FULL PAPER

Anti-Inflammatory Activity of a Novel Acetylene Isolated from the Roots of *Angelica tenuissima* Nakai

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Three polyacetylenes, one novel and two known, were isolated from the root of *Angelica tenuissima*. Using ^{1}H- and ^{13}C-NMR, COSY, HMBC, and HMQC, their structures were found to be (3*R*,8*S*)-heptadeca-1-en-4,6-diyne-3,8-diol (**1**), falcarindiol (**2**), and oplopandiol (**3**). Absolute configurations of compound **1** were established using *Mosher*'s esterification. In addition, the polyacetylenes (**1** – **3**) were evaluated for their anti-inflammatory activity. Compounds **1** and **3** showed potent inhibitory activity against lipopolysaccharide-induced nitric oxide (NO) production in RAW267.7 macrophage cells with IC_{50} values of 4.31 and 5.06 μM, respectively. Compound **1** strongly inhibited inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 in a concentration-dependent manner.

Keywords: *Angelica tenuissima*, Polyacetylenes, NO, iNOS, COX-2.

Introduction

Angelica tenuissima Nakai (*Ligusticum tenuissimum* Kitagawa), belongs to the Apiaceae family and is native to Asia. It is used in traditional medicine to treat headache, diarrhea, epilepsy, and rheumatic arthralgia [1]. Many examples of its effects are found in the literature. For example, in a model of *Parkinson*'s disease, a Korean herbal formula containing *A. tenuissima* extract has been shown to have neuroprotective effects that are partially mediated by the enhancement of autophagy [2]. In addition, a volatile extract of this plant has been shown to have antioxidant activity [3]. Chemical studies have indicated that *A. tenuissima* contains phthalides, coumarins, terpenoids, and phenylpropanoids [3][4]. The phthalide, (*Z*)-ligustilide showed anti-inflammatory activity in experiment with lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages [5]. This was the first study to report the isolation of polyacetylenes from *A. tenuissima*. Conjugated acetylenes from other plants have been shown to possess a variety of biological activities, including antitumor, anti-inflammatory, and antimicrobial effects [6]. For example, acetylenes isolated from the root of *Echinacea pallida* have anticancer activity when applied to MIA PaCa-2 and COLO320 cells [7]. Furthermore, oploxynes A and B extracted from *Oplopanax elatus* inhibited the formation of nitric oxide (NO) and prostaglandin E_2 in LPS-treated RAW 267.7 murine macrophage cells [8].

While the roots of *A. tenuissima* have shown to contain many types of compounds, the polyacetylene content of this plant has not been reported. In this study, we have isolated the novel polyacetylene, (3*R*,8*S*)-heptadeca-1-en-4,6-diyne-3,8-diol (**1**) from *A. tenuissima.* Since this compound is structurally similar to the known anti-inflammatory compounds oploxynes A and B, we hypothesized that it may have similar activity. To evaluate the anti-inflammatory effect of the polyacetylenes, we studied their inhibition of NO, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) in LPS-treated RAW 267.7 murine macrophage cells.

Results and Discussion

Elucidation of Chemical Structures

Polytacetylenes (**1** – **3**, *Fig. 1*) were purified using silica gel column chromatography (CC) and preparative HPLC. To determine the chemical structure of **1**, HR-CI-MS and 500-MHz NMR, including ^{1}H, ^{13}C, COSY, HMQC, and HMBC, were performed. The ^{1}H- and ^{13}C-NMR data are described in the *Table*. Absolute configurations of compound **1** were elucidated by *Mosher*'s esterification.

Compound **1** was obtained as a colorless oil. The molecular formula of **1** was found to be $C_{17}H_{26}O_2$ according to HR-CI-MS (obsd. $[M - H]^+$ at *m*/*z* 261.1855). The ^{1}H-NMR spectrum of compound **1** indicated the presence

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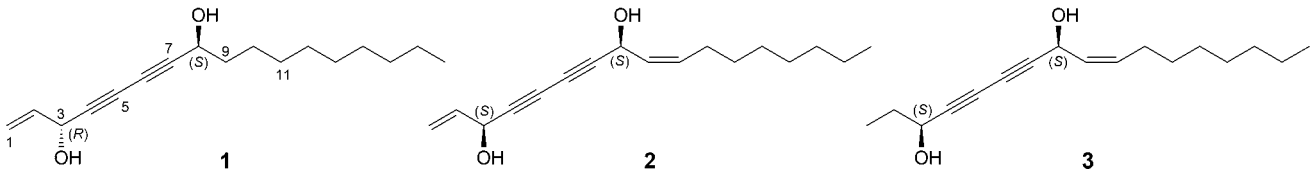

Fig. 1. Chemical structures of compounds **1** – **3**.

Table. ^{1}H- and ^{13}C-NMR spectral data of compound **1**

Position	δ(H) (J in Hz)[a])	δ(C)[b])
1	5.27 (*dt*, *J* = 10.2, 1.0), 5.48 (*ddd*, *J* = 17.0, 1.4, 1.0)	117.5
2	5.95 (*ddd*, *J* = 17.0, 10.2, 5.4)	135.9
3	4.94 (*d*, *J* = 5.3)	63.6
4	–	77.9
5	–	70.4
6	–	68.8
7	–	81.3
8	4.43 (*t*, *J* = 6.6)	63.0
9	1.72 (*dt*, *J* = 14.0, 6.7)	37.6
10[c])	1.40 – 1.46 (*m*)	25.1
11[c])	1.40 – 1.46 (*m*)	29.3
12[c])	1.26 – 1.31 (*m*)	29.4
13[c])	1.25 (*s*)	29.6
14[c])	1.25 (*s*)	29.6
15[c])	1.25 (*s*)	32.0
16[c])	1.25 (*s*)	22.8
17	0.88 (*t*, *J* = 7.0)	14.3

[a]) Recorded at 500 MHz ($CDCl_3$). [b]) Recorded at 125 MHz ($CDCl_3$). [c]) Overlapped signals. All assignments were confirmed by HMQC and HMBC experiments.

of three olefinic H-atoms (5.95, 5.48, and 5.27 ppm), two oxymethylene H-atoms (4.94 and 4.43 ppm), eight aliphatic H-atoms (1.72, 1.40 – 1.46, 1.26 – 1.28, and 1.25 ppm), and one Me (0.88 ppm). The ^{13}C-NMR spectrum showed the presence of two olefinic C-signals (135.9 and 117.5 ppm), a conjugated diyne (81.3, 77.9, 70.4, and 68.8 ppm), two oxymethylene signals (63.6, and 63.0 ppm), eight aliphatic CH_2-signals (37.6, 32.0, 29.6, 29.6, 29.4, 29.3, 25.1, and 22.8 ppm), and a Me-signal (14.3 ppm). Further analysis suggested that the two olefinic H-atom signals at 5.48 ppm (*ddd*, *J* = 17.0, 1.4, 1.0 Hz, 1 H) and 5.27 (*dt*, *J* = 10.2, 1.0 Hz, 1 H) correlated with the ^{13}C-NMR signal at 117.5 ppm (C(1)) in the HMQC spectrum. The ^{1}H,^{1}H-COSY spectrum showed that these two olefinic H-atoms correlated with protons at 5.95 (*ddd*, *J* = 17.0, 10.2, 5.4 Hz, 1 H). The oxymethylene signal at 4.94 ppm (*d*, *J* = 5.3 Hz, 1 H) correlated with the signal at 5.95 ppm. In the HMBC spectrum, the oxymethylene signal at 4.94 ppm correlated with the two olefinic C-signals at 135.9 and 117.5 ppm, and the conjugated diyne signals (81.3, 77.9, 70.4, and 68.8 ppm as shown in *Table*). In addition, the oxymethylene signal at 4.43 (*t*, *J* = 6.6 Hz, 1 H) correlated with the four alkynyl C-signals at 81.3, 77.9, 70.4, and 68.8 ppm and the aliphatic signals at 37.6 and 25.1 ppm (*Fig. 2*). Thus, the compound was identified as heptadeca-1-en-4,6-diyne-3,8-diol.

The absolute configurations of C(3) and C(8) of compound **1** were assigned on the basis of chiral derivatization followed by NMR analysis. Treatment of **1** with (*R*)-MTPACl and DMAP in pyridine yielded an (*S*)-MTPA ester (**1a**). Similar treatment of **1** with (*S*)-MTPACl afforded an (*R*)-MTPA ester (**1b**). Analysis of the ^{1}H-NMR chemical shift differences between $\delta(S)$ and $\delta(R)$ allowed the assignment of the absolute configurations of C(3) and C(8) of **1** as (*R*) and (*S*), respectively (*Fig. 3*). Therefore, the structure of compound **1** was elucidated as (3*R*,8*S*)-heptadeca-1-en-4,6-diyne-3,8-diol.

Compounds **1** – **3** had similar NMR spectra, but the numbers of C=C bonds in compounds **2** and **3** were different from that of **1**. The ^{1}H-NMR spectrum of compound **2** showed five H-atom signals at 5.92, 5.60, 5.50, 5.46, and 5.24 ppm. The olefinic H-atom signal at 5.60 ppm (*dt*, *J* = 10.6, 7.5 Hz, 1 H, H–C(9)) in compound **2** indicates a *Z*-configuration; the rest of the ^{1}H-NMR spectra was similar to that of compound **1**. Therefore, the chemical structure of **2** was determined to be falcarindiol (**2**) based on comparison with previously reported data [8]. The structure of compound **3** was determined based on comparison to that of **1** and **2**. The key difference between the spectra of compounds **1** and **3** was the presence of two olefinic H-atoms in compounds **3** (5.55 ppm, *dtd*, *J* = 11.0, 8.0, 1.0 Hz, 1 H, H–C(10); 5.47 ppm, *ddt*, *J* = 11.0, 8.5, 1.0 Hz, 1 H, H–C(9)), in place of the terminal olefinic CH_2 group seen in compound **1**. The coupling constant (11.8 Hz) between H–C(9) and H–C(10) suggested a *cis*-configuration for the C=C bond. Therefore, compound **3** was determined to be oplopandiol (**3**) in agreement with previously reported data [8].

Anti-Inflammatory Activity of Compounds **1** – **3**

Activated inflammatory cells (neutrophils, eosinophils, mononuclear phagocyte, and macrophages) express many

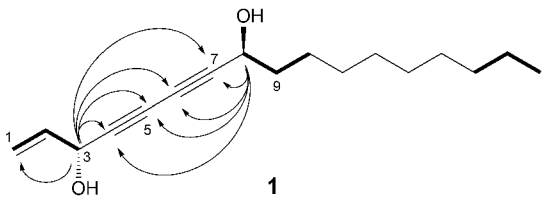

Fig. 2. ^{1}H,^{1}H COSY (—) and key HMBC (→) correlations of compound **1**.

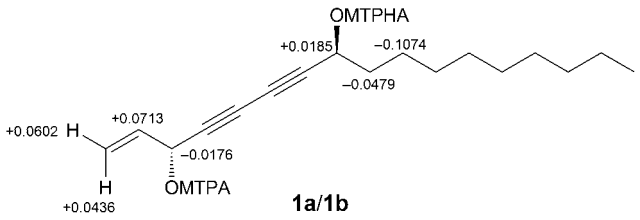

Fig. 3. $\Delta\delta(S - R)$ Values for the bis-*Mosher* esters **1a**/**1b**, in (D_5)pyridine.

biomolecules including NO, prostaglandin E_2 (PGE_2), and cytokines, such as interleukin (IL)-6, 1β, and tumor necrosis factor (TNF)-α. These signaling molecules play a role in activating macrophages and may lead to tissue damage. PGE_2 is a major inflammatory mediator and is produced by the catalysis of COX-2. NO is another important inflammatory substance. Its synthesis is controlled by several types of NOS. Accordingly, reducing iNOS and COX-2 expression, and thus inhibiting the production of NO and other inflammatory mediators, is the principle mechanism of action of anti-inflammatory agents.

The roots of *A. tenuissima* were previously reported to have an inhibitory effect on the expression of COX-2 and NO synthase in BV2 murine microglial cells [9]. Additionally, the isolated compounds, falcarindiol (**2**) and oplopandiol (**3**), were reported to have anti-inflammatory activity in LPS-treated RAW 267.7 murine macrophage cells [8]. Therefore, we evaluated their effect on the production of NO, iNOS, and COX-2.

As shown in *Fig. 4*, compounds **1** and **3** strongly inhibited the production of NO in a concentration-dependent manner with IC_{50} values of 4.31 and 5.06 μM, respectively. This effect resulted from the inhibition of LPS-induced iNOS production in macrophage cells. Compound **2** did not have any inhibitory effect. Western blotting was performed to determine the effects of compound **1** and **3** on COX-2 expression. Compounds **1** and **3** suppressed LPS-induced COX-2 expression in a concentration-dependent manner.

In summary, in this study, we have isolated for the first time a family of polyacetylenes from *A. tenuissima*. Their chemical structures were elucidated using spectroscopic methods and chemical techniques. Polyacetylenes **1** and **3** effectively suppressed proinflammatory mediators including NO, iNOS, and COX-2 and have potential as therapeutic anti-inflammatories. The results of this work also support the observation that *A. tenuissima* has anti-inflammatory activities.

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Supporting Information

Additional Supporting Information (Supplementary spectral data of compound **1** (1D and 2D NMR, HR-CI-MS)) may be found in the online version of this article: DOI: 10.1002/hlca.201500507

Experimental Part

General

TLC: silica gel *$60F_{254}$* and *RP-18 F_{254s}* silica gel plates (*Merck*, Darmstadt, Germany); detection under UV light and by spraying with vanillin-sulfuric acid reagent (1% ethanolic vanillin soln/10% ethanolic sulfuric acid) and 10% H_2SO_4 reagent, followed by heating at 110 °C for 1 min. Column chromatography (CC): silica gel (SiO_2; 70 – 230 mesh; *Merck*, Germany) and *Lichroprep RP-18* gel (40 – 63 μm; *Merck*, Darmstadt, Germany). HPLC: prep. HPLC *Gilson* system (*Gilson*, Middleton, WI, USA) *321* pump, *UV/VIS-155* detector, *FC 204* fraction collector, *Phenomenex-Luna-C18* column (250 × 21.20 mm, 10 μm.); t_R in min. Optical rotation: *JASCO DIP-1000* polarimeter (*JASCO*, Tokyo, Japan). ^{1}H- and ^{13}C-NMR spectra: *Bruker* DMX 250 (Rheinstetten, Germany) or *Jeol ECA-500* NMR spectrometer (*Jeol*, Tokyo, Japan); *(*in ppm rel. to tetramethylsilane (TMS) as internal standard, *J* in Hz at 298 K. HR-CI-MS: *Jeol* JMS-600W, chemical ionization (CI), gas chromatography/mass spectrometer (*Jeol*, Japan).

Plant Material

Angelica tenuissima roots were purchased from Yak-Ryung-Si Market in Daegu, Korea and identified by Dr. *Seung Ho Lee* at the College of Pharmacy, Yeungnam University. A voucher specimen (YU00192) has been deposited with the Natural Product Laboratory at the College of Pharmacy, Yeungnam University, Korea.

Extraction and Isolation

Dried roots of *A. tenuissima* (8.7 kg) were extracted with MeOH (25 l × 3) at r.t. The extract was concentrated *in vacuo* to afford a black gum (1.8 kg), which was dissolved in H_2O, continuously partitioned with solvents, then concentrated to give extracts of CH_2Cl_2 (334.1 g), AcOEt (24.5 g), and H_2O (1.4 kg). The CH_2Cl_2-soluble extract (334.1 g) was separated using SiO_2 vacuum liquid chromatography eluted with a gradient mixture of 100% hexanes to 100% AcOEt to afford eight fractions (*AT1 – AT8*). *Fr. AT4* (40.0 g) was separated on an open

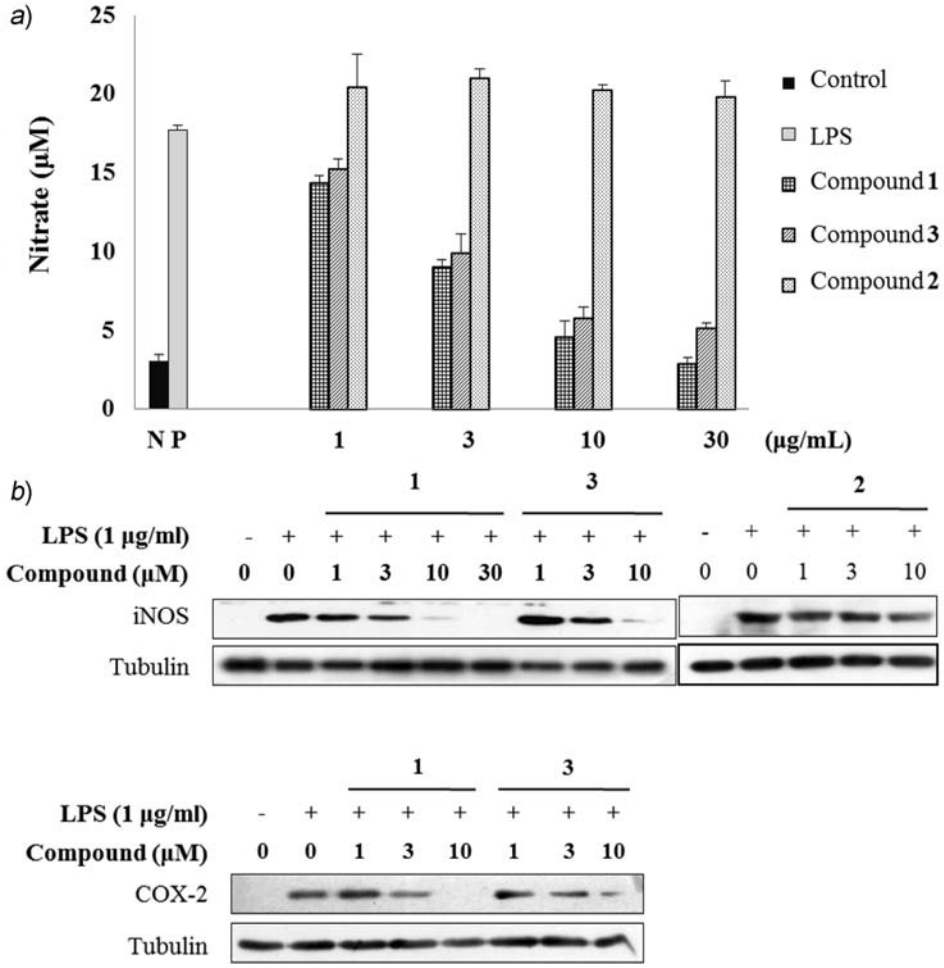

Fig. 4. Anti-inflammatory activities of compounds **1** – **3**. Inhibition of NO production a) and LPS-Induced iNOS and COX-2 expression b) in macrophage RAW264.7 cells by compounds **1** – **3**. RAW264.7 cells were pretreated for 30 min indicated concentrations of **1** – **3**, followed by stimulation with LPS (1 μg/ml) for 18 h. Total lysates were prepared, the expression levels of iNOS and COX-2 were determined by western blot analysis.

column of silica gel eluting with hexanes and AcOEt gradient mixture (10:0 – 0:10) to afford six subfractions (*AT1A* – *AT1F*). *Fr. AT1D* (3.2 g) was purified using preparative HPLC with a gradient of MeOH/H_2O (20% – 50%, 6 ml/min, 80 min) to afford compounds **1** (t_R 32.7 min; 3.2 mg), **2** (t_R 56.2 min; 728.2 mg), and **3** (t_R 57.8 min; 19.2 mg), respectively.

(3*R*,8*S*)-Heptadec-1-ene-4,6-diyne-3,8-diol (**1**). Colorless oil. $[\alpha]_D^{20}$ = +18.7 (*c* = 0.2, MeOH). NMR: see *Table*. HR-CI-MS: 261.1855 ($C_{17}H_{25}O_2^+$, $[M - H]^+$; calc. 261.1855).

Mosher*'s Esterification*. (*S*)- and (*R*)-MTPA (α-methoxy-α-(trifluoromethyl)phenylacetyl) esters of **1** were prepared using *Mosher*'s esterification method performed in NMR tubes. Compound **1** and 4-(dimethylamino) pyridine (0.2 mg) were transferred into each vial, and this mixture was dried under vacuum. (−)-(*R*) and (+)-(*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MATPCl; 6.0 μl) were immediately added to each vial as solutions in (D_5) pyridine (0.5 ml) and the vial was sealed and shaken to mix the sample and MTPACl evenly. The vial was permitted to stand at r.t. for 1 h. The soln. of derivatives was transferred to an NMR tube and monitored by ^{1}H-NMR [10].

(*S*)-MTPA Ester (= **(3*R*,8*S*)-Heptadec-1-ene-4,6-diyne-3,8-diyl (2*S*,2′*S*)-Bis(3,3,3-trifluoro-2-methoxy-2-phenylpropanoate)**; **1a**). ^{1}H-NMR (500 MHz, C_5D_5N): 6.6010 (*d*, *J* = 6.3, H–C(3)); 6.0408 – 6.1072 (*m*, H–C(2)); 5.9807 (*t*, *J* = 6.5, H–C(8)); 5.6589 (*d*, *J* = 17.4, H_a–C(8) – (1))); 5.3712 (*d*, *J* = 10.2, H_b–C(1)); 1.8419 (*dt*, *J* = 14.5, 7.2, CH_2(9)); 1.3383 (*d*, *J* = 6.7, CH_2(10)).

(*R*)-MTPA Ester (= **(3*R*,8*S*)-Heptadec-1-ene-4,6-diyne-3,8-diyl (2*R*,2′*R*)-Bis(3,3,3-trifluoro-2-methoxy-2-phenylpropanoate)**; **1b**). ^{1}H-NMR (500 MHz, C_5D_5N): 6.6186 (*d*, *J* = 6.3, H–C(3)); 5.9692 – 6.0372 (*m*, H–C(2)); 5.9622 (*t*, *J* = 6.5, H–C(8)); 5.5987 (*d*, *J* = 17.4, H_a–C(1)); 5.3276 (*d*, *J* = 10.2, H_b–C(1)); 1.8896 (*dt*, *J* = 14.5, 7.2, CH_2(9)); 1.4457 (*d*, *J* = 6.7, CH_2(10)).

Cell Culture

RAW264.7 murine macrophage cells were purchased from the Korean Cell Line Bank (*KCLB*, Seoul, Korea). The

cells were cultured in *Dulbecco*'s modified *Eagle*'s medium supplemented with 100 U/ml of penicillin, 100 U/ml of streptomycin, and 10% fetal bovine serum. The cells were incubated in an atmosphere of 5% CO_2 at 37 °C and were subcultured every 3 days.

Determination of NO Production

The level of NO production was determined by measuring the amount of nitrite present in cell culture supernatets as described previously [11][12]. Briefly, the RAW264.7 cells (1×10^5 cells/well) were treated with or without 1 μg/ml of *LPS* (*Sigma Chemical Co.*, St. Louis, MO, USA) for 24 h in the presence or absence of the test compounds (0.5 – 25 μM). The cell culture supernate (100 μl) was then reacted with *Griess* reagent (100 μl; 1% sulfanilamide in 5% phosphoric acid and 0.1% naphtylethylenediamine dihydrochloride in dist. H_2O). The absorbance at 540 nm was measured with a microplate reader (*Emax*; *Molecular Devices*, Sunnyvale, CA, USA), and the absorption coefficient was calibrated using $NaNO_2$ solution standard. The IC_{50} value was defined as the concentration of sample that reduced absorbance by 50% relative to the vehicle-treated control.

Immunoblot Analysis

Proteins were extracted from cells in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 1 mM ethylenediamine tetraacetic acid, 1 mM phenylmethyl sulfonyl fluoride, 1 μg/ml leupeptin, 1 mM sodium vanadate, and 150 mM NaCl). To measure iNOS, protein (50 μg per lane) was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane (*Millipore*, Bedford, MA, USA). The membrane was blocked with 5% skim milk, and then incubated with the corresponding antibody. The antibody for iNOS was obtained from *Santa Cruz Biotechnology* (Santa Cruz, CA, USA). The antibody for α-tubulin was obtained from *Sigma*. Antibody for IκB-α was obtained from *Cell Signaling Technology* (Danvers, MA, USA). After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions (*Amersham Pharmacia Biotec*, Little Chalfont, U.K.) [12][13].

Statistical Analysis

Values are expressed as mean ± S.E.M.

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