Tat-thioredoxin 1 reduces inflammation by inhibiting pro-inflammatory cytokines and modulating MAPK signaling

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Abstract. Thioredoxin 1 (Trx1) serves a central role in redox homeostasis. It is involved in numerous other processes, including oxidative stress and apoptosis. However, to the best of our knowledge, the role of Trx1 in inflammation remains to be explored. The present study investigated the function and mechanism of cell permeable fused Tat-Trx1 protein in macrophages and a mouse model. Transduction levels of Tat-Trx1 were determined via western blotting. Cellular distribution of transduced Tat-Trx1 was determined by fluorescence microscopy. 2',7'-Dichlorofluorescein diacetate and TUNEL staining were performed to determine the production of reactive oxygen species and DNA fragmentation. Protein and gene expression were measured by western blotting and reverse transcription-quantitative PCR (RT-qPCR), respectively. Effects of skin inflammation were determined using hematoxylin and eosin staining, changes in ear weight and ear thickness, and RT-qPCR in ear edema animal models. Transduced Tat-Trx1 inhibited lipopolysaccharide-induced cytotoxicity and activation of NF-κB, MAPK and Akt. Additionally, Tat-Trx1 markedly reduced the production of inducible nitric oxide synthase, cyclooxygenase-2, IL-1β, IL-6 and TNF-α in macrophages. In a 12-O-tetradecanoylphorbol-13-acetate-induced mouse model, Tat-Trx1 reduced inflammatory damage by inhibiting inflammatory mediator and cytokine production. Collectively, these results demonstrated that Tat-Trx1 could exert anti-inflammatory effects by inhibiting the production of pro-inflammatory mediators and cytokines and by modulating MAPK signaling. Therefore, Tat-Trx1 may be a useful therapeutic agent for diseases induced by inflammatory damage.

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Introduction

Inflammation is known to involve a series of physiological responses to various stimuli, including pathogen, chemical, and damaged cells of the host (1,2). It plays a crucial role in various diseases including neuronal diseases (3-5). Endotoxin lipopolysaccharide (LPS) can initiate inflammatory responses. Once macrophages are stimulated and activated by LPS, they can produce pro-inflammatory mediators and cytokines including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), interleukin-1beta (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), and reactive oxygen species (ROS) (6-12). ROS also play a critical rule in the inflammatory diseases. High levels of ROS can induce macrophage activation, eventually leading to the production of cytokines and the activation of inflammation signaling pathways (11,12).

Mitogen activated protein kinases (MAPKs) and nuclear factor-kappaB (NF-κB) signaling pathways are involved in LPS-induced inflammation (1,13). NF-κB plays a central role in inflammatory response through expression of inflammatory factors (14,15). In addition, MAPK family members activated by LPS are associated with the production of inflammatory factors (16,17). Thus, inhibiting MAPK and NF-κB activation might have potential as a therapeutic strategy to ameliorate inflammatory diseases.

Thioredoxin 1 (Trx1), a redox regulating protein, plays crucial roles in cell growth, cell protection against

neurotoxicity, and inhibition of apoptosis (18,19). Recently, Wu *et al* (20) have reported that Trx1 overexpression can suppress methamphetamine (METH)-induced inflammation and oxidative stress in mice spleen and suggested that Trx1 might be an important therapeutic target for METH-induced immune dysfunction. Chen *et al* (21) have shown that overexpression of Trx1 in transgenic mice can increase survival from sepsis by suppressing ER stress induced NF-κB inflammatory signaling, suggesting that Trx1 might be a potential drug target for treating sepsis. Although Trx1 plays beneficial roles in the immune system, the precise mechanism involved in the effect of Trx1 on inflammation remains unclear. In this study, we examined effects of Trx1 on inflammatory responses in macrophages and an animal model using cell permeable Tat-Trx1 fusion protein.

Materials and methods

Materials. We obtained LPS and TPA from Sigma-Aldrich. Antibodies were provided by Cell Signaling Technology. All other chemicals were used of high quality reagent grade.

Cell culture, protein treatment and western blotting. DMEM was used in the culture of Raw 264.7 cells as described previously (22,23). Tat-Trx1 protein was purified using a Ni^{2+}-NTA and PD-10 column chromatography and removed endotoxins from purified protein as described previously (24). Briefly, this protein was treated with Detoxi-Gel™ (Pierce) according to manufacturer's instruction and the proteins (<0.03 EU/ml) were confirmed using a Limulus amoebocyte lysate assay (BioWhitaker).

For concentration- and time-dependent transduction of Tat-Trx1, Raw 264.7 cells were treated with different concentrations (0.1-1 μM) of Tat-Trx1 for 1 h or with time periods (15-60 min) of Tat-Trx1 (1 μM). After transduction, the cells were further incubated and intracellular stability of Tat-Trx1 was determined by western blotting using an anti-histidine antibody. Western blotting was performed as described previously (22,25).

Western blot analysis. Equal amounts of sample proteins were separated with 15% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBST buffer (25 mM Tris-HCl, 140 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h. The membranes were immunoblotted with the indicated primary and HRP-conjugated secondary antibodies as recommended by the manufacturer. The protein bands were detected using enhanced chemiluminescent reagents (Amersham).

ROS staining. ROS staining was performed as described previously (25,26). As the optimization period of the ROS production and DNA fragmentation by LPS in Raw 264.7 cells is different, the time of ROS staining and TUNEL staining are different, respectively. Therefore, we performed the staining of ROS and TUNEL at the optimal time by LPS. To determine the optimal ROS staining time, we performed ROS staining over various times, and we confirm that the optimal time is 3 h under this experimental condition (data not shown). Tat-Trx1 (1 μM) pre-treated for 1 h and LPS (1 μg/ml) exposed for 3 h. Cells were then washed twice with PBS and stained with DCF-DA (20 μM) for 30 min. Fluorescent images were obtained by fluorescence microscopy (Nikon eclipse 80i) and the fluorescence intensity was detected with excitation at 485 nm and emission at 538 nm using a Fluoroskan ELISA plate reader (Labsystems Oy).

TUNEL staining. TUNEL staining was performed as described previously (25,26). As the optimization period of the ROS production and DNA fragmentation by LPS in Raw 264.7 cells is different, the time of ROS staining and TUNEL staining are different, respectively. Therefore, we performed the staining of ROS and TUNEL at the optimal time by LPS. To determine the optimal TUNEL staining time, we performed TUENL staining over various times, and we confirm that the optimal time is 12 h under this experimental condition (data not shown). Tat-Trx1 (1 μM) pre-treated for 1 h and LPS (1 μg/ml) exposed for 12 h. TUNEL staining was performed using a Cell Death Detection kit (Roche Applied Science, Basel, Switzerland). Fluorescent images were obtained by fluorescence microscope (Nikon eclipse 80i). Fluorescence intensity levels were measured using a Fluoroskan ELISA plate reader (Labsystems Oy) at 485 nm excitation and 538 nm emission.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Raw 264.7 cells (10^5 cells/well) were grown overnight in 12-well plate and total RNA extracted using easy-BLUE according to the manufacturer's instructions (iNtRON). Also, mouse skin tissue (50 mg) was used for total RNA extraction using TRIzol reagent (Life Technologies). The cDNA synthesis was performed using 2 μg of total RNA with cDNA Reverse Transcription Kit (Omniscript® RT Kit) as per the manufacturer's instructions (Qiagen). The mRNA levels were quantified using a SYBR Premix Ex Kit (Bio-Rad) with a CFX Real time PCR Detection Systems (Bio-Rad). The relative gene expression levels were normalized to GAPDH and calculated using the $2^{-\Delta\Delta Cq}$ method (27).

The primer sequences for PCR were as follows: COX-2 (sense: 5'-CCAGCACTTCACCCATCAGTT-3'; antisense: 5'-AAGGCGCAGTTTATGTTGTCTGT-3'), iNOS (sense: 5'-GGCTGCCAAGCTGAAATTGAAT-3'; antisense: 5'-CGTGATAGCGCTTCTGGCTCTT-3'), IL-1β (sense: 5'-TGTGTTTTCCTCCTTGCCTC-3'; antisense: 5'-TGCTGCCTAATGTCCCCTTG-3'), IL-6 (sense: 5'-AAGGAGTGGCTAAGGACCAAGAC-3'; antisense: 5'-AGTGAGGAATGTCCACAAACTGATA-3'), TNF-α (sense: 5'-CTTGTTGCCTCCTCTTTTGC TTA-3'; antisense: 5'-CTTTATTTCTCTCAATGACCCGTAG-3'), GAPDH (sense: 5'-CTTTGGCATTGTGGAAGGGCTC-3'; antisense: 5'-GCAGGGATGATGTTCTGGGCAG-3').

Experimental animal. All experiments utilized ICR mice (male, 6-8 week-old, total used animal = 25) obtained from Experimental Animal Center, Soonchunhyang University. The mice were provided with a commercial diet and water *ad libitum* under controlled temperature, humidity and lighting conditions (light/dark cycle 12:12, and 22±2˚C, 55±5%). All experimental protocols were approved by the Administrative Panel on Laboratory Animal Care of Soonchunhyang University (permit no. SCH 15-0002-3). All possible efforts

were made to avoid suffering of the rats and distress, and minimize the number used during the experiments. The euthanasia for all experimental animals was conducted utilizing general inhalants methods with Carbon dioxide (CO_2) under the guideline of our Laboratory Animal Care Permits. During the experiments of *in vivo* study, all animal health, discomforts and behavior condition were monitored minimum 1 time per each day until animal euthanasia during *in vivo* animal experiments to follow ARRIVE checklist (https://www.nc3rs.org.uk/arrive-guidelines). There is no found the death for all animals during this study.

TPA-induced ear edema. TPA-induced skin animal models were produced as described elsewhere (22,24). TPA is known as the strongest promoter of skin inflammation and well described the characteristics of TPA in skin inflammations (28). To assess the effects of Tat-Trx1 against TPA-induced ear edema, the mice were divided into five groups (n=5 per group) as follows: control group; TPA-treated group; Tat-Trx1-treated group; Trx1-treated group; and Tat peptide-treated group. TPA (1.0 μg) dissolved in 20 μl of acetone was applied to the inner and outer surfaces of the ears of the mice every day for 3 days. Tat-Trx1 (10 μg) protein was topically applied to the ears of mice every day 1 h after TPA treatment. Twenty-four hours after the final treatment with TPA and Tat-Trx1 (experimental duration=4 days), all mice (n=25) were euthanized utilizing general inhalants methods in the euthanasia chamber for fill rate of 30-70% of the chamber volume per minute with Carbon dioxide (CO_2) according AVMA Guidelines for the Euthanasia of Animals (29). Then, the ear biopsies were obtained. Ear thicknesses were measured using a digital thickness gauge (Mitutoyo Corporation). Ear weights were measured after 5 mm diameter ear biopsies were obtained from each group using a punch (Kai Industries).

Immunohistochemistry. For histological analysis, ear biopsies were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at a thickness of 5 μm, and then stained with hematoxylin and eosin (22,24).

Statistical analysis. Data are expressed as the mean ± SEM of three experiments. The statistical significance analyzed using one-way ANOVA followed by a Bonferroni post hoc test. P-value <0.05 was considered as significant.

Results

Transduction of purified Tat-Trx1 into Raw 264.7 cells. We constructed a fusion protein Tat-Trx1 and purified it. As expected, one single band was detected for the purified protein (Fig. 1A). As shown in Fig. 1B and C, Tat-Trx1 transduced into Raw 264.7 cells time-dependently and its level was maintained for 6 h. However, Trx1 did not transduce into Raw 264.7 cells. Transduced Tat-Trx1 was distributed in the cytoplasm and nucleus (Fig. 1D).

Tat-Trx1inhibits LPS-induced ROS production and DNA fragmentation. It is known that LPS can ROS production and DNA fragmentation and lead to cell death (11,12). Thus, we determined effects of Tat-Trx1 on ROS production and DNA fragmentation. As shown in Fig. 2A and B, ROS production and DNA fragmentation levels were increased in Raw 264.7 cells treated with LPS only. However, Tat-Trx1 markedly prevented such increases of ROS production and DNA fragmentation, whereas ROS production and DNA fragmentation levels showed no significant difference between Trx1 and Tat peptide treated cells.

Tat-Trx1 inhibits LPS-induced activation of MAPK and NF-κB signaling. Suppressing MAPK (p38, JNK, and ERK) and NF-κB activation is important for inhibiting inflammation in LPS-activated Raw 264.7 cells (30-32). Thus, we examined whether Tat-Trx1 could inhibit the activation of MAPK and NF-κB in LPS-treated cells. As shown in Fig. 3A and B, NF-κB (p65) and MAPK expression levels were markedly increased in LPS-exposed Raw 264.7 cells, whereas Tat-Trx1 significantly reduced expression levels of p65 and MAPK in LPS-exposed Raw 264.7 cells. The expression pattern of Akt after LPS treatment was similar to that of MAPK (Fig. 3C). However, there was no significant difference the expression of NF-κB, MAPK, and Akt in cells treated with Trx1 or Tat peptide.

We also determined levels of Bcl-2, Bax, caspase-3, and cleaved caspase-3 proteins. Bax and cleaved caspase-3 protein expression levels were increased in LPS-treated Raw 264.7 cells, whereas Tat-Trx1 reduced these protein expression levels in LPS-treated cells. In contrast, Tat-Trx1 markedly increased Bcl-2 and caspase-3 protein expression levels in LPS-treated cells, whereas there was no significant difference the expression of Bcl-2, Bax, caspase-3, and cleaved caspase-3 proteins in cells treated with Trx1 or Tat peptide (Fig. 4).

Tat-Trx1 inhibits LPS-induced inflammation. We further investigated whether Tat-Trx1 inhibited LPS-induced inflammatory responses in Raw 264.7 cells. As shown in Fig. 5A and B, LPS drastically increased the expression of pro-inflammatory mediator proteins (iNOS and COX-2) and cytokine genes (IL-1β, IL-6, and TNF-α) in Raw 264.7 cells. In contrast, Tat-Trx1 significantly reduced these inflammation factors in cells. However, Trx1 or Tat peptide did not change the expression of pro-inflammatory mediators or cytokines.

Effect of Tat-Trx1 in TPA-induced animal model. TPA-induced ear edema animal model is generally used for skin inflammation study. Thus, we examined the anti-inflammatory effect of Tat-Trx1 in TPA-induced ear edema animal model. We designed the experiment to induced mice ear edema using TPA. As shown in Fig. 6A, TPA (1 μg/ear) was topically applied to mouse ears 1 h prior to Tat-Trx1 treatment and then evaluate the effect of Tat-Trx1 on TPA-induced ear edema model. TPA markedly increased ear thickness and weight of mouse showing skin inflammation. However, Tat-Trx1 significantly inhibited skin inflammation, ear thickness, and weight (Fig. 6B and C).

We also determined the effect of Tat-Trx1 in TPA-exposed mouse model (Fig. 7). In TPA treated mouse ears, iNOS, COX-2, IL-1β, IL-6, and TNF-α mRNA expression levels were markedly increased. However, Tat-Trx1 significantly reduced these mRNA expression levels in the animal model. However, iNOS, COX-2, IL-1β, IL-6, and TNF-α mRNA expression

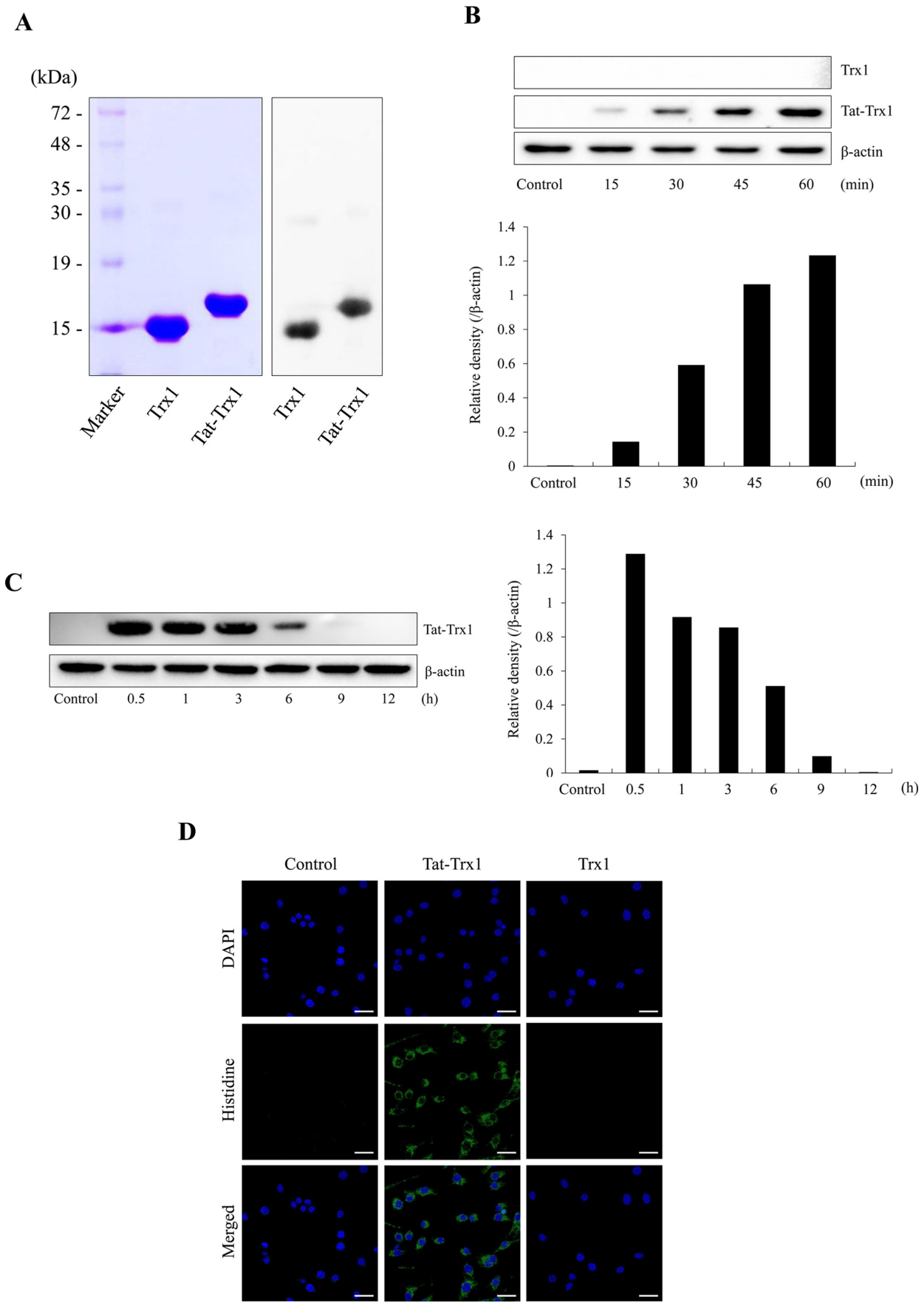

Figure 1. Purification and transduction of Tat-Trx1 protein. (A) Purified Tat-Trx1 and Trx1 proteins were identified by 15% SDS-PAGE and western blotting. (B) Cell culture media were treated with Tat-Trx1 (1 μM) for different time periods (15-60 min). (C) Intracellular stability of transduced Tat-Trx1. The cells were incubated for 12 h after transduction of Tat-Trx1 for 1 h. Transduction of Tat-Trx1 was measured by western blotting and the intensity of the bands was measured by densitometry. (D) Cells were treated with Tat-Trx1 (1 μM) for 1 h and the cellular distribution of transduced Tat-Trx1 was confirmed by fluorescence microscopy. Scale bar, 50 μm. Trx1, thioredoxin 1.

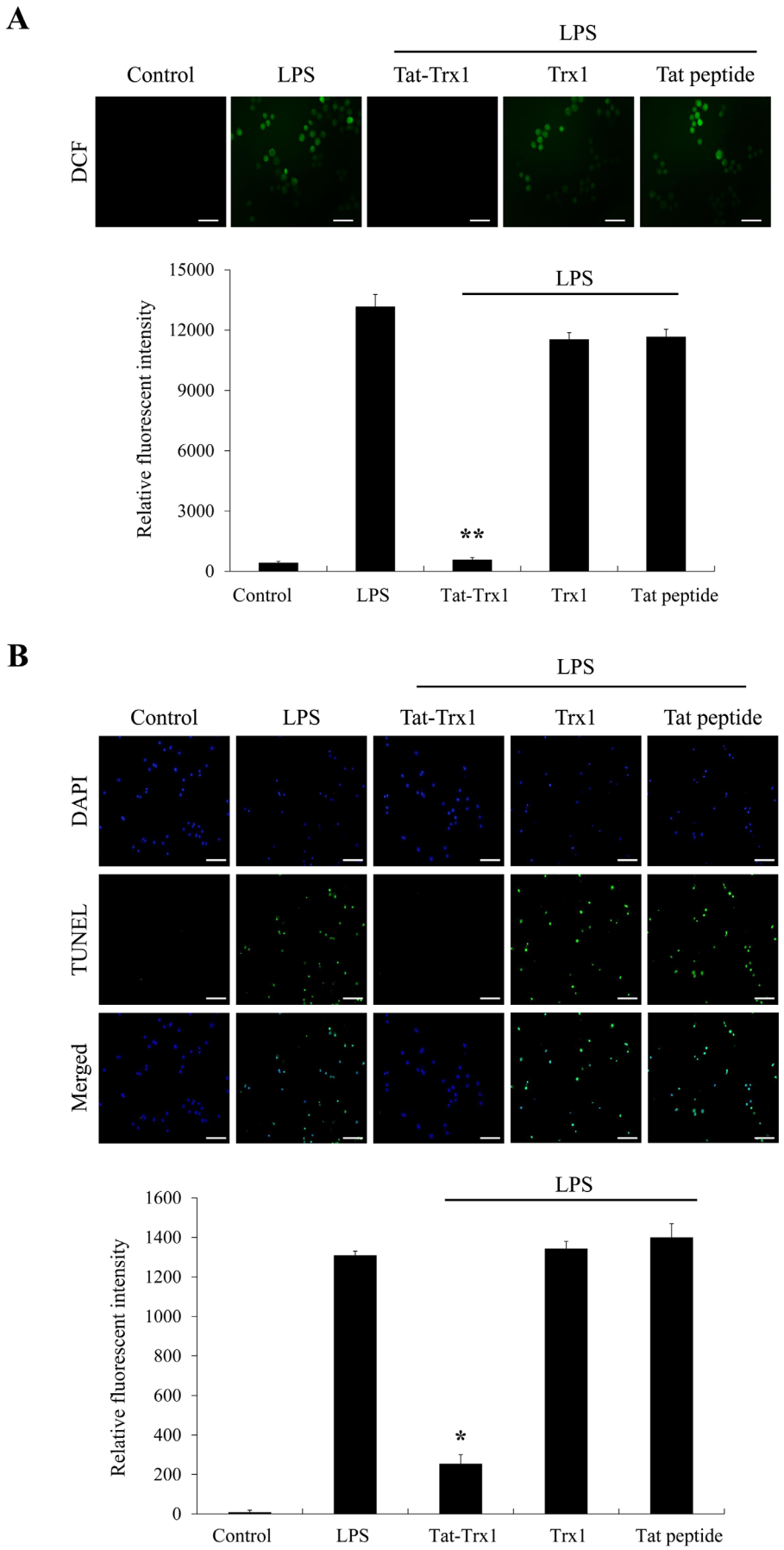

Figure 2. Effects of Tat-Trx1 on LPS-induced ROS production and DNA damage. Treatment with Tat-Trx1 (1 μM) was followed by 1 h treatment with LPS (1 μg/ml). (A) Intracellular ROS levels were measured by DCF-DA staining and (B) DNA fragmentation was detected by TUNEL staining. The fluorescence intensity was measured using an ELISA plate reader. Scale bar, 50 μm. *P<0.05 and **P<0.01 vs. LPS-treated cells. DCF-DA, 2',7'-dichlorofluorescein diacetate; LPS, lipopolysaccharide; ROS, reactive oxygen species; Trx1, thioredoxin 1.

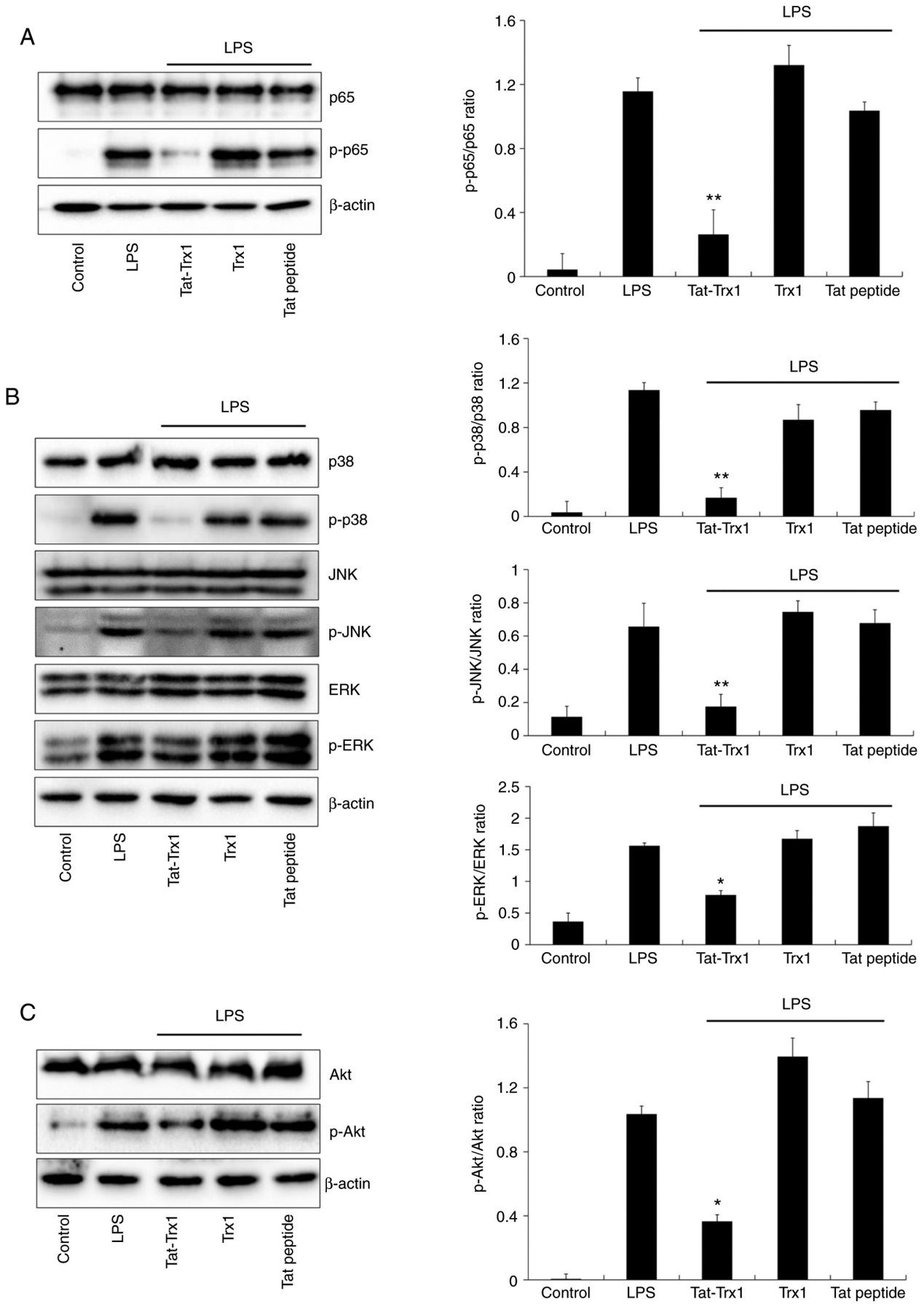

Figure 3. Effects of Tat-Trx1 on LPS-induced NF-κB (p65), MAPK (p38, JNK and ERK) and Akt phosphorylation in Raw 264.7 cells. The cells were pretreated with Tat-Trx1 (1 μM) for 1 h and then treated with LPS (1 μg/ml). Phosphorylation levels of (A) p65, (B) MAPK (p38, JNK and ERK) and (C) Akt were determined by western blotting. The band intensity was measured by densitometry. *P<0.05 and **P<0.01 vs. LPS-treated cells. LPS, lipopolysaccharide; p-, phosphorylated; Trx1, thioredoxin 1.

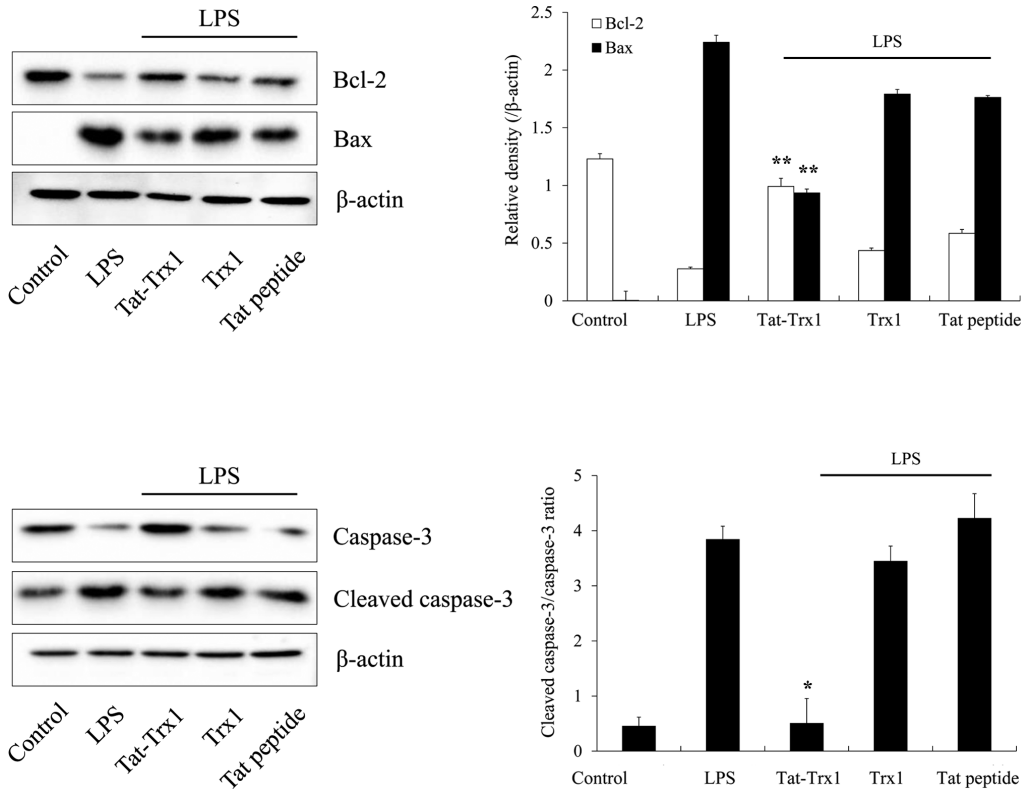

Figure 4. Effect of Tat-Trx1 on LPS-induced Bcl-2, Bax, caspase-3 and cleaved caspase-3 production in Raw 264.7 cells. The cells were pretreated with Tat-Trx1 (1 μM) for 1 h and then treated with LPS (1 μg/ml). The production levels of Bcl-2, Bax, caspase-3 and cleaved caspase-3 were determined by western blotting. The band intensity was measured by densitometry. *P<0.05 and **P<0.01 vs. LPS-treated cells. LPS, lipopolysaccharide; Trx1, thioredoxin 1.

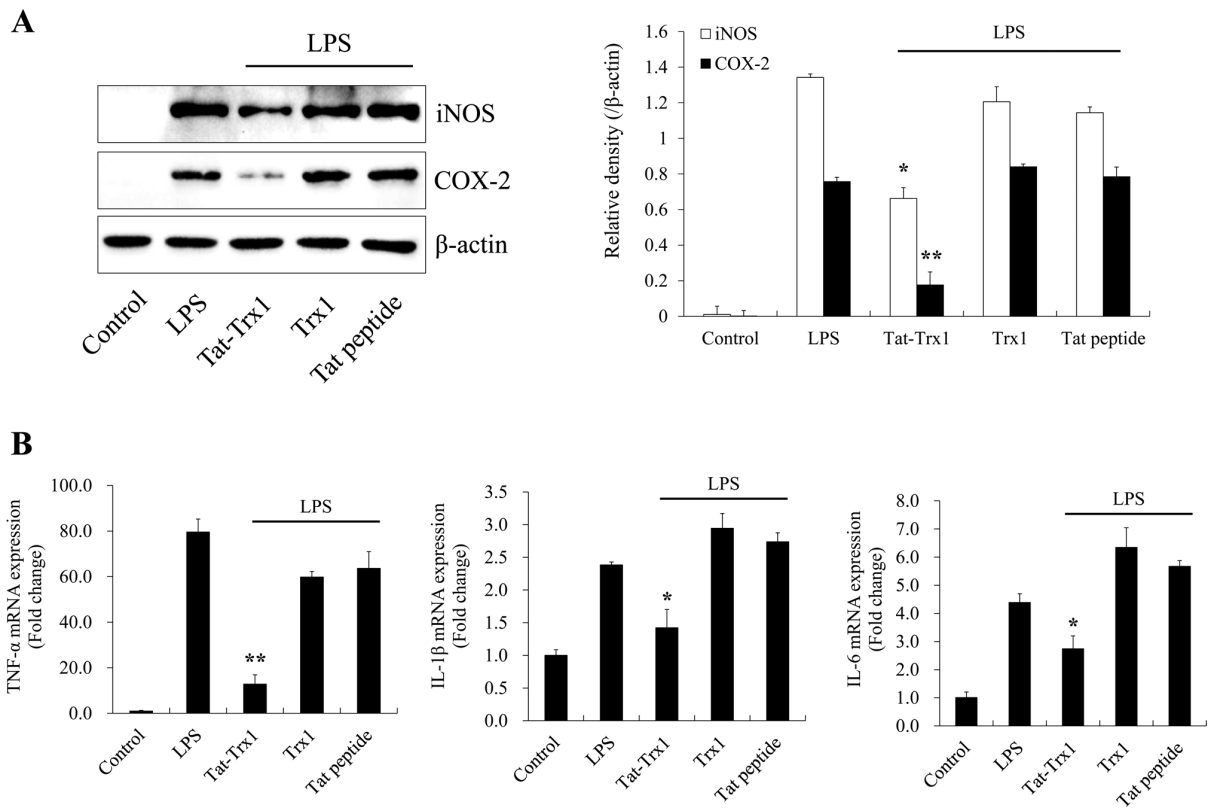

Figure 5. Effects of Tat-Trx1 on LPS-induced inflammatory responses in Raw 264.7 cells. The cells were treated with Tat-Trx1 (1 μM) for 1 h before being exposed to LPS (1 μg/ml). (A) Protein expression levels of COX-2 and iNOS were analyzed by western blotting. The band intensity was measured by densitometry. (B) mRNA levels of cytokines were determined using reverse transcription-quantitative PCR. The mRNA levels were normalized to GAPDH and subsequently represented as the fold change relative to the control group. *P<0.05 and **P<0.01 vs. LPS-treated cells. COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; Trx1, thioredoxin 1.

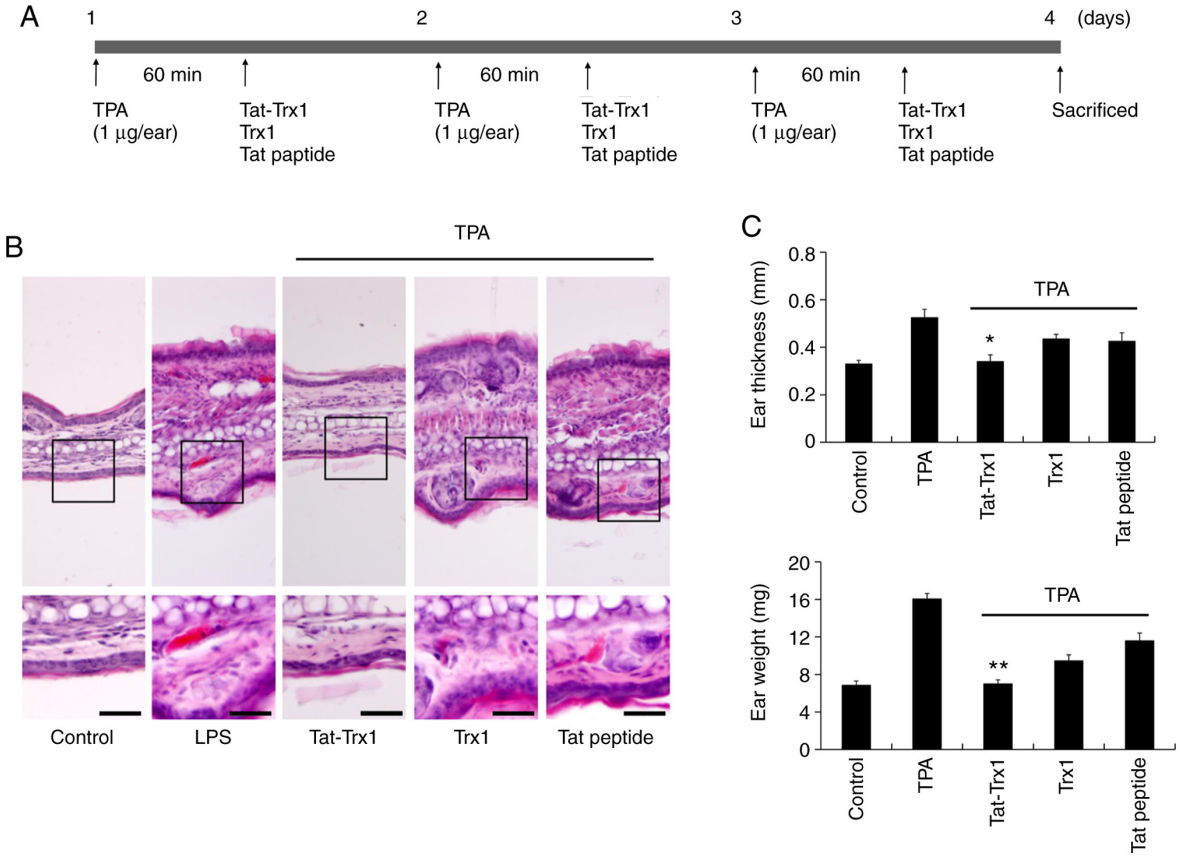

Figure 6. Effects of Tat-Trx1 on TPA-induced mice ear edema. Ears of mice were treated with TPA (1 μg/ear) once a day for 3 days. Tat-Trx1 protein (10 μg) was topically applied to mice ears 1 h prior to TPA exposure over 3 days. (A) Schematic representation of the experimental procedure. Protective effects of Tat-Trx1 were demonstrated by (B) hematoxylin and eosin staining, as well as changes in (C) ear weight and ear thickness in a TPA-induced mice ear edema model. Scale bar, 50 μm. *P<0.05 and **P<0.01 vs. TPA-treated mice. TPA, 12-O-tetradecanoylphorbol-13-acetate; Trx1, thioredoxin 1.

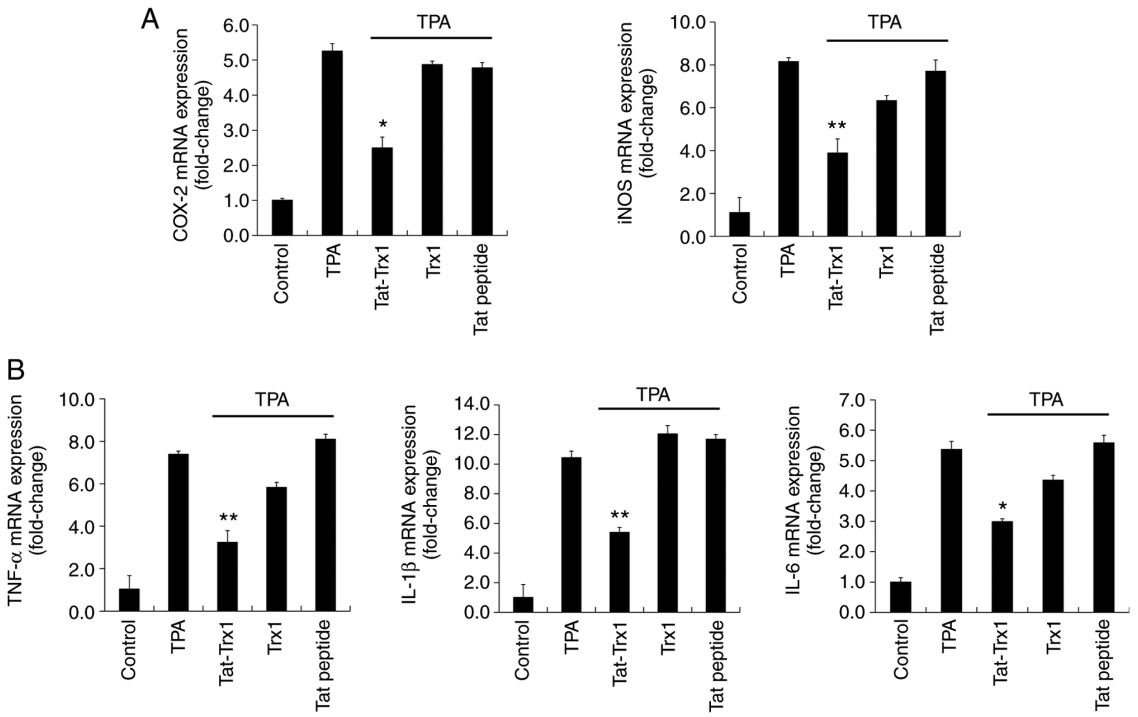

Figure 7. Effects of Tat-Trx1 on TPA-induced pro-inflammatory mediator (iNOS and COX-2) and cytokine (IL-1β, IL-6 and TNF-α) expression in mice ears. Mice were stimulated with TPA (1 μg/ear) after which Tat-Trx1 (10 μg) was topically applied to mice ear for 3 days. The mRNA levels of (A) pro-inflammatory mediators and (B) cytokines were determined using reverse transcription-quantitative PCR. The mRNA levels were normalized to GAPDH and subsequently represents as the fold change relative to the control group. *P<0.05 and **P<0.01 vs. TPA-treated mice. COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; TPA, 12-O-tetradecanoylphorbol-13-acetate; Trx1, thioredoxin 1.

levels showed no significant difference in Trx1 or Tat peptide treated group compared to those in the TPA treated group.

Discussion

Inflammatory response plays an important role in the defense against external stimuli including microbial pathogens and chemicals. Also, inflammatory response can lead to various diseases including neurodegenerative diseases (33-36). It is well known that Trx1 is a redox regulating protein with a critical function in cell growth, gene expression, and apoptosis (37,38). In addition, Trx1 exerts beneficial role in cell survival from oxidative stress due to its antioxidant and anti-inflammatory effects (39-41). Therefore, we investigated the anti-inflammatory effect of Trx1 in macrophages and an animal model. Since Tat peptide can transduce into cells cross the cellular membrane due to its potential for membrane permeability with safety and nontoxic effect, it is widely used in protein delivery into cells (42-44). In this study, we fused a Tat peptide with human Trx1 gene to produce a cell permeable Tat-Trx1 protein. Many reports have shown a beneficial effect of therapeutic transducible Tat fused proteins on cell survival both *in vitro* and *in vivo* (22,25,45-50).

LPS plays a crucial role in inducing inflammatory responses, leading to various inflammatory diseases (51). LPS can also increase intracellular ROS levels in macrophages. High levels of ROS can induce inflammatory responses (43,44). Previous studies have revealed that LPS can induce high levels of ROS and that antioxidants play a beneficial role against LPS-induced inflammation (52,53). It has been reported that Trx1 can protect cells against damage caused by oxidative stress in various diseases due to its anti-oxidant function (21,54-56). In the present study, cell permeable fusion protein Tat-Trx1 transduced into macrophages and drastically reduced LPS-induced ROS generation and DNA damage, suggesting that Tat-Trx1 could play a beneficial role in cell survival against LPS-induced damage.

Many reports have shown that LPS can induce NF-κB activation and its upstream regulator, MAPK. MAPKs pathway can regulate inflammatory responses. Activated p65 can lead to the production of pro-inflammatory mediators and cytokines (14-17,57-61). Thus, it is widely believed that NF-κB and MAPKs signaling is a promising target for treating inflammatory diseases. Here, we showed that Tat-Trx1 reduced NF-κB and MAPK activation caused by LPS, indicating that Tat-Trx1 could inhibit inflammation by regulating NF-κB and MAPKs signaling.

It is well described that TPA, a promotor of skin tumorigenesis, can induce inflammatory symptom and cancer development. In general, TPA-induced mouse model can be used to understand the inflammatory mechanism (28,62-65). Several studies have reported that TPA is a phorbol ester that promotes a direct activation of protein kinase C pathway, which results in elevated levels of prostaglandin E_2 and leukotriene B_4 in applied to the skin. Also, TPA promotes the activation of MAPK pathway that lead to the expression of several important molecules for the inflammatory responses such as adhesion molecules, chemokines, and inflammatory mediators (66-69). In addition, other studies have shown that production levels of pro-inflammatory mediators and cytokines are markedly increased in TPA-induced inflammation model (63,70,71). He *et al* (72) have shown that inflammatory responses in animal model can be quantified by measuring ear weight and ear thickness after treatment with natural compounds (*Qi-Wei-Xiao-Yan-Tang*: XYT). XYT can significantly ameliorate inflammatory responses in an animal model, suggesting that it can be used as a therapeutic agent (72). Topical application of various anti-inflammatory therapeutic agents can also significantly inhibit inflammation by suppressing inflammatory responses in a TPA-induced mouse model (73-75).

Results of the present study revealed that Tat-Trx1 markedly reduced TPA-induced ear thickness, weight, and inflammatory responses. Taken together, our results indicate that Tat-Trx1 can significantly prevent inflammation in macrophages and in an animal model by inhibiting the production of pro-inflammatory mediators and cytokines as well as NF-κB and MAPK activation. Therefore, Tat-Trx1 can be used as a therapeutic agent for treating diseases induced by inflammatory damage.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EJY, MJS, WSE and SYC were involved in the conceptualization of the study. HJY, YJC, EJS, LRL and HJC performed the experiments, and acquired and analyzed the data. HJK, YHY, DWK and DSK produced the methodology of the animal model and concurrently conducted animal studies. SHL, SL, JP and KHH were responsible for data analysis and interpretation, and participated in the drafting of the manuscript. EJY and SYC confirmed the authenticity of all the raw data. WSE, MJS and SYC were involved in the writing and editing of the manuscript and provided final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The care of animals conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research and Quarantine Service of Korea and the present study was approved by the Institutional Animal Care and Use Committee of Soonchunhyang University Cheonan, Chungcheongnam, Republic of Korea (approval no. SCH 15-0002-3).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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