Department of Biochemistry[1], Chung-Ang University College of Medicine; College of Pharmacy[2], Chung-Ang University; Department of Dermatology[3], Seoul National University Bundang Hospital, Seoul, Republic of Korea

MMS 1001 inhibits melanin synthesis via ERK activation

Hyun-E Lee[1], Jiho Song[2], Su Yeon Kim[1], Kyoung-Chan Park[3], Kyung Hoon Min[2], Dong-Seok Kim[1]

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Kyung Hoon Min, Ph.D., College of Pharmacy, Chung-Ang University, 221 Heukseok-dong, Dongjak-gu, Seoul 156-756, Korea
khmin@cau.ac.kr

Dong-Seok Kim, Ph.D., Department of Biochemistry, Chung-Ang University College of Medicine, 221 Heukseok-dong Dongjak-gu, Seoul 156-756, Korea
ds_kim@cau.ac.kr

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Melanin plays major a role in pigmentation of hair, eyes, and skin in mammals. In this study, the inhibitory effects of MMS 1001 on α-MSH-stimulated melanogenesis were investigated in B16F10 melanoma cells. MMS 1001 did not show cytotoxic effects up to 10 μM. Melanin content and intracellular tyrosinase activity were inhibited by MMS 1001 treatment in a dose-dependent manner. In Western blot analysis, MITF expression was decreased by MMS 1001. In addition, tyrosinase expressions were also reduced after MMS 1001 treatment. Further results showed that the phosphorylation of ERK was induced by MMS 1001. Moreover, a specific MEK inhibitor, PD98059, abrogated the inhibitory effects of MMS 1001 on melanin production and tyrosinase expression. These results indicate that the hypopigmentary effects of MMS 1001 resulted from the inhibition of MITF and tyrosinase expression via phosphorylation of ERK. Thus, MMS 1001 could be developed as a new effective skin-whitening agent.

1. Introduction

Melanin is a dark pigment of hair, eyes, and skin in mammals and is secreted by melanocytes at the basal layer of the epidermis of the skin. Pigmentation plays an important role in the protection against harmful DNA damages by UV radiation in humans (Nestle et al. 2009; Reelfs et al. 2010). Although melanin is a photo-protectant, which inhibits the DNA damage by absorbing UV-radiation (Agar and Young 2005), the overproduction and accumulation of melanin could be a problem resulting in several hyperpigmentary disorders, such as freckles, melasma, and age spots (Aoki et al. 2007).

Melanin synthesis is regulated by three major enzymes, including tyrosinase (Chang 2009). Tyrosinase is localized within specialized intracellular organelles, called melanosome, in the melanocytes. Tyrosinase catalyzes three different reactions; firstly, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), secondly, the oxidation of DOPA to DOPAquinone, and thirdly, the oxidation of 5,6-dihydroxyindole (DHI) to indole-quinone (Hearing and Tsukamoto 1991).

In mammals, melanin synthesis is commonly influenced by keratinocyte-secreted factors, including α-melanocyte stimulating hormone (α-MSH), stem cell factor, endothelin-1, and prostaglandin E_2 (Busca and Ballotti 2000; Miyamura et al. 2007; Park et al. 2009). α-MSH binds to melanocortin-1 receptor (MC1R) and increases cyclic AMP (cAMP) levels (Schwahn et al. 2001). cAMP is at the beginning point of several signaling cascades in melanogenesis. Protein kinase A (PKA) is a downstream effector of cAMP. PKA phosphorylates cAMP response element-binding protein (CREB), which is known to be an activator of microphthalmia-associated transcription factor (MITF) expression (Jiang et al. 2011; Lee et al. 2007). Thus, cAMP up-regulates the expression of MITF, which is the major transcription factor for tyrosinase expression.

The extracellular signal-regulated protein kinase (ERK) pathway is reported to be involved in the phosphorylation of MITF (Zhang et al. 2006). ERK activation leads to phosphorylation of MITF at Serine 73 (Hemesath et al. 1998). It is known that this phosphorylation results in MITF degradation (Jeong et al. 2011; Kim et al. 2011). Therefore, it has been reported that the activation of ERK pathway leads to subsequent down-regulation of tyrosinase expression and reduction of melanogenesis (Busca and Ballotti 2000; Kim et al. 2003; Kim et al. 2006).

To explore new hypopigmentary agents, twenty compounds have been selected from the primary screening. Among them, MMS 1001 showed the best hypopigmentary effects. Thus, in the present study, the effects of MMS 1001 on the melanin synthesis and tyrosinase activity, were investigated on B16F10 cells. This study focused on the expression of tyrosinase and MITF, and the regulation of intracellular signals related to melanogenesis.

Abbreviations: CREB, cAMP response element binding protein; DOPA, 3,4-dihydroxyphenylalanine; ERK, extracellular signal-regulated kinase; MC1R, melanocortin 1 receptor; MITF, microphthalmia-associated transcription factor; α-MSH, α-melanocyte stimulating hormone; UV, ultraviolet.

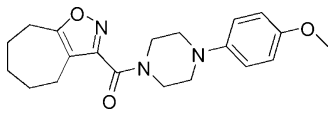


Fig. 1: The structure of MMS 1001

2. Investigations and results

2.1. Effects of MMS 1001 on cell viability in B16F10 cells

The chemical name of MMS 1001 is (4-(4-methoxyphenyl)-piperazin-1-yl) (5,6,7,8-tetrahydro-4*H*-cyclohepta[*d*]isoxazol-3-yl) methanone. The structure of MMS 1001 is shown in Fig. 1. To investigate the effect of MMS 1001 on cell viability, B16F10 cells were incubated with various concentrations of MMS 1001 (0–10 μM) for 24 h. Cell viability was determined by the crystal violet assay. MMS 1001 did not show any cytotoxicity at the concentrations range from 0.1 to 10 μM (Fig. 2A). Thus, 0.1–10 μM of MMS 1001 was used for the following experiments.

2.2. Effects of MMS 1001 on melanin synthesis and tyrosinase activity

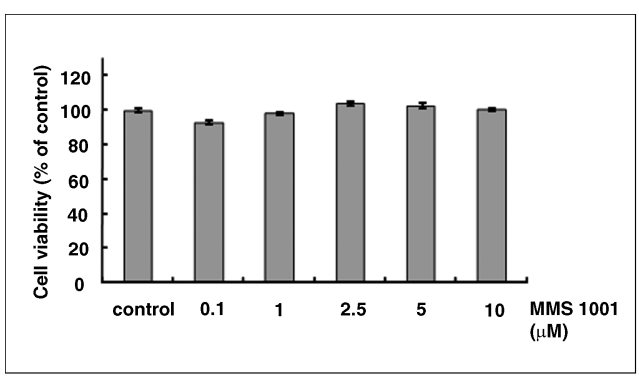
To examine the influence of MMS 1001 on α-MSH induced melanogenesis, B16F10 cells were treated with various concentrations of MMS 1001 (0–10 μM) in the presence of α-MSH (1 μM) for 3 days. The melanin contents of the α-MSH treated cells increased 2.9 times compared to the untreated control. MMS 1001 reduced melanin contents in a dose-dependent manner (Fig. 2B). Furthermore, intracellular tyrosinase activity was measured using L-DOPA as a substrate. In accordance with reduced melanin content, MMS 1001 decreased tyrosinase activity in a dose-dependent manner (Fig. 2C). To investigate whether MMS 1001 inhibits tyrosinase directly, tyrosinase activity was measured, using mushroom tyrosinase. However, MMS 1001 did not reduce tyrosinase activity directly in a cell free system (Fig. 2D).

2.3. MMS 1001 down-regulates MITF and tyrosinase expression

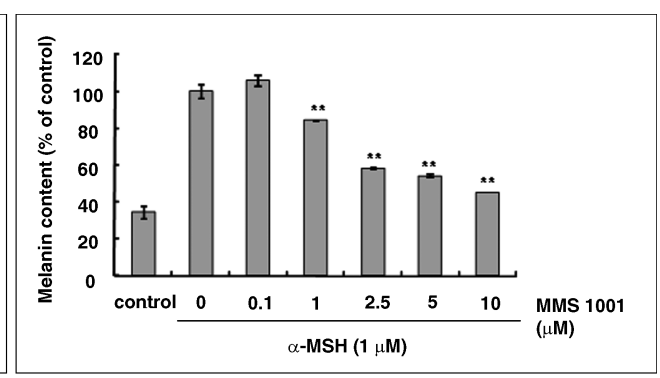
To determine whether the inhibitory activity of MMS 1001 was related to the expression levels of MITF and tyrosinase, Western blot analysis was performed with α-MSH-stimulated B16F10 cells, which were treated with MMS 1001 (5 μM). The protein level of MITF was down-regulated by MMS 1001 at 12 h and 24 h (Fig. 3A). Subsequently, the protein level of tyrosinase was also decreased at 24 h and 48 h (Fig. 3B).

2.4. MMS 1001 induces the phosphorylation of the ERK pathway

It was reported that the activation of the ERK pathway inhibited melanogenesis (Kim et al. 2003). Therefore, it was examined whether MMS 1001-induced hypopigmentation was related to the activation of the ERK signaling pathway. B16F10 cells were treated with MMS 1001 (5 μM) for 0–360 min, and then the whole cell lysate was subjected to Western blot analysis. As shown in Fig. 4, phosphorylation of MEK and ERK was clearly induced by MMS 1001. These results suggest that the activation of the ERK signaling pathway could be involved in the hypopigmentary effect of MMS 1001.



(A)



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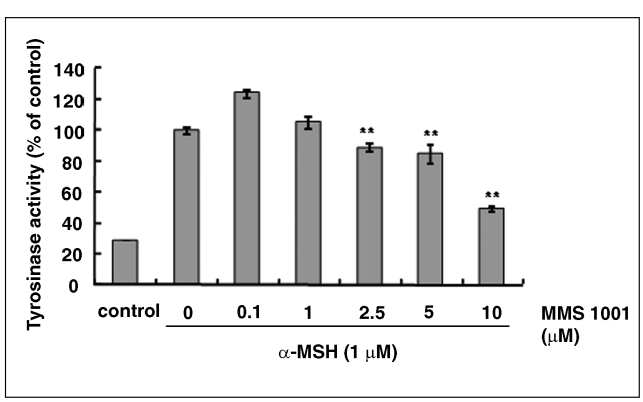
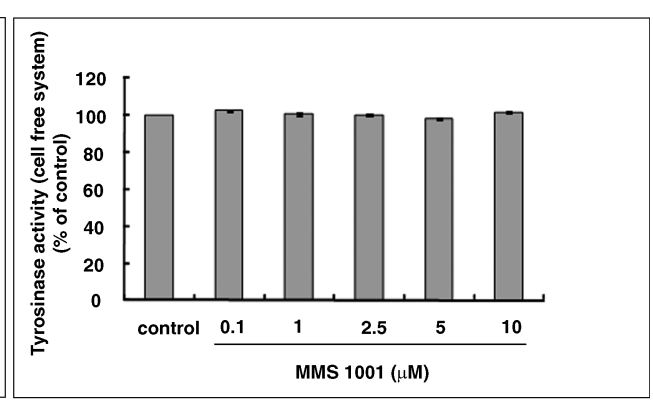


(C)



(D)

Fig. 2: Effects of MMS 1001 on melanin synthesis and tyrosinase activity. (A) B16F10 cells were treated with various concentrations of MMS 1001 (0, 0.1, 1, 2.5, 5, 10 μM) for 24 h. Cell viability was determined by the crystal violet assay as described in Materials and Methods. (B) B16F10 cells were cultured with various concentrations of MMS 1001 (0, 0.1, 1, 2.5, 5, 10 μM) for 3 days. Melanin contents were assayed as described in Materials and Methods. (C) Tyrosinase activity was determined using L-DOPA as a substrate in B16F10 cells, as described in Materials and Methods. (D) Tyrosinase activity was measured using mushroom tyrosinase in a cell free system, as described in Materials and Method. The data were averages of triplicate wells and represented as the mean ± S.D. $^{**}P < 0.01$ compared with α-MSH-treated control. The experiment was repeated independently at least two times

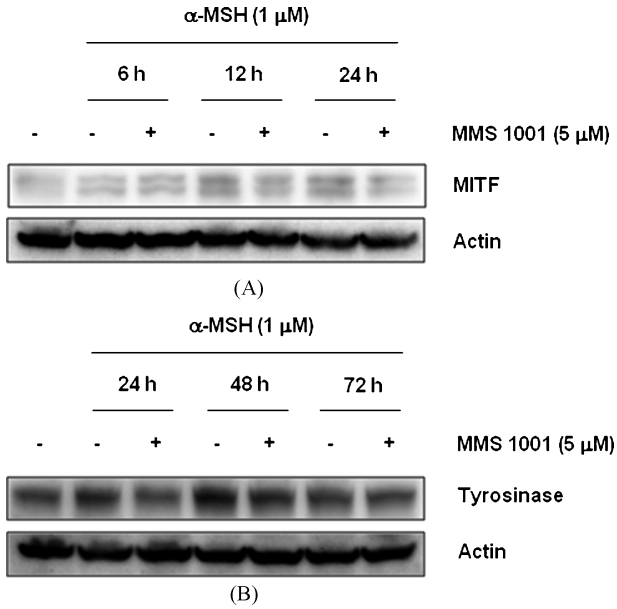


Fig. 3: Effects of MMS 1001 on the expression of melanogenic proteins. B16F10 cells were treated with MMS 1001 (5 μM) in the presence of α-MSH (1 μM) for the indicated periods of time. Thereafter, the expression levels of MITF (A) and tyrosinase (B) were measured by Western blot analysis. Equal amounts of protein loading were confirmed by actin expression

2.5. Effects of a specific MEK inhibitor, PD98059, on melanogenesis

Since MMS 1001 activated the ERK signaling pathway, it was investigated using PD98059, a selective inhibitor of MEK, whether the ERK signaling pathway was related to melanogenesis. PD98059 treatment significantly restored MMS 1001-induced melanin decrease in the presence of α-MSH (Fig. 5A). In addition, the effect of PD98059 on the expression of tyrosinase was examined. As shown in Fig. 5B, α-MSH-treatment increased the expression of tyrosinase. However, MMS 1001-treated cells decreased the expression of tyrosinase induced by α-MSH. Furthermore, PD98059 recovered the down-regulation of tyrosinase by MMS 1001. These results suggest that the phosphorylation of ERK by MMS 1001 contributes to the reduced melanin synthesis via decreased tyrosinase protein expression.

3. Discussion

Although melanin plays an important protective role against UV radiation, accumulation of melanin pigment could cause hyperpigmentary skin disorders. Thus, hypopigmentary effects of MMS 1001 were studied in B16F10 cells for the treatment of various hyperpigmentary disorders and cosmetic applications.

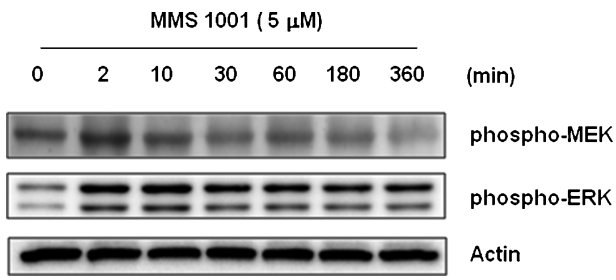


Fig. 4: Effects of MMS 1001 on the phosphorylation of MEK and ERK. B16F10 cells were exposed to 5 μM MMS 1001 for the indicated time (0–360 min). After treatment, the phosphorylation of MEK and ERK was examined by Western blot analysis. Equal amounts of protein loading were confirmed by actin expression

Melanin contents are decreased by MMS 1001 in α-MSH-stimulated B16F10 cells (Fig. 2A). Based on the results, Western blot analyzed the mechanisms behind MMS 1001 regulating the molecular signaling that is involved in melanogenesis.

MITF plays a key role in melanogenesis, as the major transcriptional activator of the tyrosinase expression (Eves et al. 2006; Hennessy et al. 2005). Thus, α-MSH stimulated B16F10 cells, which demonstrated increased levels of MITF expression. However, MMS 1001 treatment reduced the MITF protein expression, induced by α-MSH (Fig. 3A).

Tyrosinase is a rate-limiting enzyme in melanogenesis (Sulaimon and Kitchell 2003). In several studies, many skin-whitening agents were reported to decrease melanogenesis, by directly inhibiting tyrosinase activity (Kim et al. 2005). However, MMS 1001 did not show direct inhibitory effect. Instead, MMS 1001 decreased intracellular tyrosinase activity in a dose-dependent manner (Fig. 2C). Furthermore, MMS 1001 treatment decreased tyrosinase expression after α-MSH treatment (Fig. 3B). These data indicated that MMS 1001 might regulate the signal transduction pathways involved in tyrosinase expression.

The Akt pathway and CREB phosphorylation are involved in the tyrosinase expression (Das et al. 2005). Therefore, it was examined, whether MMS 1001 affected phosphorylation of Akt and/or CREB. The results showed that MMS 1001 did not influence phosphorylation of Akt and CREB (data not shown).

It has been reported that the activation of ERK leads to phosphorylation of MITF at Ser73, which is responsible for MITF degradation (Kono et al. 2006; Menon and Sudheer 2007). In the present study, the phosphorylation of ERK and MEK, an upstream kinase of ERK was examined by Western blot analysis. The results showed that phosphorylation of ERK and MEK were induced by MMS 1001 (Fig. 4). The specific MEK/ERK signaling inhibitor, PD98059, blocked the hypopigmentary effect that is induced by MMS 1001, which abrogated the down-regulation of tyrosinase expression by MMS 1001 (Fig. 5B), indicating that MMS 1001-induced ERK phosphorylation leads to the decreased tyrosinase expression.

In conclusion, MMS 1001 inhibits melanin synthesis and tyrosinase activity in α-MSH-stimulated B16F10 cells. Furthermore, MMS 1001 suppresses the expression of MITF and tyrosinase via phosphorylation of MEK and ERK. These results suggest that MMS 1001 may provide a beneficial effect for inhibiting melanin synthesis and could be useful as a new skin-whitening agent.

4. Experimental

4.1. Materials

MMS 1001 was newly synthesized and stored at −20 °C as stock solution (50 mM). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Dulbecco's modified Eagle's medium (DMEM), and trypsin-EDTA were purchased from WelGENE (Daegu, South Korea). 3,4-Dihydroxy-L-phenylalanine (L-DOPA) and mushroom tyrosinase were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). PD98059 was purchased from Calbiochem (La Jolla, CA, USA), and antibodies specific for phospho-ERK1/2 (Thr202/Tyr204, #9101S) and phospho-MEK (MAPK/ERK kinase) (Ser217/221, #9121) were from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific for tyrosinase (C-19) and actin (I-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and microphthalmia Ab-1 (C5, MS-771-P0) was obtained from NeoMarkers (Fremont, CA, USA). Secondary antibodies, which are specific for anti-goat IgG (PI-9500), anti-mouse IgG (PI-2000), and anti-rabbit IgG (PI-1000), were purchased from Vector Laboratories (Burlingame, CA, USA).

4.2. Cell culture

B16F10 murine melanoma cells were obtained from Korean Cell Line Bank (Seoul, Korea). The cells were maintained in DMEM supplemented with

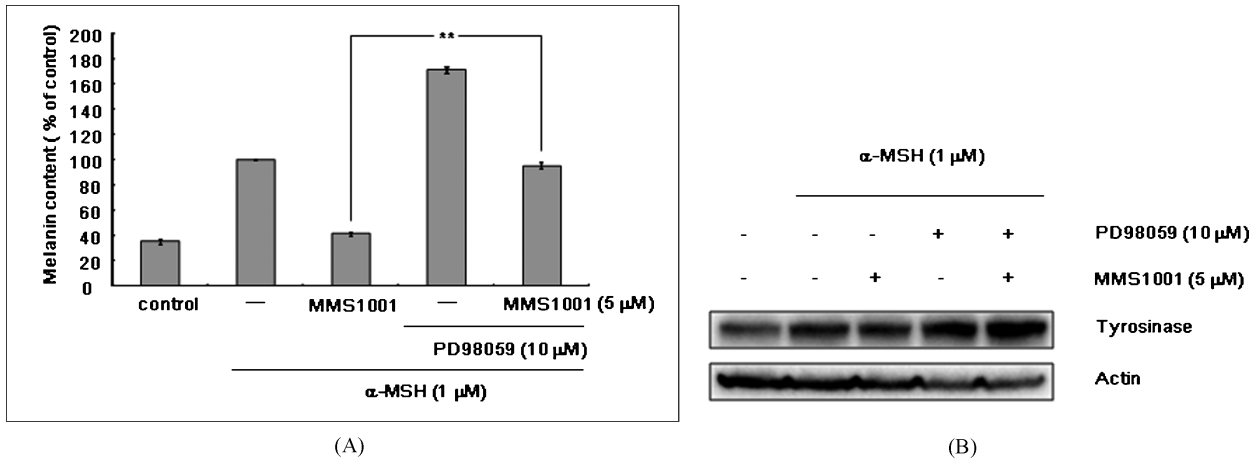


Fig. 5: Effects of PD98059 on melanogenesis and tyrosinase expression. B16F10 cells were exposed to 5 μM MMS 1001 and 10 μM PD98059 in the presence of 1 μM α-MSH. (A) After 3 days, Melanin contents were measured as described in Materials and Methods. The data were averages of triplicate wells and represented as the mean ± S.D. $^{**}P < 0.01$ compared with the MMS 1001-treated group. The experiment was repeated independently at least two times. (B) After 24 h, the expression level of tyrosinase was examined by Western blot analysis. Equal amounts of protein loading were confirmed by actin expression

10% (v/v) FBS, 50 μg/ml of streptomycin, and 50 μg/ml of penicillin in 5% CO_2 at 37 °C

4.3. Cell viability assay

Cell viability was determined using a crystal violet assay. After incubating cells with MMS 1001 for 24 h, the media were removed, and the cells were stained with 0.1% crystal violet in 10% ethanol for 5 min, at room temperature. The cells were then rinsed 4 times with distilled water, and the crystal violet retained by adherent cells was extracted with 95% ethanol. Absorbance was determined at 590 nm, using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).

4.4. Measurement of melanin content

Extracellular melanin release was measured as described previously (Smalley and Eisen 2000), with a slight modification. Briefly, B16F10 cells were incubated at a density of 5×10^4 cells in 6-well plates overnight. α-MSH (1 μM) was then added and cells were treated with increasing concentrations of MMS 1001 in phenol red-free DMEM for 3 days. Two hundred μl aliquots of media were then placed in 96-well plates and the optical density (OD) of each culture well was measured, using an ELISA reader at 400 nm. The cells were then counted using a hemocytometer. Melanin production was expressed as the percentage of α-MSH-treated controls.

4.5. Tyrosinase activity

Tyrosinase activity was assayed as DOPA oxidase activity. B16F10 cells were incubated at a density of $5x10^4$ cells in 6-well plates, and incubated with MMS 1001 in DMEM for 3 days. Cells were washed with PBS and lysed with lysis buffer (0.1 M phosphate buffer [pH 6.8] containing 1% Triton X-100). Cells were then disrupted by freeze-thawing, and lysates were clarified by centrifugation at 13,000 rpm for 30 min. After quantifying protein content using a protein assay kit (Bio-Rad, Hercules, CA, USA), the cell lysates were adjusted to the same amount of protein with lysis buffer, 90 μl of each lysate was pipetted into the wells of a 96-well plate, and 10 μl of 10 mM L-DOPA was added. Control wells contained 90 μl of lysis buffer and 10 μl of 10 mM L-DOPA. After incubation at 37 °C for 20 min, DOPAchrome formation was monitored by measuring absorbance at 475 nm, using an ELISA reader.
A cell-free assay system was used to determine the direct effect of MMS 1001 on tyrosinase activity. Seventy μl of phosphate buffer containing MMS 1001 was mixed with 20 μl of 53.7 units/ml mushroom tyrosinase, after which 10 μl L-DOPA was then added. Following incubation at 37 °C for 20 min, the absorbance was measured at 475 nm.

4.6. Western blot analysis

Cells were lysed in cell lysis buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitors [Complete^{TM}; Roche, Mannheim, Germany], 1 mM Na_3VO_4, 50 mM NaF and 10 mM EDTA). Twenty μg of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride (PVDF) membranes, which were then blocked with 5% dried milk in Tris-buffered saline, which contained 0.5% Tween 20. Blots were then incubated with the appropriate primary antibodies at a dilution of 1:1000, and further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Thermo Scientific Inc., Bremen, Germany). The images of the blotted membranes were obtained, using a LAS-1000 lumino-image analyzer (Fuji Film, Tokyo, Japan).

4.7. Statistics

The statistical significance of the differences between groups was assessed by analysis of variance (ANOVA), followed by the Student's t-test. *P* values of < 0.01 were considered significant.

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