Baicalein protects C6 glial cells against hydrogen peroxide-induced oxidative stress and apoptosis through regulation of the Nrf2 signaling pathway

EUN-OK CHOI[1,2], JIN-WOO JEONG[2], CHEOL PARK[3], SU HYUN HONG[5], GI-YOUNG KIM[6], HYE-JIN HWANG[2,4], EUN-JU CHO[1] and YUNG HYUN CHOI[2,5]

[1]Department of Food and Nutrition, College of Human Ecology, Pusan National University, Busan 609-735; [2]Anti-Aging Research Center and Blue-Bio Industry RIC, Departments of [3]Molecular Biology and [4]Food and Nutrition, College of Natural Sciences and Human Ecology, Dongeui University, Busan 614-714; [5]Department of Biochemistry, Dongeui University College of Korean Medicine, Busan 614-052; [6]Laboratory of Immunobiology, Department of Marine Life Sciences, Jeju National University, Jeju 690-756, Republic of Korea

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Abstract. Baicalein, a flavonoid originally obtained from the roots of *Scutellaria baicalensis* Georgi, has been reported to possess various biological properties. Although several studies have demonstrated the anti-oxidative activity of baicalein, its neuroprotective mechanisms have not been clearly established. The present study aimed to detect the effects of baicalein against hydrogen peroxide (H_2O_2)-induced neuronal damage in C6 glial cells and to investigate the molecular mechanisms involved in this process. The results demonstrated that baicalein effectively inhibited H_2O_2-induced growth and reactive oxygen species (ROS) generation. We noted that Baicalein also attenuated the H_2O_2-induced formation of comet tail, phosphorylation of p-γH2A.X, loss of mitochondrial membrane potential (MMP or ΔΨm), and changes to apoptosis-related protein expression, which suggests that it can prevent H_2O_2-induced cellular DNA damage and apoptotic cell death. Furthermore, treatment with baicalein effectively induced the expression of nuclear factor-erythroid 2-related factor 2 (Nrf2) as well as heme oxygenase-1 (HO-1) and thioredoxin reductase 1 (TrxR1) in a concentration and time-dependent manner. Moreover, the protective effects of baicalein against H_2O_2-induced DNA damage and apoptosis were abolished by zinc protoporphyrin (ZnPP) IX, a HO-1 inhibitor, and auranofin, a TrxR inhibitor. In addition, we noted that the cytoprotective effects of baicalein were attenuated by transient transfection with Nrf2-specific small interfering RNA (siRNA). The findings of our present study suggest that baicalein enhances cellular antioxidant defense capacity through the inhibition of ROS generation and the activation of the Nrf2 signaling pathway, thus protecting C6 cells from H_2O_2-induced neuronal damage.

Correspondence to: Professor Yung Hyun Choi, Department of Biochemistry, Dongeui University College of Korean Medicine, 52-57 Yangjeong-ro, Busanjin, Busan 614-052, Republic of Korea
E-mail: choiyh@deu.ac.kr

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Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), are generated during normal cellular metabolism, and they play critical roles in the signal transmission mechanism (1,2). However, H_2O_2 also exerts genotoxicological effects, as it produces new free radicals and causes damage to the main cellular components (3,4). Moreover, the excess production of ROS increases oxidative damage, leading to cellular dysfunction and cell death (5,6). Oxidative stress is mainly caused by neurodegenerative disorders, including dementia, which has attracted significant attention; as brain cells are damaged, the interaction between neurons is inhibited, resulting in disability of memory and cognitive functions (7,8). Glial cells, which outnumber neurons in the brain, are non-neuronal cells that maintain homeostasis as well as supporting and protecting neurons in the central nervous system (CNS) (9). Therefore, the attenuation of oxidative stress and the inhibition of apoptosis in glial cells are critical for protection from neurodegenerative disorders. Thioredoxin reductase 1 (TrxR1) is a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidoreductase that decreases the active site of cytosolic thioredoxin-1 (Trx1) from the disulfide form to the biologically active dithiol form (10). Trx1 contributes to antioxidative activity by donating electrons to peroxiredoxins for the reduction of H_2O_2 (11,12).

In addition, the heme oxygenase (HO) pathway has been reported to be active in the CNS and to operate as an underlying protective mechanism of cells exposed to an oxidizing agent (13). Moreover, the enhancement of HO-1 protein

expression has been associated with protection against stress conditions, such as oxidative stress (14). HO-1 gene expression is mainly regulated by the nuclear factor-erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway, and the induction of this enzyme protects cells against oxidative stress-induced damage and apoptosis (15,16). The Kelch-like ECH-associated protein-1 (Keap1) controls Nrf2 activation and nuclear accumulation by binding to the Nrf2 protein and targeting it for proteosomal degradation (17). Nrf2 is released from Keap1 repression under conditions of oxidative stress, and it translocates to the nucleus where it increases several antioxidant genes, such as HO-1 and TrxR1 (15,18,19). Thus, we suggest that the activation of Nrf2 is crucial for the cytoprotective mechanism against oxidative stress.

Baicalein, a flavonoid originally obtained from the roots of a traditional Chinese herb, *Scutellaria baicalensis* Georgi, has been widely used in the treatment of inflammation, hypertension, cardiovascular disease, bacterial infection and cancer (20,21). Moreover, the neuroprotective effects of baicalein have been demonstrated in several experimental models, such as models of Alzheimer's disease (22,23), ischemic stroke (24,25), and Parkinson's disease (26,27). However, an association between the Nrf2 pathway with the neuroprotective role of baicalein against oxidative stress in glial cells has not previously been demonstrated. Thus, in the present study, we investigated the neuroprotective effect exerted by baicalein and also its mechanisms which protect against H_2O_2-induced neuronal damage in a model using C6 glial cells.

Materials and methods

Reagents and antibodies. In the present study, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) were all purchased from Welgene, Inc. (Daegu, Korea). Baicalein (5,6,7-trihydroxyflavone; purity 98%), H_2O_2, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine-iodide (JC-1), zinc protoporphyrin (ZnPP) IX and auranofin were all purchased from Sigma Chemical Co. (St. Louis, MO, USA); 2',7'-dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes, Inc. (Eugene, OR, USA); primary antibody against phosphorylated histone variant H2A.X (p-γH2A.X; #9718) was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA); β-actin (sc-1616), poly(ADP-ribose) polymerase (PARP; sc-7150), X-linked inhibitor of apoptosis protein (XIAP; sc-11426), Nrf2 (sc-13032), Keap1 (sc-15246), HO-1 (sc-7696) and TrxR1 (sc-28321) antibodies were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The secondary antibodies against goat anti-rabbit IgG-HRP (sc-2004), goat anti-mouse IgG-HRP (sc-2005) and bovine anti-goat IgG-HRP (sc-2350) were all purchased from Santa Cruz Biotechnology, Inc.

Cell culture and treatment. C6 glial cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in DMEM supplemented with 10% heat-inactivated FBS and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37˚C in a humidified incubator in an atmosphere of 5% CO_2 in air. Baicalein was dissolved in dimethyl sulfoxide (DMSO) as a stock solution at 100 mM, which was then diluted with DMEM to the desired concentration prior to use. H_2O_2 was diluted in DMEM to a final concentration of 0.5 mM.

Cell viability assay. C6 cells were seeded in 6-well plates at a density of $2x10^5$ cells/well. After incubation for 24 h, the cells were pretreated with various concentrations of baicalein for 1 h prior to incubation in the absence or presence of H_2O_2 for 24 h. An MTT working solution (0.5 mg/ml) was added to the culture plates and incubated for 3 h at 3˚C. The culture supernatant was removed from the wells, and DMSO was added to dissolve the formazan crystals completely. The absorbance of each well was measured at 540 nm using a microplate reader (Dynatech Laboratories, Chantilly VA, USA). The effect of baicalein on cell growth was assessed as the percentage of cell viability, in which the vehicle-treated cells (0.05% DMSO) were considered 100% viable.

Comet assay (single-cell gel electrophoresis). The cell suspension was mixed with 0.5% low melting agarose (LMA) at 37˚C, and the mixture was spread on fully frosted microscopic slides pre-coated with 1% normal melting agarose. After solidification of the agarose, the slides were covered with 0.5% LMA and then immersed in a lysis solution (2.5 M NaCl, 100 mM Na-ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, 1% Triton X-100, and 10% DMSO, pH 10.0) for 30 min at 4˚C. The slides were then placed in a CometAssay Electrophoresis System Starter Kit (KORMED, Seongnam, Korea) containing 300 mM NaOH and 10 mM Na-EDTA (pH 13.00) for 40 min to allow for DNA unwinding and examination of alkali-labile damage. An electrical field was then applied (300 mA, 20 V) for 20 min at 4˚C to draw the negatively charged DNA toward the anode. After electrophoresis, the slides were washed three times for 5 min at 4˚C in a neutralizing buffer (0.4 M Tris, pH 7.5). The slides were then stained with 20 μg/ml PI and observed using a fluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany). The images were also analyzed using an image analysis system (Komet 5.5; Kinetic Imaging, Liverpool, UK) to evaluate the degree of DNA damage. The tail length and the tail moment were used as measures of the extent of DNA damage. One hundred cells were randomly selected from one sample (two slides were made for one sample, 50 randomly selected cells per slide) and then measured. We calculated the values of the mean tail length for each sample and the percentage values of the cells in five ranges of tail length: undamaged cells without a tail, cells with a tiny tail, cells with a dim tail (28), cells with a clear tail, and only tail. The ranges of tail length and tail moment were divided arbitrarily in the present study.

Measurement of intracellular ROS. To assess the generated ROS, the cells were incubated with 10 μM DCFH-DA for 20 min at room temperature in the dark to monitor ROS production. ROS levels in the cells were monitored with a flow cytometer (Becton-Dickinson, San Jose, CA, USA) using CellQuest Pro software, as previously described (29).

Measurement of apoptosis. For quantitative assessment of the induced cell apoptotic rate, a fluorescein-conjugated Annexin V (Annexin V-FITC) staining assay was performed according to the manufacturer's instructions (BD Biosciences

Pharmingen, San Jose, CA, USA). Briefly, the cells in each sample were stained with 5 μl Annexin V-FITC and 5 μl PI. After incubation for 15 min at room temperature in the dark, the degree of apoptosis was quantified by flow cytometer as a percentage of the Annexin V-positive and PI-negative cells (Becton-Dickinson) using CellQuest Pro software (29).

Measurement of mitochondrial membrane potential (MMP; $\Delta\Psi m$). The mitochondrial transmembrane electrochemical gradient was measured using JC-1 staining. Briefly, the cells were collected and incubated with 10 μM JC-1 solution for 15 min at 37˚C in the dark. The fluorescence intensity of the red/green ratio was quantified by flow cytometer (Becton-Dickinson) using CellQuest Pro software, as previously described (30).

Protein extraction and western blot analysis. After removing the media, the cells were washed with ice-cold phosphate-buffered saline (PBS) and gently lysed for 20 min in an ice-cold lysis buffer (40 mM Tris, pH 8.0, 120 mM, NaCl, 0.5% nonidet-P40, 0.1 mM sodium orthovanadate, 2 μg/ml leupeptin, and 100 μg/ml phenymethylsulfonyl fluoride). The supernatants were collected, and the protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For western blot analysis, equal amounts of protein extracts were denatured by boiling at 95˚C for 5 min in a sample buffer [0.5 M Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.1% bromophenol blue and 10% β-mercaptoethanol] at a ratio of 1:1. The samples were stored at -80˚C or immediately used for western blot analysis. Aliquots containing 30 μg of total protein were separated by denaturing SDS-polyacrylamide gel electrophoresis and transferring electrophoretically to nitrocellulose membranes (Amersham Biosciences, Arlington Heights, IL, USA). The membranes were then blocked with 5% skim milk and incubated overnight at 4˚C with primary antibodies, probed with enzyme-linked secondary antibodies for 1 h at room temperature, and detected using an enhanced chemiluminescence (ECL) detection system (both from Amersham Biosciences).

Small interfering RNA (siRNA) transfection. Nrf2 siRNA and control siRNA were both purchased from Santa Cruz Biotechnology, Inc. The siRNAs were transfected into C6 cells according to the manufacturer's instructions using Lipofectamine® RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA). The cells were seeded in 6-well culture plates for transfection and incubated with 50 nM control or Nrf2 siRNA for 24 h in serum-free OPTI-MEM media (Invitrogen). After 24 h transfection, the cells were incubated under the experimental conditions.

Statistical analysis. Data are expressed as the means ± standard error of the mean (SEM). The comparison between groups was undertaken using ANOVA, and significance was analyzed using Duncan's multiple range test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Baicalein prevents H_2O_2-induced growth inhibition in C6 cells. We first determined the effect of baicalein on the viability of C6 cells using the MTT assay. As shown in Fig. 1A, the results demonstrated that baicalein (25-300 μM) alone for 24 h had no detectable effect on C6 cell survival. To determine the protective effects of baicalein on H_2O_2-induced cytotoxicity in C6 cells, the cells were pre-treated with baicalein for 1 h and exposed to H_2O_2 for an additional 24 h. As shown in Fig. 1B, the treatment of C6 cells with 0.5 mM H_2O_2 for 24 h resulted in approximately a 43% loss of cellular viability compared with the control cells. However, the cytotoxic effect of H_2O_2 was blocked by pretreating cells with baicalein (25-100 μM).

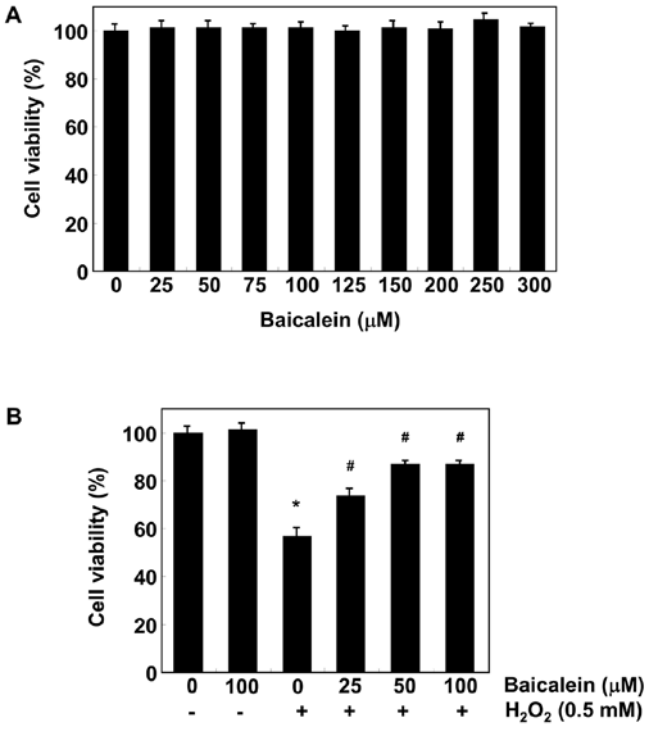

Figure 1. Effects of baicalein on H_2O_2-induced growth inhibition in C6 cells. The cells were treated with various concentrations of baicalein for 24 h (A) or pretreated with the indicated concentrations of baicalein for 1 h, and further incubated in the presence and absence of 0.5 mM H_2O_2 for 24 h (B). Cell viability was estimated using an MTT reduction assay. The data are represented as the means ± SEM obtained from three independent experiments (*P<0.05 compared with the untreated control group; $^\#$P<0.05 compared with H_2O_2-treated group).

Baicalein attenuates H_2O_2-induced ROS generation and DNA damage in C6 cells. To examine the inhibitory effect of baicalein on H_2O_2-induced ROS production, C6 cells were stimulated with 0.5 mM H_2O_2 for 30 min in the presence and absence of baicalein, and the intracellular ROS level was then determined. As expected, increased ROS generation was detected in cells after stimulation with H_2O_2 alone (Fig. 2A). However, pretreatment with baicalein significantly reduced H_2O_2-induced ROS production. We further examined the effects of baicalein on DNA damage caused by H_2O_2 using a Comet assay and western blot analysis. As indicated in Fig. 2B, treatment with H_2O_2 alone significantly increased the number of DNA breaks, resulting in an increase in fluorescence intensity in the tails of the comet-like structures, which was associated with an increase in the tail length and tail moment (Table I). However, these phenomena were prevented by pretreatment with baicalein. In addition, the western blot analysis revealed that the

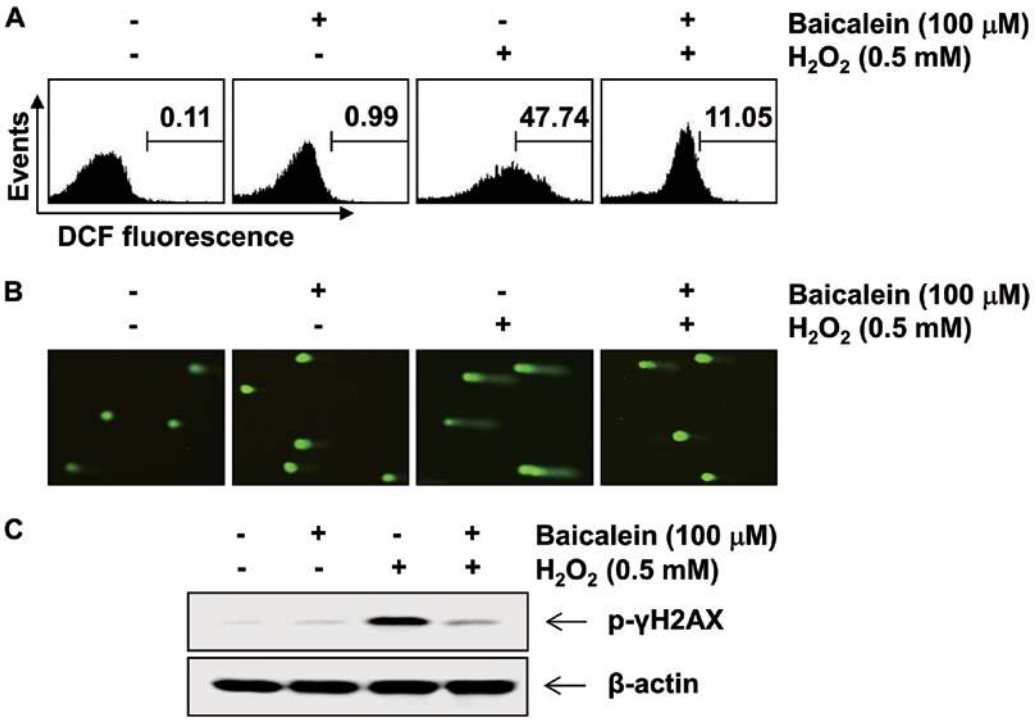

Figure 2. Effects of baicalein on H_2O_2-induced reactive oxygen species (ROS) generation, DNA damage, and γH2A.X phosphorylation in C6 cells. The cells were pretreated with the indicated concentration of baicalein for 1 h and then stimulated with or without 0.5 mM H_2O_2 for 30 min. The cells were incubated at 37˚C in the dark for 20 min with a culture medium containing 10 μM DCFH-DA to monitor ROS production. ROS generation was measured by flow cytometry (A). The cells were pretreated with baicalein for 1 h and further incubated in the presence and absence of 0.5 mM H_2O_2 for 24 h. The comet assay was performed to detect cellular DNA damage, and representative photographs of the comets were taken using a fluorescence microscope (x200 original magnification) (B). Cellular proteins isolated in cells grown under the same conditions as (B) were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with specific antibodies against p-γH2A.X and β-actin (C), as a loading control.

Table I. Preventive effect exerted by baicalein on H_2O_2-induced DNA damage in C6 cells (means ± SEM).

Compounds	Scored cells	Tail moment	Tail length
Control	100	2.27±0.81	64.4±4.97
Baicalein (100 μM)	100	1.50±1.23	53.16±8.13
H_2O_2 (0.5 mM)	100	42.09±4.34	156.55±9.04
Baicalein+H_2O_2	100	4.75±1.72	97.66±6.21

level of p-γH2A.X, a classic marker of DNA double-strand break formation (31), in C6 cells treated with H_2O_2 alone was markedly increased. However, pretreatment with baicalein was found to inhibit the increase in p-γH2A.X expression caused by treatment with H_2O_2 (Fig. 2C).

Baicalein reduces H_2O_2-induced loss of MMP and apoptosis in C6 cells. As mitochondrial permeability is critical for the oxidative stress-induced apoptotic pathway (32), we evaluated the effect of baicalein on the MMP of C6 cells using flow cytometry. After incubation with H_2O_2 alone, the loss of MMP was markedly increased compared to the untreated control (Fig. 3A), which indicated mitochondrial damage and dysfunction. By contrast, pretreatment with baicalein effectively prevented the loss of MMP induced by H_2O_2 in a concentration-dependent manner. We also examined the ability of baicalein to protect against H_2O_2-triggered C6 cell apoptosis using Annexin V/PI-staining. The flow cytometry results indicated that the percentage of apoptotic cells treated with 0.5 mM H_2O_2 was approximately 51% (Fig. 3B), which was significantly reduced by pretreatment with baicalein. Furthermore, we determined the effects of baicalein on the expression of apoptosis-related proteins PARP and XIAP. As illustrated in Fig. 3C, the degradation of PARP and the downregulation of XIAP were observed in H_2O_2-treated C6 cells. However, pretreatment with baicalein effectively protected against these changes.

Baicalein upregulates Nrf2, HO-1 and TrxR1 expression in C6 cells. The fact that Nrf2 signaling regulates cellular antioxidant response has been well documented (33). We sought to determine whether signaling was associated with baicalein-mediated neuroprotection. Western blot analysis indicated that treating C6 cells with baicalein induced the expression of Nrf2 protein in a concentration- and time-dependent manner, which was associated with the downregulation of Keap1 (Fig. 4). Concomitant with the induction of Nrf2, the levels of HO-1 and TrxR1 were also markedly increased after treatment with baicalein in C6 cells.

Induction of HO-1 and TrxR1 is involved in the protective effect exerted by baicalein against H_2O_2 treatment in C6 cells. In order to investigate the role of HO-1 induction in the baicalein-mediated neuroprotective effects exerted against oxidative stress, we inhibited HO-1 activity using ZnPP, a specific inhibitor of HO-1. As shown in Fig. 5A and B, in the presence of ZnPP, the protective effects of baicalein on H_2O_2-induced

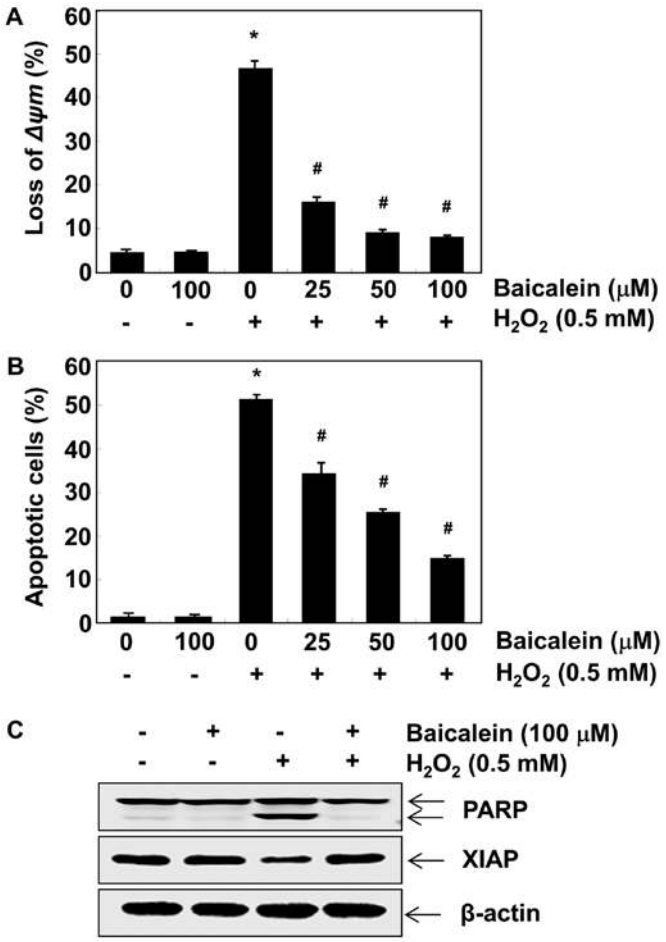

Figure 3. Effects of baicalein on H_2O_2-induced loss of mitochondrial membrane potential (MMP), apoptosis, poly(ADP-ribose) polymerase (PARP) degradation, and X-linked inhibitor of apoptosis protein (XIAP) inhibition in C6 cells. The cells were pretreated with various concentrations of baicalein for 1 h, and then incubated with and without 0.5 mM H_2O_2 for 24 h (A and B). The cells were collected and incubated with 10 μM JC-1 for 15 min at 37˚C in the dark. The cells were then washed once with PBS, and mean JC-1 fluorescence intensity was detected using a flow cytometer (A). The cells were stained with FITC-conjugated Annexin V and PI for flow cytometric analysis (B). The percentage of apoptotic cells was determined by counting the percentage of Annexin V-positive cells. The data are represented as the means ± SEM values obtained from three independent experiments (*P<0.05 compared with untreated control group; $^\#$P<0.05 compared with H_2O_2-treated group). Cells were pretreated with 100 μM baicalein for 1 h and then incubated with and without 0.5 mM H_2O_2 for 24 h. The cells were lysed, and the cellular proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with specific antibodies against PARP and XIAP (C). β-actin was used as the loading control.

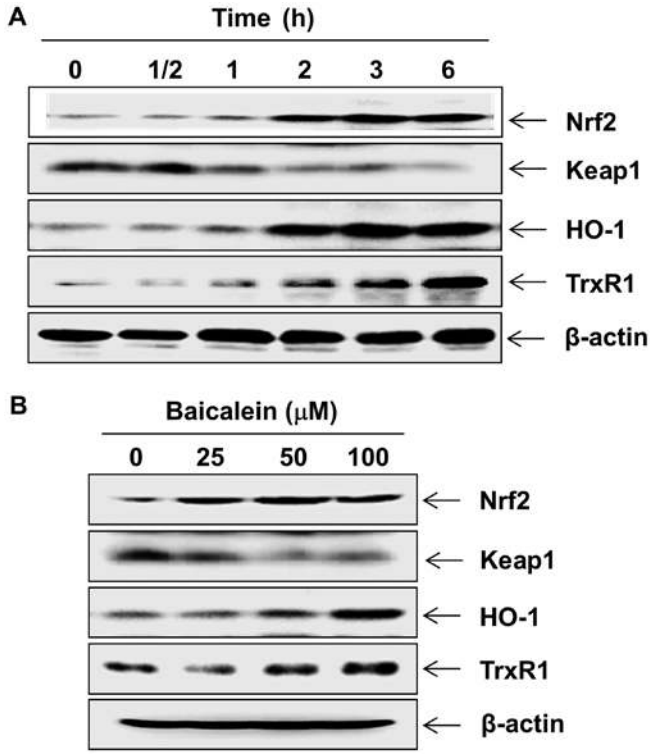

Figure 4. Effects of baicalein on the levels of nuclear factor-erythroid 2-related factor 2 (Nrf2), Kelch-like ECH-associated protein-1 (Keap1), heme oxygenase-1 (HO-1), and thioredoxin reductase 1 (TrxR1) in C6 cells. The cells were incubated for the indicated periods of time with 100 μM baicalein (A) or with various concentrations of baicalein for 6 h (B). Cellular proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with specific antibodies against Nrf2, Keap1, HO-1 and TrxR1. β-actin was used as the loading control.

loss of MMP and reduction of cell viability were significantly attenuated. Furthermore, ZnPP blocked the protection provided by baicalein against H_2O_2-induced degradation of PARP, phosphorylation of γH2A.X, and downregulation of XIAP as well as apoptosis (Fig. 5C and D). To determine whether the protective effect of baicalein was related to its inductive effect on TrxR1 expression, we blocked TrxR1 activity using auranofin, a selective TrxR1 inhibitor. The results indicated that auranofin also reversed the inhibition of loss of MMP and apoptotic activity caused by baicalein in H_2O_2-stimulated C6 cells (Fig. 6).

Baicalein upregulates HO-1 and TrxR1 expression via Nrf2 activation in C6 cells. It has previously been reported that HO-1 and TrxR1 are regulated through the Nrf2 cascade (19,34-36). We developed an Nrf2 gene knockdown model using siRNA transfection to demonstrate the contribution of Nrf2 signaling to the negative effects of baicalein on H_2O_2-induced cytotoxicity. Western blot analysis indicated that Nrf2 siRNA reduced the baicalein-induced expression of Nrf2 compared with untransfected control and control siRNA-transfected cells) (Fig. 7A). The baicalein-induced expression of HO-1 and TrxR1 was also blocked by Nrf2 siRNA, which is evidence that the augmentation of HO-1 and TrxR1 was mediated by Nrf2. Furthermore, Nrf2 siRNA significantly attenuated the protective effects of baicalein against the H_2O_2-induced reduction of cell viability and apoptosis (Fig. 7B and C), which was associated with the disappearance of the potential of baicalein to protect against H_2O_2-induced PARP degradation, γH2A.X phosphorylation and XIAP reduction (Fig. 7D).

Discussion

The excessive production of ROS, which causes oxidative damage to proteins, lipids and DNA, is one of the most prominent factors related to neurodegeneration (37). The mitochondrial electron transport system is a major source of intracellular ROS generation (32), whereby the mitochondria play a pivotal role in the process of ROS-mediated cell death. Moreover, H_2O_2 directly induces mitochondrial dysfunction, followed by a rapid efflux of intracellular ROS, which increases

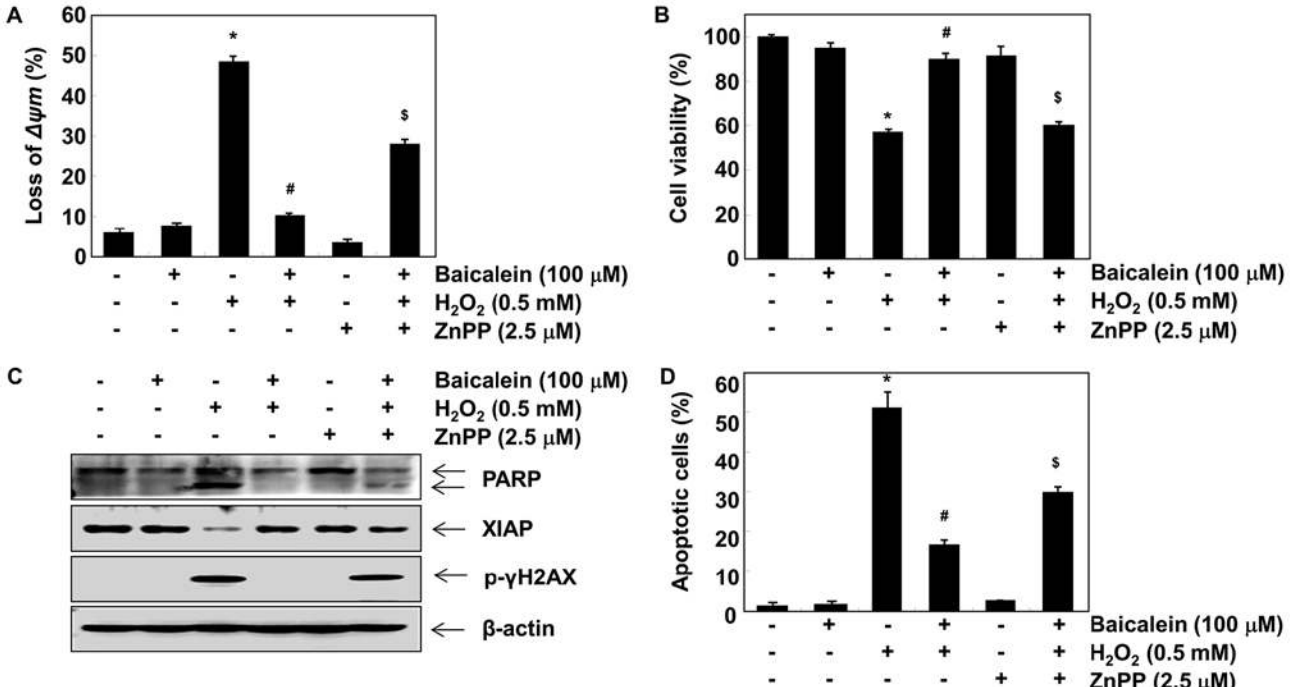

Figure 5. Effects of a heme oxygenase-1 (HO-1) inhibitor on the baicalein-mediated attenuation of mitochondrial membrane potential (MMP) loss, growth inhibition, and apoptosis caused by H_2O_2 in C6 cells. The cells were pretreated for 1 h with 100 μM baicalein and then treated for 24 h with or without 0.5 mM H_2O_2 in the absence or presence of 2.5 μM zinc protoporphyrin (ZnPP). After incubation with 10 μM JC-1 for 20 min, JC-1 fluorescence intensity was detected using a flow cytometer (A). Cell viability was assessed using an MTT reduction assay (B). Cellular proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with specific antibodies against poly(ADP-ribose) polymerase (PARP), X-linked inhibitor of apoptosis protein (XIAP) and p-γH2A.X (C). β-actin was used as the loading control. To quantify the degree of apoptosis, the cells were stained with FITC-conjugated Annexin V and propidium iodide (PI) for flow cytometric analysis (D). The data are represented as the means ± SEM values obtained from three independent experiments (*P<0.05 compared with untreated group; $^\#$P<0.05 compared with H_2O_2-treated group; $^\$$P<0.05 compared with H_2O_2 and baicalein-treated group).

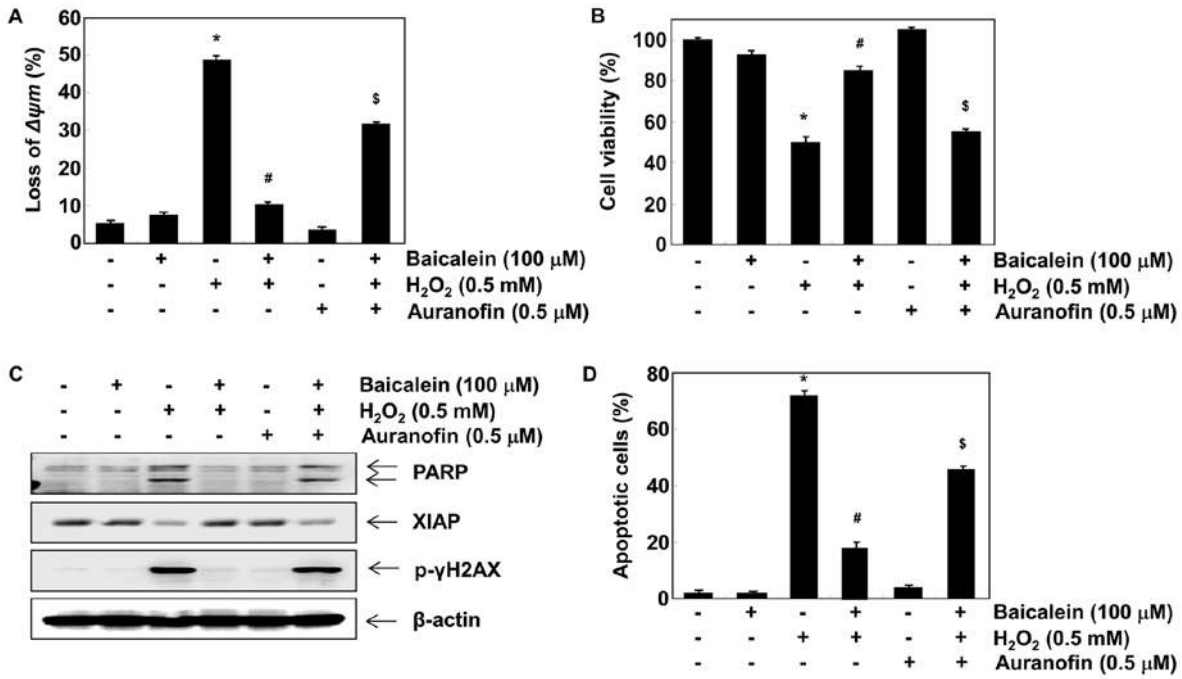

Figure 6. Effects of a thioredoxin reductase 1 (TrxR1) inhibitor on the baicalein-mediated attenuation of loss of mitochondrial membrane potential (MMP), growth inhibition, and apoptosis by H_2O_2 in C6 cells. The cells were pretreated for 1 h with 100 μM baicalein and then treated for 24 h with or without 0.5 mM H_2O_2 in the absence or presence of 0.5 μM auranofin. After incubation with 10 μM JC-1 for 20 min, JC-1 fluorescence intensity was detected using a flow cytometer (A). Cell viability was assessed using an MTT reduction assay (B). Cellular proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with specific antibodies against poly(ADP-ribose) polymerase (PARP), X-linked inhibitor of apoptosis protein (XIAP) and p-γH2A.X (C). β-actin was used as the loading control. To quantify the degree of apoptosis, the cells were stained with FITC-conjugated Annexin V and propidium iodide (PI) for flow cytometry analysis (D). The data are represented as the means ± SEM values obtained from three independent experiments (*P<0.05 compared with the untreated group; $^\#$P<0.05 compared with H_2O_2-treated group; $^\$$P<0.05 compared with H_2O_2- and baicalein-treated group).

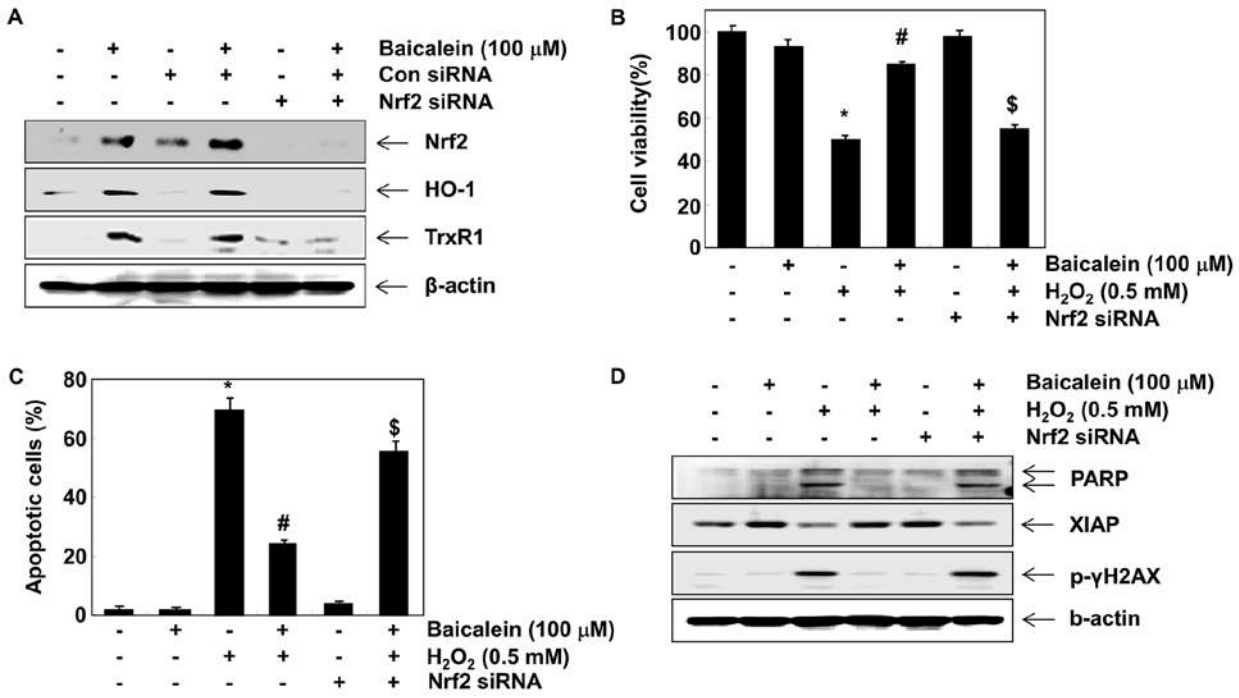

Figure 7. Nuclear factor-erythroid 2-related factor 2 (Nrf2)-mediated induction of heme oxygenase-1 (HO-1) and thioredoxin reductase 1 (TrxR1) by baicalein in C6 cells. The cells were transfected with a control (Con siRNA, as a negative control for RNA interference) and Nrf2 siRNA. After 24 h, the cells were treated with 100 μM baicalein for 1 h and then stimulated with or without 0.5 mM H_2O_2 for 24 h. Cellular proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies (A and D). β-actin was used as the loading control. Cell viability was assessed using an MTT reduction assay (B). To quantify the degree of apoptosis, the cells were stained with FITC-conjugated Annexin V and propidium iodide (PI) for flow cytometry analysis (C). The results are in mean ± SEM values obtained from three independent experiments (*P<0.05 compared with the untreated group; $^\#$P<0.05 compared with H_2O_2-treated group; $^\$$P<0.05 compared with H_2O_2- and baicalein-treated group).

the permeabilization and depolarization of the mitochondrial membrane. This event likely facilitates the rapid disruption of MMP and the release of apoptosis-inducing factors that activate the caspase-dependent signaling cascades in the induction of apoptosis (38). Therefore, the search for functional food or bioactive compounds that act against oxidative stress is critical for the prevention and cure of neurodegenerative disorders. The purpose of the present study was to determine whether baicalein blocked H_2O_2-induced oxidative stress in C6 cells or not. The results demonstrated that treatment of C6 cells with H_2O_2 caused the marked intracellular accumulation of ROS and the loss of MMP, and further inhibited cell survival, leading to apoptosis. However, when the C6 cells were pre-treated with baicalein, the H_2O_2-induced generation of ROS, loss of MMP, reduction of cell viability, and apoptosis were significantly attenuated, as previously reported in studies on other neuron-like cells (27). Thus, we presume that baicalein improves mitochondrial function through eliminating the overproduction of ROS induced by H_2O_2 and thereby reducing H_2O_2-induced apoptosis. In addition, our results showed that H_2O_2 treatment increased DNA tail moment and length in the comet assay and expression of p-γH2A.X, which are widely used markers for the detection of DNA damage (31). However, in the present study, both events were abolished by baicalein, indicating that it protected against H_2O_2-induced apoptosis in the C6 cells by reducing the amount of DNA damage caused by the destructive impact of oxidative stress in the C6 cells.

Apoptosis, which is programmed cell death, is a tightly regulated cell suicide response that facilitates the correct development and homeostasis of multicellular organisms; in mammalian cells, two major apoptotic pathways (the cell death receptor-mediated and mitochondrial-mediated apoptotic pathways) have been studied (39). Previous research has indicated that the impairment of MMP and ROS generation is closely linked to the initiation of caspase-dependent apoptotic signaling in many types of cells (5). Caspases, which are a family of cysteine acid proteases, are the central regulators of the execution of cell death in response to various apoptotic stimuli (40). In the final stage of apoptosis, both pathways induce the activation of executioner caspases, such as caspase-3 and -7, which results in the degradation of substrate proteins, such as PARP, a biochemical hallmark of cell apoptosis (39). The activation of caspases may also be regulated by a variety of proteins, including members of the inhibitor of apoptosis proteins (IAP) family, which promote cell survival after a wide variety of apoptotic stimuli elicited via intrinsic and extrinsic pathways through selectively binding with caspases, thus inhibiting caspase activity and apoptosis (39). Moreover, previous research has indicated that H_2O_2 induces apoptosis through the activation of caspases in addition to the inhibition of IAP family proteins (41). In the present study, western blot analyses revealed that baicalein effectively blocked the H_2O_2-induced cleavage of PARP, a downstream target protein of the activated caspase-3 in C6 cells. Under the same conditions, baicalein also rescued the H_2O_2-induced downregulation of XIAP, a representative member of the IAP family, compared with the control. Although further molecular studies are needed, our findings indicate that baicalein potentially prevents H_2O_2-induced apoptosis through the inactivation of caspase cascades in C6 cells.

Previous research has suggested that Nrf2, a master cellular sensor for oxidative stress, and its repressor, Keap1, play indispensable roles in protecting a variety of tissues from a wide array of toxic insults, including oxidative stress. Under normal conditions, Nrf2 is inactive and bound in the cytosol by Keap1 (35). The dissociation of Nrf2 from Keap1 is a prerequisite for nuclear translocation, and the subsequent DNA binding of Nrf2 is necessary to regulate the inducible expression of cytoprotective phase II enzymes (15,18). In the present study, we found that baicalein increased Nrf2 protein expression and decreased Keap1 in a concentration- and time-dependent manner in C6 cells. These results are consistent with those of previous studies (42-44), and were associated with the induction of HO-1 and TrxR1. However, the inhibition of HO-1 function using ZnPP significantly weakened the inhibitory effects of baicalein on H_2O_2-induced MMP loss, growth inhibition, and apoptosis by blocking PARP cleavage, XIAP inhibition, and γH2A.X phosphorylation. Moreover, pre-treatment with a TrxR1 inhibitor also markedly abrogated the protective effects of baicalein against H_2O_2-induced oxidative stress. In addition, the knockdown of Nrf2 by Nrf2-targeted siRNA completely cancelled out baicalein-induced HO-1 and TrxR1 expression, suggesting that Nrf2 is a critical upstream regulator of the baicalein-mediated induction of HO-1 and TrxR1. Furthermore, the removal of Nrf2 also halted the baicalein-induced restoration of H_2O_2-mediated growth inhibition and the apoptosis of C6 cells. These results suggest that the Nrf2-dependent induction of HO-1 and TrxR1 by baicalein, at least in part, may participate in the protection against oxidative stress in C6 cells.

In conclusion, the results of our present study clearly demonstrated that baicalein exerted a protective effect against H_2O_2-induced DNA damage, growth inhibition, and apoptosis in C6 cells. Baicalein also successfully suppressed the accumulation of intracellular ROS, leading to substantial regain of MMP, at least in part, through the activation of Nrf2 signaling and the induction of HO-1 and TrxR1. These findings suggest that baicalein has a potential neuroprotective value as an antioxidant agent.

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