both acute and chronic inflammatory processes [6]. In the skin, stimulation of these signaling cascades leads to induce abnormal expression of cell adhesion molecules and cytokine/chemokine dysregulation which are believed to increase infiltration of monocytes/T cells into the site of inflammation [7,8]. In addition, activation of NF-κB induces expression of nitric oxide synthase, and enzymes that control prostaglandin synthesis, which help to induce the inflammation responses [9,10].

Cellular defenses against ROS composed of antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase, and low molecular mass antioxidants such ascorbic acid, glutathione and tocopherols [11]. SOD catalyzes the decomposition of superoxide to generate hydrogen peroxide which is then subsequently converted to water and oxygen by glutathione peroxidase and catalase [12]. Thus, these enzymes are vital for maintaining a balanced cellular redox state and are thought to have a beneficial effect against oxidative stress in various diseases [13].

Recombinant enzymes such as SOD and catalase have been used to protect against oxidative stress [14–18], with limited efficacy due to the lack of their efficient uptake into cells. We could potentially overcome this limitation by generating a cell permeable SOD fused with a peptide derived from HIV-1 Tat protein transduction domain (PTD) that is capable of delivering protein into cells [19]. Previous studies have reported that PTD derived from Tat can mediate delivery of the target protein to various tissues in mice, including the brain [20,21]. A cell permeable SOD, Tat-SOD has been extensively studied in vitro and in vivo, and has been shown to be beneficial in several oxidative stress models such as ischemia and diabetes [19,22–24].

The availability of cell-permeable SOD prompted us to study the possibility of its topical application to control ROS-mediated inflammatory diseases. In this study, we examine the effect of Tat-SOD on TPA-induced skin inflammation. We show that topical application of Tat-SOD to mice ears significantly inhibited TPA-induced ear edema as well as TPA-induced expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and enzymes (COX-2, iNOS) in mice. Furthermore, treatment of Tat-SOD after TPA application resulted in significant reduction in TPA-induced activation of NF-κB and MAPKs such as ERK and p38. These data indicate that cell-permeable SOD mediated by HIV-1 Tat PTD can be used as a therapeutic agent for the treatment of skin inflammation by topical application.

2. Materials and methods

2.1. Animal and cell culture

Male 6-to-8-week-old ICR mice were purchased from the Experimental Animal Center, Hallym University, Chunchon, South Korea. The animals were housed at constant temperature (23 °C) and relative humidity (60%) with a fixed 12-h light:12-h dark cycle and free access to food and water. The animals used in this experiment were treated according to the "Principles of Laboratory Animal Care" (NIH Publication No. 86-23). The immortalized human keratinocyte cell line, HaCaT, was maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.2. Reagents

TPA, Indomethacin, AA-861, 2′,7′-Dichlorofluorescin diacetate (DCF-DA) and prednisolone were purchased from Sigma (St. Louis, MO, USA). Primary antibodies against COX-2 and actin (Santa Cruz, CA, USA), and phosphor-IκBα, phosphor-p38, phosphor-ERK, or phosphor-JNK, total-IκBα, total-p38 MAPK, total-ERK, and total-JNK (Cell Signaling Technology, Beverly, MA, USA) were obtained commercially. HRP-conjugated anti-rabbit or goat antibodies were supplied by Sigma (St. Louis).

2.3. Purification and introduction of Tat-SOD fusion proteins into cells

Expression and purification of SOD fusion proteins were carried out as described previously [19,22]. SOD fusion proteins were expressed by inducing *E. coli* BL21 cells transformed with plasmids encoding the SOD or Tat-SOD fusion proteins with IPTG. To prepare SOD fusion proteins, the induced cells were pelleted and lysed in a binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9). The fusion proteins were purified by affinity chromatography on a Ni^{2+}-IDA column, followed by desalting with a PD10 column (Amersham). The SOD preparation eluted from column was subjected to a Detoxi-Gel™ Endotoxin Removing Gel (Pierce, Rockford, IL, USA) to remove endotoxin. Endotoxin levels for the SOD preparation were below the detection limit (<0.1 EU/ml) as tested by Limulus Amoebocyte Lysate assay (BioWhitaker, Walkersville, MD, USA). The purified SOD fusion proteins dissolved in PBS containing 20% glycerol were then aliquoted and stored at −80 °C. To introduce the Tat-SOD fusion proteins into HaCaT cells, the cells were plated at 6×10^5 cells/well in 6-well plates. When the cells were 70% confluent, the cells were incubated with fresh medium containing various concentrations of the SOD fusion proteins for 1 h. The cells were washed with PBS,

exposed to trypsin without EDTA (Gibco-BRL) for 10 min and washed again with PBS.

2.4. TPA-induced skin inflammation

The skin inflammation was induced in the right ear of mouse by triple topical application of TPA. Five mice in a group were used. TPA (1.0 μg) dissolved in 20 μl of acetone was applied to the inner and outer surfaces of the mice ears every 24 h for 3 days. SOD fusion protein or prednisolone as a reference compound was topically applied to the ears of mice at 1 h after TPA treatment. On day 2, TPA was reapplied, and SOD was applied at 1 h later. On day 3, TPA was applied and 1 h later, SOD was applied to the same area. At 24 h after the last treatment with SOD, ear thickness was measured using a digital thickness gauge (Mitutoyo Corporation, Japan). Mice were sacrificed and ear biopsies were obtained with a punch (a diameter of 5 mm, Kai Industries Co. Ltd., Gifu, Japan) and weighed. Ear biopsies were frozen in liquid N_2, and homogenized in a buffer and stored at −70 °C until use.

2.5. Measurement of ROS levels

ROS levels of mice ears were determined using the ROS-sensitive dye 2′,7′-dichlorofluorescein diacetate (DCF-DA), which is converted by ROS into the highly fluorescent 2′,7′-dichlorofluorescein (DCF) [25]. Ear biopsies were frozen in liquid nitrogen and homogenized vigorously in 0.1 M phosphate buffer (pH 7.4), and their homogenate was incubated with DCF-DA (10 μM) for 30 min. The level of DCF fluorescence, reflecting the concentration of ROS, was measured at 485 nm excitation and 538 nm emission by a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland).

2.6. Western blot

Ear biopsies were homogenized vigorously in tissue protein extraction reagent buffer with protease inhibitor cocktail (Pierce), and their homogenate was incubated on ice for 15 min in the presence of 0.1% Triton X-100. Lysates from HaCaT cells were prepared by incubating cells in lysis buffer (125 mM Tris–HCl, pH 6.8, 2% SDS, 10% v/v, glycerol.) at 4 °C for 30 min. Protein concentration was determined by Bio-Rad protein assay. Samples of 50 μg proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and the proteins were electrotransferred to a nitrocellulose membrane, which was blocked with 10% dry milk in PBS. The membrane was probed with the indicated antibodies, and the immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham) as recommended by the manufacturer.

2.7. Histology

For histological analysis, ear biopsies were fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 4 μm, and then stained with hematoxylin and eosin. Stained tissue sections were examined to analyze the infiltration of inflammatory cells using standard bright-field optics (Zeiss, AXIOIMAGER M1, Göttingen, Germany).

2.8. RT-PCR analysis

Total RNA was isolated from ear biopsies using a Trizol reagent kit (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions [26]. The RNA (2 μg) was reversibly transcribed with 10,000 U of reverse transcriptase and 0.5 μg/μl oligo-$(dT)_{15}$ primer (Promega, Madison, WI, USA). PCR amplification of cDNA aliquots was performed with the following sense and antisense primers (5′ → 3′): TNF-α sense, ACA AGC CTG TAG CCC ACG; TNF-α antisense, TCC AAA GTA GAC CTG CCC; IL-1β sense, TGC AGA GTT CCC CAA CTG GTA CAT C; IL-1β antisense, GTG CTG CCT AAT GTC CCC TTG AAT C; IL-6 sense, CAA GAA AGA CAA AGC CAG AGT CCT T; IL-6 antisense, TGG ATG GTC TTG GTC CTT AGC C; COX-2 sense, ACT CAC TCA GTT TGT TGA GTC ATT C; COX-2 antisense, TTT GAT TAG TAC TGT AGG GTT AAT G; ICAM-1 sense, TCG GAG GAT CAC AAA CGA AGC; ICAM-1 antisense, AAC ATA AGA GGC TGC CAT CAC G; MIP-2 sense, GAA CAA AGG CAA GGC TAA CTG A; MIP-2 antisense, AAC ATA ACA ACA TCT GGG CAA T; MCP-1 sense, ACT GAA GCC AGC TCT CTC TTC CTC; MCP-1 antisense, TTC CTT CTT GGG GTC AGC ACA GAC; beta-actin sense, GGA CAG TGA GGC CAG GAT GG; and beta-actin antisense, AGT GTG ACG TTG ACA TCC GTA AAG A. PCR was performed in 50 μl of 10 mmol/l Tris–HCl (pH 8.3), 25 mmol/l $MgCl_2$, 10 mmol/l dNTP, 100 U of *Taq* DNA polymerase, and 0.1 μmol/l 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.

2.9. Measurement of cytokines and PGE_2

For measurement of TNF-α, IL-1β, IL-6 and PGE_2 in the ear samples in TPA-induced skin edema model, ear biopsy samples were taken using 5 mm ear punch. For TNF-α, IL-1β and IL-6, the biopsies were homogenized vigorously in tissue protein extraction reagent buffer with protease inhibitor cocktail (Pierce), and their homogenate was incubated on ice for 15 min in the presence of 0.1% Triton X-100. In case of PGE_2, the biopsies were homogenized in PBS containing indomethacin (10 mM), AA-861 (10 mM) and protease inhibitor cocktail. Their 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 ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. HaCaT cells were cultured in the presence of TPA for 12 h, and TNF-α and IL-1 β in the supernatants were measured by ELISA kits.

2.10. Electrophoretic mobility shift assay (EMSA)

Mouse skin was treated with PBS or SOD fusion protein for 60 min after TPA treatment, and then nuclear extracts of ear biopsies were prepared and analyzed for NF-κB binding activity by EMSA as described previously [27]. An NF-κB consensus oligonucleotide (Promega) 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 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 dithiothreitol) 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 on a 6% native polyacrylamide gel in TBE buffer (89 mM Tris–HCl, 89 mM boric acid, and 2 mM EDTA).

2.11. *Statistical analysis*

The results were expressed as the mean ± S.E.M. from at least three independent experiments. The values were evaluated via one-way ANOVA, followed by Duncan's multiple range tests using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. *Effects of Tat-SOD on TPA-induced ear edema in mice*

Mouse ear edema induced by TPA has been used as a model for the acute/chronic inflammation. Multiple topical application of TPA to ICR mouse ear was shown to induce a chronic type of skin inflammation consisting of edema, epidermal hyperplasia and infiltration of inflammatory cells [28]. Triple application of TPA over 3 days to mouse ears induced inflammatory cell infiltration and increases in ear weight as well as ear thickness. All these parameters were significantly reduced by prednisolone, a reference drug (Fig. 1). We previously demonstrated that Tat-SOD fusion proteins can penetrate into the living animal skin with their biological activity [29]. Topical application of cell-permeable Tat-SOD to mice ears 1 h after TPA application once a day for 3 days afforded significant protection against TPA-induced edema and hyperplasia in a dose-dependent manner, as judged by ear thickness (Fig. 1A) and ear weight (Fig. 1B). Topical application of control SOD, which lacks protein transduction domain, showed no or little effect on these parameters. Post-application of Tat-SOD was also found to significantly inhibit infiltration of inflammatory cells, while control SOD had a minimal effect (Fig. 1C). To ensure that the Tat moiety itself is not affecting TPA-induced inflammation, an additional control of Tat-GFP (Green fluorescent protein) was used in our preliminary experiments. Tat-GFP did not affect TPA-induced ear edema, as judged by measurement of increase in the ear thickness and weight (data not shown). Next, we examined the effect of Tat-SOD on

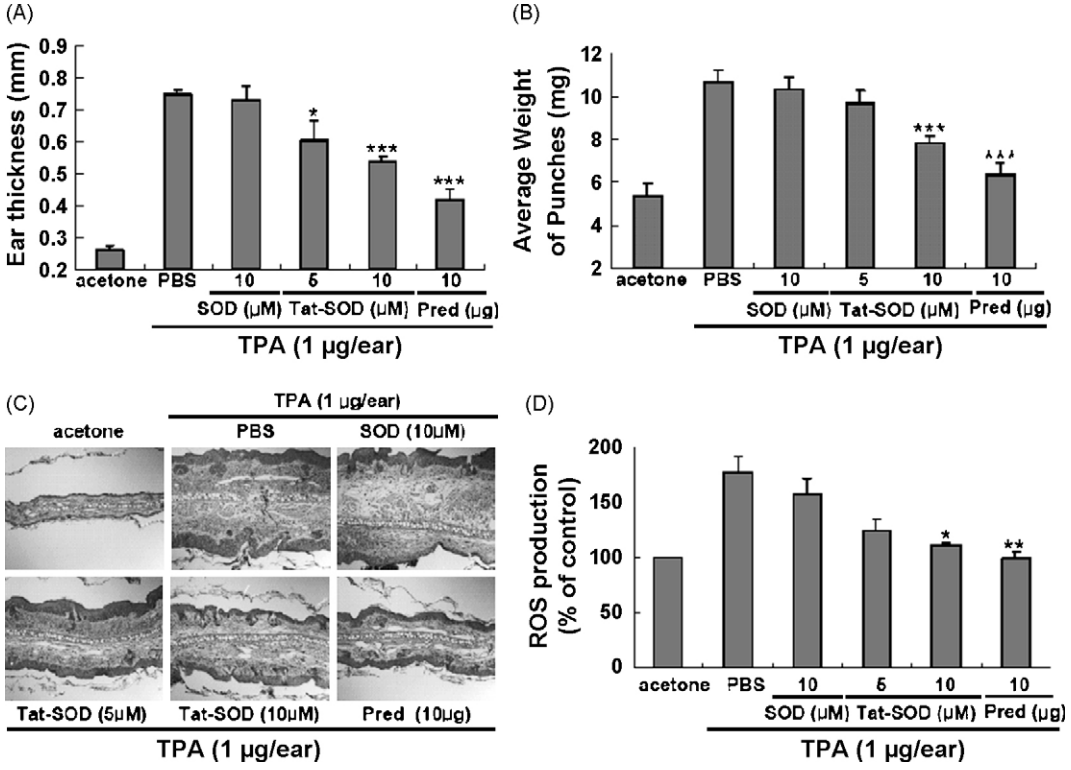

Fig. 1 – Inhibitory effect of Tat-SOD on TPA-induced ear edema. Ear of ICR mice (five mice in each group) was treated with TPA (1 μg/ear) once a day for 3 days. PBS or SOD fusion protein was topically applied to mice ears 1 h after TPA treatment during 3 days. The mice were sacrificed 24 h after the last SOD treatment. Inhibition of TPA-induced ear edema by topical application of Tat-SOD was analyzed by measuring changes in ear thickness (A) and ear weight (B). Data are mean ± S.D. from five ear samples. For histological analysis, ear skin sections were prepared as described in Section 2 and then stained with hematoxylin and eosin (original magnification, ×100) (C). The homogenate of ear biopsies was prepared and ROS levels of the homogenate were measured after staining with a fluorescent dye, DCF-DA by an ELISA plate reader (D). The data are the mean ± S.E.M. of three separate experiments. In (A), (B) and (D), $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with mice treated with TPA alone.

ROS generation induced by TPA, mice ears were treated with TPA for 1 h and then exposed to Tat-SOD for 1 h and the levels of ROS in tissue homogenates were evaluated by using DCF-DA as a probe. Tat-SOD inhibited TPA-induced increase of ROS levels in mice ears whereas the control SOD had minimal effect (Fig. 1D). These results suggest that Tat-SOD can efficiently remove ROS generation by TPA stimulation in mice ears.

3.2. *Effects of Tat-SOD on TPA-induced production of pro-inflammatory cytokines in mice ears*

Since it has been reported that the pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 might be involved in TPA-induced skin inflammation [30–33], we next examine the effects of Tat-SOD on pro-inflammatory cytokine production in TPA-stimulated mice ears. ELISA was employed using the ear biopsies. Topical application of TPA alone results in an increase in TNF-α, IL-1β and IL-6 protein levels. Post-treatment of Tat-SOD after TPA application significantly reduced production of TNF-α, IL-1β and IL-6 protein, while control SOD had no or minimal effects on the levels of these cytokines (Fig. 2A–C). We further examined the effects of Tat-SOD on pro-inflammatory gene expression in TPA-stimulated mice ears by RT-PCR using the ear biopsies. Post-treatment with Tat-SOD inhibited markedly TPA-induced mRNA expression of theses cytokines in a dose-dependent manner, while control SOD had a minimal effect (Fig. 2D). These results suggest that the inhibition of production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in TPA-stimulated mice ear by Tat-SOD is mediated through the transcriptional downregulation of theses target genes. Since adhesion molecules and chemokines contribute to the recruitment of inflammatory cells to the site of inflammation, we examined the effect of Tat-SOD on TPA-induced expression of ICAM-1, MIP-2 and MCP-1 by RT-PCR. Tat-SOD was able to significantly inhibit TPA-induced expression of ICAM-1, MIP-2 and MCP-1 (Fig. 2D).

3.3. *Effects of Tat-SOD on TPA-induced cyclooxygenase-2 expression and prostaglandin E_2 production in mice ears*

Activation of COX-2 and subsequent PGE_2 production on TPA-induced skin inflammation has been extensively studied. In order to examine whether Tat-SOD can inhibit COX-2 expression and PGE_2 production in vivo, we topically applied Tat-SOD on TPA-treated mice ears and analyzed COX-2 expression and PGE_2 production using the ear biopsies. Topical

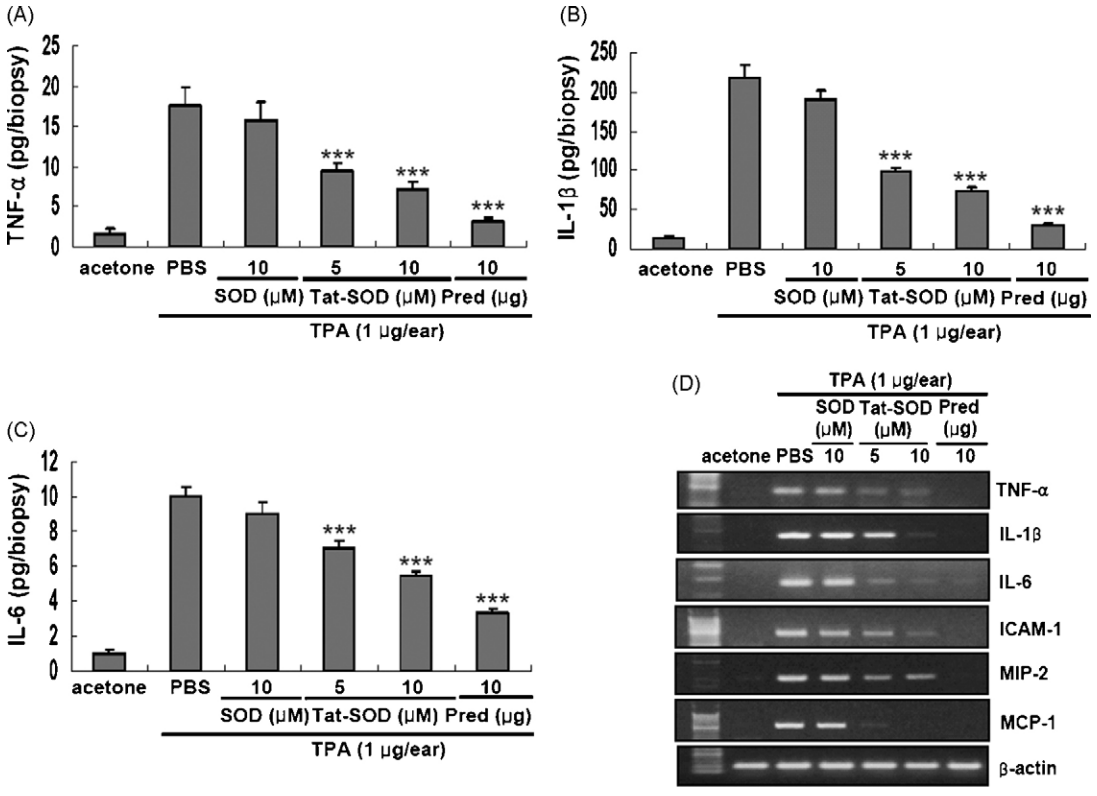

Fig. 2 – Inhibitory effect of Tat-SOD on TPA-induced production of pro-inflammatory cytokines in mice ears. Ear of ICR mice (five mice in each group) was treated with TPA (1 μg/ear) once a day for 3 days. Tat-SOD was topically applied to mice ears 1 h after TPA treatment during 3 days. The mice were sacrificed 24 h after the last SOD treatment. Five ear samples were homogenized in a sample buffer. Supernatant fractions of homogenates from ear biopsies were examined for cytokine production using ELISA duplication assays (A–C). Values are mean ± S.E.M. from five ear samples. Total RNA was extracted from ear biopsies. TNF-α, IL-1β, IL-6, ICAM-1, MIP-2, MCP-1 and β-actin mRNA were analyzed by RT-PCR using specific primers (D). The data shown are representative of three independent experiments (five mice in each group). $^{***}P < 0.001$ compared with mice treated with TPA alone.

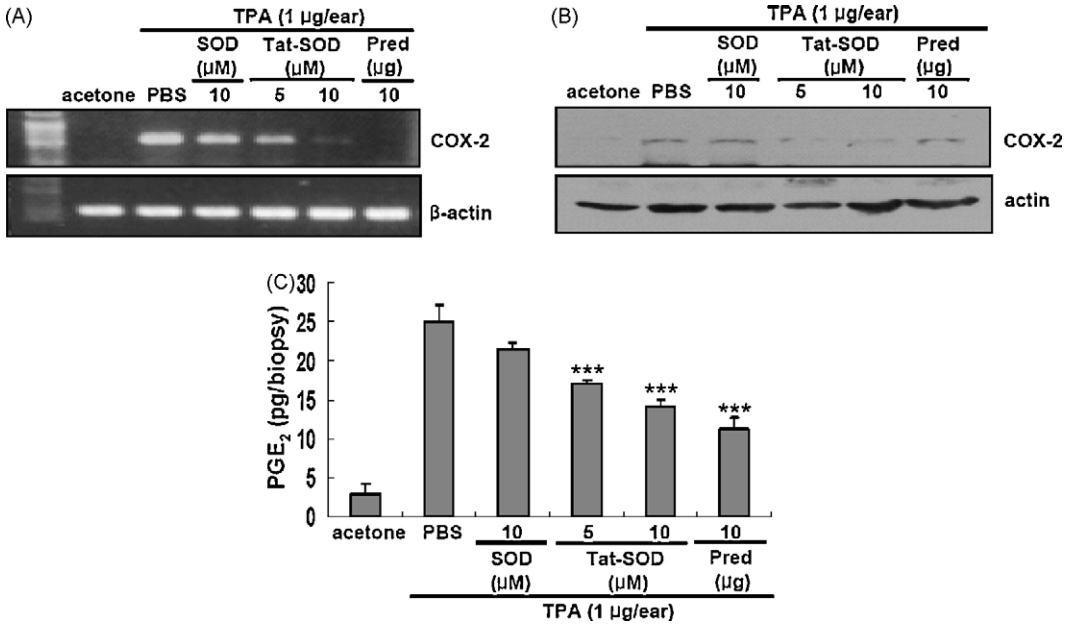

Fig. 3 – Inhibitory effect of Tat-SOD on TPA-induced cyclooxygenase-2 expression and prostaglandin E_2 production in mice ear. Mice were stimulated with TPA and treated with Tat-SOD. Five ear biopsies were homogenized as described in Section 2. (A) Total RNA was extracted. COX-2 mRNA was analyzed by RT-PCR using specific primers. (B) Mice ear extracts were prepared and analyzed for COX-2 protein expression. The data shown are representative of three independent experiments (five mice in each group). (C) The production of PGE_2 in the supernatant was also evaluated by PGE_2 ELISA duplication assays. Data are mean ± S.E.M. from five ear samples. $^{***}P < 0.001$ compared with mice treated with TPA alone.

application of Tat-SOD to mice ears after TPA application significantly inhibited COX-2 mRNA and protein expression, as judged by RT-PCR and Western blot analysis, while control SOD had a minimal effect (Fig. 3A and B). Although iNOS mRNA was weakly induced, topical application of Tat-SOD to mice ears after TPA application significantly inhibited iNOS mRNA (data not shown). Topical application of TPA alone resulted in an increase in PGE_2 concentration. Tat-SOD significantly inhibited TPA-induced PGE_2 production, while control SOD showed a slight inhibition of PGE_2 production (Fig. 3C).

3.4. *Effects of Tat-SOD on TPA-induced proinflammatory cytokines, COX-2 expression and PGE_2 production in HaCaT human keratinocytes*

Since the keratinocyte is one of major cell types involved in skin inflammatory responses [34], we further examined the effects of Tat-SOD on the proinflammatory mediators in the human keratinocytes. Human keratinocyte cell line, HaCaT cells were treated with TPA and the culture supernatants were harvested and analyzed for the production of TNF-α and IL-6 by ELISA. Tat-SOD significantly inhibited the production of TNF-α and IL-6 in HaCaT cells (Fig. 4A and B). Furthermore, Tat-SOD significantly inhibited TPA-induced PGE_2 production and COX-2 expression in HaCaT cells (Fig. 4C and D). These results suggest that Tat-SOD is capable of modulating TPA-induced pro-inflammatory mediators in the keratinocytes. We next examined the introduction of SOD by Tat PTD. Tat-SOD fusion proteins were added to HaCaT cells at concentrations ranging from 0.1 to 1 μM for 1 h, and the amounts of protein taken up were analyzed by Western blotting. As shown in Fig. 4E, the level of Tat-SOD increased with concentration, whereas the control SOD did not.

3.5. *Effects of SOD on TPA-induced activation of NF-κB in mice ears*

Since TPA has been reported to induce activation of NF-κB, a redox-sensitive transcription factor [35,36], which is known to regulate expression of various pro-inflammatory genes in mice ears [4], we attempted to determine the effect of Tat-SOD on TPA-induced activation of NF-κB. Nuclear extracts from TPA-stimulated ear biopsies were analyzed by using EMSA. Post-treatment with Tat-SOD resulted in a decrease in TPA-induced DNA binding activity of NF-κB in a dose-dependent manner (Fig. 5A). Next, we examined the regulatory effect of Tat-SOD on TPA-induced signal cascade of NF-κB activation, such as IκBα phosphorylation and IκBα degradation. As shown in Fig. 5B, Tat-SOD significantly inhibited TPA-induced IκBα phosphorylation and degradation in mice ears, while control SOD had the minimal effect.

3.6. *Inhibition of TPA-induced MAP kinase activation by Tat-SOD in mice ear*

The mitogen-activated protein kinase (MAPK) signaling cascades have been shown to be involved in activation of NF-κB upon TPA stimulation [4,36]. We further investigated the regulatory effect of Tat-SOD on the activity of MAP kinases

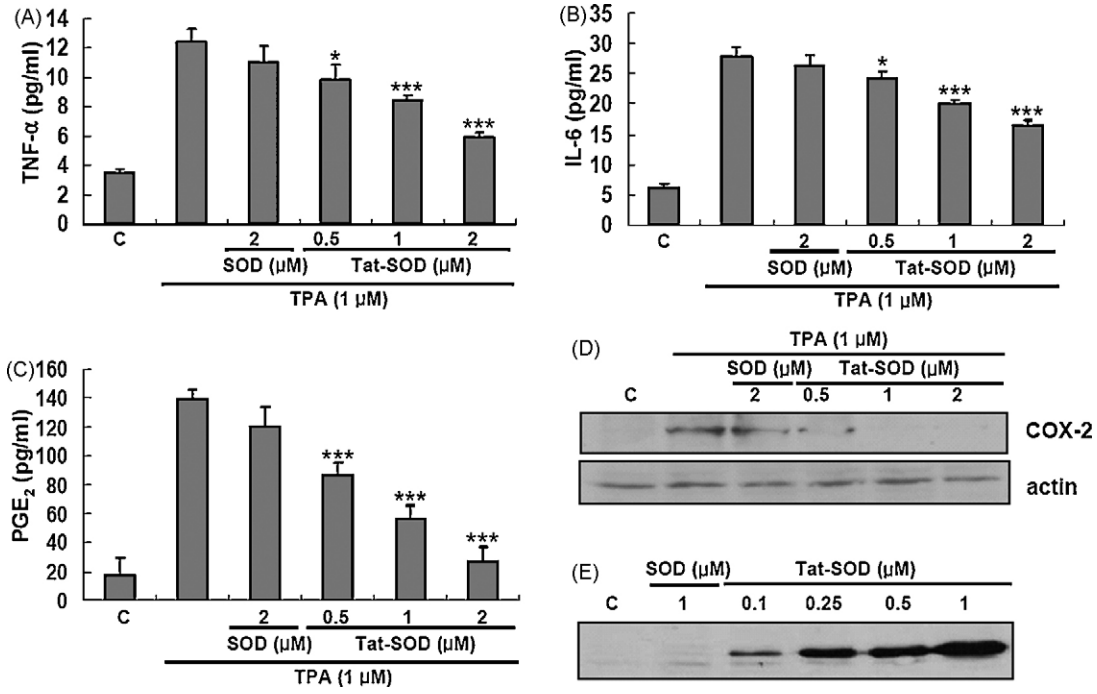

Fig. 4 – Inhibitory effect of Tat-SOD on TPA-induced pro-inflammatory cytokines, COX-2 expression and PGE_2 production in HaCaT human keratinocytes. HaCaT cells were stimulated with 1 μM TPA for 12 h with or without pretreatment with Tat-SOD or SOD fusion protein for 1 h. Culture medium was harvested and analyzed for TNF-α (A) and IL-6 (B) by ELISA. The production of PGE_2 in the supernatant was also evaluated by ELISA. Data are mean ± S.E.M. from five ear samples (C). Cell lysates were prepared and analyzed for COX-2 protein expression by Western blot (D). SOD fusion proteins were added to the culture medium for 1 h. Cell lysates were then analyzed by Western blotting to determine the level of uptake of SOD protein (E). The data shown are representative of 3 independent experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with mice treated with TPA alone.

such as p38, JNK and ERK protein kinase. To examine the effect of Tat-SOD on TPA-induced MAPK activation, Tat-SOD was topically applied on TPA-treated mouse ear and then MAP kinase activation with cellular extracts from ear biopsies was analyzed by Western blot analysis using phospho-specific antibodies against MAPK proteins (Fig. 6). Tat-SOD decreased TPA-induced phosphorylation of ERK and p38 MAP kinase in a dose-dependent manner, while it has a minimal effect on JNK levels which was not changed upon TPA stimulation under our experimental conditions.

4. Discussion

Since the involvement of ROS in the various skin inflammatory diseases has been extensively studied [1], in this study, we tested the potential therapeutic role of cell-permeable SOD, Tat-SOD in the TPA-induced inflammation in vitro and in vivo. The data in the present study showed that the skin inflammation induced by TPA was inhibited by Tat-SOD. Evidence for this protective role of Tat-SOD against skin inflammation was provided by the findings that topically applied Tat-SOD after TPA treatment resulted in reduction of ear thickness and weight, inhibition of monocyte infiltration, and suppression of pro-inflammatory cytokine production, COX-2 activation and PGE_2 production in mice ears. We also found that Tat-SOD was able to inhibit TPA-induced activation of NF-κB and MAP kinases such as ERK and p38 in mice ears. Furthermore, we demonstrated the ability of Tat-SOD to reduce cytokine expression and activation of COX-2 and PGE_2 production in human keratinocytes.

Considerable evidences suggest that generation of ROS plays a critical role in the development of skin inflammation induced by TPA [18]. In addition, previous studies reported that the level of SOD was decreased on treatment with TPA and decreased activity would favor the proinflammatory responses [37,38], suggesting the role of SOD in TPA-induced skin inflammation. Antioxidant enzymes such as SOD have been used as potential therapeutic agents for ROS-mediated diseases, with limited efficacy due to the lack of their efficient delivery into cells and tissues [17,18]. Recently, this problem was overcome by utilizing a peptide derived from HIV-1 Tat protein transduction domain (PTD) that is capable of efficiently delivering target proteins into cells [20,39]. A cell permeable SOD, Tat-SOD has been shown to be beneficial in several oxidative stress models such as ischemia and diabetes [22–24].

The characteristic features of TPA-induced acute/chronic inflammation are the edema, epidermal hyperplasia and infiltration of inflammatory cells in mouse skin [28]. As shown in Fig. 1A and B, Tat-SOD inhibited TPA-induced increase in ear thickness and weight when topically applied at 60 min

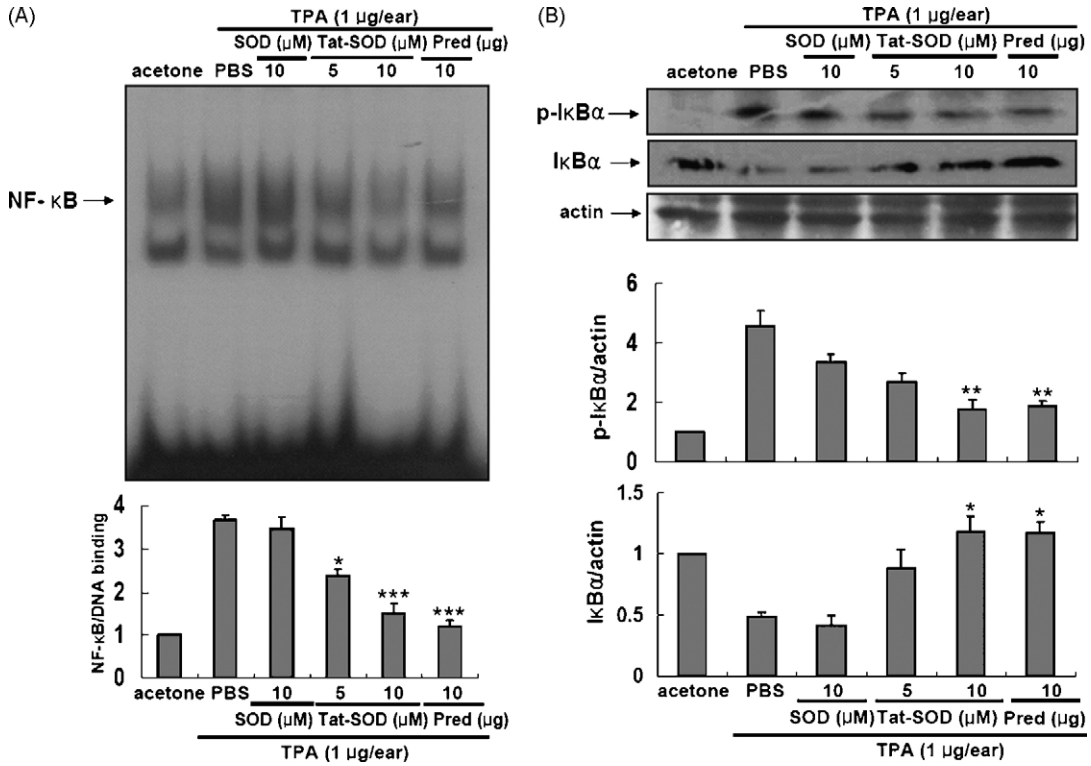

Fig. 5 – Effect of Tat-SOD on TPA-induced activation of NF-κB in mouse ear. Ear of ICR mice (five mice in each group) was treated with TPA (1 μg/ear). PBS or SOD fusion protein was topically applied to mice ears 1 h after TPA treatment. The mice were sacrificed 1 h after the last SOD treatment. DNA-binding activity of NF-κB in the nuclear extracts of the mouse ear biopsies was measured by EMSA (A). Phosphorylation and degradation of IκBα were analyzed by Western blot analysis (B). The data shown are representative of three independent experiments (five mice in each group). $^{*}P < 0.05$ $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with mice treated with TPA alone.

after TPA treatment, while control SOD, which lacks protein transduction domain, had minimal effect. Tat-SOD applied 30 min before TPA treatment has the same effect in inhibiting edema (data not shown). In addition, topically applied Tat-SOD significantly inhibited the infiltration of monocytes into the skin which represents one of the early steps to inflammatory events within the skin (Fig. 1C). Although the underlying action mechanisms by which Tat-SOD inhibits TPA-induced monocyte infiltration into skin need to be elucidated, Tat-SOD may exert its inhibitory effect on monocyte infiltration by suppressing TPA-induced expression of adhesion molecules such as ICAM-1 as well as chemokines such as MIP-2 and MCP-1 (Fig. 2D). Taken together, these observations suggest that the inhibitory effects of Tat-SOD on TPA-induced skin inflammation may be due, at least in part, to interference with formation of ear edema and suppression of monocyte infiltration.

We performed experiments to explore the effect of Tat-SOD on the TPA-induced expression of pro-inflammatory cytokines, COX-2 and iNOS and PGE_2 production in mice ears. Post-treatment with Tat-SOD significantly inhibited mRNA and protein expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in mice ears with TPA-induced skin inflammation. Furthermore, Tat-SOD suppressed TPA-induced expression of COX-2 and subsequent PGE_2 production in mice ears.

Previous studies have reported that TPA-induced ROS mediates various cellular functions such as NF-κB activation, MAPK activation, which were responsible for expression of proinflammatory cytokines [4]. Growing body of evidences suggested that ROS are capable of activating NF-κB in both the canonical and non-canonical pathway in cell-type specific manner (reviewed in ref. [40]). The classical pathway of NF-κB activation involves the activation of the IκB-kinase (IKK) complex, which contains two kinase subunits (IKKα and IKKβ) and a regulatory subunit IKKγ/NEMO. In the presence of low or physiological levels of ROS, the IKK complex phosphorylates IκBα on Ser32 and Ser36, resulting in its ubiquitination and subsequent proteasomal degradation [40]. This allows NF-κB to translocate into the nucleus, where it activates the transcription of target genes such as proinflammatory genes. The non-canonical pathway of NF-κB activation involves NF-κB inducing kinase (NIK)- and IKKα-dependent processing of p100 into p52, which binds DNA in association with its partners, like RelB and stimulates target genes. We performed experiments to further elucidate the molecular mechanisms underlying the anti-inflammatory activity of Tat-SOD. The effect of Tat-SOD on the TPA-induced activation of NF-κB and MAPKs were examined in mice ears. As shown in Fig. 5, TPA-induced NF-κB activation and IκBα phosphorylation/degradation were significantly inhibited in mice ears treated with Tat-SOD, indicating the functional consequence of ROS

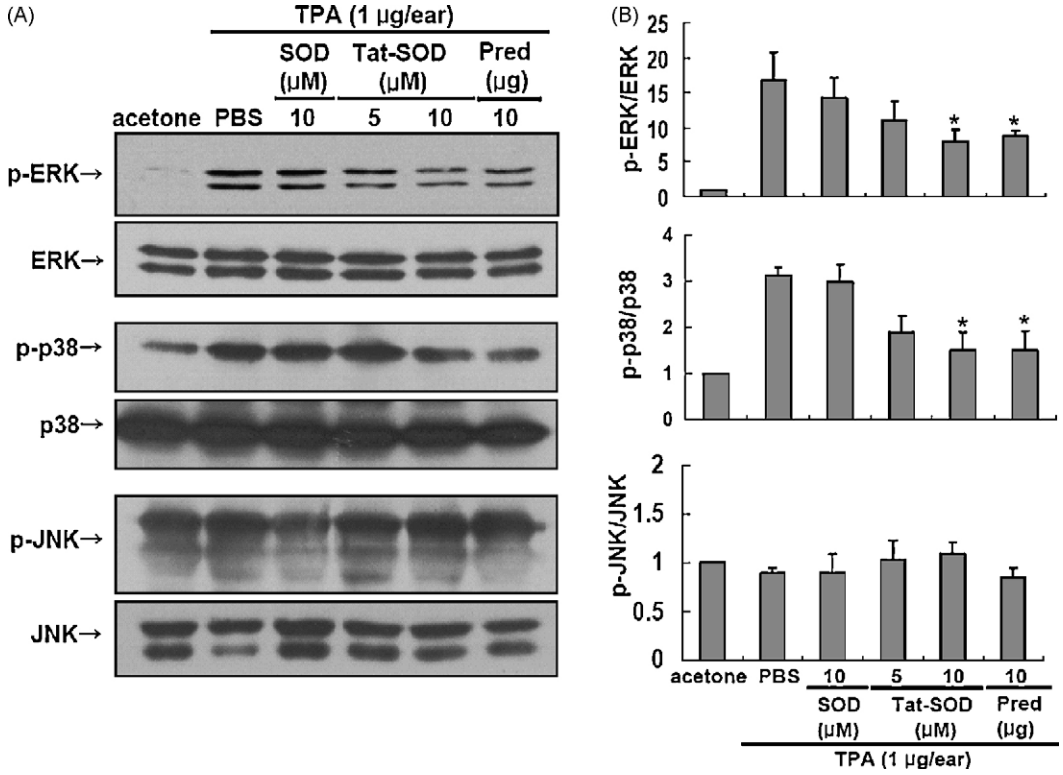

Fig. 6 – Inhibition of TPA-induced MAP kinase activation by Tat-SOD in mice ear. Ear of ICR mice (five mice in each group) was treated with TPA (1 μg/ear). PBS or SOD fusion protein was topically applied to mice ears 1 h after TPA treatment. The mice were sacrificed 1 h after the last SOD treatment. Extracts from ear biopsies were prepared and analyzed for MAP kinase protein activation (A). The data shown are representative of three independent experiments (five mice in each group). The level of proteins detected by Western blot was quantified by densitometer (B). *$P < 0.05$ compared with mice treated with TPA alone.

generation by TPA. Furthermore, post-treatment of Tat-SOD after TPA application significantly inhibited activation of MAPKs such as ERK, and p38, whereas it did not affect JNK activation (Fig. 6). These results indicate that Tat-SOD has anti-inflammatory activities by regulating the signaling pathways leading to activation of NF-κB and MAPKs such as ERK and p38.

In summary, we have provided evidence that Tat-SOD has the topical anti-inflammatory activities by assessing the protective effects of Tat-SOD on TPA-induced skin inflammation. The anti-inflammatory effects of Tat-SOD depend on the down-regulation of TPA-induced expression of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 and COX-2 and the inhibition of TPA-induced activation of NF-κB and MAPK such as ERK and p38. The observed inhibition of TPA-induced skin inflammation by Tat-SOD suggests that cell-permeable SOD may be useful as therapeutic agents for various ROS-associated inflammatory skin diseases.

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