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Zinc(II) Ion Promotes Anti-inflammatory Effects of rhSOD3 By Increasing Cellular Association

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Running Title: Zinc(II) Ion Promotes Anti-inflammatory Effects

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Abstract

Recently, we demonstrated that **superoxide dismutase 3 (SOD3)** is a strong biomedicine candidate. SOD3 accomplished anti-oxidant function without cell penetration and inhibited inflammatory responses via non-enzymatic functions. SOD3 has the heparin binding domain associating cell surface. Interestingly, we have found that Zn^{2+} promotes transduction effects of **recombinant human SOD3 (rhSOD3)** by increasing uptake via **heparin binding domain (HBD)**. We showed an uptake of rhSOD3 from media to cell lysate via HBD, resulting in an accumulation of rhSOD3 into the nucleus, which is promoted by the presence of Zn^{2+}. As a result, we showed the increased inhibitory effects of rhSOD3 on NF-kB and **STAT3** signal under the presence of Zn^{2+} which is elevated association of rhSOD3 into the cells. These results suggested that the optimized procedure can help to enhance the inflammatory efficacy of rhSOD3, as a novel biomedicine.

Key Words: Anti-inflammatory, Cytopermeability, Heparin Binding Domain, Superoxide dismutase 3, Zinc (II) ion

INTRODUCTION

Superoxide dismutase (SOD) is an antioxidant enzyme, which functions to remove reactive oxygen species from cellular milieu and to preserve cells. SOD functions to dismutase of two superoxide radical to hydrogen peroxide and oxygen. SOD is classified into SOD1 containing copper and zinc atoms, SOD2 containing manganese atom, and SOD3 is located in the cell surface or the extracellular fluid. Especially, SOD3 contains copper and zinc atoms, similar to SOD1, but is characterized in that a **heparin-binding domain (HBD)** is existed in the C-terminal end. **It has been reported that the HBD of SOD3 acts as a signal of nuclear localization so that it is located within the nuclei of thymuses and testis cells so as to protect genomic DNA from oxidative stress and to regulate the DNA transcription sensitive to oxidation-reduction reaction (1).**

Since SOD3 has the HBD, SOD3 may function to protect cell membranes by binding to the cell membranes. It is well known that SOD3 plays a role in the body's defense mechanism in serums and extracellular matrices (2). Gene therapy with SOD3 improves aorta restenosis in rabbits and alleviates collagen-induced arthritis in mice (3). Recently, it has been reported that SOD3 could inhibit telomere shortening, a cell aging phenomenon in human fibroblasts and could extend the replicative life span of human fibroblast (4). Adenoviral expression of SOD3 was found to efficiently suppress the growth of melanoma and breast carcinoma cells (5, 6). Furthermore, in human skin fibroblasts and vascular smooth muscle cells, it has been demonstrated that inflammatory cytokines such as IFN-γ, IL-4, and IL-1α, up-regulate the expression of SOD3 protein and mRNA (7, 8). Conversely, SOD3 is down-regulated by both TNF-α and TGF-β (7, 8). However, the specific molecular mechanisms remain to be elucidated, particularly with regard to IFN-γ-induced SOD3 expression.

Certain proteins can effectively enter cells through cellular membranes is found, studies to use such proteins as transport means to transduce useful substances into cells are now actively performed. Typical examples of such proteins include HIV Tat protein, ANTP, VP22 protein, PEP-1 peptide, and the like (9). It has been known that the cell-transduction ability of such proteins is caused by the properties of a protein transduction domain (PTD) with the activity capable of crossing a cell membrane phospholipid bilayer (10). **The HBD of SOD3 has similar properties of other cell penetrating peptide, like highly positive charged residues. SOD3 has proteolytic variant 209E missing the HBD at the C-terminal end.** We have compared cellular uptake of rhSOD3 and 209E to investigate role of HBD in cellular uptake. In this study, we found that Zn^{2+}, which is one of cofactor of SOD3, promoted cellular uptake and anti-inflammatory effects of rhSOD3. Therefore, optimized procedure can help to develop rhSOD3 as a novel biomedicine.

RESULT

Effect of cellular uptake efficiency by metal 2+ ions in HaCaT and 293T cells

Since SOD3 can show anti-inflammatory effects by scavenging extra cellular superoxide molecules, we tried to elevate enzymatic activity of SOD by adding **its catalytic cofactors, zinc and copper ions.** To examine the potential effect of metal 2+ ions, recombinant human SOD3 (rhSOD3) was added into HaCaT or 293T cells and were incubated for 1 h under the presence of $CuSO_4$ or $ZnCl_2$. Interestingly, when $ZnCl_2$ was added to the HaCaT or 293T cells, rhSOD3 remained in the media was decreased, while rhSOD3 associated to the cell was increased by a dose-dependent manner **(Fig. 1A, left)**, indicating that zinc ions **can increase cellular association or uptake of rhSOD3.** However, $CuSO_4$ didn't show any changes of rhSOD3 level either media or cells. In the same condition, **rh209E, which is missing HBD,** wasn't changed at all in cell lysates in HaCaT or 293T cells **(Fig. 1B, lower, right)**, **indicating that increase of cellular association or uptake of rhSOD3 by zinc ions is mediated by HBD**.

Localization of exogenous rhSOD3 in the HaCaT

In order to investigate the transduction effect of zinc ions between HBD and cell line, we have studied the internalization of exogenously added fusion proteins using **HaCaT, which expressing cell surface GAGs such as heparin sulfates.** We identified surface-bound and internalized rhSOD3 in HaCaT using fluorescence microscopy. **The punctate was a typical membrane signal by fluorescence microscopy.** rhSOD3 were found to be internalized and strikingly increased the internalization of SOD3 in the presence of Zn^{2+} **(Fig. 2A, middle lane)**. These results suggested that **Zn^{2+} enhanced the internalization of rhSOD3.** However,

rh209E which is deficient of HBD, was failed to internalize in the present or absent Zn^{2+} (Fig. 2A, right lane), supporting that HBD is critical for cellular internalization. rh209E could be not internalized in the cell line that lacks the ability to interact cell surface heparin sulfate proteoglycans (HSPGs) (11), suggesting that Zn^{2+} plays a crucial, deterministic role in the uptake of rhSOD3 into the HaCaT, which is expressed on the cell surface such as **heparin sulfates.**.

Zn^{2+} promotes inflammatory responses of SOD3 by inhibition of phosphorylation of NF-kB and STAT3 in the HaCaT

First, we examined whether Zn^{2+} can promote cellular efficacy of rhSOD3 **to regulate NF- kB signal in HaCaT.** NF-kB signal is known to be one of the important in the inflammatory responses. The phosphorylation of NF-kB p65 was strongly inhibited by incubation with rhSOD3 and $ZnCl_2$, whereas $ZnCl_2$ itself exhibited no inhibitory effect (Fig. 3A, 3B). When the cells were treated with $ZnCl_2$ and rhSOD3, p-NF-kBp65 was inhibited in a dose-dependent manner (Fig. 3B). These results suggested that Zn^{2+} could promote anti-inflammatory activity of SOD3 through the inhibition of the p-NF-kB 65. After the HaCaT were stimulated by TNFα and IFNγ, **we have investigated STAT3 which is induced tyrosine phosphorylation by cytokines. The pSTAT3 was strongly inhibited by treatment with rhSOD3, whereas the pSTAT3 was not changed with rh209E (Fig. 3C- lane 1).** Compared to the levels of tyrosine phosphorylation, the total levels of these proteins were not altered **(Fig. 3C- lane 2)**. **To determine the effect of $ZnCl_2$, we analyzed mRNA expression level of IL-1β, IL-1α, TNFα, IL-8 and IL-6 in HaCaT. After the HaCaT were stimulated by TNFα and IFNγ, the cells were treated with rhSOD3 or rh209E under the present or absent $ZnCl_2$.** As shown in Fig.

4, in combination with rhSOD3 and $ZnCl_2$, the levels of inflammatory cytokines could be reduced by $ZnCl_2$, effectively. However, in combination with rh209E and $ZnCl_2$, the levels of inflammatory cytokines could not be reduced by $ZnCl_2$. Quantitative analysis showed that Zn^{2+} inhibited inflammatory responses of SOD3 caused in response to IL-1β, IL-1α, TNFα, IL-8 and IL-6 altered (Fig. 4).

Zn^{2+} enables penetration of rhSOD3 into epidermis of mouse skin

To evaluate effects of Zn^{2+}, rhSOD3 is translocated to dorsal skin, the sections with a thickness of 30 μm were made with cryotome and then were observed under confocal microscope (Supple. 1). When we applied to the skin with FITC-conjugated rhSOD3 and $ZnCl_2$, the signals of rhSOD3 were found in the epidermis. However, we only applied to the skin with FITC-conjugated rhSOD3, we could not find the signals of rhSOD3 (Supple. A).To confirm the same dose of FITC-conjugated rhSOD3, we have analyzed by Western blot analysis (Supple. B). It suggested that Zn^{2+} enhanced the skin permeation. This property of Zn^{2+} might enhance the penetration of rhSOD3 into viable layers of the skin. This result indicated that Zn^{2+} could elevate delivery of HBD proteins into the skin.

DISCUSSION

The potential of SOD3 as an important therapeutic drug for treating various diseases has been evaluated; however, various limitations have hindered the achievement of a homogenous, active form of hSOD3 (12, 13, 14). The anti-oxidative effect of SOD3 is well established (15, 16, 17, 18). However, the role of SOD3 in immune responses remains unexplored, recently, we demonstrated a unique role of SOD3 in controlling immune response (19).

SOD3 is a secretory protein and is composed of two subunits. One is full length of SOD3, the other is 209E which is lacked HBD. HBD of SOD3 could be eliminated by intracellular proteolytic cleavage before secretion (20). When we have treated rh209E to the cells, we couldn't find the 209E signal in the cell lysate. However, full length rhSOD3 was found in the cell lysate by Zn^{2+} dose dependent manner. These results suggested that HBD of SOD3 played a role in the internalization of SOD3 under the presence of Zn^{2+}, in particular, Zn^{2+} enhanced the internalization of SOD3 from the media to cell lysate. HBD of SOD3 is likely to chelate Zn^{2+} to permit interaction with the negatively charged oligosaccharides.

Interestingly, the expression of cytokine such as IL-1β, IL-1α, TNF-α, IL-8, and IL-6 were decreased by rhSOD3 and rh209E. These recombinant proteins are effective enzymes to reduce the mRNA level of cytokines after treatment with TNFα and IFNγ to the HaCaT. However, rhSOD3 is significantly effective enzyme to reduce the inflammatory cytokines in the presence of Zn^{2+}. The inhibitory effect of rhSOD3 on inflammatory cytokine is dependent on the presence of Zn^{2+} which might be elevated internalization of rhSOD into the cell and translocation into nuclear region. These results suggested that Zn^{2+} promotes anti-inflammatory effects of rhSOD3 by increasing cellular association via HBD of rhSOD3. In addition, Zn^{2+} could elevate delivery of HBD proteins into the skin, which possess with HBD, similar to

SOD3. It is a useful enhancer in the delivery of small and large therapeutics having poor skin penetration, especially protein possessed HBD. The optimized procedure can help to overcome fundamental barriers in the development of HBD proteins including rhSOD3, as a novel biomedicine.

MATERIALS AND METHODS

Preparation of recombinant SOD3

The recombinant SOD3 was prepared as described previously (21). 293T cells were transient transfected with SOD3 construct for 48 h. The supernatant was collected, and purified using a column containing Ni-NTA agarose (Qiagen), and dialysis. **The purified SOD3 activity was measured with a SOD assay kit (Dojindo) as described previously (22).** To inject into the mice or treatment *in vitro*, SOD3 was filtered to eliminate endotoxin. **SOD3 has two isoforms such as full length and proteolytic variant 209E which is wanted HBD at the C-terminal end.** The full length human SOD3 and proteolytic cleaved form 209E variant, from Met1 to Glu227, containing a C-terminal His6 tag was inserted into pcDNA3.1 (Invitrogen) using HindIII and EcoRI or HindIII and XbaI, respectively. Plasmids encoding hSOD3 and 209E variants were transfected into 293T-EBNA cells with Attractene (Qiagen) based on the manufacturer's instructions. One day after transfection, the media were replaced with serum-free Dulbecco's Modified Eagle Medium (DMEM).

Protein expression and purification

Five days after transfection, culture media containing rhSOD3 were collected, filtrated, and loaded onto HiTrap Chelating HPcolumn (GE Healthcare). After loading, the column was washed with more than 50 column volumes of washing buffer, 50 mM $NaPO_4$, 500 mM NaCl, and 30 mM imidazole. Then, rhSOD3 and rh209E were eluted by increasing the elution buffer containing 500 mM imidazole, followed by dialysis in PBS. The concentration of purified rhSOD3 was determined based on a bovine serum albumin standard curve with a protein assay dye (Bio-Rad).

Activity assay for SOD

To measure the enzymatic activity of rhSOD3, the rate of superoxide radical formation was quantified by spectrophotometer. A 20 μl sample was mixed with 200 μl of 200 μM xanthine (Sigma) and 50 μM WST-1 (Dojindo) in PBS. After adding 0.0005 unit XOD (Sigma), the increase in the formazan dye was immediately recorded using a colorimetric method at A450. The generation of a formazan dye was determined kinetically, and absolute SOD activity was determined from the dilution factor exhibiting 50% inhibition (IC50) on the inhibition curve. **The purified SOD3 activity was measured with a SOD assay kit (Dojindo) as described previously (22).**

Western blot analysis

Western blot was performed as previously described (23). Briefly, cells were lysed with radio-immuno-precipitation assay buffer (2 mM EDTA, 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM sodium vanadate, 10 mM NaF, 1 mM PMSF, 1% Triton X-100, 10% glycerol, and a protease inhibitor cocktail) and harvested immediately. The samples were loaded onto sodium dodecyl sulfate-polyacrylamide gels for electrophoresis and subsequently transferred onto polyvinylidene fluoride membranes obtained from Millipore (Bedford, MA, USA). After membranes were blocked, they were incubated with specific primary antibodies overnight at 4^{o}C with gentle agitation. The membranes were washed and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Bands were detected using ECL Plus western blotting detection reagents from Amersham Biosciences Co (Piscataway, NJ, USA).

Measurements of nucleus accumulation for rhSOD3

In order to visualize the rhSOD3 and rh209E of distribution within the cell, two kinds of the SOD3 proteins were labeled with FITC (Fluorescein isothiocyanate). Specifically, PBS phosphate buffered saline (PBS) was labeled by reaction for 2 h at room temperature with FITC-labeled rhSOD3 protein in 1 mg to 100 mg on the buffer solution. We have prepared rhSOD3 or rh209E with FITC-labeled protein solution, in order to remove the unreacted FITC by high-speed liquid chromatography devices for protein purification (FPLC, Fast Protein Purification Liquid Chromatography) and then purified by desalting columns below were used in the experiment. HaCaT was on a 8 mm cover slip to $1X10^4$ cells / well in dispensing a rear, 10% FBS and 100 units/㎖ penicillin and 100 g /㎖ streptomycin and 1% by adding a IMDM (Isocove's modified Dulbecco's medium, using the GIBCO) medium and incubated under the conditions of 5% CO_2, 37℃. When the cells were attached to a coverslip at 30 to 40% confluent, the cells were starved as serum free and cultured for 24 h. When the cells were confluent 50~60 % confluent, the cells were treated with rhSOD3 or rh209E of 10 μg protein in isolated and **purified labeled with FITC and Hoechst33342 fluorescence staining according to the method above and in the cell culture medium with confocal fluorescence microscopy** (confocal laser scanning microscope, Carl Zeiss LSM 510).

Anti-inflammatory effects of rhSOD3

At 70% confluence, HaCaT were starved with serum-free DMEM for 6 h prior to treatment with 10 ng/ml TNF-α and 100 U/ml IFN-γ. After purified rhSOD3 and 209E was added to cells with 200U/mL, and added various concentration of $ZnCl_2$ (0-10 μM). And then, the cells were harvested after 24 h incubation by directly adding SDS sample buffer containing protease

inhibitors. p-STAT3, STAT3, GAPDH, and rhSOD3 were analyzed by western blot analysis with anti-p-STAT3, p-STAT3, GAPDH (Santa Cruz Biotechnology, CA, USA) and anti-hSOD3 (AbCam, Cambridge, UK) antibodies.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from cells or tissues using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and complementary DNA was synthesized using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Quantitative real-time PCR was performed using the KAPA SYBR fast qPCR Kit (KAPA biosystems, Woburn, MA, USA) as previously described (24) and the results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. The PCR conditions were 1 cycle at 95 ^{o}C for 5min, followed by 35 cycles at 96^{o}C for 20 s, 60^{o}C for 20 s and 72^{o}C for 20 s, and ending with one cycle at 72 ^{o}C for 5 min. Primers used in this experiment were purchased from Qiagen.

Histological analysis

After shaving on mouse dorsal skin, the mixture of rhSOD3 2000 unit and $ZnCl_2$ 50mM was treated to 50 μl. **rhSOD3 treatment group was applied to the mouse back skin by rhSOD3 2000 unit.** After treated 1 h, mouse back skin were fixed with 4% paraformaldehyde in phosphate buffered saline for 24 h, washed with tap-water, dehydrated with grade ethanol and then embedded in paraffin. The paraffin blocks were cut in 4-μm thick sections, mounted on glass slides, dewaxed, rehydrated with grade ethanol and then stained with FITC and **Hoechst33342** fluorescence staining. Analysis was carried out using a fluorescence attached microscope (Olympus, Tokyo, Japan).

Statistical Analysis

Data were presented as means ± SEM, and statistical comparisons between groups were made with the unpaired two-sided t-test. All experiments were performed at least three times.

Acknowledgments

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Figure Legends

Fig. 1. Zn^{2+} induces uptake of rhSOD3 via heparin binding domain

The HaCaT (A) and 293T cells (B) were cultured with purified rhSOD3 or rh209E **under various concentrations (0, 10, 25, 50 μM)** of $CuSO_4/ZnCl_2$. Amounts of rhSOD3 or rh209E in HaCaT and 293T cells were compared between media and cell lysates by western blot analysis.

Fig. 2. Zn^{2+} promotes nuclear accumulation by not rh209E but rhSOD3 in HaCaT

After incubation with/without $ZnCl_2$, HaCaT were washed and stained with secondary antibodies labeled with FITC, prior to and after cell permeabilization, respectively. Green labeled surface-bound fraction of rhSOD3 was evident in intercellular contacts, whereas intracellular fraction of rh209E manifests that did not locate in cytosol and nuclear region. **Counterstaining was labeled with Hoechst33342 which stained nucleic acid** (A). The data were represented the mean ± SEM of fluorescent intensity (B, C). **Statistical comparisons between groups were made with the unpaired two-sided t-test. All experiments were performed at least three times. Differences with # P<0.005 versus Control group, and *P<0.05 versus rhSOD3 treatment group were considered statistically significant.**

Fig. 3. Inhibition of NF-kB p65 and STAT3 signal through transduction effect not rh209E but rhSOD3 by Zn^{2+} in HaCaT

The cells were treated both TNF-α (10 ng/ml) and IFNγ (100U/ml) and various concentration of $ZnCl_2$ (0-10 μM) (A). Purified rhSOD3 was added to cells with 200U/ml (B). Amounts of p-NF-kB P65 in cytosolic was analyzed by western blot analysis. After purified rhSOD3 and rh209E was added to cells with/without 200U/mL, and added with/without $ZnCl_2$ (10 μM) (C).

All proteins were analyzed by western blot analysis.

Fig. 4. Zn^{2+} reduces inflammatory cytokine by treating with rhSOD3 or rh209E in HaCaT

HaCaT were treated with 10ng/ml TNF-α and 100 U/ml IFN-γ. After purified rhSOD3 or rh209E were added to cells with 200U/mL, and added various concentration of $ZnCl_2$ (10μM). Quantitative real-time PCR was performed using the KAPA SYBR fast qPCR Kit (KAPA biosystems, Woburn, MA, USA) as previously described (24). **The data were represented the mean ± SEM. Statistical comparisons between groups were made with the unpaired two-sided t-test. All experiments were performed at least three times. Differences with # *P*<0.005 versus control group (-), and **P*<0.05 and ***P*<0.001 versus TNFα+IFNγ treatment group (+) were considered statistically significant.**

Supplementary 1. Zn^{2+} enables penetration of rhSOD3 into epidermis of mouse skin

rhSOD3 treatment group was applied to the mouse back skin by the mixture of rhSOD3 2000 unit and $ZnCl_2$ 50mM or rhSOD3. The paraffin blocks were stained with FITC and Hoechst33342 fluorescence staining. Arrows are indicated epidermis of mouse back skin. Confocal images of skin penetration of rhSOD3 with/without $ZnCl_2$ (50mM) (A). The proteins of back skin were measured skin penetration of rhSOD3 with/without $ZnCl_2$ (50mM) by western blot analysis (B).

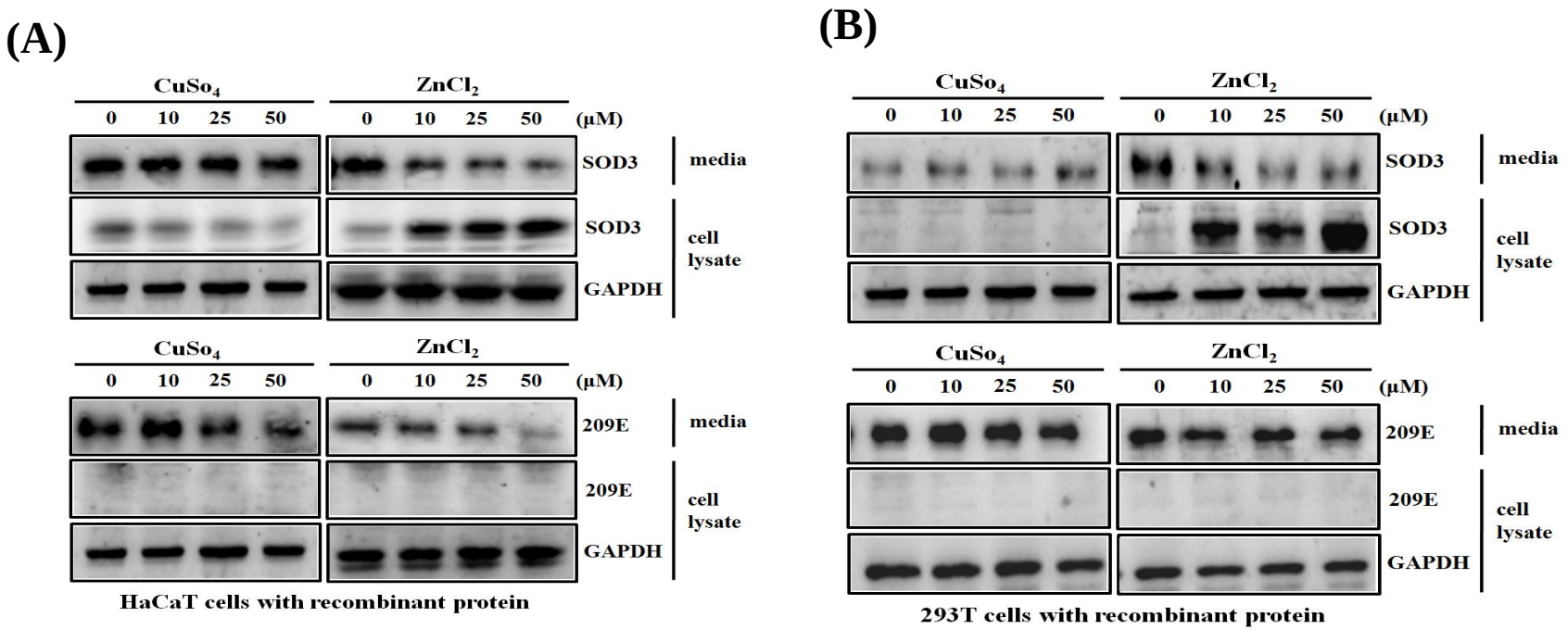


Figure 1. Zinc ions induce uptake of SOD3 via heparin binding domain

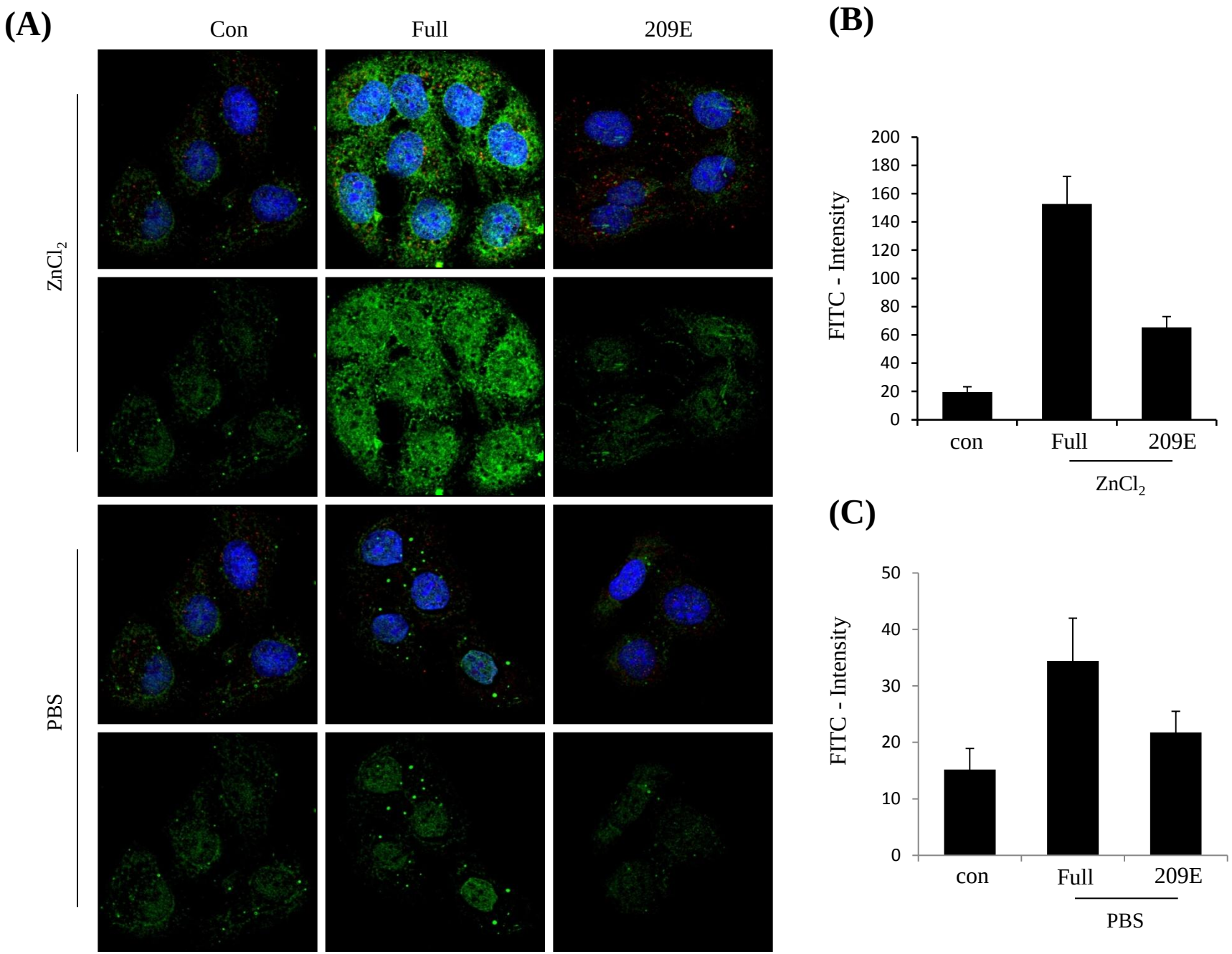


Figure 2. Zinc ions promote endothelial endocytosis of SOD3

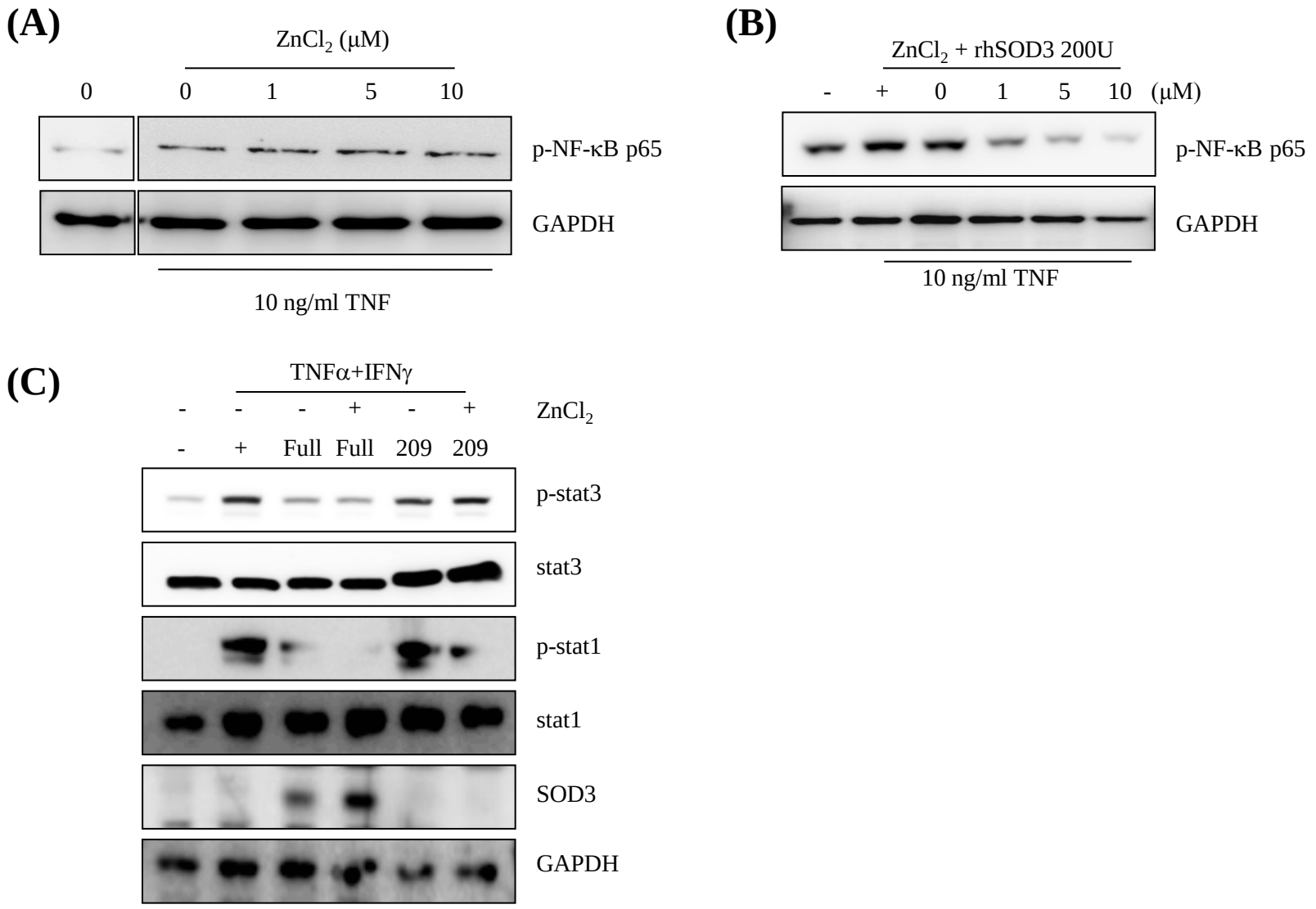


Figure3. Inhibition of NF-kBp65 and STAT signaling cascades through transduction effect of SOD3 by Zn^{2+} in HaCaT

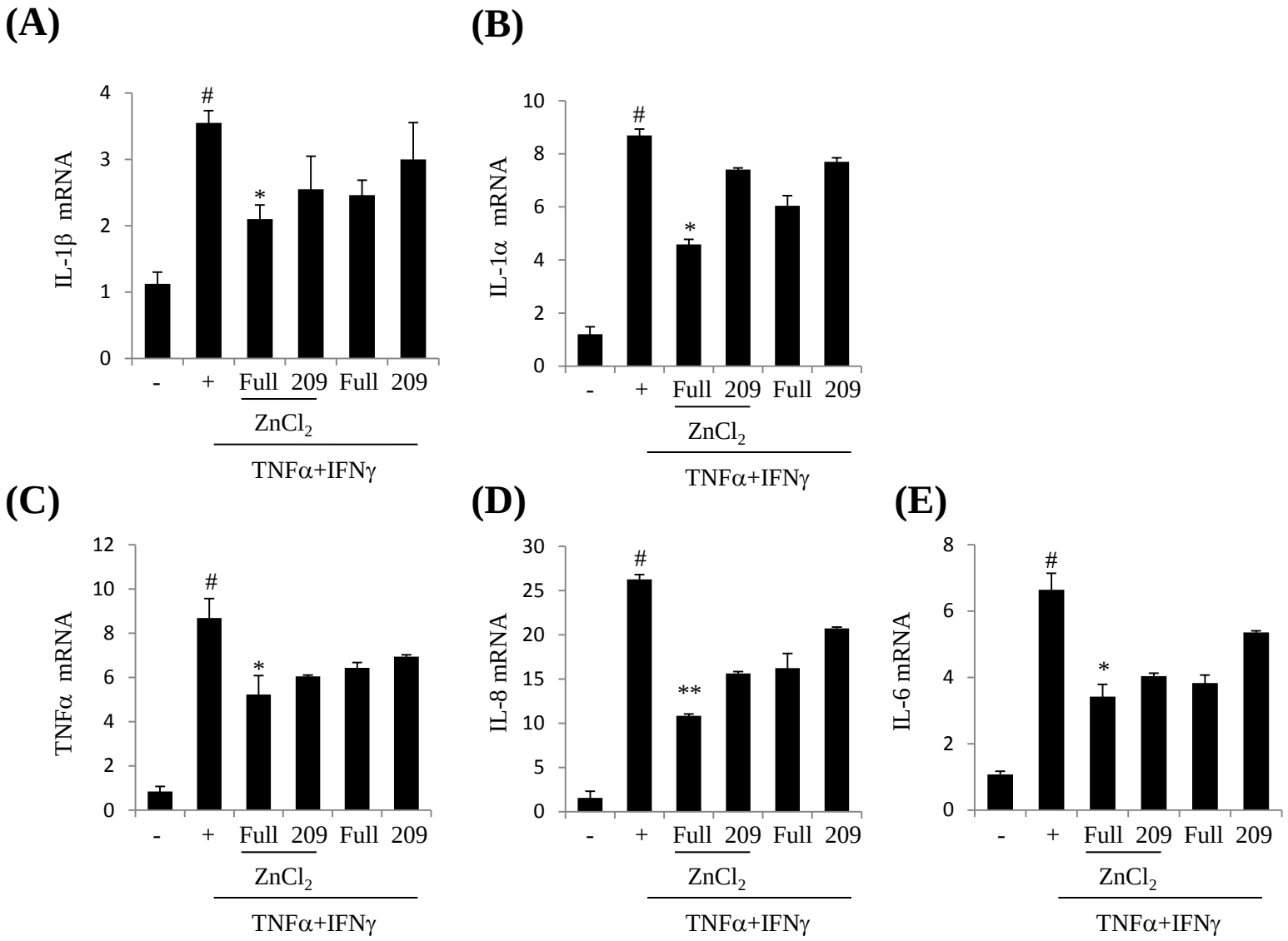


Figure 4. Zn^{2+} reduced inflammatory cytokine in HaCaT

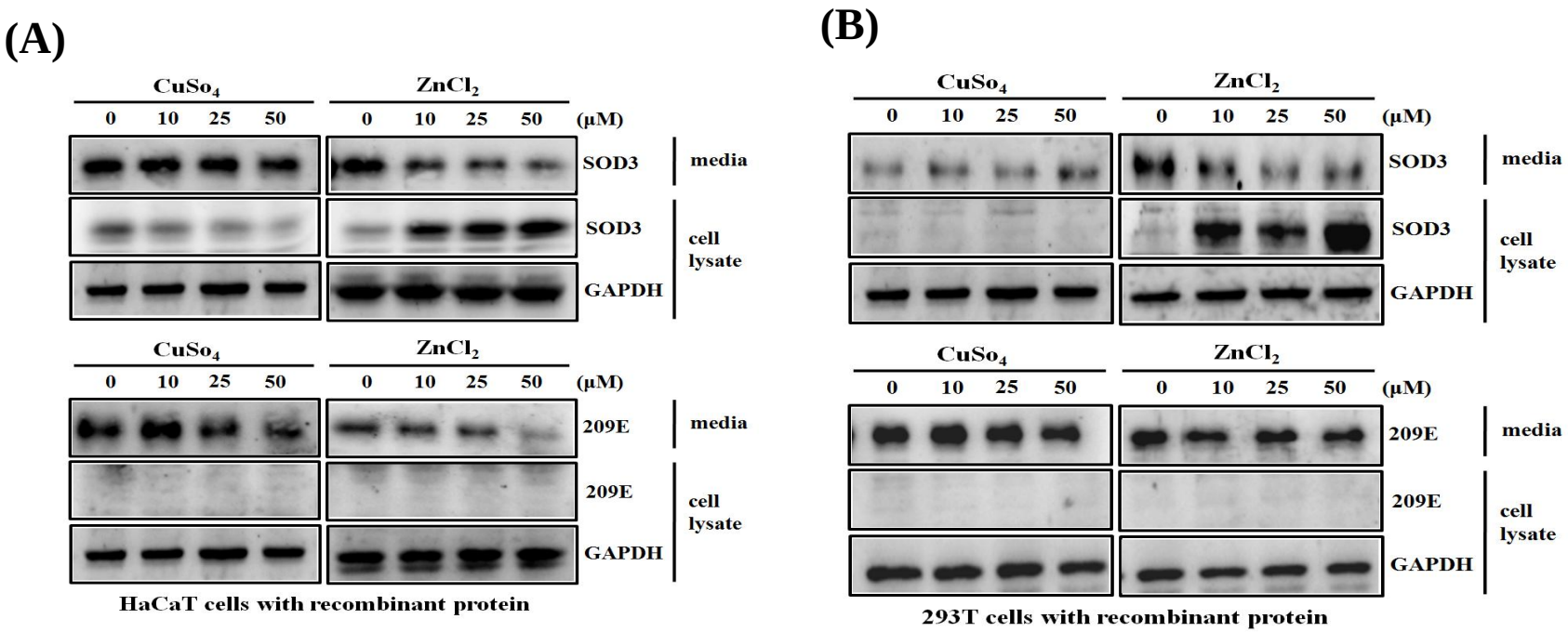
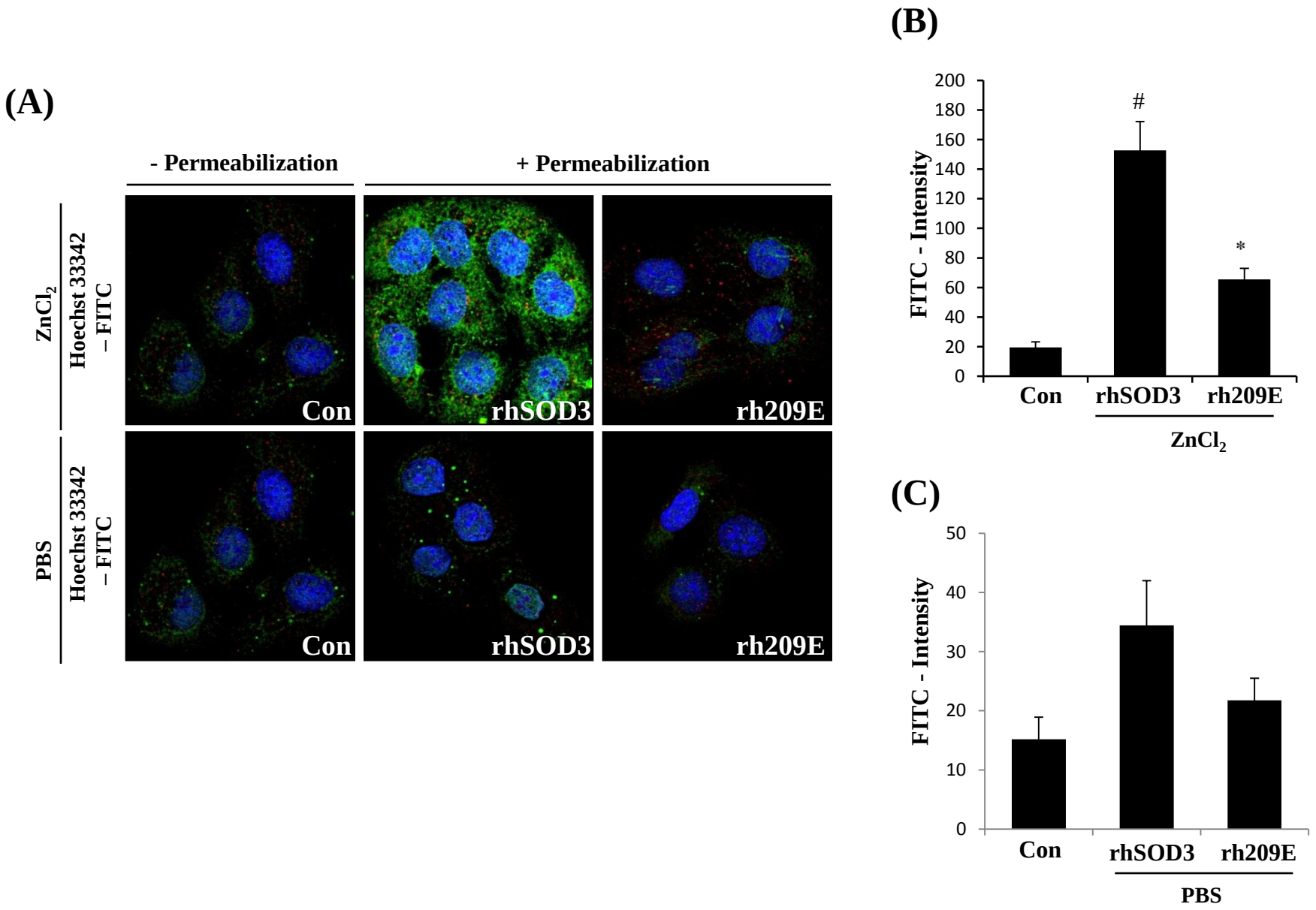


Figure 1. Zn^{2+} induces uptake of rhSOD3 via heparin binding domain



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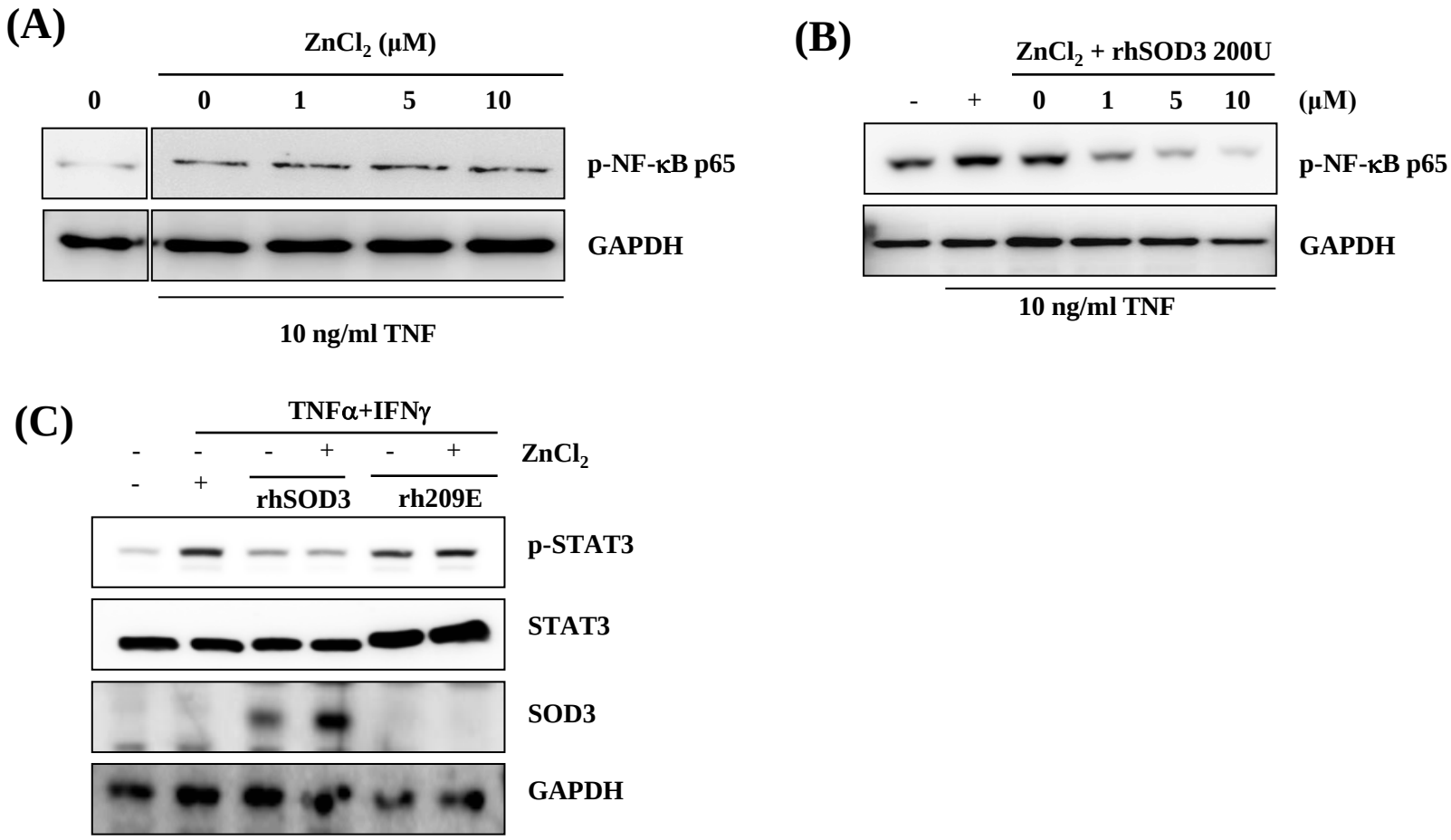

Figure 3. Inhibition of NF-kBp65 and STAT3 signal through transduction effect not rh209E but rhSOD3 by Zn^{2+} in HaCaT

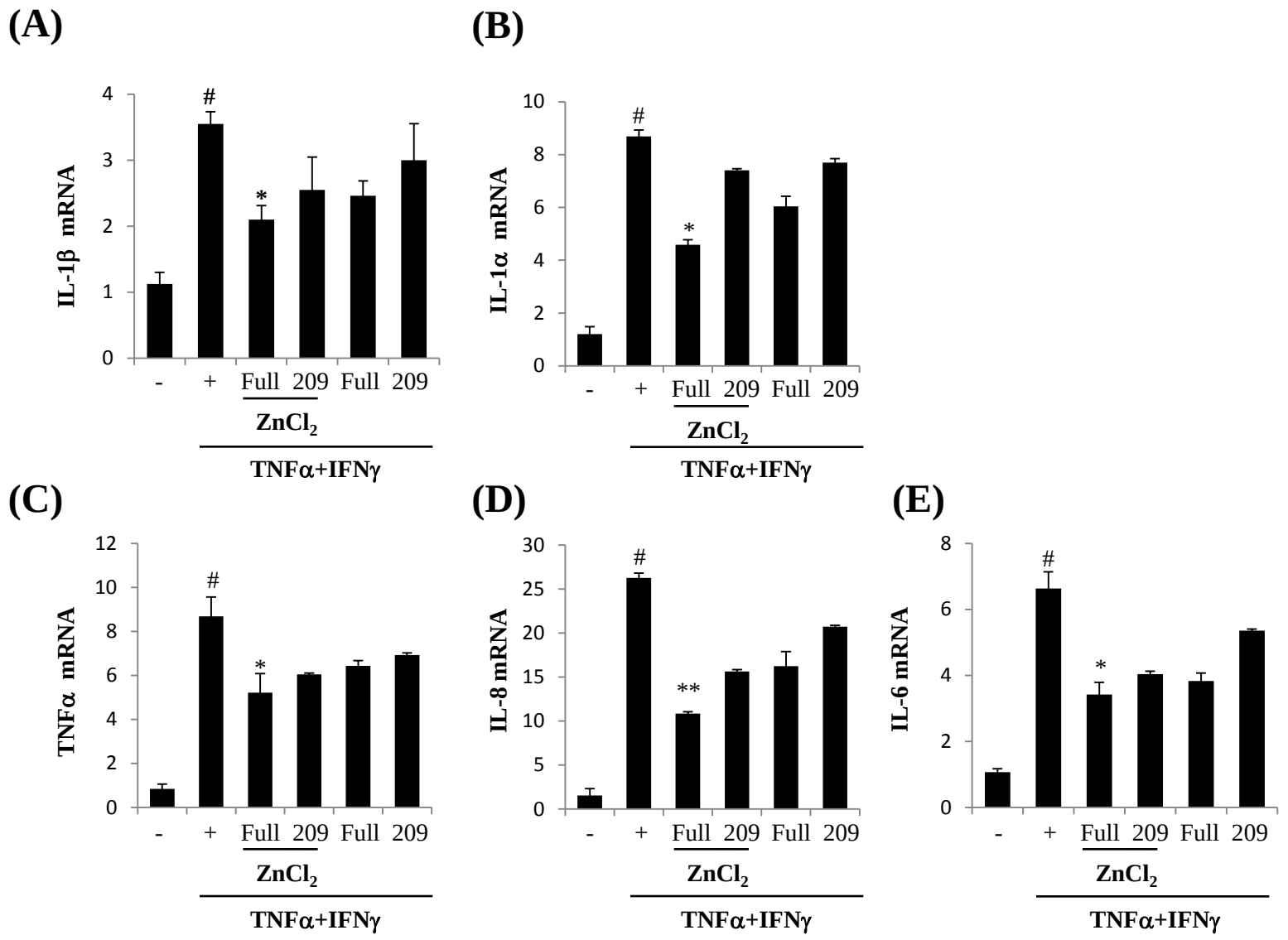

Figure 4. Zn^{2+} reduces inflammatory cytokine by treating with rhSOD3 or rh209E in HaCaT