Hypopigmentary Action of Dihydropyranocoumarin D2, a Decursin Derivative, as a MITF-Degrading Agent

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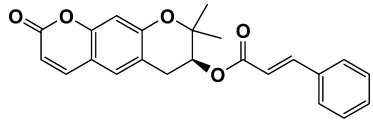
In this study, the decursin derivative dihydropyranocoumarin D2 (**1**) was selected for its effects on melanogenesis using a spontaneously immortalized mouse melanocyte cell line (Mel-Ab). The results showed that **1** effectively inhibited melanin synthesis in a concentration-dependent manner, but that it did not inhibit tyrosinase in a cell-free system. In addition, the changes in ERK, Akt, and microphthalmia-associated transcription factor (MITF) in response to treatment with **1** were assessed. The results revealed that ERK was dramatically up-regulated and MITF was down-regulated in response to treatment with **1**, but that Akt was unchanged. Therefore, the effects of **1** on melanogenesis were examined in the absence or presence of PD98059 (a specific inhibitor of the ERK pathway). PD98059 restored hypopigmentation and the down-regulation of MITF induced by **1**. Finally, MITF down-regulation by **1** was clearly restored by both chloroquine, a lysosomal proteolysis inhibitor, and MG132, a proteasome inhibitor.

Angelica gigas Nakai (Umbelliferae) is a monocarpous biennial or short-lived perennial plant found in forests, in grasslands, and along stream banks in mainland China, Japan, and Korea. The roots of *A. gigas* are used in traditional Chinese medicine. In addition, it has been reported that decursin from *A. gigas* has antitumor and antibacterial activities.[1,2] Furthermore, decursin exhibits protective effects against glutamate-induced neurotoxicity, which suggests that it has antioxidative stress activity.[3]

The production and distribution of melanin pigment is a major determinant of skin and hair color. Tyrosinase is a major melanosomal enzyme involved in melanogenesis that catalyzes the rate-limiting reaction of the melanogenic process.[4,5] Therefore, melanin production depends primarily on the expression and activation of tyrosinase.[6]

It was recently reported that an ethanol extract of *A. gigas* roots effectively inhibited isobutylmethylxanthine-induced melanogenesis in B16 melanoma cells.[7] However, to the best of our knowledge, there have been no studies conducted to evaluate the effects of decursin on melanogenesis, even though it is one of the primary constituents of *A. gigas*. Therefore, decursin derivatives were screened for their inhibitory effects on tyrosinase, and dihydropyranocoumarin D2 (**1**) was selected as a promising lead compound. In the present study, we have investigated the hypopigmentary effects and the possible mechanisms by which **1** inhibits melanogenesis.



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Interestingly, **1** did not inhibit tyrosinase activity in an in vitro assay. Therefore, the major signaling pathways related to melanogenesis were investigated. Microphthalmia-associated transcription factor (MITF) is known to regulate tyrosinase expression and melanin synthesis at the transcription level.[8−10] Furthermore, it has been reported that activation of the extracellular signal-regulated kinase (ERK) pathway leads to MITF degradation and subsequent inhibition of melanin synthesis.[11−13] In the present study, the effects of **1** on melanin synthesis and tyrosinase activity, including the signaling pathways of MITF degradation, were examined in a spontaneously immortalized mouse melanocyte cell line (Mel-Ab).

Results and Discussion

Cytotoxicity of Dihydropyranocoumarin D2 (1) in Mel-Ab Cells. The effects of several decursin derivatives on melanogenesis were tested to identify new skin-whitening agents, and **1** was found to induce a strong hypopigmentary effect. To determine the toxicity of **1**, Mel-Ab cells were treated with increasing doses of this compound (1, 10, 50, 100 μM), after which the cell viability was measured using a crystal violet assay. The results revealed that **1** has no cytotoxic effect on Mel-Ab cells at the concentrations tested (data not shown).

Dihydropyranocoumarin D2 (1) Decreases Melanogenesis in Mel-Ab Cells. To investigate the hypopigmentary effect of **1**, Mel-Ab cells were treated with **1** (1−100 μM) for four days, after which the cells were photographed under a phase-contrast microscope. The results revealed that treatment with **1** strongly reduced melanin synthesis (Figure 1A). In addition, the melanin content was measured. Consistent with the microscopic observations, **1** significantly inhibited melanogenesis in a dose-dependent manner (Figure 1B).

Tyrosinase plays a major role in regulating the rate-limiting step involved in melanin synthesis;[14] therefore, mushroom and plant tyrosinases have been used to identify potent tyrosinase inhibitors in many studies.[15,16] However, some substances inhibit melanogenesis by regulating the transcription of tyrosinase without inhibiting tyrosinase activity directly.[12,13,17] Therefore, the tyrosinase activity of cells treated with **1** was measured. The results showed that the tyrosinase activity decreased as a function of the concentration of **1** (Figure 2A). These findings demonstrated that **1** regulated tyrosinase and subsequently suppressed melanin synthesis in Mel-Ab cells. To determine if **1** inhibits tyrosinase activity directly, its effects on mushroom tyrosinase were measured in a cell-free system. The results revealed that **1** did not affect

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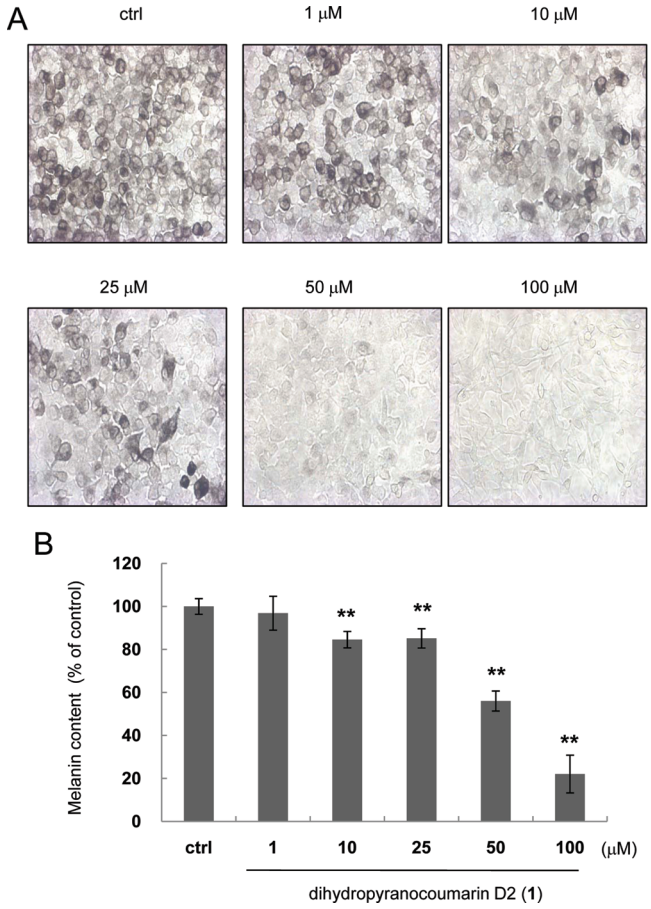

Figure 1. Effects of dihydropyranocoumarin D2 (**1**) on melanogenesis. Mel-Ab cells were cultured with 1−100 μM **1** for four days. (A) Phase-contrast photomicrographs were then taken using a digital video camera. The results shown are an example of three replicate experiments. (B) The melanin content was measured as described in the Experimental Section. The results shown are the average of three replicate experiments ± SD (**$p < 0.01$ when compared to an untreated control).

tyrosinase activity directly (Figure 2B), although it strongly inhibited melanogenesis (Figure 1B). Taken together, these findings demonstrate that **1** inhibits melanogenesis, possibly through the down-regulation of tyrosinase.

Dihydropyranocoumarin D2 (1) Activates ERK and Down-regulates MITF and Tyrosinase. It has been reported

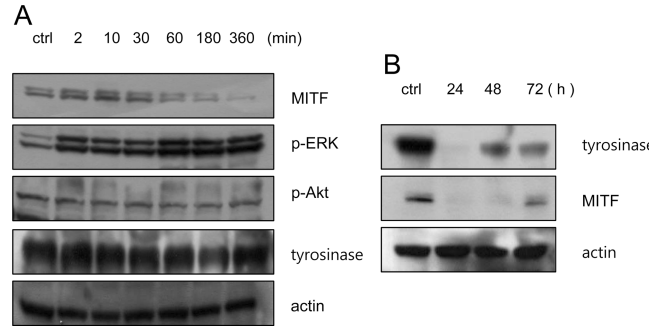

Figure 3. Effects of dihydropyranocoumarin D2 (**1**) on ERK, Akt, and MITF. (A) Following 24 h of serum starvation, Mel-Ab cells were treated with 50 μM **1** for the indicated times. Whole cell lysates were then subjected to western blot analysis using antibodies against phospho-specific ERK (p-ERK), phospho-specific Akt (p-Akt), MITF, and tyrosinase. (B) Cells were cultured with 50 μM **1** for 24−72 h, and the whole cell lysates were then subjected to western blot analysis with antibodies against MITF and tyrosinase. Equal protein loadings were confirmed using anti-actin antibody.

that ERK and Akt activation play important roles in the regulation of melanin synthesis.[18−20] Additionally, ERK activation has been shown to reduce tyrosinase gene transcription.[11] Moreover, we have confirmed that ERK pathway activation is involved in decreased melanin production in human and mouse melanocytes.[12,13,17,21] Thus, **1** was evaluated to determine if it influenced the ERK and Akt signaling pathway. As shown in Figure 3A, **1** activated ERK in a time-dependent manner, while Akt was not affected. Interestingly, ERK activation was not transient, but was sustained for at least 360 min. In accordance with the ERK activation, the MITF level was down-regulated by **1** at 60−360 min. These findings suggested that the reduced levels of melanogenic protein were responsible for decreased melanogenesis. MITF is a major transcription factor responsible for regulation of the expression of melanogenic enzymes such as tyrosinase, TRP-1, and TRP-2.[22−24] Since **1** did not affect the tyrosinase activity directly in a cell-free system, the MITF and tyrosinase protein levels were evaluated following treatment with **1**. The results showed that **1** (50 μM) reduced both the MITF and tyrosinase protein levels (Figure 3B). These findings suggest that the down-regulation of both MITF and tyrosinase is related to **1**-induced hypopigmentary effects.

Effect of the ERK Pathway Inhibition on Melanin Synthesis. Previous studies have shown that ERK is an important kinase involved in melanin synthesis because ERK activation induces the phosphorylation and subsequent degradation of

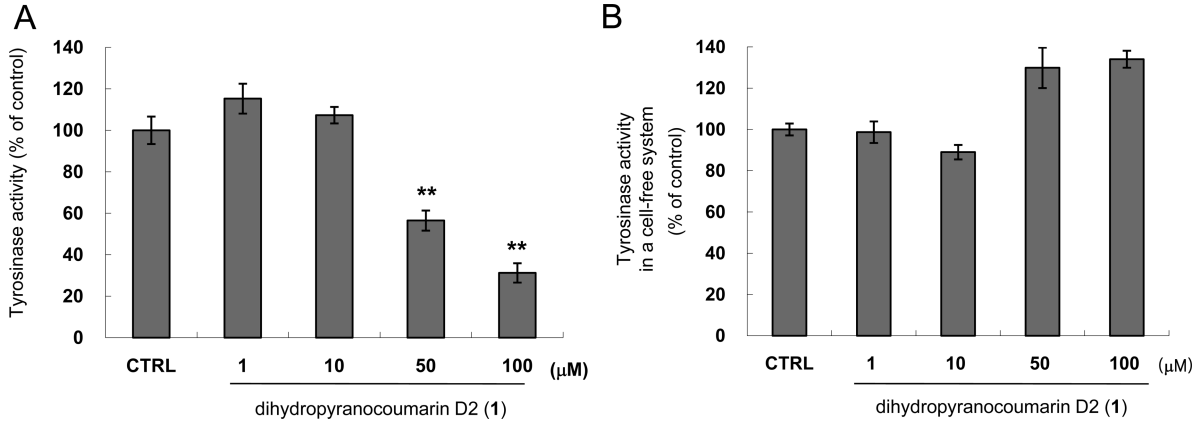

Figure 2. Effects of dihydropyranocoumarin D2 (**1**) on tyrosinase activity. (A) Tyrosinase activity in a cell-free system was measured as described in the text. Mushroom tyrosinase was used for a tyrosinase activity assay in a cell-free system. (B) Mel-Ab cells were cultured with 1−100 μM **1** for four days, and the cellular tyrosinase activity was then measured as described in the text. The results shown are the average of three replicate experiments ± SD (**$p < 0.01$ when compared to an untreated control).

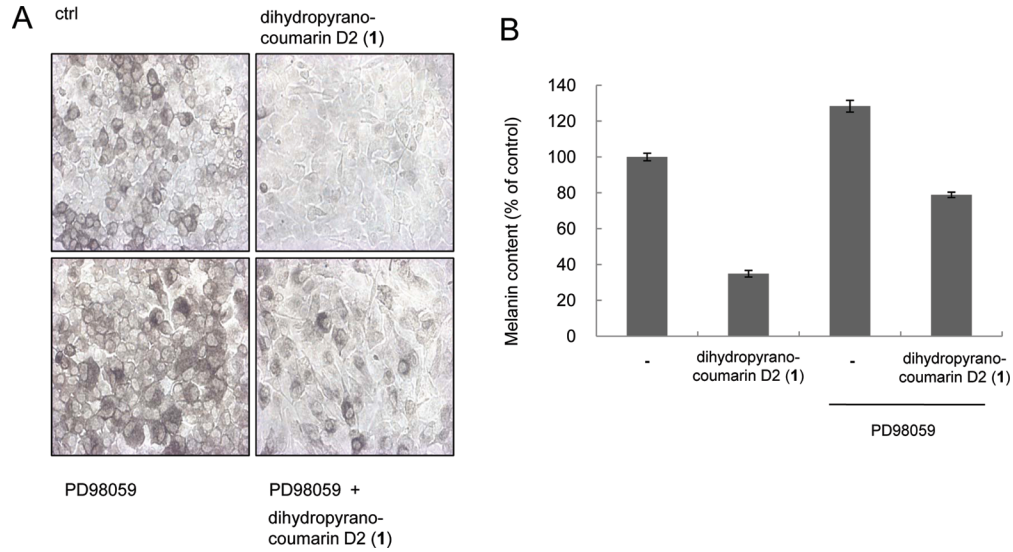

Figure 4. Effects of dihydropyranocoumarin D2 (**1**) on melanogenesis in the presence of PD98059. Mel-Ab cells were pretreated with 20 μM PD98059 for 1 h, after which they were cultured with 50 μM **1** for four days. (A) Phase-contrast photographs were then taken using a digital video camera. (B) The melanin content was measured as described in the Experimental Section. Each determination was made in triplicate, and the data shown represent the mean ± SD.

MITF.[18,19,25] Our group has also reported that sphingosine-1-phosphate immediately activates ERK and causes MITF degradation within 3 h of sphingosine-1-phosphate treatment.[13] In the present study, **1** also decreased the levels of MITF. These results suggest that MITF and tyrosinase down-regulation is mediated by **1**-induced ERK activation. To investigate the relationship between **1**-induced hypopigmentation and the ERK pathway, Mel-Ab cells were cultured for four days in the absence or presence of PD98059 (a specific inhibitor of the ERK pathway), after which they were photographed under a phase-contrast microscope (Figure 4A). The results showed that PD98059 abrogated the hypopigmentary effect induced by **1**. In addition, the melanin synthesis was also measured, and the results were consistent with the microscopic observations (Figure 4B).

Dihydropyranocoumarin D2 (1) Decreased the MITF Protein Levels via Both Lysosomal and Proteosomal Degradation. Previously, we suggested that MITF degradation and subsequent tyrosinase down-regulation were related to ERK activation.[13,26] However, the mechanism by which ERK regulates MITF degradation and expression has not been completely elucidated. It has been reported that MITF can be down-regulated via ubiquitin-dependent proteasome degradation and that MITF degradation results in decreased melanin synthesis.[19,25] Thus, we verified the down-regulation of MITF by **1** to determine if this resulted from MITF degradation. To accomplish this, a proteasome inhibitor, MG132, and/or the lysosomal proteolysis inhibitor chloroquine was used. Specifically, cycloheximide was added to Mel-Ab cells to inhibit protein synthesis, after which the cells were pretreated with MG132 and/or chloroquine for 1 h followed by 3 h of treatment with **1**. The MITF levels were then analyzed by western blot analysis. As shown in Figure 5, the decrease in MITF levels in response to treatment with **1** was restored by pretreatment with MG132 or chloroquine. In addition, co-treatment of MG132 and chloroquine almost completely blocked the MITF down-regulation. On the basis of these results, it may be suggested that **1** leads to MITF degradation via both a ubiquitin-proteasome pathway and a lysosomal proteolysis pathway. In addition, PD98059 blocked **1**-induced ERK activation and MITF degradation. These results suggest that prolonged ERK activation in response to **1** contributes to the down-regulation of melanin synthesis via MITF degradation.

In summary, the results of the present study demonstrate that **1** activates the ERK pathway in a prolonged manner and leads to degradation of MITF and down-regulation of tyrosinase at the protein level, which contributes to the inhibition of melanin synthesis. Thus, the results obtained suggest that compound **1** can be considered as a potential skin-whitening agent by acting as an MITF suppressor.

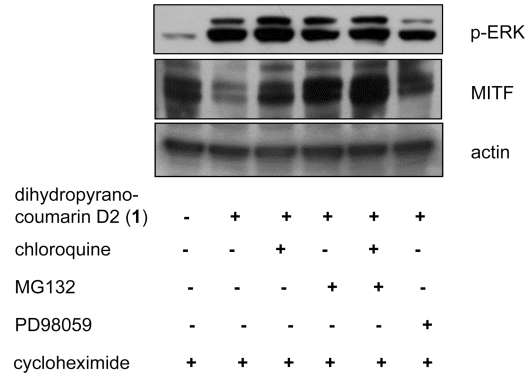

Figure 5. Effect of the inhibition of protein degradation or the ERK pathway on MITF expression. Following serum starvation, Mel-Ab cells were pretreated with 25 μg/mL of cyclohexamide for 1 h as indicated. The cells were also pretreated with 25 μM MG132, 100 μM chloroquine, or 20 μM PD98059 for 1 h, after which they were treated with 50 μM **1** for 3 h. Whole cell lysates were then subjected to western blot analysis using antibodies against phospho-specific ERK or MITF. Equal protein loadings were confirmed using anti-actin antibody.

Experimental Section

General Experimental Procedures. Compound **1** and (*S*)-(+)-decursinol[27] were synthesized at Chungnam National University. Cholera toxin (CT), 12-*O*-tetradecanoylphorbol 13-acetate (TPA), synthetic melanin, L-DOPA, chloroquine, and mushroom tyrosinase were obtained from Sigma (St Louis, MO), MG132 and cycloheximide were obtained from Calbiochem (San Diego, CA), and PD098059 was obtained from Cell Signaling Technology (Beverly, MA). Antibodies recognizing phospho-specific ERK1/2 (Thr202/Tyr204, number 9101S) and phospho-specific Akt (Ser473, #9271S) were purchased from Cell Signaling Technology, microphthalmia Ab-3 (C5+D5, MS-773-P0) was obtained from NeoMarkers (Fremont, CA), and tyrosinase (C-19) and actin (I-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Synthesis of Dihydropyranocoumarin D2 (1). Two drops of *N,N*-dimethyl formamide and thionyl chloride (4.44 mL, 60.9 mM) were added to a solution of *trans*-cinnamic acid (1.81 g, 12.2 mM) in 20 mL of anhydrous benzene. After refluxing for 5 h at 70−80 °C, the reaction solution was cooled to room temperature and concentrated under reduced pressure to obtain cinnamoyl chloride. (*S*)-(+)-Decursinol (2 g, 8.12 mM) was added to a solution of cinnamoyl chloride and pyridine (1.97 mL, 24.4 mM) in anhydrous dichloromethane at room temperature. After stirring for 2 h, the reaction mixture was concentrated under reduced pressure. The resulting residue was then purified by silica gel column chromatography (5−30% EtOAC in *n*-hexane) to obtain (*S*)-(+)-3-phenylacrylic acid 2,2-dimethyl-8-oxo-3,4-dihydro-2*H*,8*H*-pyrano[3,2-*g*]chromen-3-yl ester (**1**) (1.5 g, 49.3%) as a white solid: $[\alpha]_D^{25}$ +42.0 (*c* 3, $CHCl_3$); mp 136−137 °C; ESIMS *m/z* 377 $(M + H)^+$; $R_f = 0.40$ (50% EtOAc/hexane).[28] The purity (>99.5%) of **1** was determined by chiral stationary phase HPLC analysis [HPLC (Shimadzu LC-6AD, Kyoto, Japan), column (Chiralcel OD-H 0.46 cm Φ × 25 cm, Daicel Chemical Industries, Ltd., Co., Osaka, Japan), solvent (2-propanol/*n*-hexane, 1:6), flow rate 1 mL].

Cell Culture of Mel-Ab Cells. The Mel-Ab cell line is a mouse-derived spontaneously immortalized melanocyte cell line that produces large amounts of melanin.[29] Mel-Ab cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 nM TPA, 1 nM CT, 50 μg mL^{-1} streptomycin, and 50 U mL^{-1} penicillin at 37 °C under 5% CO_2.

Cell Viability. Cell viability was determined using a crystal violet assay.[29] Briefly, the cells were incubated with **1** for 24 h, after which the culture medium was removed and the cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature and then rinsed four times with distilled water. The crystal violet retained by the adherent cells was then extracted with 95% ethanol. Finally, the absorbance was determined at 590 nm using an ELISA reader (Tecan, Salzburg, Austria).

Measurement of Melanin Content and Microscopy. The melanin content was measured using a slight modification of the method described by Tsuboi et al.[30] Briefly, the cells were treated with various concentrations of **1** for four days. The cell pellets were then dissolved in 1 mL of 1 N NaOH at 100 °C for 30 min and centrifuged for 20 min at 16000*g*, after which the optical density of the supernatants was measured at 400 nm using an ELISA reader. Before measuring the melanin content, the cells were observed under a phase-contrast microscope (Olympus Optical Co., Tokyo, Japan) and photographed using a CoolSNAP$_{cf}$ digital video camera system (Roper Scientific, Inc., Tucson, AZ) supported by the RS Image software (Roper Scientific).

Tyrosinase Activity. The tyrosinase activity was assessed using a slight modification of the method described by Busca et al.[31] Briefly, Mel-Ab cells were cultured in six-well plates and then incubated with **1** at various concentrations for four days. The cells were then washed with ice-cold phosphate-buffered saline and lysed with 0.1 M phosphate buffer (pH 6.8) containing 1% Triton X-100. The cells were then disrupted by freezing and thawing, after which the lysates were clarified by centrifugation at 10000*g* for 5 min. After quantifying the protein levels and adjusting the concentrations with lysis buffer, 90 μL of each lysate containing the same amount of protein was placed in each well of a 96-well plate, and 10 μL of 10 mM L-DOPA was then added to each well. The control wells contained 90 μL of 0.1 M phosphate buffer (pH 6.8) and 10 μL of 10 mM L-DOPA. The plates were incubated at 37 °C, and the absorbance at 475 nm was measured every 10 min for at least 1 h using an ELISA plate reader. A cell-free assay system was also used to examine the direct effects of **1** on tyrosinase activity. Phosphate buffer (70 μL) containing various concentrations of **1** was combined with 20 μL of 10 μg mL^{-1} mushroom tyrosinase, and 10 μL of 10 mM L-DOPA was then added to each well. After incubation at 37 °C, the absorbance was measured at 475 nm.

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, protease inhibitors (Complete, Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA], after which 10 μg of protein per lane was separated by SDS-polyacrylamide gel electrophoresis. The separated proteins were then blotted onto PVDF membranes, which were subsequently saturated with 5% dried milk in Tris-buffered saline containing 0.4% Tween 20. Next, the blots were incubated with the appropriate primary antibodies at a dilution of 1:1000 and then further incubated with horseradish peroxidase-conjugated secondary antibody. The bound antibodies were detected using an enhanced Chemiluminescence Plus kit (Amersham International, Little Chalfont, UK).

Statistical Analysis. Differences among treatments were assessed by analysis of variance (ANOVA) followed by Dunnett's test. A p value of <0.01 was taken to indicate statistical significance.

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