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PEP-1-GLRX1 protein exhibits anti-inflammatory effects by inhibiting the activation of MAPK and NF-κB pathways in Raw 264.7 cells

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Glutaredoxin 1 (GLRX1) has been recognized as an important regulator of redox signaling. Although GLRX1 plays an essential role in cell survival as an antioxidant protein, the function of GLRX1 protein in inflammatory response is still under investigation. Therefore, we wanted to know whether transduced PEP-1-GLRX1 protein inhibits lipopolysaccharide (LPS)- and 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced inflammation. In LPS-exposed Raw 264.7 cells, PEP-1-GLRX1 inhibited cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), activation of mitogen activated protein kinases (MAPKs) and nuclear factor-kappaB (NF-κB) expression levels. In a TPA-induced mouse-ear edema model, topically applied PEP-1-GLRX1 transduced into ear tissues and significantly ameliorated ear edema. Our data reveal that PEP-1-GLRX1 attenuates inflammation *in vitro* and *in vivo*, suggesting that PEP-1-GLRX1 may be a potential therapeutic protein for inflammatory diseases. [BMB Reports 2020; 53(2): 106-111]

INTRODUCTION

Inflammatory response is known to be a defense against external harmful factors, such as microbial pathogens and chemicals. However, an excessive inflammatory response contributes to various disorders, including cardiovascular diseases, cancer, arthritis, and neuronal diseases (1, 2). It is well known that macrophages, which are important immune cells, regulate the inflammatory response and produce pro-inflammatory mediators and cytokines. These mediators and cytokines contribute to the pathogenesis of inflammation (3-6). Therefore, Gue *et al*. (2016) suggest that inhibition of pro-inflammatory mediators and cytokines is important for preventing the progression of inflammatory diseases (7).

Nuclear factor-kappaB (NF-κB) is well known to modulate the inflammatory response. NF-κB is located in the cytoplasm as a complex with IκB under normal conditions. However, NF-κB is translocated to the nucleus under IκB degradation when exposed to inflammatory stimuli, such as LPS (8). Also, other studies have shown that NF-κB is an essential pathway associated with pro-inflammatory mediator production in the inflammatory response (9, 10). In addition, mitogen-activated protein kinase (MAPK) has been considered to be a typical molecular target for the development of anti-inflammatory agents. Several studies have reported that the activation of MAPKs leads to increased production of the pro-inflammatory mediators. Therefore, NF-κB and MAPKs signaling pathways have been considered to be potential targets for anti-inflammatory drugs (9, 11-13).

Human glutaredoxin (GLRX) is a small molecular-weight protein and a member of the thioredoxin family. In humans, GLRX1 is located in the cytosol, whereas GLRX2 is located in the mitochondria (14). Several studies have described GLRX1 as being distributed in various tissues and as regulating redox-dependent signaling pathways. GLRX1 has a protective role against oxidative stress (15-17). Also, Cater *et al*. (2014) have shown that GLRX1 plays an important role as an antioxidant protein and protects against copper-induced toxicity and neuronal cell death (18). We also have reported that PEP-1-GLRX1 markedly protects against hippocampal neuronal cell damage by inhibiting oxidative stress (19). In this study, we investigated the function of PEP-1-GLRX1 against

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inflammatory responses *in vitro* and *in vivo*.

RESULTS AND DISCUSSION

Transduction of PEP-1-GLRX1 into Raw 264.7 cells

PTDs including PEP-1 peptide are known to be small peptides that can transduce the plasma membrane either alone or combined with various macromolecules, such as proteins (20). PEP-1-GLRX1 protein was produced as described previously (19). Purified PEP-1-GLRX1 was confirmed through SDS-PAGE and Western blotting with a histidine antibody (Fig. 1A). Next, we measured the transduction efficiency of purified protein into Raw 264.7 cells. Figs. 1B-1D show that PEP-1-GLRX1 concentration- and time-dependently transduced into the cells. Transduced PEP-1-GLRX1 levels remained for up to a maximum of 9 h. We have also reported that this protein was transduced into HT-22 cells (19). However, the transduced levels of this protein into HT-22 cells remained longer than in Raw 264.7 cells.

Effect of PEP-1-GLRX1 on expression of COX-2 and iNOS

Since protein transduction is important for the development of therapeutic proteins, PTD-fused protein transduction can be used for the intracellular application of therapeutic proteins (20-24). In previous studies, we have demonstrated that PTD-fusion proteins were transduced into various cells (25-30). In this study, we first identified the distribution of transduced PEP-1-GLRX1 using fluorescence analysis. Green fluorescence signals were strongly observed in the cells treated with PEP-1-GLRX1, whereas cell treated with control GLRX1 did not show the fluorescence signals (Fig. 2A). Next, to identify the location of transduced PEP-1-GLRX1, we prepared nuclear and cytosolic fractions from the cells and did Western blotting using subcellular specific marker antibodies. Fig. 2B shows that transduced PEP-1-GLRX1 was distributed into the cytosol and nuclei of the cells.

To find out whether PEP-1-GLRX1 has anti-inflammatory roles against LPS-exposed Raw 264.7 cells, we examined the expression of COX-2 and iNOS levels. Treatment with LPS markedly increased COX-2 and iNOS expressions levels more than did those in the control. PEP-1-GLRX1 treatment significantly reduced the COX-2 and iNOS expression levels in the LPS-treated cells (Fig. 2C). These results suggest that transduced PEP-1-GLRX1 has anti-inflammatory effects by inhibiting pro-inflammatory mediators. Chung *et al*. (2010) showed that pro-inflammatory mediators increased in GLRX1 knockout mice more than in wild-type mice in a cigarette smoke-induced lung inflammation model, suggesting that the GLRX1 plays an important role in lung inflammation (31). In addition, other studies have shown that a pro-inflammatory response was increased in the lens and heart of GLRX1-deficient mice (32, 33). On the other hand, Aesif *et al*. (2011) reported that ablation of the GLRX1 protein attenuates LPS-induced pro-inflammatory responses by controlling S-glutathionylation-sensitive signaling pathways, suggesting that GLRX1 expression is critical for activating alveolar macrophages in an LPS-induced lung inflammation mice model (34).

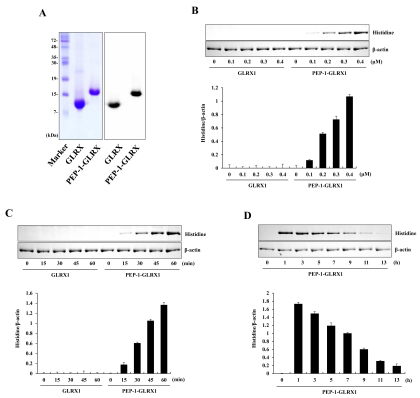

Fig. 1. Purification and transduction of PEP-1-GLRX1 protein into Raw 264.7 cells. Purified PEP-1-GLRX1 and control GLRX1 proteins were confirmed by 15% SDS-PAGE and Western blot analysis (A). Transduction of PEP-1-GLRX1 proteins into Raw 264.7 cells. PEP-1-GLRX1 protein (0.1-0.4 μM) was added to the culture media for 1 h (B), PEP-1-GLRX1 protein (0.4 μM) was added to the culture media for 15-60 min (C). The stability of transduced PEP-1-GLRX1 protein was assessed after various time periods. The cells were treated with PEP-1-GLRX1 protein (0.4 μM), incubated with 1-13 h, and analyzed by Western blot analysis (D). The band intensities were measured by densitometer. The data are presented as mean values ± SD (n = 3).

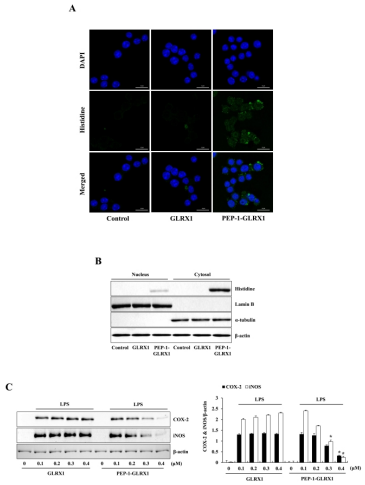

Fig. 2. Effects of transduced PEP-1-GLRX1 protein on LPS-induced inflammatory response in Raw 264.7 cells. The cells were treated with PEP-1-GLRX1 protein (0.4 μM) for 1 h, and the cellular distribution of transduced PEP-1-GLRX1 protein was confirmed by fluorescence microscopy (A). Scale bar = 50 μm. Subcellular localization of PEP-1-GLRX1 (B). The nuclear and cytosolic extracts were prepared from transduced and normal Raw 264.7 cells and analyzed by Western blotting. The cells were pretreated with PEP-1-GLRX1 protein (0.4 μM) for 1 h and then treated with LPS (1 μg/ml). Expression levels of COX-2 and iNOS protein were measured by Western blot analysis (C). The band intensity was measured by densitometer. The data are presented as mean values ± SD (n = 3). *P < 0.01 compared with LPS-treated cells.

Effect of PEP-1-GLRX1 on the NF-κB and MAPK signaling pathways

NF-κB and MAPK signaling pathway promote the production of pro-inflammatory mediators. NF-κB is one of the key transcription factors that control gene expression of pro-inflammatory mediators and cytokines (35, 36). Also, MAPK signaling pathways (ERK, p38, JNK) are crucial for NF-κB activation and regulate the inflammatory response (37, 38). Therefore, we measured the effects of PEP-1-GLRX1 against LPS-induced phosphorylation of NF-κB and MAPKs in Raw 264.7 cells. In the LPS-treated cells, phosphorylated NF-κB and MAPKs expression levels were increased more than in the control. In contrast, PEP-1-GLRX1 significantly reduced phosphorylated NF-κB and MAPKs expression levels more than in the LPS-treated cells. Control GLRX1 did not alter phosphorylated NF-κB and MAPKs expression levels (Fig. 3). These results suggest that transduced PEP-1-GLRX1 inhibits inflammatory responses by regulating NF-κB and MAPKs signaling pathways. In agreement with our results, Ryu *et al*. (2018) have reported that transduced PEP-1-GLRX1 inhibited phosphorylation of MAPK signaling in H_2O_2-exposed HT-22 cells (19). Other studies have reported that phosphorylated IκBα and p-65 protein expression levels were markedly increased in cigarette-smoke-induced lung inflammation GLRX1 knockout mice (31). In addition, several studies have reported that the phosphorylated NF-κB and MAPKs expression levels were increased in LPS-treated macrophages (39-41). In contrast, overexpression of GLRX1 decreased S-glutathionylation IKKβ Cys179 and increased NF-κB activation after oxidation induced by oxidative stress, suggesting that cellular content of GLRX1 regulates the activation of NF-κB under oxidative stress (42). Also, other studies have demonstrated that the cellular content of GLRX1 is regulated by the proinflammatory stimuli. GLRX1 level was increased in bronchial epithelial cells of in mice with allergic airway inflammation, activation of NF-κB and GLRX1 is also increased concomitant with activation of NF-κB in the retinal glial cells (43-45). Therefore, these findings suggest that NF-κB and GLRX1 may be regulated in a coordinated fashion. However, the role of GLRX1 in regulation of NF-κB signaling is not completely understood yet. Further studies are needed.

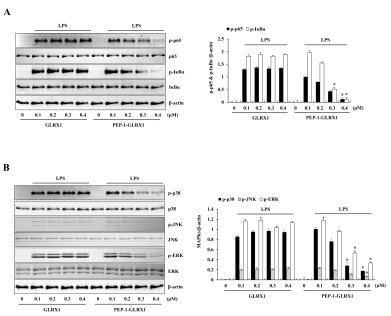

Fig. 3. Effects of transduced PEP-1-GLRX1 protein on LPS-induced NF-κB and MAPK phosphorylation in Raw 264.7 cells. The cells were pretreated with PEP-1-GLRX1 protein (0.4 μM) for 1 h and then treated with LPS (1 μg/ml). Phosphorylation of NF-κB (A) and MAPK (B) levels was measured by Western blot analysis. The band intensity was measured by densitometer. The data are presented as mean values ± SD (n = 3). *P < 0.01 compared with LPS-treated cells.

Effect of PEP-1-GLRX1 on mouse-ear edema

PTDs can effectively transduce exogenous macromolecules into cells and tissues (46). TPA is known as an inducer of skin inflammation, and several studies have used it to find out the effects of different treatments against skin inflammation (47-50). Therefore, we wanted to find out whether PEP-1-GLRX1 was transduced into mice ear tissue and whether it demonstrated protective effects against TPA-induced edema. After mice ears were treated with PEP-1-GLRX and TPA as described in Methods, we confirmed PEP-1-GLRX1 levels using immunohistochemical analysis. As shown in Fig. 4A, there were no changes in the His antibody staining in the control-, TPA-, and TPA + control GLRX1-treated groups. However, fluorescent signals were markedly increased in the PEP-1-GLRX1-treated groups compared to that in the control and other groups.

Further, we measured the effects of PEP-1-GLRX1 against TPA-induced mouse-ear edema. As shown in Fig. 4B, TPA-treated mice showed markedly increased ear thickness and weights compared to those in the control mice. PEP-1-GLRX1 drastically reduced ear thickness and weights more than in TPA-treated mice. However, there were no significant changes in the control GLRX1-treated mice compared to the TPA-treated mice. In addition, PEP-1-GLRX1 drastically reduced infiltration of monocytes, which is an early event in skin inflammation. Control

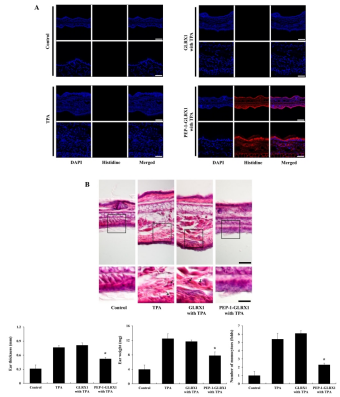

Fig. 4. Effects of PEP-1-GLRX1 protein on ear edema in a TPA-induced mice model of inflammation. Ears of mice were exposed to TPA (1 μg/ear), and PEP-1-GLRX1 protein (10 μg) was topically applied to mice ears 1 h after TPA treatment for 3 days. Transduced PEP-1-GLRX1 protein was confirmed by Histidine antibody (A). Scale bar = 50 μm (top panel) and 25 μm (bottom panel). The effects of PEP-1-GLRX1 protein against TPA-induced ear edema was analyzed by hematoxylin and eosin immunostaining, changes of ear thickness and ear weights, and monocyte infiltration folds (B). Scale bar = 50 μm (top panel) and 25 μm (bottom panel). *P < 0.01 compared with TPA-treated mice. The data are presented as mean values ± SD (*n* = 5).

GLRX had a minimal effect. These results indicate that PEP-1-GLRX1 plays an anti-inflammatory role in TPA-induced skin inflammation. We have demonstrated that cell-permeable antioxidant PTD-fusion protein markedly inhibits TPA-induced inflammation in a mice model (27, 48). In addition, other studies have reported that antioxidant proteins play protective roles in inflammation (51, 52). However, the exact function of GLRX1 in inflammation requires further study.

In summary, we have demonstrated that transduced PEP-1-GLRX1 markedly inhibits inflammation *in vitro* and *in vivo* by the reduction of inflammatory responses. Therefore, we suggest that PEP-1-GLRX1 may have applications in inflammatory disorders.

MATERIALS AND METHODS

Materials and cell culture

We obtained PEP-1-GLRX1 and control GLRX1 protein were prepared as described previously (19). Histidine, COX-2, iNOS, p-p65, p65, p-IκBα, IκBα, p-p38, p38, p-JNK, JNK, p-ERK, ERK, and β-actin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Beverly, MA, USA). LPS and TPA was purchased from Sigma-Aldrich (St. Louis, MO, USA). We obtained male ICR mice (4-6 weeks old) from the Experimental Animal Center at Hallym University. All other agents were of the highest grade available unless otherwise stated.

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

Purification and transduction of PEP-1-GLRX1 protein

PEP-1-GLRX1 and control GLRX1 proteins were purified as described previously (19). To remove endotoxins from purified proteins, we treated the proteins with Detoxi-Gel™ (Pierce, Rockford, IL, USA) according to the manufacturer's instruction and confirmed the proteins (< 0.03 EU/ml) using a Limulus amoebocyte lysate assay (BioWhitaker, Walkersville, MD, USA) (27, 48). Then, the protein concentration was measured using the Bradford assay (53).

To detect the transduction of PEP-1-GLRX1, we treated Raw 26.7 cells with various concentrations of PEP-1-GLRX1 and control GLRX1 (0.1-0.4 μM) for 1 h. Also, the cells were treated with PEP-1-GLRX1 and control GLRX1 (0.4 μM) for various times (15-60 min). Then the cells were treated with trypsin-EDTA, washed with phosphate-buffered saline (PBS), and harvested for Western blot analysis.

Western blot analysis

We did Western blot analysis as described previously (27, 48). Equal amounts of sample proteins were separated with 15% SDS-PAGE and transferred to a nitrocellulose membrane, which was blocked with 5% nonfat dry milk in TBST buffer (25 mM Tris-HCl, 140 mM NaCl, 0.1% Tween 20, *p*H 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, Franklin Lakes, NJ, USA).

Fluorescence microscopy analysis

Fluorescence microscopy analysis was done as described previously (27, 48). Raw 264.7 cells were grown on coverslips and treated with 0.4 μM of PEP-1-GLRX1 for 1 h at 37°C. Then, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 5 min at room temperature. Then the cells were permeabilized and blocked for 40 min with 3% bovine serum albumin, 0.1% Triton X-100 in PBS (PBS-BT) and washed with PBS-BT. The primary antibody (Histidine) was diluted 1:2000 and incubated for 1 h at room temperature. The secondary antibody (Alexa Fluor 488, Invitrogen) was diluted 1:15000 and incubated for 1 h at room temperature in the dark. Nuclei were stained for 2 min with 1 μg/ml DAPI (Roche, Mannheim, Germany). We analyzed the distributions of fluorescence using a fluorescence microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan).

Subcellular fractionation of the transduced cells

The nuclear and cytosolic fractions were prepared as previously described (54). Transduced Raw 264.7 cells were washed with PBS, acid-washed with 0.2 M glycine-HCl, *p*H 2.2, and trypsinized for 10 min at 37°C. The cells were harvested after washing with cold PBS and pelleted. The cells were then resuspended in 1 ml of NP-40 buffer by gentle pipetting and incubated on ice for 10 min. Cells were spun through a sucrose cushion at 1000 *g* for 10 min, and the cytosolic fractions were collected from the supernatants. Pellets were washed with 1 ml of NP-40 buffer to completely remove cytosolic fractions. The nuclei were lysed in a lysis buffer (50 mM Tris-HCl, *p*H 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μg/ml PMSF, 1% Triton X-100). The resulting nuclear and cytosolic lysates were analyzed by Western blotting.

TPA-induced mouse ear edema model

Male ICR mice were housed at a constant temperature (23ºC) and relative humidity (60%) with a fixed 12 h light:12 h dark cycle and had 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 and Quarantine Service of Korea and were approved by the Hallym Medical Center Institutional Animal Care and Use Committee.

TPA-induced mouse-ear edema models were prepared as described previously (27, 48). To examine the effects of PEP-1-GLRX1 against TPA-induced ear edema, we divided the mice into four groups (n = 5 per group). The experimental groups were as follows: (1) normal control mice; (2) TPA-induced ear-edema mice; (3) TPA + control GLRX1-treated mice; and (4) TPA + PEP-1-GLRX1-treated mice. 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. PEP-1-GLRX1 (10 μg) protein

was topically applied to the ears of mice every day 1 h after TPA treatment. After the final treatment with TPA and PEP-1-GLRX1, we sacrificed the mice to obtain ear biopsies . Ear thicknesses were measured using a digital thickness gauge (Mitutoyo Corporation, Toyko, Japan). Ear weights were measured after 5-mm diameter ear biopsies were obtained from each group using a punch (Kai Industries, Gifu, Japan). For histological analysis, ear biopsies were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at a thickness of 5 μm, and then stained with Histidine and hematoxylin and eosin.

Statistical analysis

Data represent the mean of three experiments ± SD. Differences between groups were analyzed by one-way analysis of variance followed by a Bonferroni's post-hoc test using GraphPad Prism software (version 5.01; GraphPad Software Inc., San Diego, CA, USA); $P < 0.05$ was considered to indicate a statistically significant difference.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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