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Journal of Cellular Biochemistry 113:1987–1997 (2012)

Journal of Cellular Biochemistry

The Cytoprotective Effect of Butin Against Oxidative Stress is Mediated by the Up-Regulation of Manganese Superoxide Dismutase Expression through a PI3K/Akt/Nrf2-Dependent Pathway

Rui Zhang,[1] Sungwook Chae,[2] Jun Hwa Lee,[3] and Jin Won Hyun[1*]

[1] *School of Medicine and Applied Radiological Science Research Institute, Jeju National University, Jeju 690-756, South Korea*

[2] *Department of Herbal Resources Research, Korea Institute of Oriental Medicine, Daejeon 305–811, South Korea*

[3] *Samsung Changwon Hospital, Sungkyunkwan University School of Medicine, Changwon 630-723, South Korea*

ABSTRACT

Butin (7,3′,4′-trihydroxydihydroflavone), a flavonoid with antioxidant activity, was recently reported to protect cells against H_2O_2-induced apoptosis, oxidative DNA damage and oxidative mitochondrial dysfunction. The objective of the present study was to elucidate the mechanism by which butin protects mitochondria. The antioxidant function of manganese superoxide dismutase (Mn SOD) is important in preventing oxidative stress. While exposure to H_2O_2 reduced the expression of Mn SOD in Chinese hamster lung fibroblast (V79-4), the addition of butin restored Mn SOD expression at both the mRNA and protein levels, resulting in increased Mn SOD activity. The transcription factor NF-E2-related factor 2 (Nrf2) regulates Mn SOD gene expression by binding to the antioxidant responsive element (ARE). Butin enhanced the nuclear translocation and ARE-binding activity of Nrf2, which was decreased by H_2O_2. The siRNA-mediated knockdown of Nrf2 attenuated butin-induced Mn SOD expression and activity. Further, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, Akt) contributed to the ARE-driven Mn SOD expression. Butin activated PI3K/Akt and exposure to either LY294002 (a PI3K inhibitor), Akt inhibitor IV (an Akt-specific inhibitor), or Akt siRNA suppressed the butin-induced activation of Nrf2, resulting in decreased Mn SOD expression and activity. Finally, the cytoprotective effect of butin against H_2O_2-induced cell damage was suppressed by the siRNA-mediated knockdown of Mn SOD. These studies demonstrate that butin attenuates oxidative stress by activating Nrf2-mediated Mn SOD induction via the PI3K/Akt signaling pathway. J. Cell. Biochem. 113: 1987–1997, 2012.

KEY WORDS: BUTIN; MANGANESE SUPEROXIDE DISMUTASE; NF-E2-RELATED FACTOR 2; PHOSPHATIDYLINOSITOL 3-KINASE/PROTEIN KINASE B

Oxidative stress resulting from an increase in reactive oxygen species (ROS) and/or a concomitant decline in antioxidant scavenging capacity may damage proteins, lipids, nucleic acids, and other cellular structures [Valko et al., 2006; Kang et al., 2009b]. Because ROS formation occurs naturally, mammalian cells have developed several adaptive mechanisms to either limit ROS formation or to detoxify ROS. These mechanisms employ either antioxidant compounds or antioxidant enzymes [Chen and Kunsch, 2004]. Antioxidant enzymes not only provide a major mechanism by which cells combat the toxicities of ROS, but antioxidant enzyme induction is also highly effective and sufficient for protecting cells against oxidative stress and the toxic and neoplastic effects of many toxicants and carcinogens [Dröge, 2002]. Among the various antioxidant enzymes, the protective functions of manganese superoxide dismutase (Mn SOD) against oxidative stress have recently been emphasized [Belikova et al., 2009].

Mn SOD, the primary antioxidant enzyme that scavenges superoxide anion in mitochondria, is essential for aerobic life [Weisiger and Fridovich, 1973]. Lack of Mn SOD expression results in dilated ventricular cardiomyopathy, neonatal lethality, and neurodegeneration [Li et al., 1995; Lebovitz et al., 1996]. The overexpression of Mn SOD has been shown to protect against oxidative stress-induced cell death and tissue injury [Kiningham et al., 1999; Yen et al., 1999]. The transcriptional regulation of Mn

Grant sponsor: Korea Government; Grant number: 2011-0003772.

*Correspondence to: Jin Won Hyun, School of Medicine, Jeju National University, Jeju 690-756, South Korea.
E-mail: jinwonh@jejunu.ac.kr

Manuscript Received: 28 June 2011; Manuscript Accepted: 10 January 2012
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 17 January 2012
DOI 10.1002/jcb.24068 • © 2012 Wiley Periodicals, Inc.

SOD is mediated predominantly by the redox-sensitive transcription factor NF-E2-related factor 2 (Nrf2) [Na et al., 2008]. The activity of Nrf2 is regulated by its association with the cytoskeleton Kelch-like ECH-associated protein 1 (Keap1), which sequesters Nrf2 in the cytosol. When Nrf2 dissociates from Keap1, Nrf2 can translocate to the nucleus where it binds to the antioxidant responsive element (ARE) in conjunction with other transcription factors [Nguyen et al., 2003]. The ARE is a *cis*-acting enhancer sequence that mediates the transcriptional activation function of Nrf2 in response to oxidative stress [Rushmore et al., 1991]. An ARE is found in the promoter regions of genes encoding many antioxidant enzymes, including Mn SOD [Kwak et al., 2001]. Therefore, genes regulated by an ARE encode proteins that help to control cellular redox status and protect cells from oxidative damage [Hayes and McLellan, 1999]. Recent results demonstrated that the nuclear translocation of Nrf2 requires the activation of several signal transduction pathways, including the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, Akt) pathway [Nakaso et al., 2003]. PI3K and Akt are important enzymes involved in the transduction of various signals from the cell surface to the nucleus. The PI3K/Akt pathway is associated with the modulation of ARE-driven gene expression via Nrf2 activation [Li et al., 2007].

A variety of edible phytochemicals are able to activate Nrf2 signaling, thereby up-regulating a set of enzymes, including NADP(H): Quinone oxidoreductase-1, SOD, glutathione S-transferase, heme oxygenase-1, and γ-glutamate-cysteine ligase [Lee and Surh, 2005]. Therefore, it has been suggested that the use of antioxidant compounds may prevent or alleviate diseases, particularly those for which oxidative stress is the main cause. Flavonoids, which are important constituents of the human diet, are a group of naturally occurring polyphenolic compounds found ubiquitously in vegetables and fruits. Flavonoids are also found in medicinal plants; herbal remedies containing flavonoids have been used worldwide in folk medicine [Di Carlo et al., 1999]. Recently, much attention has been focused on the potential uses of flavonoid-based drugs for the prevention and therapy of free radical-mediated human diseases, such as atherosclerosis, ischemia, inflammation, neuronal degeneration, and cardiovascular diseases [van Acker et al., 2000]. Because of the antioxidant and free radical scavenging properties of flavonoids, their biological activities have been studied intensively [Saric et al., 2009].

Butin (7,3′,4′-trihydroxydihydroflavone) has been isolated from several medicinal herbs, such as *Dalbergia odorifera*, *Adenanthera pavanina*, and *Vernonia anthelmintica Willd* [Tian et al., 2004; Liu et al., 2005; Su et al., 2007], and is reported to possess biological properties such as skin-whitening and contraception [Bhargava, 1986; Lee et al., 2006]. Recently, we demonstrated that butin protected cells against H_2O_2-induced damage [Zhang et al., 2008], protected DNA against oxidative damage [Kang et al., 2009a], and reduced oxidative stress-induced mitochondrial dysfunction and mitochondria-dependent apoptosis [Zhang et al., 2010, 2011]. To the best of our knowledge, few studies have investigated the mechanism underlying the protective properties of butin in mitochondria. Therefore, in the present study, we investigated the ability of butin to up-regulate Mn SOD expression via the activation of the PI3K/Akt/Nrf2 pathway.

MATERIALS AND METHODS

REAGENTS

Butin was purchased from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan) and dissolved in dimethylsulfoxide (DMSO); the final concentration of DMSO did not exceed 0.02%. 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) were purchased from Sigma Chemical Company (St. Louis, MO). Primary rabbit polyclonal Mn SOD antibody was purchased from Stressgen Corporation (Victoria, Canada) and primary rabbit polyclonal Nrf2, β-actin antibodies, and Akt inhibitor IV were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary anti-phospho Akt (Ser 473) and Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA). LY294002 was provided by Calbiochem (San Diego, CA). The ARE-luciferase reporter gene was kindly provided by Professor Young-Joon Surh of Seoul National University (Seoul, Korea). Other chemicals and reagents were of analytical grade.

CELL CULTURE

Chinese hamster lung fibroblasts (V79-4) and human Chang liver cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2 and cultured in Dulbecco's modified Eagle's and RPMI 1640 medium, respectively, containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 units/ml).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated from cells using Trizol (GibcoBRL, Grand Island, NY). RT-PCR was performed as described previously [Kang et al., 2006]. The PCR conditions for Mn SOD and for the housekeeping gene GAPDH were as follows: 30 cycles of 94°C for 15 s, 60°C for 30 s, and 68°C for 60 s. The primer pairs (Bionics, Seoul, South Korea) were the following: Mn SOD sense 5′-GAC CTG CCT TAC GAC TAT GG-3′ and antisense 5′-GAC CTT GCT CCT TAT TGA AG-3′; GAPDH sense 5′-GTG GGC CGC CCT AGG CAC CAG G-3′ and antisense 5′-GGA GGA AGA GGA TGC GGC AGT G-3′. Amplified products were resolved on 1% agarose gels, stained with ethidium bromide, and photographed under ultraviolet light.

WESTERN BLOTTING ANALYSIS

Cells were harvested, washed twice with PBS, lysed on ice for 30 min in 100 μl of lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and then centrifuged at 13,000*g* for 15 min. The supernatants were collected and the protein concentrations were determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 min, electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gel, and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), which were subsequently incubated with primary antibodies. The membranes were further incubated with a horseradish peroxidase-conjugated secondary immunoglobulin G antibody (Pierce, Rockford, IL). Protein bands were detected using an enhanced chemiluminescence western blotting detection kit

(Amersham, Little Chalfont, Buckinghamshire, UK) and subsequent exposure to X-ray film.

MITOCHONDRIA EXTRACT PREPARATION AND MEASUREMENT OF Mn SOD ACTIVITY

Cells were seeded in culture dishes at a concentration of 1.5×10^5 cells/ml and treated with butin at 10 μg/ml 16 h later. After 1 h, 1 mM H_2O_2 was added to the plates and incubated for 24 h. Mitochondria were extracted by mitochondrial fraction kit (Active Motif, Japan) according to the manufacturer's instructions. Protein concentration was measured using a Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as a standard. Mn SOD activity was measured using a colorimetric assay kit (Abcam, Cambridge, MA) according to the manufacturer's protocol. The kit utilizes water-soluble tetrazolium salt (WST-1), which produces a water-soluble formazan dye upon reduction with superoxide anion, and the product that was detected at 450 nm. SOD activity was calculated on the basis of the percent inhibition of superoxide anion.

IMMUNOCYTOCHEMISTRY

Cells plated on coverslips were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 2.5 min. Cells were exposed to blocking buffer (3% bovine serum albumin in PBS) for 1 h and incubated for 2 h with Nrf2 antibody diluted in blocking buffer. Antigen-bound primary Nrf2 antibody was detected by 1 h incubation with a 1:500 dilution of FITC-conjugated secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA). After washing with PBS, the stained cells were mounted onto microscope slides with mounting medium containing DAPI (Vector, Burlingame, CA). Images were collected using the LSM 510 program on a Zeiss confocal microscope.

NUCLEAR EXTRACT PREPARATION AND ELECTROPHORETIC MOBILITY SHIFT ASSAY

Cells were seeded in culture dishes at a concentration of 1.5×10^5 cells/ml, and 16 h later, were treated with butin at 10 μg/ml. After 1 h, 1 mM H_2O_2 was added to the plates and incubated for 12 h. Cells were harvested and lysed on ice with 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, and 1% NP-40) for 4 min. After 10 min of centrifugation at 3,000*g*, the pellets were suspended in 50 μl of extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 300 mM NaCl, 0.2 mM EDTA, 1 mM DTT, and 1 mM PMSF), incubated on ice for 30 min and centrifuged at 13,000*g* for 5 min. The supernatants were harvested as nuclear protein extracts and stored at −70°C after determining protein concentration. A synthetic double-stranded oligonucleotide probe containing an ARE sequence was labeled at its 5′ end with [γ-^{32}P]-ATP and T4 polynucleotide kinase. The double-stranded sequence was 5′-TTT TCT GCT GAG TCA AGG GTC CG-3′ and 3′-AAA AGA CGA CTC AGT TCC AGG C-5′. The probe (50,000 cpm) was incubated with 6 μg of the nuclear extracts at 4°C for 30 min in a final volume of 20 μl containing 12.5% glycerol, 12.5 mM HEPES (pH 7.9), 4 μM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA and 1 mM DTT with 1 μg of poly (dI-dC). Binding products were resolved in 5% polyacrylamide gels and the bands were visualized by autoradiography.

TRANSIENT TRANSFECTION WITH THE ARE PROMOTER AND LUCIFERASE ASSAY

Cells were transiently transfected with a plasmid harboring the ARE promoter using the transfection reagent DOTAP according to the manufacturer's instructions (Roche, Mannheim, Germany). After overnight transfection, cells were treated with 10 μg/ml of butin. Cell were then washed twice with PBS and lysed with reporter lysis buffer. After vortexing and centrifuging at 12,000*g* for 1 min at 4°C, the supernatant was stored at −70°C for the luciferase assay. After 20 μl of the cell extract was mixed with 100 μl of the luciferase assay reagent at room temperature, the mixture was placed in a luminometer to measure the light produced.

TRANSIENT TRANSFECTION OF SMALL INTERFERING RNA (siRNA)

Cells were seeded at 1.0×10^5 cells/well in a 24-well plate and allowed to reach approximately 50% confluence on the day of transfection. The siRNA constructs used were a mismatched siRNA control (siControl), siRNA against Nrf2 (siNrf2, Santa Cruz Biotechnology), siRNA against Akt (siAkt, Dharmacon, Lafayette, CO) and siRNA against Mn SOD (siMn SOD, Santa Cruz Biotechnology). Cells were transfected with 10–50 nM siRNA using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 24 h after transfection, the cells were treated with 10 μg/ml of butin for 12 or 24 h and assayed by western blotting, Mn SOD activity, and MTT.

CELL VIABILITY

The effect of butin on cell viability was determined by an MTT assay, which is based on the reduction of tetrazolium salt by mitochondrial dehydrogenase in viable cells [Carmichael et al., 1987]. Cells transfected with siRNA were seeded in a 96-well plate at a density of 1×10^5 cells/ml and treated with 10 μg/ml of butin followed 1 h later by 1 mM of H_2O_2. After incubating for 24 h at 37°C, 50 μl of the MTT stock solution (2 mg/ml) was added to each well to attain a total reaction volume of 250 μl. After incubation for 4 h, the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl DMSO, and the absorbance at 540 nm was measured on a scanning multi-well spectrophotometer. To determine the effect of butin on rotenone (inhibitor of mitochondrial complex I), cells were pretreated with butin and exposed to 1 μM of rotenone for 24 h. Cell viability was measured using the MTT assay.

ROS SCAVENGING ACTIVITY

To detect intracellular ROS, the DCF-DA method was used [Rosenkranz et al., 1992]. Cells were seeded in a 96-well plate at a concentration of 1×10^5 cells/ml and 16 h later, were treated with 10 μg/ml of butin for 30 min prior to adding of 1 mM H_2O_2 or 1 μM rotenone. Cells were incubated for 30 min or 12 h at 37°C, respectively. The fluorescence of 2′,7′-dichlorofluorescein was detected at 485 nm excitation and at 535 nm emission, using a PerkinElmer LS-5B spectrofluorometer.

COLONY FORMATION ASSAY

Cells were seeded into 60 mm dishes to produce approximately 400 colonies per dish. After treatment of 10 μg/ml of butin and 1 mM H_2O_2 or 1 μM rotenone, the cultures were incubated for 10

days. Colonies were fixed with 75% ethanol and 25% acetic acid and stained with trypan blue. Colonies containing 50 or more cells were considered viable.

HYDROGEN PEROXIDE SCAVENGING ACTIVITY

This assay is based on the ability of butin to scavenge the H_2O_2 in 2,2′-azino-di(3-ethyl-benzthiazoline-6-sulphonic acid)-peroxidase medium (ABTS) [Muller, 1975]. 20 μl of butin and 20 μl of 1 mM H_2O_2 were mixed with 20 μl of 0.1 M phosphate buffer (pH 5.0) in a 96-well plate and incubated at 37°C for 5 min. Then 30 μl of ABTS and 30 μl of peroxidase (1 unit/ml) were added to the 96-well plate and incubated at 37°C for 10 min. The absorbance was measured at 405 nm using a spectrophotometer.

STATISTICAL ANALYSIS

All measurements were made in triplicates and all values were represented as mean ± standard error of the mean (SEM). The results were subjected to an analysis of variance (ANOVA) using the Tukey test to assess the statistical significance. A value of $P < 0.05$ was considered statistically significant.

RESULTS

BUTIN RESTORED MN SOD mRNA AND PROTEIN EXPRESSION AND Mn SOD ACTIVITY

Treatment of cells with 1 mM H_2O_2 decreased Mn SOD mRNA and protein expression. However, butin at 10 μg/ml restored Mn SOD mRNA and protein expression (Fig. 1A,B), resulting in a significant increase in Mn SOD activity (Fig. 1C).

BUTIN INCREASED THE LEVEL OF NRF2 TRANSCRIPTION FACTOR

Most of the genes encoding phase II detoxifying and antioxidant enzymes have an ARE sequence in their promoter region. Nrf2 is an important transcription factor that regulates ARE-driven Mn SOD gene expression. We investigated whether butin up-regulates Mn SOD by activating Nrf2. Butin treatment restored Nrf2 protein expression, which is attenuated by H_2O_2 treatment (Fig. 2A), resulting in the translocation of Nrf2 from the cytosol to the nucleus (Fig. 2B). Moreover, butin-treated cells significantly elevated the level of Nrf2 binding to the ARE sequence, as assessed by a gel shift assay using nuclear extract (Fig. 2C). To confirm the functional relevance of Nrf2 binding to the Mn SOD ARE sequence, an ARE-promoter construct containing the Nrf2 binding DNA consensus site linked to a luciferase reporter gene was used. As illustrated in Figure 2D, butin increased the transcriptional activity of Nrf2. These results further suggested that butin up-regulated the activation of Nrf2. To confirm the requirement of Nrf2 for butin-induced Mn SOD expression, cells were transfected with siNrf2 RNA for 24 h prior to butin treatment. As shown in Figure 2E,F, butin-induced Mn SOD expression and activity were markedly inhibited by siRNA-mediated Nrf2 knock-down. These results indicate that Nrf2 is partially required for butin-induced Mn SOD activation.

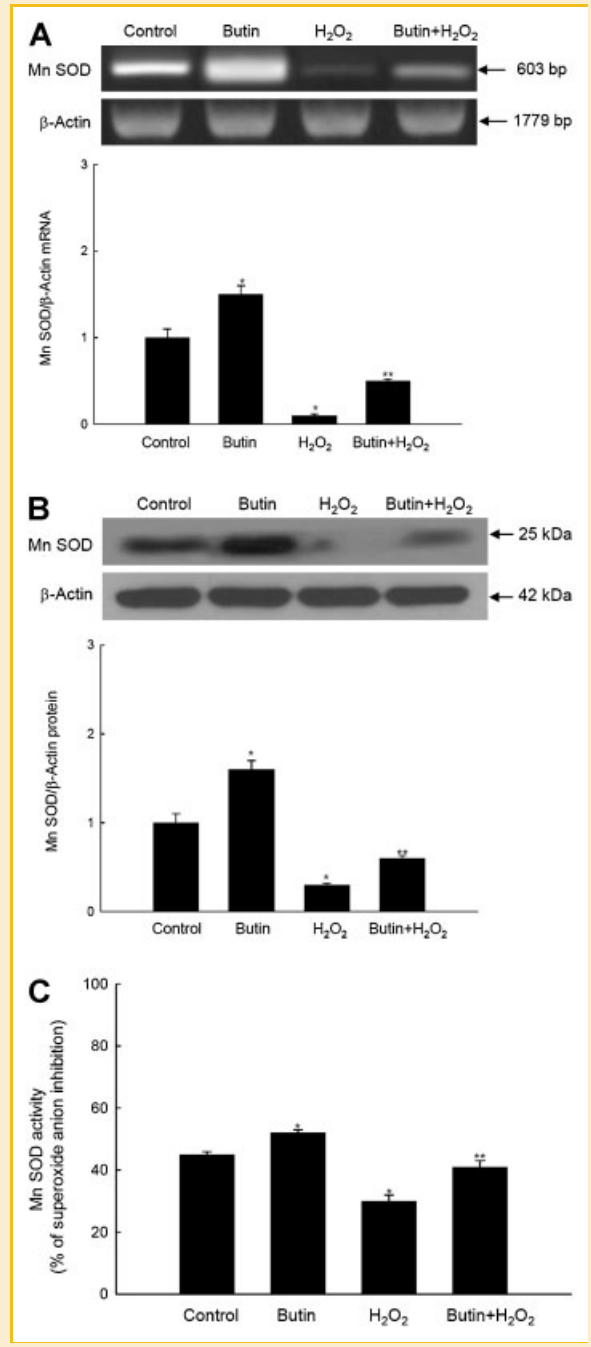

Fig. 1. Effect of butin on Mn SOD mRNA expression, protein expression, and enzyme activity. A: Cells were treated with butin at 10 μg/ml, and 1 h later, 1 mM H_2O_2 was added for 12 h. Total RNA was extracted and Mn SOD mRNA expression was analyzed by RT-PCR and quantified. *Significantly different from control cells ($P < 0.05$) and **significantly different from H_2O_2-treated cells ($P < 0.05$). B: Cell lysates were electrophoresed and the expression of Mn SOD protein was detected using a Mn SOD-specific antibody and quantified. *Significantly different from control cells ($P < 0.05$) and **significantly different from H_2O_2-treated cells ($P < 0.05$). C: Mn SOD activity was measured using a colorimetric assay kit. *Significantly different from control cells ($P < 0.05$) and **significantly different from H_2O_2-treated cells ($P < 0.05$).

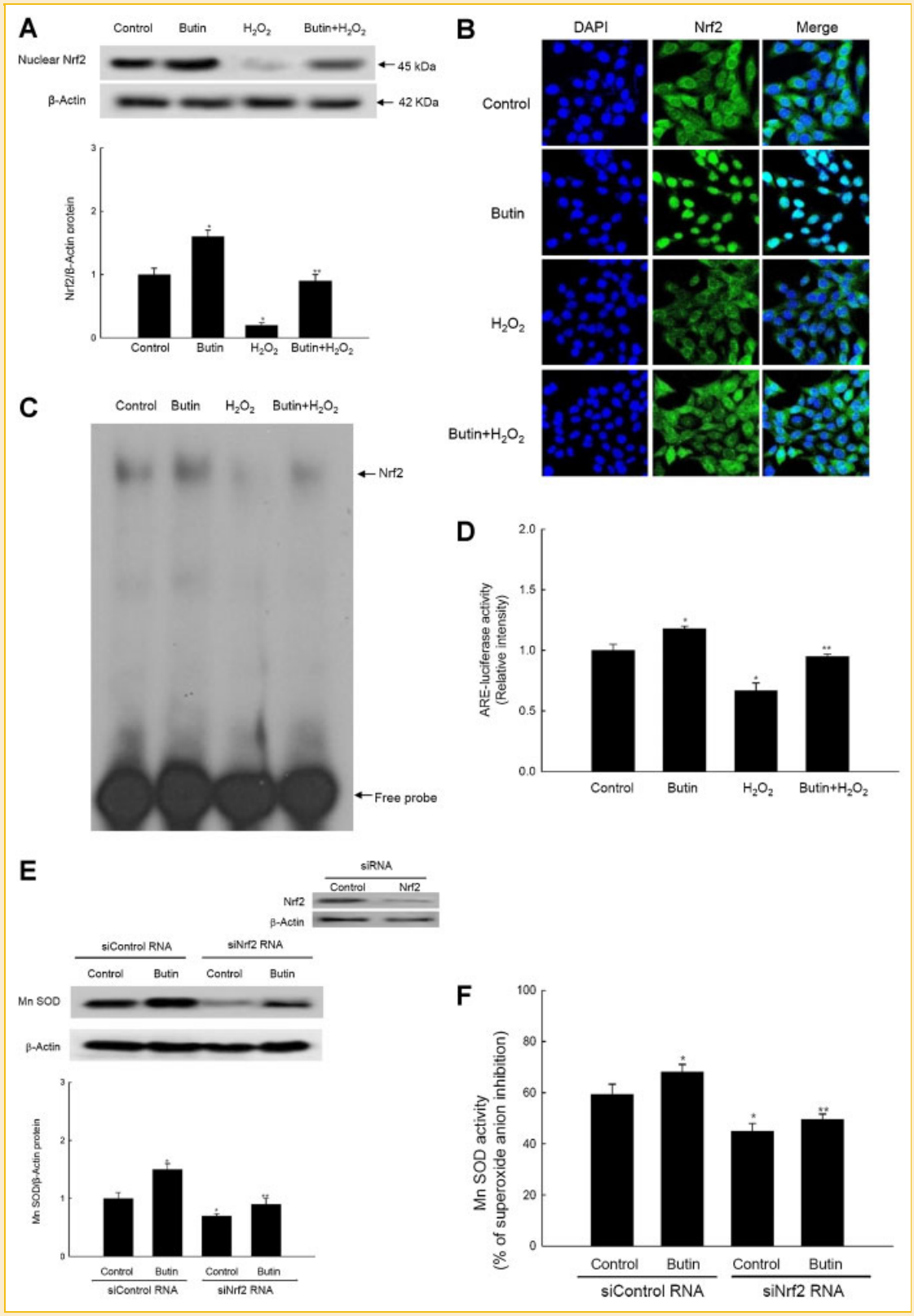

Fig. 2. Effect of butin on Nrf2 level. A: Nuclear extracts were prepared after treatment with 10 μg/ml of butin, and 1 h later, 1 mM H_2O_2 was added for 12 h. Western blot of nuclear lysates was detected with a Nrf2-specific antibody and quantified. *Significantly different from control cells ($P < 0.05$) and **significantly different from H_2O_2-treated cells ($P < 0.05$). B: Confocal imaging using FITC-conjugated secondary antibody staining indicates the location of Nrf2 (green) by the anti-Nrf2 antibody, DAPI staining indicates the nucleus (blue), and the merged image indicates the nuclear location of Nrf2. C: The electrophoretic mobility shift assay performed for the detection of the ARE-binding activity of Nrf2. D: The transcriptional activity of Nrf2 was assessed using an ARE-luciferase construct and a luminometer. *Significantly different from control cells ($P < 0.05$) and **significantly different from H_2O_2-treated cells ($P < 0.05$). Cells were transfected in 10–50 nM siControl RNA and siNrf2 RNA using Lipofectamine™ 2000 according to the manufacturer's instruction. At 24 h after transfection, the cells were treated with 10 μg/ml of butin for 24 h, and (E) the expression of Mn SOD protein was examined by western blot analysis and quantified and (F) the activity of Mn SOD was detected by a colorimetric assay kit. *Significantly different from control in siControl RNA-transfected cells ($P < 0.05$) and **significantly different from butin treatment in siControl RNA-transfected cells ($P < 0.05$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

BUTIN ACTIVATED Nrf2-DRIVEN Mn SOD VIA PI3K/Akt PHOSPHORYLATION

To elucidate the upstream signaling pathway involved in butin-mediated Nrf2 activation and Mn SOD induction, we assessed the activation of Akt, a major signaling enzyme involved in protection against cellular oxidative stress. The activation of Akt by butin was detected by western blot with an antibody specific for phospho Akt. As shown in Figure 3A, exposure to butin increased the phosphorylation of Akt in H_2O_2-treated cells. We then determined whether the Akt pathway is involved in the induction of Nrf2 activation and Mn SOD expression. Cells were pre-incubated for 30 min with the PI3K inhibitor LY294002 or with Akt inhibitor IV and then treated with butin for 12 h or 24 h. An increase in nuclear Nrf2 accumulation and Mn SOD protein expression occurred following butin treatment. Inhibition of the PI3K/Akt pathway dramatically reduced the capacity of butin to increase Nrf2 and Mn SOD protein levels (Fig. 3B). In addition, induction of activity of Mn SOD by butin was also suppressed via inhibition of the PI3K/Akt pathway (Fig. 3C). To further confirm these observations, cells were transfected with siAkt RNA. As shown in Figure 3D, the nuclear translocation of Nrf2 and the expression of Mn SOD were markedly inhibited in siAkt RNA-transfected cells, regardless of butin treatment. Furthermore, the activity of Mn SOD was also dramatically reduced in siAkt RNA-transfected cells no matter treatment with or without butin (Fig. 3E). These results indicate that the PI3K/Akt pathway is partially required for the induction of Nrf2 nuclear accumulation, Mn SOD expression, and its activity.

Mn SOD INVOLVED IN CELL DAMAGE INDUCED BY OXIDATIVE STRESS

As shown in Figure 4A, Mn SOD protein expression was markedly inhibited in siMn SOD RNA-transfected cells regardless of butin treatment. To determine whether the increased level of Mn SOD activity induced by butin confers cytoprotection against oxidative stress, cells were transfected with siMn SOD RNA for 24 h prior to butin treatment. siMn SOD RNA attenuated the protective effect of butin against H_2O_2-induced cytotoxicity (Fig. 4B).

BUTIN SCAVENGED ROS PRODUCTION AND INCREASED CELL SURVIVAL IN BOTH V79-4 LUNG FIBROBLASTS AND HUMAN CHANG LIVER CELLS

Since more studies need to be conducted in various cell line types to address the question whether the protective effect of butin is generally applicable, the study in V79-4 lung fibroblasts and human Chang liver cells were performed. As shown in Figure 5A, butin significantly scavenged ROS production induced by H_2O_2 in these two cell lines. In addition, butin also restored cell viability and colony formation reduced by H_2O_2 in both cells (Fig. 5B,C). To investigate whether butin shows the protective effect against H_2O_2, and another oxidative stress stimulator, rotenone (a well-known inhibitor of mitochondrial complex I), ROS generation, cell viability, and colony formation were assessed. Indeed, butin showed the similar protective effects on rotenone induced oxidative damage compared with the treatment of H_2O_2 (Fig. 5D–F).

BUTIN SCAVENGED HYDROGEN PEROXIDE

To confirm whether butin could react directly to detoxify H_2O_2, we assessed the scavenging effect of butin on hydrogen peroxide. Butin at 10 μg/ml showed 16% scavenging activity. N-acetyl cysteine (NAC), a well-known ROS scavenger, used as a positive control and showed 95% scavenging activity (Fig. 6). These data indicated that butin could directly scavenge hydrogen peroxide.

DISCUSSION

Flavonoids, including butin, are known to possess powerful antioxidant properties, which are attributed to the presence of phenolic hydroxyl groups in the flavonoid structure. These compounds usually contain aromatic hydroxyl groups that are responsible for the antioxidant activities of flavonoids [van Acker et al., 2000]. Recently, we demonstrated that butin protected cells against H_2O_2-induced apoptosis by scavenging ROS and activating antioxidant enzymes, by activating the PI3K/Akt/oxoguanine glycosylase 1 pathway and by inhibiting mitochondrial dysfunction [Zhang et al., 2008, 2010, 2011; Kang et al., 2009a]. In the present study, we demonstrated that butin modulated Mn SOD induction via the PI3K/Akt/Nrf2 pathway. To our knowledge, this is the first report demonstrating that butin can regulate Mn SOD expression.

Oxidative stress refers to the unbalanced redox equilibrium between free radical production and the ability of cells to defend against them. One feasible way to alleviate free radical-mediated-cellular injury is to augment the oxidative defense capacity through the intake of antioxidants. Moreover, the induction of endogenous phase II detoxifying enzymes or antioxidant proteins seems to be a reasonable strategy to prevent disease aggravation. Although the superoxide anion is not considered to be highly reactive when compared to other ROS, its toxicity may derive, at least in part, from its location in mitochondria where its attack on critical respiratory chain targets is facilitated. Mn SOD is a mitochondrial matrix enzyme that scavenges ROS and protects the cell against the insults of oxidative stress [Li et al., 1995]. Therefore, many studies have suggested that Mn SOD plays a key role in cell survival and that Mn SOD is necessary for the maintenance of mitochondrial integrity in cells exposed to oxidative stress [Oberley, 2004]. The transcriptional regulation of Mn SOD is predominantly mediated by the redox-sensitive transcription factor Nrf2 [Na et al., 2008]. The ability of Nrf2 to up-regulate the expression of antioxidant genes via ARE-binding suggests that increasing Nrf2 activity may provide a useful system for combating oxidative insults. Nrf2, a member of the cap'n'collar family of bZIP transcription factors, can act as a master regulator in the ARE-driven transactivation of antioxidant genes [Lee and Surh, 2005]. A distinct set of Nrf2-regulated proteins detoxifies xenobiotics, reduces oxidized proteins, maintains cellular reducing equivalents, disrupts redox cycling reactions, and counteracts the noxious effects of ROS [Jaiswal, 2004]. Nrf2 is

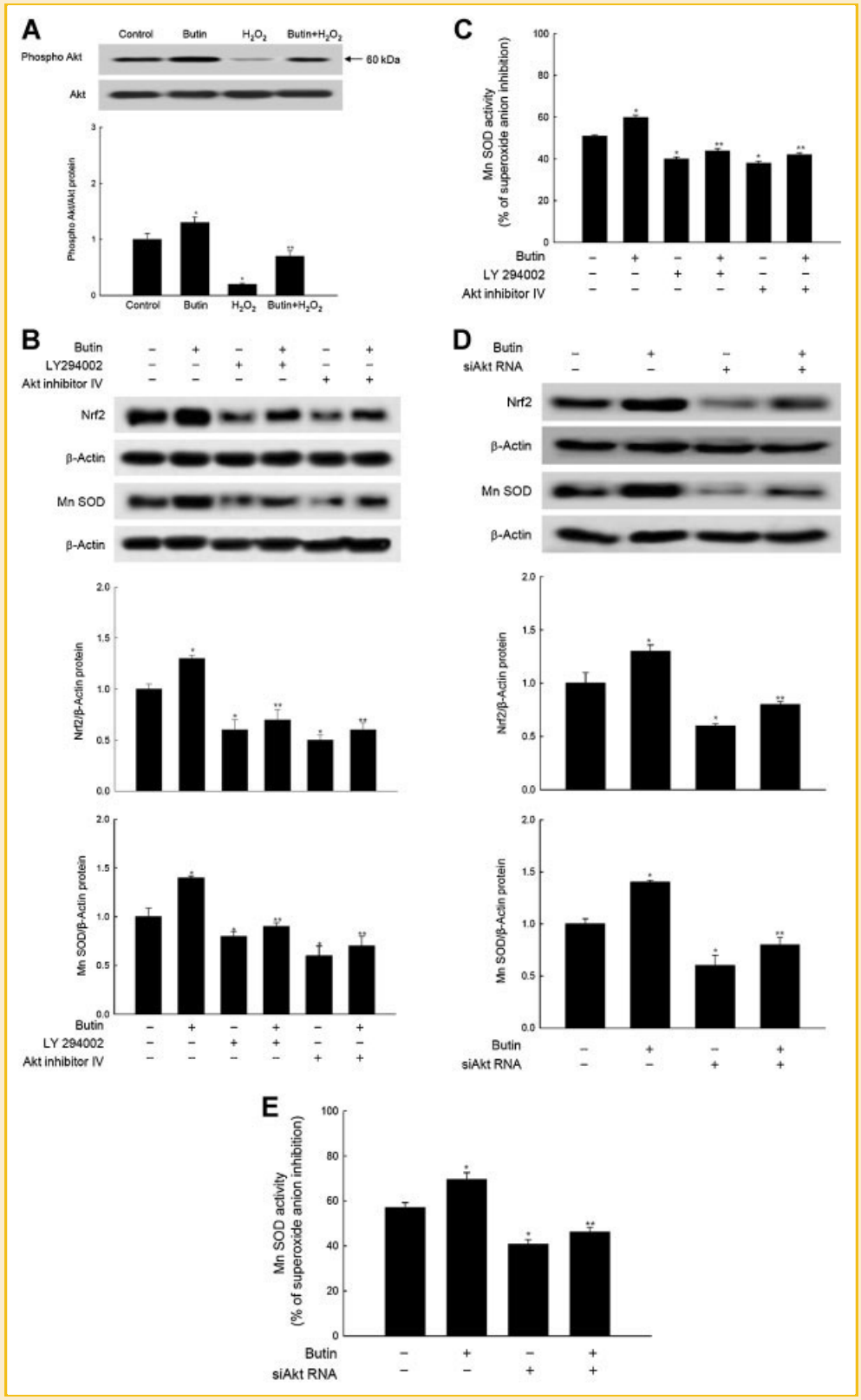

Fig. 3. Induction of Mn SOD and activation of Nrf2 by butin via phosphorylation of PI3K/Akt. A: Cell lysates were subjected to electrophoresis and phospho Akt and Akt were detected using specific antibodies and quantified. *Significantly different from control cells ($P < 0.05$) and **significantly different from H_2O_2-treated cells ($P < 0.05$). After treatment with LY294002 or Akt inhibitor IV, (B) cell lysates were subjected to electrophoresis and nuclear Nrf2 and Mn SOD were detected using specific antibodies and quantified, and (C) Mn SOD activity was measured by a colorimetric assay kit. *Significantly different from control cells ($P < 0.05$) and **significantly different from butin-treated cells ($P < 0.05$). Cells were transfected in 10–50 nM siControl RNA and siAkt RNA. At 24 h after transfection, the cells were treated with 10 μg/ml of butin for 12 or 24 h, and (D) the nuclear Nrf2 and Mn SOD protein expressions were examined by western blot analysis and quantified, and (E) Mn SOD activity was detected by a colorimetric assay kit. *Significantly different from control in siControl-transfected cells ($P < 0.05$) and **significantly different from butin treatment in siControl-transfected cells ($P < 0.05$).

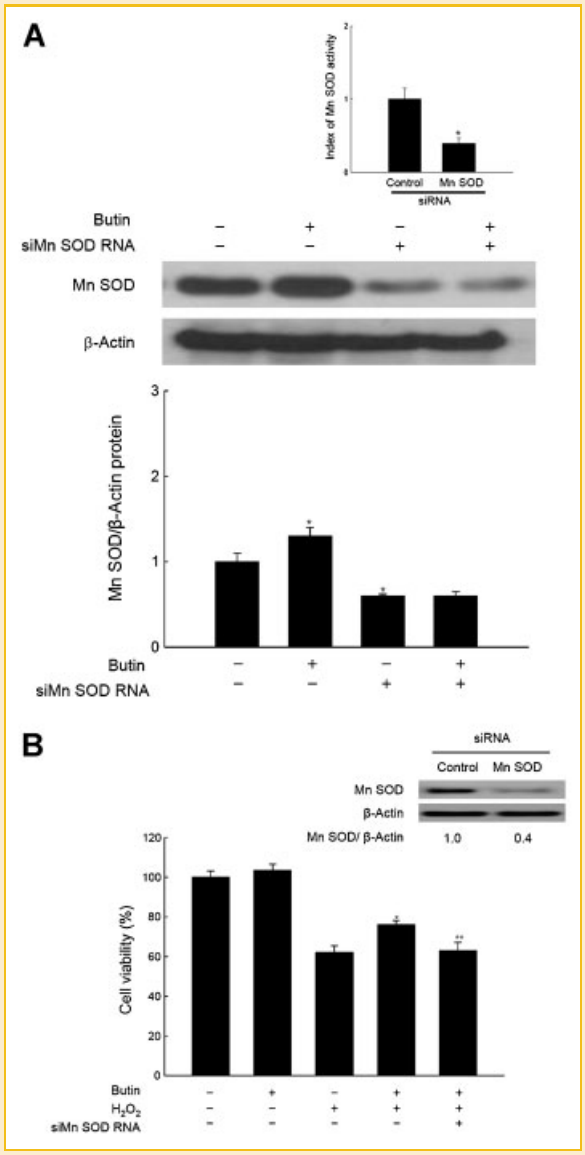

Fig. 4. Cytoprotective effect of butin against H_2O_2-induced cell death via up-regulation of Mn SOD activity. A: Cells were transfected in 10–50 nM siControl RNA and siMn SOD RNA. At 24 h after transfection, the cells were treated with 10 μg/ml of butin for 24 h and the expression of Mn SOD protein was examined by western blot analysis and quantified. *Significantly different from control in siControl-transfected cells ($P < 0.05$). B: Cells were transfected in 10–50 nM siMn SOD RNA, followed by 1 h of incubation with butin and exposure to 1 mM H_2O_2 for 24 h. Cell viability was measured using the MTT assay. *Significantly different from H_2O_2 treatment in siControl-transfected cells ($P < 0.05$) and **significantly different from butin plus H_2O_2 treatment in siControl-transfected cells ($P < 0.05$).

sequestered in the cytoplasm as an inactive complex with its cytosolic repressor Keap1. The dissociation of Nrf2 from Keap1 is a prerequisite for the nuclear translocation and subsequent DNA binding of Nrf2. After forming a heterodimer with a small Maf protein inside the nucleus, the active Nrf2 binds to *cis*-acting ARE, also known as the Maf recognition element [Juan et al., 2005]. In addition to the dissociation of the Nrf2-Keap1 complex, which is facilitated by upstream kinase-mediated signals, the covalent modification of multiple cysteine residues in Keap1 by electrophiles or inducers of detoxifying enzymes also contributes to the release of Nrf2 from Keap1 [Dinkova-Kostova et al., 2002]. The multiple mechanisms of Nrf2 activation by signals mediated via one or more of the upstream kinases, including MAPKs, PI3K/Akt, and PKC, were reviewed recently [Lee and Surh, 2005]. Our study indicates that butin up-regulated Mn SOD expression by increasing the nuclear levels and ARE-binding activity of Nrf2, which were attenuated by H_2O_2. The mechanisms leading to nuclear translocation of Nrf2 include its release from Keap1 into the cytosol. However, because the half-life of Nrf2 is short, these mechanisms should also include the stabilization of the Nrf2 protein [Jain et al., 2008]. Our results demonstrate that butin increased the level of Nrf2, suggesting that butin may delay Nrf2 degradation. Some reports demonstrated that MnSOD is not only regulated by Nrf2, but also by other transcription factors such as CCAAT/enhancer binding protein β (C/EBPβ) [Ranjan and Boss, 2006] and Forkhead Box Protein O 3a (FOXO3a) [Kops et al., 2002]. Furthermore, NF-kB is known to exert a cytoprotective effect against tumor necrosis factor-alpha (TNF-α) induced apoptosis. Delhalle et al. [2002] also reported NF-kB-dependent Mn SOD expression protects adenocarcinoma cells from TNF-α-induced apoptosis. Thus, knockdown of Nrf2 may partially affect Mn SOD expression. Most studies of the regulation of phase II gene expression have focused on the role of the PI3K/Akt pathway. The activation of the PI3K/Akt pathway is a key step in diverse biological processes, including cell proliferation, growth, and survival [Huang et al., 2004]. Our results demonstrate that the Nrf2-mediated increase in Mn SOD protein induced by butin is dependent on the activation of PI3K/Akt; LY294002 (a PI3K inhibitor), Akt inhibitor IV, and siAkt RNA all decreased the butin-induced accumulation of Nrf2 and Mn SOD. These results show that the PI3K/Akt pathway is regulated by butin and that these pathways participate in the induction of Nrf2, thereby regulating Mn SOD expression. In addition, we showed that siMn SOD RNA can partially reverse the protective effects of butin, providing further evidence that Mn SOD plays a cytoprotective role. Furthermore, Nrf2/ARE signaling regulates antioxidant genes such as glutathione reductase, glutathione peroxidase, catalase, in addition to detoxifying enzymes (known as "phase 2" enzymes) such as glutathione-S-transferase and NAD(P)H:quinone oxidoreductase 1 [Kwak et al., 2001]. Therefore, several other pathways may be involved in the oxidative stress resistance induced by butin.

In order to investigate whether the protective effect of butin are generally applicable, the effect of butin on another cell line (human Chang liver cells) and on inhibitor of mitochondrial complex I (rotenone) was also determined. Butin did not exhibit significant differences on cell types and oxidative stimulator types. Furthermore, we confirmed that butin could react directly to detoxify H_2O_2; thus, we hypothesized that the capacity of butin to direct scavenge H_2O_2 at least in part was involved in its protective effect against oxidative stress-induced cell death.

In summary, the present results suggest that butin protects cells against oxidative stress-induced cell death. This protection possibly occurs via increased activation of PI3K/Akt, which appears to be

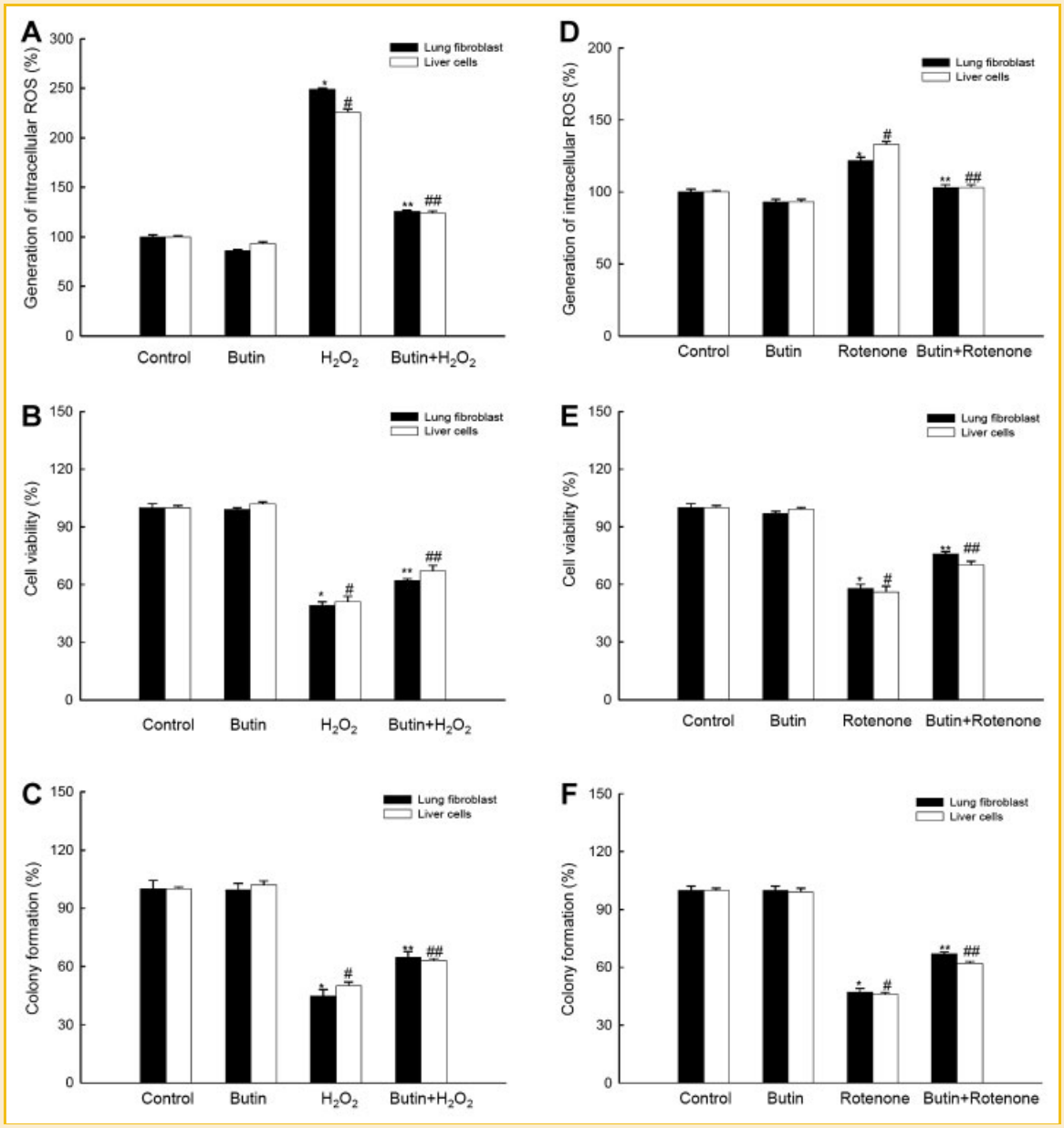

Fig. 5. Effects of butin on ROS production and cell survival in both V79-4 lung fibroblasts and human Chang liver cells. A: Cells were treated with butin for 1 h prior to treatment with H_2O_2 for 30 min. The intracellular ROS was detected by DCF-DA using a fluorescence spectrophotometer. *Significantly different from control ($P < 0.05$) and **significantly different from H_2O_2-treated V79-4 lung fibroblasts ($P < 0.05$). #Significantly different from control ($P < 0.05$) and ##significantly different from H_2O_2-treated human Chang liver cells ($P < 0.05$). B: Cells were treated with butin for 1 h prior to treatment with H_2O_2 and incubated for 24 h. Cell viability was measured using the MTT assay. *Significantly different from control ($P < 0.05$) and **significantly different from H_2O_2-treated V79-4 lung fibroblasts ($P < 0.05$). #Significantly different from control ($P < 0.05$) and ##significantly different from H_2O_2-treated human Chang liver cells ($P < 0.05$). C: After treatment of butin and H_2O_2, cells were allowed to grow for 10 days. The resulting colonies were stained with trypan blue and counted. *Significantly different from control ($P < 0.05$) and **significantly different from H_2O_2-treated V79-4 lung fibroblasts ($P < 0.05$). #Significantly different from control ($P < 0.05$) and ##significantly different from H_2O_2-treated human Chang liver cells ($P < 0.05$). D: Cells were treated with butin for 1 h prior to treatment with 1 μM rotenone. The intracellular ROS was detected by DCF-DA using a fluorescence spectrophotometer. *Significantly different from control ($P < 0.05$) and **significantly different from rotenone-treated V79-4 lung fibroblasts ($P < 0.05$). #Significantly different from control ($P < 0.05$) and ##significantly different from rotenone-treated human Chang liver cells ($P < 0.05$). E: Cells were treated with butin and exposed to 1 μM rotenone for 24 h. Cell viability was measured using the MTT assay. *Significantly different from control ($P < 0.05$) and **significantly different from rotenone-treated V79-4 lung fibroblasts ($P < 0.05$). #Significantly different from control ($P < 0.05$) and ##significantly different from rotenone-treated human Chang liver cells ($P < 0.05$). F: After treatment of butin and rotenone, cells were allowed to grow for 10 days. The resulting colonies were stained with trypan blue and counted. *Significantly different from control ($P < 0.05$) and **significantly different from rotenone-treated V79-4 lung fibroblasts ($P < 0.05$). #Significantly different from control ($P < 0.05$) and ##significantly different from rotenone-treated human Chang liver cells ($P < 0.05$).

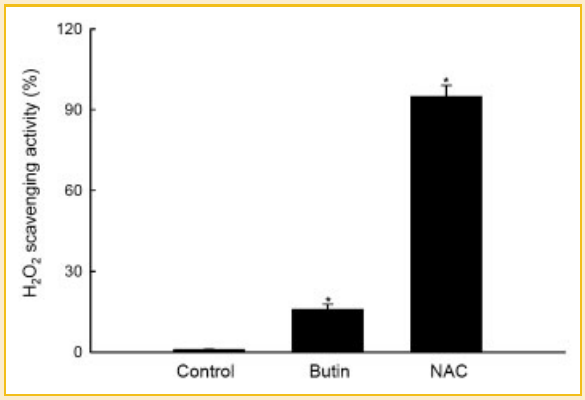

Fig. 6. Effect of butin on hydrogen peroxide scavenging. Scavenging of hydrogen peroxide was assessed spectrophotometrically using ABTS. N-acetyl cysteine (1 mM, NAC) was used as a positive control. *Significantly different from control cells ($P < 0.05$).

responsible for the nuclear translocation of Nrf2, the subsequent binding of Nrf2 to ARE, and the up-regulation of Mn SOD gene expression.

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