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Trichodimerol and sorbicillin induced apoptosis of HL-60 cells is mediated by reactive oxygen species

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In this study, two secondary metabolite compounds, trichodimerol and sorbicillin were isolated from the mycelial mass of the marine fungus *Trichothecium* sp.. It was found that trichodimerol and sorbicillin exhibited strong cytotoxic activity with IC_{50} values from 6.55 μM to 28.55 μM on three cancer cell lines, HL-60, U937 and T47D. Then HL-60 cells were employed for apoptotic assay. The two compounds could significantly increase the levels of activated caspase-3/7 in a dose-dependent manner and remarkably increase sub-G_1 fraction in the cell cycle using flow cytometry, indicating that trichodimerol and sorbicillin potently induced apoptosis in HL-60 cells. Trichodimerol or sorbicillin induced ROS levels also showed dose-dependent increases in HL-60 cells as measured by 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), while trichodimerol or sorbicillin induced apoptosis was effectively blocked by the ROS inhibitor *N*-acetyl-L-cysteine (NAC). Western blot results showed that trichodimerol or sorbicillin could increase phosphorylated p38 levels and decrease ERK and phosphorylated ERK levels in a concentration-dependent manner. Our findings suggest that the pro-apoptosis effects of trichodimerol and sorbicillin were mediated by ROS, while activation of p38 and inhibition of ERK may be involved in these effects.

1. Introduction

Marine microorganisms have attracted more and more attention as promising sources for novel antitumor agents (Williams 2009). As an important component of marine microorganisms, marine derived fungi also represent high potential for the discovery of new cytotoxic metabolites (Boris et al. 2013). This has been proven in our previous investigations on several marine fungi (Huang et al. 2006; Sun et al. 2006; Yao et al. 2009). The fungus *Trichothecium* sp. was isolated from the roots of mangrove forest collected in the tidal flat of South China Sea, and then be cultivated and extracted. The primary bioassay of the acetone extract from the mycelial mass showed strong inhibition against *Alternaria solani*. Repeated column chromatography of the acetone extract afforded two biosynthetically related secondary metabolites, trichodimerol and sorbicillin.

Recently more than 30 monomeric and dimeric sorbicillin-related natural products have been discovered in only a few fungal genera (*Trichoderma*, *Verticillium*, and *Penicillium*) (Maskey et al. 2005). These secondary metabolite compounds show promising biological activities such as cytotoxicity (Liu et al. 2005) and inhibition of the biosynthesis of β-1,6-glucan (Kontani et al. 1994). The most intriguing biological activity was reported for trichodimerol, which was firstly isolated from a fungus of the genus *Trichoderma* (Andrade et al. 1992). It has been demonstrated that trichodimerol could inhibit lipopolysaccharide (LPS)-induced production of tumor necrosis factor α (TNF-α) in cultured human monocytes and, therefore, to have potential for the treatment of septic shock (Warr et al. 1996). Although trichodimerol and sorbicillin have attracted more and more attention, few detailed studies on their antitumor activities are reported and related mechanisms of action remain unclear.

Inducing cancer cell apoptosis has become a new possibility for tumor treatment (Schulze-Bergkamen and Krammer 2004). Reactive oxygen species (ROS) act as important signaling molecules in cancer cell apoptotic processes (Circu and Aw 2010; Raj et al. 2011). Recent reports suggest that mitogen-activated protein kinases (MAPK), including p38 MAPK, ERK, and JNK, play an important role in ROS mediated apoptosis (Ravindran et al. 2011).

Here we report the cytotoxic activity of trichodimerol and sorbicillin (Fig. 1) on three cancer cell lines, HL-60, U937 and T47D and their pro-apoptotic activity against HL-60. The ROS-related mechanisms underlying apoptosis of HL-60 cells induced by trichodimerol and sorbicillin were further investigated.

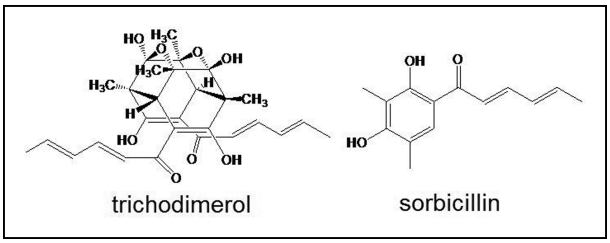

Fig. 1: The structure of trichodimerol and sorbicillin.

2. Investigations and results

2.1. Cytotoxicity of trichodimerol and sorbicillin in three cancer cell lines

Three cancer cell lines, HL-60, U937 and T47D were treated with various concentrations of trichodimerol or sorbicillin for 24 h with vincristine as positive control. The cytotoxic activities were then evaluated by SRB assay to determine IC_{50} values. As shown in the Table, both trichodimerol and sorbicillin displayed significant cytotoxic activities against three cancer cell lines with IC_{50} values between 6.55 μM and 28.55 μM.

2.2. Ttrichodimerol and sorbicillin induce apoptosis in HL-60 cells

The ability of trichodimerol and sorbicillin to induce apoptosis in HL-60 cells was assessed by measuring the levels of activated caspase-3/7 with a Caspase-Glo kit. It was found that after 24 h incubation both trichodimerol and sorbicillin could significantly increase the levels of activated caspase-3/7 in a concentration-dependent manner with highest levels at 20 μM (Fig. 2A; trichodimerol, about 6.5-fold above control, $P < 0.001$; sorbicillin, about 5.7-fold above control, $P < 0.001$), indicating potent pro-apoptotic effects of trichodimerol and sorbicillin. The induction of apoptosis was confirmed by determination of sub-G_1 fractions using a flow cytometric approach (Fig. 2B). After treatment with 20 μM trichodimerol or sorbicillin for 24 h, cells were harvested and assessed by flow cytometry. The significantly increased sub-G_1 peak of apoptotic cells in the cell cycle indicated that trichodimerol and sorbicillin potently induced apoptosis in HL-60 cells.

2.3. Trichodimerol and sorbicillin increase ROS production in HL-60 cells

The compounds were assessed for their ability to generate ROS in HL-60 cells using the fluorescent probe 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA). HL-60 cells were treated with various concentrations of trichodimerol or sorbicillin for 2 h. Cells were then harvested, stained with DCFH-DA, and processed for flow cytometry analysis. Concentration-dependent ROS induction was observed with highest ROS levels at 20 μM for trichodimerol and sorbicillin (Fig. 2C; trichodimerol, about 3.6-fold above control, $P < 0.001$; sorbicillin, about 3.3-fold above control, $P < 0.001$).

Table: The results of cytotoxic activity test[a]

Compound	HL-60	U937	T-47D
Trichodimerol	6.55	10.74	20.18
Sorbicillin	10.96	11.35	28.55
Vincristin	3.42	2.95	15.60

[a]IC_{50} values, μM, cells were treated for 24 h and determined by the SRB method. Data represent mean values of three independent experiments.

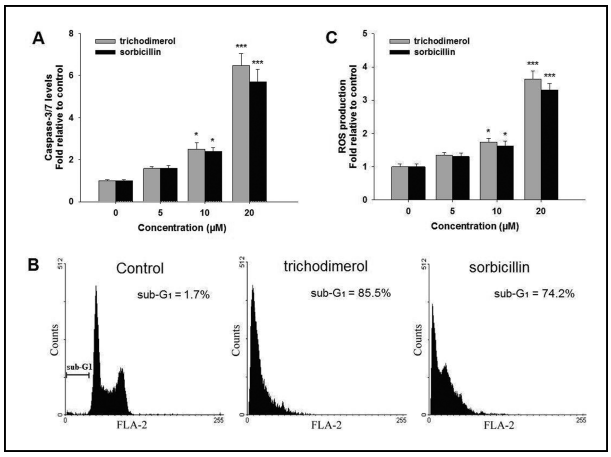

Fig. 2: (A) Induction of apoptosis by trichodimerol or sorbicillin. HL-60 cells were treated with various concentrations of trichodimerol or sorbicillin for 24 h. Cells were then processed to measure levels of active caspase-3/7 (apoptotic markers) using the Caspase-Glo kit. Results are presented as mean ± SD from 3 independent experiments and normalized to untreated cells. (*$P < 0.05$, ***$P < 0.001$ versus control). (B) Induction of sub-G_1 fractions by trichodimerol or sorbicillin in HL-60 cells. After treatment with trichodimerol or sorbicillin for 24 h at the concentration of 20 μM, cells were harvested by trypsinization, and assessed by flow cytometry. Data were analyzed using ModFit LT version 4.0 software. (C) Induction of ROS in HL-60 cells by trichodimerol or sorbicillin. Cells were treated with various concentrations of trichodimerol or sorbicillin for 2 h. Cells were then harvested, stained with DCFH-DA, and processed for flow cytometry analysis to detect ROS levels. Results are presented as mean ± SD from 3 independent experiments and normalized to untreated cells. (*$P < 0.05$, ***$P < 0.001$ versus control).

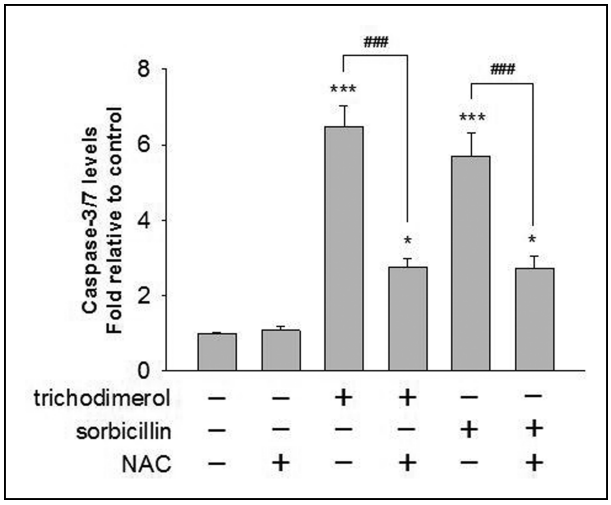

Fig. 3: NAC inhibited apoptosis induced by trichodimerol or sorbicillin. HL-60 cells were treated with 20 μM trichodimerol or sorbicillin for 24 h, with and without 2 h pretreatment with 10 mM NAC. Cells were then processed to measure levels of active caspase-3/7 using the Caspase-Glo kit. Results are presented as mean ± SD from 3 independent experiments and normalized to untreated cells. (*$P < 0.05$, ***$P < 0.001$ versus control; ###$P < 0.001$ with NAC pretreatment versus without NAC pretreatment as indicated).

2.4. ROS inhibitor NAC attenuated apoptosis induced by trichodimerol and sorbicillin

To test whether the cell apoptosis observed was mediated *via* ROS production, we pretreated cells in the presence and absence of the ROS scavenger *N*-acetyl-L-cystein (NAC) and assessed cell apoptosis by measuring the levels of activated caspase-3/7 with a Caspase-Glo kit. When 10 mM NAC was added to cells before treatment with trichodimerol or sorbicillin, a remarkable reduction in caspase-3/7 activity induced by trichodimerol or sorbicillin was observed (Fig. 3; trichodimerol, 42%, $P < 0.001$; sorbicillin, 48%, $P < 0.001$).

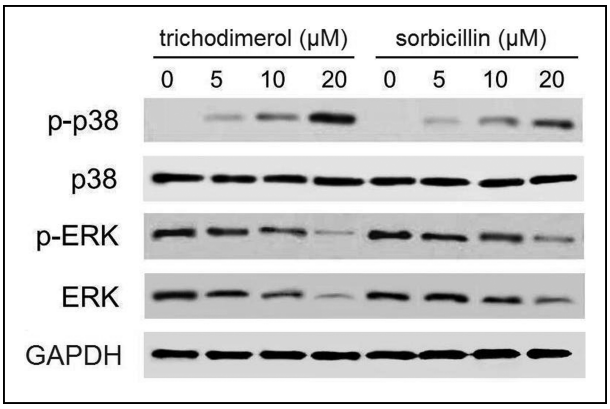

Fig. 4: Activation of p38 and inhibition of ERK induced by trichodimerol or sorbicillin. HL-60 cells were treated with various concentrations of trichodimerol or sorbicillin. Expression of proteins was analyzed by Western blot analysis, and GAPDH was used as a loading control.

2.5. Trichodimerol and sorbicillin activate p38 MAPK and inhibit ERK in HL-60 cells

Research suggested that the production of ROS in cells closely affect MAPK pathways, including p38 MAPK, ERK, and JNK (Kim et al. 2006), which play important roles in cell apoptosis. Thus we determined the protein levels of p38, phosphorylated p38, ERK, and phosphorylated ERK, in treated HL-60 cells 6 h after treatment with trichodimerol or sorbicillin. It was found that trichodimerol or sorbicillin could increase phosphorylated p38 levels in a concentration-dependent manner, but no significant changes in p38 levels were observed. In contrast, both ERK and phosphorylated ERK levels were decreased in a concentration-dependent manner after treatment with trichodimerol or sorbicillin (Fig. 4).

3. Discussion

Apoptosis induced by anticancer agents has been shown to correlate with their therapeutic effect. Inducing cancer cell apoptosis has become a new therapeutic strategy in tumor treatment. Recently researchers are making efforts to find novel small molecules that show potent apoptosis induction. Marine derived fungi have been proven to be a rich source for the discovery of new cytotoxic and pro-apoptotic compounds. In this regard, the cytotoxic and pro-apoptotic effects of two marine derived secondary metabolites, trichodimerol and sorbicillin, were examined. The results demonstrated that trichodimerol and sorbicillin exhibited strong cytotoxic effects against three cancer cell lines, HL-60, U937 and T47D. Their cytotoxicity in HL-60 cells was associated with the ability of apoptosis induction, and we further attempted to investigate the mechanisms underlying trichodimerol and sorbicillin induced apoptosis.

ROS play a central role in apoptosis of many kinds of cells. ROS leakage from mitochondria as a consequence of damage incurred during the process of apoptosis is a primary trigger of further apoptosis in mammalian cells. Meanwhile exogenous ROS, such as moderate levels of H_2O_2, are able to induce apoptosis in different types of cells (Trachootham et al. 2009). More importantly, several studies have suggested that endogenously produced ROS, commonly triggered by anti-tumor agents, play a key role in the apoptosis of tumor cells (Kim et al. 2011; Yang et al. 2014). In this case, the role of intracellular ROS formation in trichodimerol and sorbicillin induced apoptosis of HL-60 cells were examined. After treatment with trichodimerol or sorbicillin, significantly increased ROS levels were observed in dose-dependent manners. Moreover, while pretreating cells with the ROS inhibitor NAC, cell apoptosis was remarkably attenuated. These results suggested that trichodimerol and sorbicillin could increase ROS levels in HL-60 cells, and the apoptosis induced by trichodimerol or sorbicillin was partly mediated by ROS.

Several cellular signaling pathways may participate in ROS-mediated cell apoptosis; among which, the MAPK pathway, including p38 MAPK, ERK, and JNK, plays an important role. Previous reports have suggested that p38 MAPK and ERK act oppositely in cell apoptosis (Wada and Penninger 2004). Phosphorylation of pro-apoptotic Bax protein mediated by p38 MAPK leads to initiation of mitochondrion-dependent apoptosis in cells treated with various stimulants (Wagner and Nebreda 2009). By contrast, activated ERKs phosphorylate Bcl-2 and Mcl-1, two members of the anti-apoptotic Bcl-2 family, lead to inhibition of apoptosis (Siddiqa et al. 2008; Konopleva et al. 2012). In this study, treatment of HL-60 cells with trichodimerol or sorbicillin led to significant changes in the levels of activated p38 MAPK and ERK. They increased the levels of phosphorylated p38 MAPK and decreased the levels of phosphorylated ERK, consistent with previous research where activation of p38 and inhibition of ERK was observed in tumor cell apoptosis induced by different reagents (Iwama et al. 2001; Tan et al. 2008).

Taken together, our results clearly show that trichodimerol and sorbicillin could exhibit strong cytotoxic activity on three cancer cell lines, and remarkably induce apoptosis in HL-60 cells. Their pro-apoptotic effects were mediated by ROS, while activation of p38 and inhibition of ERK may be involved in these effects. Our findings suggest that marine derived monomeric and dimeric sorbicillin-related natural products may have a potential in the development of new chemotherapeutic agents. However, the detailed mechanisms of tumor cell apoptosis induced by trichodimerol and sorbicillin require further investigation.

4. Experimental

4.1. Materials and reagents

Trichodimerol and sorbicillin (purity > 98%, analyzed by HPLC) were separated and purified as described below in 4.2 and 4.3. p38, phosphorylated p38, ERK, phosphorylated ERK and relative secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), *N*-acetyl-L-cysteine (NAC), sulphorhodamine B (SRB) and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytic grade.

4.2. Fungus material, cultivation and extraction

The fungus strain was isolated from roots of mangroves collected in the South China Sea, in March of 2005, and identified as *Trichothecinm* sp. by Prof. Li Tian (the First Institute of Oceanography, State Oceanic Administration of China). A voucher specimen (No.CAAN053032) is deposited in the key Laboratory of Marine Biology of State Oceanography Administration of China. The strain was cultured on seed medium at 25 °C on a rotary shaker for 12 days. The culture medium contained potato decoction 100 mL, corn decoction 10 mL, dextrose 7 g, aerated seawater 600 mL, and distilled water 300 mL. On the thirteenth day, the fermentation broth including cells was harvested and then centrifuged to separate the mycelial mass from the aqueous layer. The mycelial mass was exhaustively extracted with acetone (five times) and the resulting solution was evaporated in vacuum to get a crude extract (92 g).

4.3. Isolation and characterization of trichodimerol and sorbicillin

The extract was subjected to gradient elution in petroleum ether/acetone (100:1 to 1:1) on a silica gel column to give a series of fractions. The third fraction (20:1) was chromatographed over Sephadex LH-20 column (Pharmadex, $CHCl_3$/MeOH 1:1) and further purified on reversed-phase prep-HPLC (Shimazu, ODS-C_{18}, MeOH/H_2O 75:25), to give trichodimerol (160 mg). The fifth fraction (10:1) was chromatographed over a Sephadex LH-20 column ($CHCl_3$/MeOH 1:1) and further purified on reversed-phase silica gel (Chromatorex C_{18}, MeOH/H_2O 7:3), to afford sorbicillin (235 mg).

Their structures were established on the basis of spectral data and confirmed by comparison with literature reports.

Trichodimerol (Andrade et al. 1992): yellow needle-like crystal (MeOH); [α]-186°(c 0.1, MeOH), ESI-MS m/z 497[M + H]$^+$. ^{1}H-NMR (600 MHz, DMSO-d_6) δ:16.46 (1H, s, 5-OH), 7.21(1H, dd, J = 14.7 Hz, 10.8 Hz, H-9), 6.74 (1H, s, 3-OH), 6.43 (2H, m, H-8, 10), 6.27 (1H, dq, J = 14.7 Hz, 6.6 Hz, H-11), 3.00 (1H, s, H-1), 1.86 (3H, d, J = 6.6 Hz, H-12), 1.27 (3H, s, H-14), 1.26 (3H, s, H-13); ^{13}C-NMR (150 MHz, DMSO-d_6)δ: 200.3(C-7), 174.6(C-5), 142.5(C-9), 140.1(C-11), 131.1(C-10), 119.4 (C-8), 104.2(C-3), 103.5(C-6), 78.7(C-2), 59.4(C-4), 56.5(C-1), 21.5 (C-13), 19.5(C-14), 18.7(C-12).

Sorbicillin (Trifonov et al. 1982): orange needle-like crystal (MeOH); ^{1}HNMR (300 MHz, CD_3OD)δ: 1.89 (3H, d, J = 6.6 Hz, H-12), 2.07(3H, s, 3-CH_3), 2.17(3H, s, 5-CH_3), 6.28 (1H, dq, J = 15.0 Hz, 6.6 Hz, H-11), 6.40 (1H, m, H-10), 7.10(1H, d, J = 15.0 Hz, H-8), 7.40(1H, dd, J = 15.0 Hz, 10.7 Hz, H-9), 7.54(1H, s, H-6); ^{13}C-NMR (75 MHz, CD_3OD)δ: 8.0(3-CH_3), 16.4(5-CH_3), 18.9(C-12), 123.4(C-8), 132.0(C-10), 141.7(C-11), 145.3(C-9), 112.0(C-1), 114.0(C-3), 117.2(C-5), 130.0(C-6), 162.2(C-4), 163.7(C-2), 193.9(C-7).

4.4. Cell culture and cytotoxic activity test

The three cancer cell lines, HL-60, U937 and T47D, were obtained from the American Type Culture Collection (ATCC), and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), in a humidified atmosphere of 5% CO_2 in air, at 37 °C. Cell inhibition was measured by SRB assay (Skehan et al. 1990). Briefly, cell suspensions were plated in 96-well plates at a density of 3×10^5 cells/mL. Then the two test compounds and vincristine solutions at different concentrations were added to each well and further incubated for 24 h. Following drug exposure, the cells were fixed with 12% trichloroacetic acid and the cell layer was stained with 0.4% SRB. The absorbance of SRB solution was measured at 515 nm. All experiments were performed three times. Concentration-response curves were then generated and the IC_{50} values were calculated from the linear portion of log concentration-response curves.

4.5. Apoptosis assay

Apoptosis in HL-60 cells was assessed by measuring the levels of activated caspase-3/7 with a Caspase-Glo kit (Promega, Madison, WI, USA) following the manufacturer's protocol. Cells were seeded at 1×10^3 cells/well in 96-well plates in 60 μL. After 24 h incubation to allow cells to settle and, in some cases, a 2 h pretreatment with NAC, different concentrations of compounds were added and cells were incubated for 24 h before processing. Luminescence was detected using a luminometer plate reader (Luminoskan Ascent, Thermo Electron, Helsinki, Finland).

The induction of apoptosis was confirmed by a flow cytometric approach using propidium iodide (PI) as fluorescent stain (Liu et al. 2007). Briefly, cells were plated in 24-well plates (3×10^5 cells/well), in triplicate, and then treated with trichodimerol or sorbicillin for 24 h at the concentration of 20 μM. Cells were harvested, washed twice with PBS, and the cellular DNA stained with 200 μL PI (50 μg/mL, RNase 1 μg/mL, Triton X-100 0.1%). After incubation at 4 °C for 20 min, the cells were analyzed by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA, USA).

4.6. ROS production measurement

ROS production was examined by flow cytometry using DCFH-DA as described before (Zegura et al. 2004). DCFH-DA can readily diffuse into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within the cells. ROS produced by the cells can oxidize DCFH to the highly fluorescent compound 2',7'-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of ROS produced by the cells. Briefly, the HL-60 cells were incubated with 20 μM DCFH-DA. After 30 min incubation at 37 °C, 5% CO_2 in the dark, DCFH-DA was removed and the cells were treated with trichodimerol or sorbicillin for 2 h. Cells were then harvested and analyzed by flow cytometry on a FACS Calibur flow cytometer.

4.7. Western blot analysis

Cultures of HL-60 cells at a density of 3×10^5 cells/mL were harvested, washed with ice-cold PBS, and suspended in 0.5 mL lysis buffer (10 mM Tris-HCl pH 7.6, 100 mM NaCl, 1.0 mM EDTA and 100 mg/L PMSF) containing protease inhibitor (Complete tablets, Roche Diagnostics). Lysates were centrifuged at 8000 × g for 10 min, and the protein concentrations were determined by bicinchoninic acid (BCA) protein quantified kit (Pierce, Rockford, IL, USA). Cellular total proteins (30 μg) were separated by 12% sodium dodecylsulfate (SDS)-polyacrylamide gels and transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% nonfat milk for 2 h at room temperature, and then incubated for 2 h at room temperature with monoclonal primary antibodies (anti-p38, anti-phospho-p38, anti-ERK and anti-phospho-ERK). The treated membrane was washed with PBS and incubated with peroxidase-conjugated secondary antibody for an additional hour at room temperature. The membranes were then treated with an enhanced chemiluminescence (ECL) and signals were detected by exposing membranes to X-ray films. Relative signal intensity was quantified with a Bio Image Intelligent Quantifier system (Nihon-Bio Image Ltd, Tokyo, Japan). Expression levels of the GAPDH were used for standardization.

4.8. Statistical analysis

SPSS10.0 statistical software was used for data analysis. Results are expressed as mean ± SD. Statistical significance was determined by using the Student's *t*-test. Differences were considered significant when $P < 0.05$.

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