2011 International Conference on Bioscience, Biochemistry and Bioinformatics
IPCBEE vol.5 (2011) © (2011) IACSIT Press, Singapore

Levosulpiride, (S)-(-)-5-Aminosulfonyl-N-[(1-Ethyl-2-Pyrrolidinyl) Methyl]-2-Methoxybenzamide, Enhances the Transduction Efficiency of PEP-1- Ribosomal Protein S3

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***Abstract*—Levosulpiride, (S)-(-)-5-Aminosulfonyl-N-[(1-ethyl-2-pyrrolidinyl) methyl]-2-methoxybenz- amide, is a dopamine D2 receptor anta- gonist, and has anti-emetic, anti-dyspeptic, anti-psychotic, and anti-depressant activities. This study investigated the effect of levo sulpiride on the transduction of the protein transduction domain fu sion PEP-1 ribosomal protein S3 (PEP-1-rpS3) and examined its influence on the stimulation of the therapeutic properties of PEP-1-rpS3. PEP-1-rpS3 transduction into HaCaT human keratinocytes and mouse skin were stimulated by levosulpiride in a way that did not directly affect the cell viability. Following 12-O-tetradecanoylphorbol-13-acetate(TPA)-induced inflamm -ation in mice, levosulpiride alone was ineffective in reducing TPA-induced edema and in inhibiting the elevated productions of inflammatory mediators and cytokines such as cycloxygenase-2, inducible nitric oxide synthase, interleukin-6 and -1β, and tumor necrosis factor-α. Anti-inflammatory activity by PEP-1-rpS3 + levosulpiride was significantly more potent than PEP-1-rpS3 alone. These results suggest that levosulpiride may be useful in enhancing the therapeutic effect of PEP-1-rpS3 against various inflammatory diseases.**

Keywords-component: Levosulpiride, Inflammation, PEP-1-rpS3, TPA, Protein transduction

I. INTRODUCTION.

Inflammatory diseases such as rheumatoid arthritis, atherosclerosis, and psoriasis are triggered by a large number of factors. Triggering is characterized by the increased expressions of pro-inflammatory cytokines such as interleukin-1beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) and inflammatory mediators such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [1-3].

Ribosomal protein S3 (rpS3) is a component of the 40S ribosome subunit. In addition to its role in the translation process, rpS3 is involved in several extra-ribosomal functions such as DNA repair and the oxidative stress signaling pathway [4]. Also, rpS3 is a subunit of nuclear NF-κB complexes, which regulate key genes in inflammation, cell proliferation, cell death, and development [5, 6].

Protein transduction domains (PTDs) such as Tat and PEP-1 have the ability to deliver various hydrophilic macromolecules including therapeutic proteins [7, 8].

Levosulpiride, (S)-(-)-5-Aminosulfonyl-N-[(1-ethyl-2-pyrrolidinyl) methyl]-2-methoxy- benzamide, is an (-)-enantiomer of sulpiride that exhibits anti-emetic, anti-dyspeptic, anti-psychotic, and anti-depressant activities. Levosulpiride specifically blocks dopamine D2 receptors in the central nervous system (CNS) and gastrointestinal tract. Also, dopaminergic receptors are involved in epidermal barrier homeostasis [9, 10].

In this study, we investigated whether levosulpiride increases the transduction efficiency of PEP-1-rpS3 proteins into cells in vitro and in vivo, and the influence on the anti-inflammatory effect of PEP-1-rpS3 against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation. Although the mechanism is not fully understood, the results indicate that levosulpiride may be exploited to enhance the transduction efficiency of therapeutic PTD fusion proteins for a variety of disorders

II. METHODS

A. Cell viability test

To assess cell viability, HaCaT cells were seeded into wells of a 6-well plate at grown to 70% confluence. The cells were pretreated with levosulpiride (250 ng/ml) for 1 h. After removal of levosulpiride, cells were treated with various concentrations of PEP-1-rpS3 (0.5-2 μM) for 1 h. Then, the cells were washed with Dulbecco's phosphate buffered saline (DPBS) and treated with TPA (4.0 μg/ml) for 12 h. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT).

B. Transduction of PEP-1-rpS3 into human keratinocytes

The cells were pretreated with levosulpiride (250 ng/ml) for 1 h and exposed to various concentrations (0.5-2 μM) of PEP-1 fusion proteins and for various incubation times (10-60 min). Then, the cells were harvested and cell extracts were prepared for Western blot analysis.

C. TPA-induced skin inflammation and histology

Male 6–8 week old ICR mice were purchased from the Hallym University Experimental Animal Center. All animals were treated according to the "Principles of Laboratory Animal Care" (NIH Publication No. 86-23), and handling and experimental protocols were approved by the Hallym Medical Center Institutional Animal Care and Use Committee.

Skin inflammation was induced in the right ear of each mouse (n=5) by topical application of TPA (1.0 μg) dissolved in 20 μl of acetone to the inner and outer surfaces of the mice ears. Levosulpiride (250 ng/ear) was applied to the same surfaces and PEP-1-rpS3 (3 μM) was topically applied 1 h later. The treatments were repeated for three consecutive days. On the fourth day, ear thicknesses and weights of each group (n= 5) were measured using a digital thickness gauge (Mitutoyo, Tokyo, Japan) and a 5-mm diameter punch.

For histological analysis, ear biopsy samples were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxyline and eosin.

D. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from ear biopsy samples was isolated using Trizol reagent according to the manufacturer's instructions. cDNA was synthesized from total RNA (2 μg) using reverse transcriptase (1,000 U) and 0.5 μg/μl of oligo-(dT) primer. The cDNA were PCR-amplified with the following specific primers: IL-6 antisense, 5'-TGGATGGTCTTGGTCC TTAGCC-3'; IL-6 sense, 5'-CAAGAAAGACAAAGC CAGAG TCCTT-3'; IL-1β antisense, 5'-GTGCTGCCTA ATGTCCCCTTGAATC-3'; IL-1β sense, 5'-TGCAG AGTTCCCCAACTGGTACATC-3';TNF-α antisense, 5'-TGGCACCACTAGTTG GTTGTCTTT-3'; TNF-α sense, 5'-AAGTTCCCAAATGGCCTCCC-3';and β-actin antisens 5'-GGACAGTGAG GCCAGGATGG-3'; β-actin sense, 5'-AGTGTGACGTTGA CATCCGTAAAGA-3'. After PCR was performed, the products were resolved on a 1% agarose gel and visualized with UV light after ethidium bromide staining.

III. RESULT

A. Effect of PEP-1-rpS3 on TPA-induced cell toxicity by co-treatment with levosulpiride

Previously, we reported that transduced PEP-1-rpS3 is associated with a marked decrease in DNA lesions in UV-exposed mouse skin, amelioration of ischemic damage by the reducing DNA fragmentation and lipid peroxidation, and reduced expression of various pro-inflammatory cytokines *in vitro* and *in vivo* (Choi et al., 2006; Hwang et al., 2008; Ahn et al., 2010). To investigate the effect of levosulpiride (Fig. 1A) on the cell viability, a MTT-based assay was done. The inflammatory inducer TPA (1.0 ug/ml) decreased viability of HaCaT human keratinocytes by 50% as compared to the control populations. Levosulpiride had no effect on the cell toxicity induced by TPA. On the other hand, PEP-1-rpS3 increased the viability of HaCaT cells by approximately 70, 85, 90, and 92%, of control, respectively (Fig. 1B). Additionally, co-treatment with levosulpiride (250 ng/ml) and PEP-1-rpS3 slightly increased cell viability, compared to the treatment with PEP-1-rpS3 alone. Collectively, the data supported the notion that levosulpiride might enhance the transduction of PEP-1-rpS3 into HaCaT cells without affecting cellular viability, with PEP-1-rpS3 consequently protecting from TPA-induced cell death.

B. Effect of levosulpiride on transduction of PEP-1-rpS3 into the HaCaT human keratinocytes

To identify the effect of levosulpiride on the transduction of PEP-1-rpS3 into HaCaT cells, cells were incubated with levosulpiride (250 ng/ml) 1 h prior to PEP-1-rpS3 treatment. Transduction of PEP-1-rpS3 into cells was assessed by Western blotting analysis. The intracellular transduction of PEP-1-rpS3 increased in dose- and time-dependent manners (Fig. 2A). Levosulpiride (250 ng/ml) significantly increased the transduction efficiency of PEP-1-rpS3 into HaCaT cells, compared to samples not treated with levosulpiride. In addition, the effect of levosulpiride on the transduction of PEP-1-GFP (as a positive control protein) was examined. Transduction of PEP-1-GFP was consistent with that of PEP-1-rpS3 (Fig. 2B). The data supported the suggestion that levosulpiride enhances the *in vitro* transduction efficiency of PEP-1 fusion proteins.

C. Effect of levosulpiride on transduction of PEP-1-rpS3 into mouse skin

To next examine the effect of levosulpiride on the transduction of PEP-1-rpS3 into mouse skin, PEP-1-rpS3 (3 μM) was topically applied to mouse skin 1 h after levosulpiride treatment. The distribution of PEP-1-rpS3 in mouse skin was detected histologically. PEP-1-rpS3 fusion proteins were detected in the dermis as well as the epidermis of the subcutaneous layer, and penetrated more deeply in the dermis of the subcutaneous layer in levosulpiride treated samples (Fig. 3). These results were evidence that levosulpiride enhances the transduction of PEP-1-rpS3 *in vivo* as well as *in vitro*.

D. Enhancement of in vivo anti-inflammatory activity of PEP-1-rpS3 by levosulpiride

To assess whether the increased transduction of PEP-1-rpS3 in mouse skin resulted in increased suppression of TPA-induced inflammation, TPA (1.0 μg) was applied to mice ears for 3 days. Inflammatory cell infiltration and distinct increases in ear thickness and weight were evident (Figs. 4A and 4B). Application of levosulpiride did not significantly reduce ear weight and thickness. Topical application of PEP-1-rpS3 produced a clear reduction in ear weight and thickness. Additionally, PEP-1-rpS3 inhibited TPA-induced edema in the presence of levosulpiride as compared with the results from mice treated solely with PEP-1-rpS3. The histology results from the control group (Fig. 4C, panel 1) were like those in PEP-1-rpS3 and levosulpiride co-treated groups (Fig. 4C, panel 5). These

results provided evidence that levosulpiride significantly improves the inhibition of TPA-induced inflammation by PEP-1-rpS3 *in vivo*.

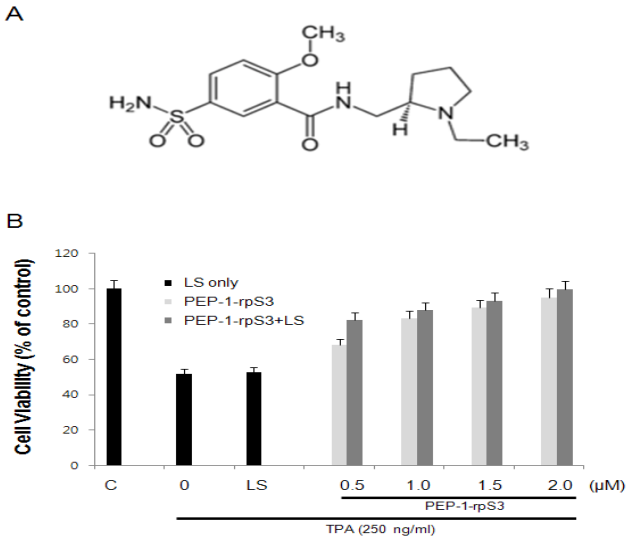
E. Effect of PEP-1-rpS3 on the expression level of inflammatory mediators and cytokines in the presence of levosulpiride

To examine whether the expressions of the inflammatory mediators COX-2 and iNOS in the TPA-induced edema mice model were changed upon exposure to PEP-1-rpS3 and/or levosulpiride, the levels of these inflammatory mediators were determined in ear biopsies by Western blot analysis (Fig. 5A). Exposure to TPA led to a marked increase in their levels (lane 2), while levosulpiride had no influence (lane 3). Treatment with PEP-1-rpS3 (lane 4) was effective in decreasing the elevated levels of COX-2 and iNOS induced by TPA. In addition, the elevated levels of COX-2 and iNOS were decreased in the presence of both levosulpiride and PEP-1-rpS3 (lane 5) more significantly than when treated with PEP-1-rpS3 alone. The next experiment evaluated the inflammatory cytokines TNF-α, IL-1β, and IL-6 in the TPA-induced edema mice model using RT-PCR. The levels of these cytokines increased after treatment with TPA (Fig. 5B, lane 2). Treatment with levosulpiride did not reduce their levels (lane 3). PEP-1-rpS3 slightly reduced the production of all three cytokines induced by TPA (lane 4). However, the anti-inflammatory activity of PEP-1-rpS3, which inhibited the expression of the pro-inflammatory cytokines, was markedly increased in the presence of levosulpiride (lane 5). These results provided support for the suggestion that levosulpiride could increase the transduction of PEP-1-rpS3 into mice skin, with the subsequent PEP-1-rpS3-mediated marked reduction of the levels of the inflammatory mediators and pro-inflammatory cytokines, compared to sample not treated with levosulpiride

IV. SUMMARY

We demonstrated that levosulpiride enhance the transduction efficiency of PEP-1-rpS3 in vitro and in vivo without side effects. Also, anti-inflammatory effects of PEP-1-rpS3 combined with levosulpiride more increased than the PEP-1-rpS3 alone. Thus, levosulpiride may be useful as enhancer of the transduction of PTD fusion therapeutic protein.

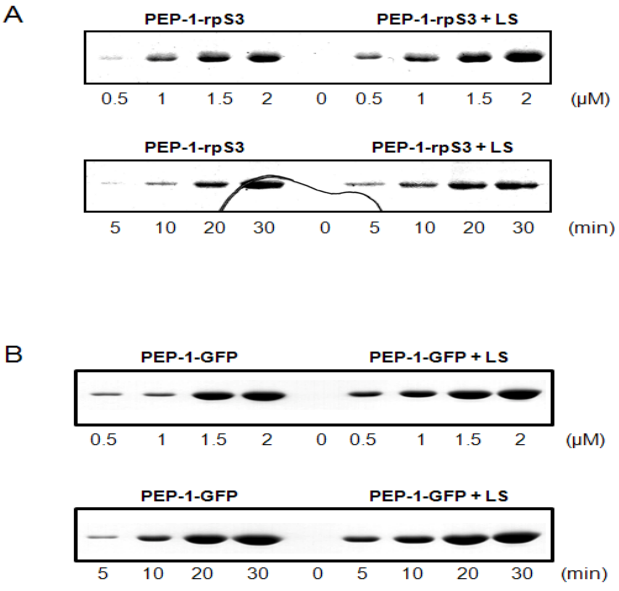
Effect of levosulpiride on the viability of human HaCaT Keratino cytecells.



(A) The structure of levosulpiride, (S)-(-)-5-Aminosulfonyl-N-[(1-ethyl-2-pyrrolidinyl) methyl]-2-methoxybenzamide. (B) Effect of levosulpiride on the viability of HaCaT cells. Cells were pretreated with levosulpiride (250 ng/ml) and then incubated with PEP-1-rpS3 for 2 h. Cell toxicity was induced by TPA (250 ng/ml). Cell viability was assessed using a MTT assay and is expressed as a percentage of control without TPA treatment. C, untreated control; LS, levosulpiride.

Dose- and time-dependent transduction of PEP-1-rpS3 into

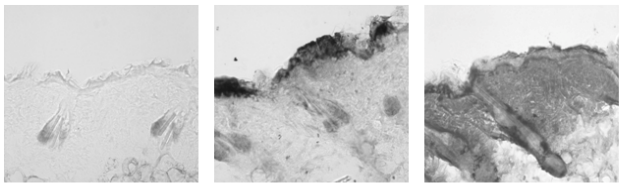
HaCaT cells in the presence of levosulpiride



For dose-dependent transduction of (A) PEP-1-rpS3 and (B) PEP-1-GFP, cells were incubated with various concentrations of PEP-1 fusion proteins. When treated with levosulpiride, cells were pretreated with levosulpiride

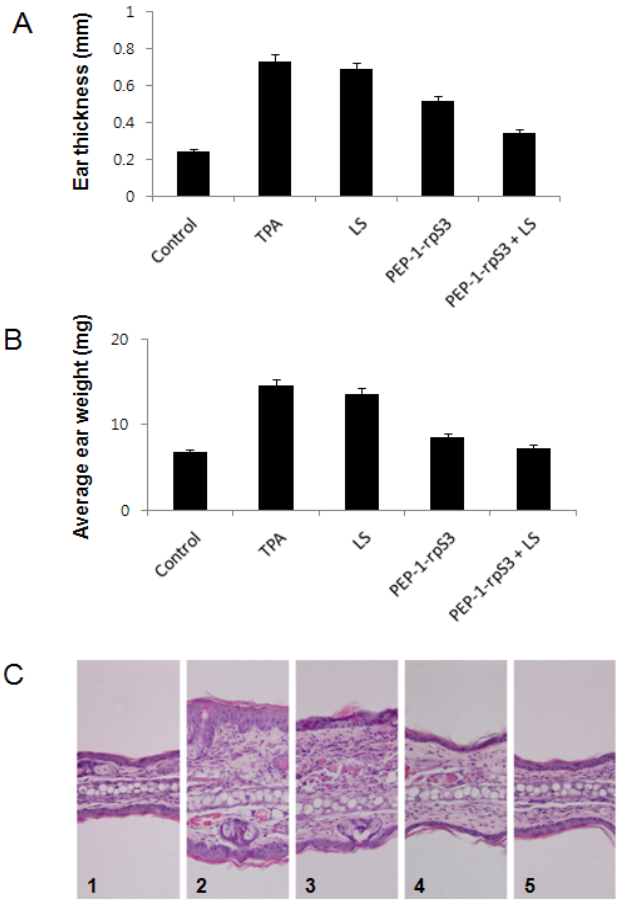
(250 ng/ml) 1 h prior to treatment with PEP-1 fusion proteins. For time-dependent transduction of (A) PEP-1-rpS3 and (B) PEP-1-GFP, cells were incubated with PEP-1-fusion proteins (1 μM) alone or with PEP-1 fusion proteins and levosulpiride (250 ng/ml) for 0–30 min. Transduced PEP-1 fusion proteins were analyzed by Western blotting.

Localization of transduced PEP-1-rpS3 in mouse skin



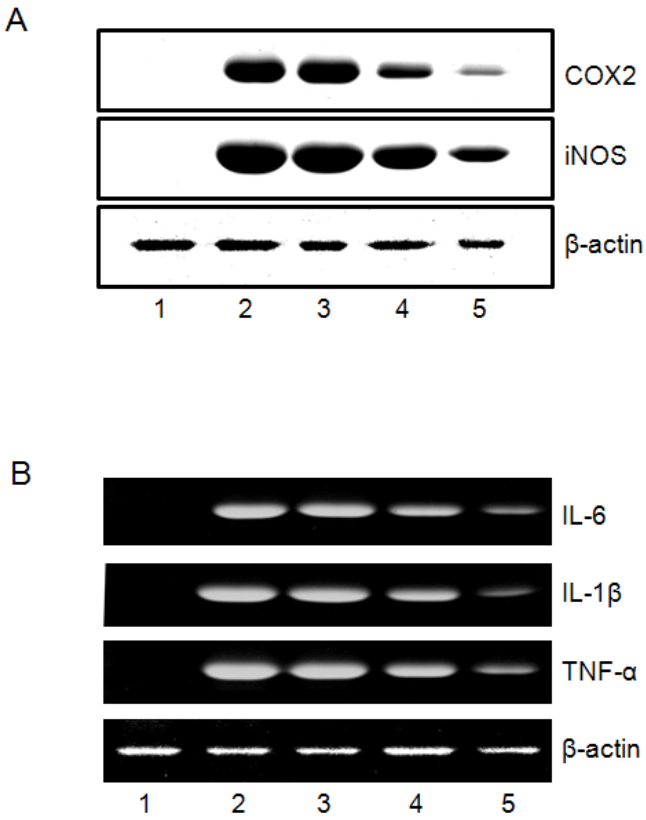
PEP-1-rpS3 (3 μM) was topically applied onto the shaved area of mouse dorsal skin for 60 min. Skin tissues were immunostained with rabbit anti-histidine IgG (1:400) and then stained with biotinylated goat anti-rabbit IgG (1:200). The sections were visualized with 3, 3'-diaminobenzidine and observed microscopically. LS, levosulpiride.

Inhibitory effect of PEP-1-rpS3 on TPA-induced ear edema in the presence of levosulpiride



The ears of mice were pretreated with TPA (1.0 μg/ear) once a day for 3 days. Then, levosulpiride was topically applied to mice ears followed by treatment of PEP-1-rpS3. (A) Ear thickness and (B) ear weight were measured after administration of PEP-1-rpS3. (C) Histochemical analysis of mouse ear tissue. 1, Untreated control; 2, TPA-treated skin; 3, levosulpiride treated skin after TPA treatment; 4, PEP-1-rpS3 treated skin after TPA treatment; 5, levosulpiride and PEP-1-rpS3 treated skin after TPA treatment. LS, levosulpiride.

Inhibitory effect of PEP-1-rpS3 on levels of pro-inflammatory cytokines and mediators in a TPA induced inflammation mice model



The ears of mice were pretreated with TPA (1.0 μg/ear) once a day for 3 days. Then, levosulpiride was topically applied to mice ears followed by treatment of PEP-1-rpS3. (A) The levels of COX-2 and iNOS expression were analyzed by Western blotting. (B) Total RNA was extracted from ear biopsies. The levels of IL-6, IL-1β, TNF-α, and β-actin mRNA were analyzed by RT-PCR using specific primers. Lanes are as follows: lane 1, Untreated control; lane 2, TPA-treated skin; lane 3, levosulpiride treated skin after TPA treatment; lane 4, PEP-1-rpS3 treated skin after TPA treatment; lane 5, levosulpiride, and PEP-1-rpS3 treated skin after TPA treatment

ACKNOWLEDGEMENTS

This work was supported by the Priority Research Centers Program grant (2009-0093812) and by the Regional Research Universities Program/Medical & Bio-material Research Center grant through the National Research

Foundation of Korea funded by the Ministry of Education, Science and Technology.

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