Levosulpiride, (S)-(-)-5-Aminosulfonyl-N-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxybenzamide, enhances the transduction efficiency of PEP-1-ribosomal protein S3 in vitro and in vivo

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INTRODUCTION

Many proteins with poor transduction efficiency were reported to be delivered to cells by fusion with protein transduction domains (PTDs). In this study, we investigated the effect of levosulpiride on the transduction of 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 was stimulated by levosulpiride in a manner that did not directly affect the cell viability. Following 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice, levosulpiride alone was ineffective in reducing TPA-induced edema and in inhibiting the elevated productions of inflammatory mediators and cytokines, such as cyclooxygenase-2, inducible nitric oxide synthase, interleukin-1β, interleukin-6, tumor necrosis factor-alpha (TNF-α), and inflammatory mediators such as cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) (5-8). 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 (9, 10).

Levosulpiride, (S)-(-)-5-Aminosulfonyl-N-[(1-ethyl-2-pyrrolidinyl)methyl]-2-methoxybenzamide, is an (-)-enantiomer of sulpiride that exhibits anti-emetic, anti-dyspeptic, anti-psychotic, and anti-depressant activities (16). Levosulpiride specifically blocks dopamine D2 receptors in the central nervous system (CNS) and gastrointestinal tract (17). Also, dopaminergic receptors are involved in epidermal barrier homeostasis (18).

In this study, we investigated whether levosulpiride increases the transduction efficiency of PEP-1-rpS3 proteins 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.
RESULTS

Effect of PEP-1-rpS3 on TPA-induced cell toxicity by co-treatment with levosulpiride
We previously reported that transduced PEP-1-rpS3 can reduce DNA lesions in UV-exposed mouse skin, ameliorate ischemic damage by reducing DNA fragmentation and lipid peroxidation, and decrease expression of various pro-inflammatory cytokines in vitro and in vivo (15, 19 and 20). To investigate the effects of levosulpiride on cell viability, a MTT-based assay was performed. The structure of levosulpiride is shown in Fig. 1A. The inflammatory inducer TPA (4.0 μg/ml) decreased the viability of HaCaT human keratinocytes by 46%, as compared to the control populations. However, levosulpiride had no effect on cell toxicity induced by TPA. PEP-1-rpS3 increased the viability of HaCaT cells in a concentration-dependent manner, and when coadministered with levosulpiride (250 ng/ml), PEP-1-rpS3 enhanced cell viability, compared to the treatment with PEP-1-rpS3 alone (Fig. 1B). 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 against TPA-induced cell death.

Effect of levosulpiride on transduction of PEP-1-rpS3 into HaCaT cells and mouse skin
We identified the role of levosulpiride as an enhancer of the transduction of PEP-1-rpS3 into HaCaT human keratinocytes and mouse skin. The intracellular transduction of PEP-1-rpS3 increased in a dose-dependent manner (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).

To examine the influence of levosulpiride on the in vivo transduction of PEP-1-rpS3, mouse skin was treated with levosulpiride 1 h prior to addition of PEP-1-rpS3. PEP-1-rpS3 was detected in the dermis as well as the epidermis of the subcutaneous layer, and penetrated more deeply in the dermis of the subcutaneous layer pre-treated with levosulpiride (Fig. 2C). These results suggest that levosulpiride enhanced the trans-
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Fig. 3. 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 mouse ears, followed by treatment with PEP-1-rpS3. (A) Ear thickness and (B) ear weight were measured after administration of PEP-1-rpS3. *P < 0.01 compared with mice treated with TPA alone. (C) Histochemical analysis of mouse ear tissue. 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.

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 inflammatory mediators and cytokines in the TPA-induced edema mouse model were modulated by treatment with PEP-1-rpS3 and/or levosulpiride, their levels were determined by Western blot and RT-PCR analysis. As shown in Fig. 4A, exposure to TPA led to a marked increase in the levels of COX-2 and iNOS, while levosulpiride had no influence. Treatment with PEP-1-rpS3 was effective in decreasing the elevated levels of COX-2 and iNOS induced by TPA. However, in the presence of both levosulpiride and PEP-1-rpS3, a more significant down-regulation of these genes was observed. Also, the RT-PCR data showed that reduction in ear weight and thickness. Additionally, PEP-1-rpS3 inhibited TPA-induced edema more significantly in the presence of levosulpiride, as compared with results from the set of mice treated solely with PEP-1-rpS3. The histological results from the control group were similar to those in the PEP-1-rpS3 and levosulpiride co-treated groups (Fig. 3C). These results provide evidence that levosulpiride significantly improved the inhibition of TPA-induced inflammation by PEP-1-rpS3 in vivo.
the levels of IL-6, IL-1β, and TNF-α increased after treatment with TPA (Fig. 4B). Treatment with levosulpiride did not result in any change. PEP-1-rpS3 effectively reduced the production of all three cytokines induced by TPA. In addition, 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. These results provided further support for the suggestion that levosulpiride may have increased the transduction of PEP-1-rpS3 into mouse skin.

**DISCUSSION**

12-O-Tetradecanoylphorbol-13-acetate (TPA) promotes skin carcinogenesis, which is related to inflammatory responses including the development of edema, hyperplasia, and various diseases, as well as the induction of pro-inflammatory cytokines, generation of reactive oxygen species (ROS), overexpression of COX-2, and inducible nitric oxide synthase (iNOS) (5, 6, 21-23). Since inhibition of the production of these inflammatory mediators may be a new paradigm for preventing a variety of inflammatory diseases, much research has focused on the discovery and application of therapeutic drugs, including the use of proteins. However, there are still a myriad of problems concerning the use of therapeutic proteins as drugs, which include unfavorable pharmacokinetic parameters caused by their large molecular size and charge (24).

Ribosomal protein S3 (rpS3) is involved in the DNA repair process by virtue of its DNA deoxyribophosphodiesterase activity and DNA glycosylase activity (25). Previously, we reported construction of a PTD fusion protein expression vector of PEP-1-rpS3, and demonstrated the efficient transduction ability of PEP-1-rpS3 in vivo as well as in vitro (19). Sulpiride is well-known for possessing anti-emetic, anti-dyspeptic, and anti-psychotic attributes (16, 17). In particular, levosulpiride is the levorotatory enantiomer of sulpiride and a highly selective antagonist of D2 receptors. The present study was undertaken to investigate whether the transduction efficiency and anti-inflammatory activity of PEP-1-rpS3 were modulated by levosulpiride.

Presently, PEP-1-rpS3 improved the viability of HaCaT cells in a concentration-dependent manner (Fig. 1B). Addition of levosulpiride to TPA-treated cells produced no additional toxicity, while PEP-1-rpS3 enhanced the viability of HaCaT cells in the presence of levosulpiride, compared with the absence of levosulpiride. The observations that the transduction of PEP-1-rpS3 into HaCaT cells and mouse skin was increased in the presence of levosulpiride both in vitro and in vivo (Fig. 2) provide evidence that the presence of levosulpiride somehow increases PEP-1-rpS3 transduction and, consequently, increases cell viability.

A TPA-induced inflammation animal model was utilized to assess the influence of levosulpiride on the anti-inflammatory activity of PEP-1-rpS3. PEP-1-rpS3 inhibited ear swelling and the elevation of pro-inflammatory cytokines and inflammatory mediators (Fig. 3 and 4), which are distinct characteristics of inflammation. In the presence of levosulpiride, PEP-1-rpS3-mediated inhibition of inflammation was more profound than in the absence of levosulpiride. The collective data strongly supports the idea that the anti-inflammatory activity of PEP-1-rpS3 is closely associated with the levosulpiride-mediated increased transduction of PEP-1-rpS3.

Maintenance of skin barrier homeostasis is very important because epidermal hyperplasia and inflammation arise from the disruption of the skin barrier or from a dry condition of the skin. Metabotropic and ionotropic receptors play important roles in maintaining the homeostasis of the skin barrier through the regulation of intracellular cyclic AMP concentration (26). The dopamine 2-like receptor family is composed of dopaminergic D2, D3, and D4 receptors, and is a class of metabotropic G protein coupled receptors. D2-like receptor agonists accelerate barrier recovery, whereas D2-like receptor antagonists delay recovery, prompting the suggestion that dopaminergic receptors participate in epidermal barrier homeostasis (18). Although the mechanism of levosulpiride that enhances the transduction of PEP-1-rpS3 is unclear, it is conceivable that the topical treatment of skin with TPA disrupts the skin barrier homeostasis and thereby induces inflammation, and consequently, levosulpiride could delay recovery of the disrupted skin barrier homeostasis. Accordingly, PEP-1-rpS3 could be efficiently transduced into the epidermis of the subcutaneous layer of skin. In further studies, to clearly demonstrate that skin barrier homeostasis is related to the transduction mechanism of PTD proteins, it will be necessary to examine whether agonists of dopaminergic D2 receptors affect the transduction of PTD fusion proteins.

In conclusion, the collective results of this study indicate the potential of levosulpiride as an effective enhancer of the transduction of various PTD-fused therapeutic proteins.

**MATERIALS AND METHODS**

**Materials**
Levosulpiride and TPA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against COX-2, iNOS, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents, unless otherwise stated, were obtained from Sigma-Aldrich and were of the highest analytical grade available.

**Cell culture and cell viability test**
HaCaT human keratinocytes were grown in DMEM containing 10% FBS and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin). Cells were maintained at 37°C under a humidified atmosphere of 95% air/5% CO₂. To assess cell viability, cells were seeded into 6-well plates, and after reaching 70% confluency, 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, followed by treatment with TPA (4.0 μg/ml) for another 12 h. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as previously described (27).

Transduction of PEP-1-fusion proteins into human keratinocytes

PEP-1-rpS3 and PEP-1-green fluorescent protein (PEP-1-GFP) were purified as previously described (19). To detect the transduction of PEP-1 fusion proteins into HaCaT cells, the cells were grown to desired confluence in 6-well plates. The cells were pretreated with levsulpiride (250 ng/ml) for 1 h, and then exposed to various concentrations (0.5-2 μM) of PEP-1 fusion protein. The cells were then harvested and cell extracts were processed for Western blot analysis.

TPA-induced skin inflammation and histology

Male 6-8 week old ICR mice were purchased from the Hallym University Experimental Animal Center. The animals were housed at a constant temperature (23°C) and relative humidity (60%) with alternating 12 h cycles of light and dark. They were provided with food and water ad libitum. All animals were treated according to the “Principles of Laboratory Animal Care” (NIH Publication No. 85-23), 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 mouse ears as previously described (21). Levsulpiride (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) with 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 hematoxylin and eosin. For Western blot analysis, proteins from ear biopsy samples were prepared by vigorous homogenization, followed by centrifugation at 10,000 x g for 10 min.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from ear biopsy samples was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) 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'-TGGATGGTCTTGG-3'; TNF-α antisense, 5'-TGCAGAGTTCCCCAACTGGTACAT-3'; IL-1 β-actin antisense, 5'-TGGCACCACTAGTTGGTTGTCTT-3'; TNF-α sense, 5'-AGTGTGACGTTGACATCCGTAAAGA-3'. After PCR was performed, the products were resolved on a 1% agarose gel and visualized with UV light after ethidium bromide staining.

Statistical analysis

Data were expressed as the means ± SD. Comparisons between groups were made with ANOVA, followed by Dunnett’s test. A value of P < 0.01 was considered to be statistically significant.

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