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Lignan compounds and 4,4′-dihydroxybiphenyl protect C2C12 cells against damage from oxidative stress

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Abstract

Lignan compounds are known to have various biological activities, especially antioxidative effects. We investigated whether lignan compounds show antioxidative activity in myoblast C2C12 cells. Among 14 lignan compounds investigated, two lignans containing two phenolic functional groups, namely Gomisin J and GR-12, prevented hydrogen peroxide (H_2O_2)-induced cell death. A simple compound, 4,4′-dihydroxybiphenyl, which was found to be a common component of Gomisin J and GR-12, also largely prevented H_2O_2-induced cell death and almost completely prevented H_2O_2-induced increases in p38 MAPK phosphorylation. Our present results provide a useful in vitro system for clarifying the molecular mechanisms of lignan-mediated antioxidative effects and evaluating lead molecules toward the development of therapeutic drugs.

Keywords: Gomisin J; GR-12; Furoguaiacin; 4,4′-Dihydroxybiphenyl; Oxidative stress; C2C12

Reactive oxygen species (ROS) are oxygen free radicals that possess one or more unpaired electron(s). These unstable molecules, which have electron(s) available to react with various substrates, strongly damage various biological molecules, including proteins, lipids, and DNA, thereby resulting in further damage to cells and tissues [1–3]. ROS are normal products of several natural processes, such as oxygen metabolism and the inflammatory response [4]. As an example, ATP synthesis in the electron transfer system of mitochondria results in the generation of several radicals. ROS have been implicated in many other events, such as signal transductions and the aging process, as well as in many diseases, including Parkinson's disease, Alzheimer's disease, and cancer [5–7].

In skeletal muscle, ROS can be produced both exogenously and endogenously [8]. Acute exercise has been shown to lead to necrotic injury of skeletal muscle cells, which can be attributed to ROS [9], whereas mild and chronic exercise may induce antioxidative effects [10]. On the other hand, hindlimb immobilization was found to cause an increase in endogenous hydrogen peroxide (H_2O_2) generation in the cytoplasm [11]. Many lines of evidence have shown that dystrophic muscle cells are exposed to oxidative stress [12,13]. Muscle atrophy occurs under conditions of reduced muscle activity, such as denervation, hindlimb suspension, space flight and bed rest, and also in numerous diseases, such as cancer, diabetes mellitus, and sepsis [14–16]. On these diverse occasions, elevated protein degradation activity through the ubiquitin–proteasome pathway is observed [16]. Treatment of muscle cells with H_2O_2 also causes muscle protein degradation via the ubiquitin–proteasome pathway [17]. These data suggest that the effects of ageing in muscle, including decreases in the muscle mass, strength, tension, and velocity, may result from the accumulation of ROS over a long period of time.

Lignan compounds in the plant kingdom constitute a large and diverse group of phenylpropanoid dimers, in which phenylpropane units are linked by a carbon–carbon bond between positions 8 and 8′. These components are known to have various biological activities, such as

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anticancer, bactericidal, antiviral, anti-inflammatory, neuroprotective, and antioxidative effects [18–21]. However, no evidence of any protective function of lignans for in vitro systems derived from skeletal muscle has been produced.

In the present study, we investigated whether lignan compounds have antioxidative activity in cultured muscle cells, specifically C2C12 murine myoblast cells. In the course of screening 14 lignan compounds, we found that treatment with two lignans, GR-12 and Gomisin J, largely protected against H_2O_2-induced cell death. This study is the first to show an antioxidative effect of natural compounds on myoblast cells.

Materials and methods

Chemicals. Fourteen lignan compounds, including Gomisin A [22], Gomisin J [22], and GR-12, were kindly provided by Tsumura Central Research Institute (Ibaraki, Japan). All the lignan compounds were dissolved in dimethyl sulfoxide (DMSO) as 100 mM stock solutions.

Cell culture. Mouse C2C12 myoblast cells and Neuro2A cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

Measurement of cell viability. C2C12 cells were plated on 96-microwell cell-culture plates (5×10^3 cells/well in 100 μl of medium) and grown for 24 h. Thereafter, H_2O_2 was added either with or without other compounds at the indicated concentrations, and the cells were incubated for another 6 h. Cell viability was evaluated using an MTT assay kit (Roche), while cell cytotoxicity was estimated by measuring the amount of lactate dehydrogenase (LDH) released from damaged cells into the culture media using an LDH assay kit (Promega). Both procedures were carried out according to the manufacturer's instructions.

Western blot analysis. C2C12 cells were washed twice with ice-cold PBS (−) and lysed with lysis buffer containing 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, protease inhibitors (10 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate, and 10 mM β-glycerophosphate). Protein concentrations were determined using the BCA protein assay (Pierce). The proteins were separated in a 10% SDS–PAGE gel and electro-transferred onto a Hybond-P PVDF membrane (Amersham Biosciences). After blocking with 5% BSA dissolved in wash buffer (0.1% Tween 20 in Tris–HCl-buffered saline), the membranes were incubated with anti-phospho-ERK1/2 (Santa Cruz Biotechnology), anti-phospho-JNK, anti-phospho-p38 MAPK, anti-ERK1/2, anti-JNK or anti-p38 MAPK (Cell Signaling Technology) antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The reactive bands were visualized with enhanced chemiluminescence (ECL) substrates.

Statistical analysis. Statistical significance was determined by one-way analysis of variance (ANOVA). If a significant difference was found, the group means were compared by Dunnett's multiple comparison of means test. The IC_{50} values for 4,4′-dihydroxybiphenyl were obtained by nonlinear regression (curve fitting) followed by a sigmoidal dose–response calculation with a variable slope (Prism version 3.0; GraphPad Software).

Results and discussion

First, we investigated whether lignan compounds could protect against oxidative stress-induced cell death in murine myoblast C2C12 cells using the MTT assay. Screening of the 14 lignan compounds revealed that Gomisin J and GR-12 (α-guaiaconic acid, furoguaiacin) significantly protected against the cell death induced by 200 μM H_2O_2 (Figs. 1 and 2A). In contrast, Gomisin A, which was previously reported to have several activities, such as protection against the neurotoxicity induced by glutamine in cortical neuronal cells [21] and in liver regeneration [23], had no positive effect against cell death. The antioxidative activity of Gomisin J with 200 μM H_2O_2 was dose-dependent: $50.4 \pm 1.2\%$ viability at 1 μM, $65.4 \pm 2.5\%$ viability at 5 μM, and $75.5 \pm 1.3\%$ viability at 10 μM (Fig. 2B). On the other hand, GR-12 only showed significant antioxidative activity at 10 μM ($87.5 \pm 2.5\%$ viability; Fig. 2B). C2C12 cell growth was not altered by 6 h of incubation with Gomisin J or GR-12 at 10 μM (data not shown). Another assay system measuring the level of LDH release

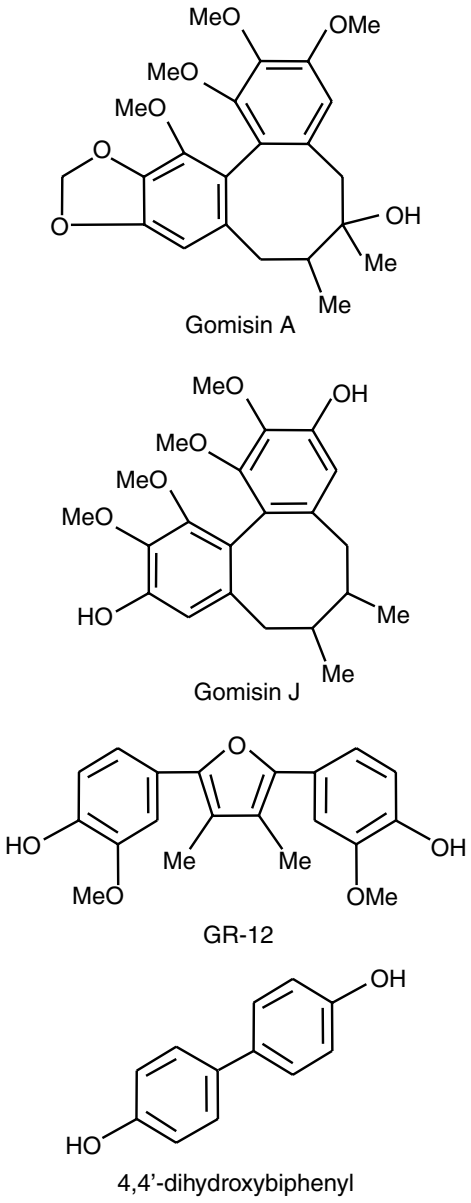

Fig. 1. Chemical structures of the lignan compounds Gomisin A, Gomisin J, GR-12, and 4,4′-dihydroxybiphenyl.

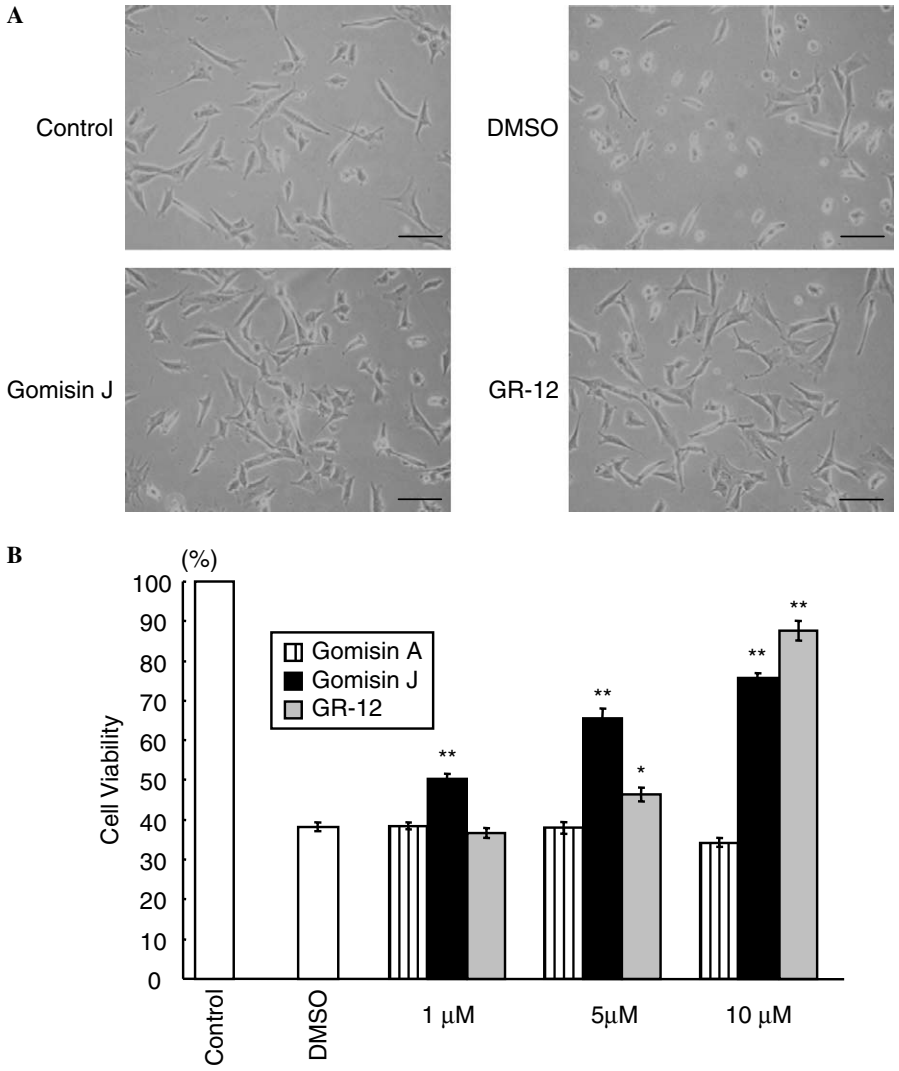

Fig. 2. Gomisin J and GR-12 protect C2C12 cells against H_2O_2-induced cell death. (A) Phase-contrast microscopy images of C2C12 cells after 6 h of treatment with 200 μM H_2O_2 in the absence (0.1% DMSO) or presence of 10 μM Gomisin J or 10 μM GR-12. Control cells were treated with sterilized water. Scale bars = 50 μm. (B) C2C12 cells were treated with 200 μM H_2O_2 for 6 h in the absence (0.1% DMSO) or presence of Gomisin A, Gomisin J or GR-12 at the indicated concentrations. Control cells were not treated with H_2O_2. Cell viability was measured using the MTT assay. The data represent means ± SEM of three or four experiments performed in triplicate. Significant differences from DMSO treatment were detected. $^{*}P < 0.05$, $^{**}P < 0.01$, one-way ANOVA followed by Dunnett's test.

revealed that Gomisin J and GR-12 at 10 μM drastically prevented H_2O_2-induced cell death (Fig. 3A). H_2O_2 reacts with Fe^{2+} to form OH radicals (Fenton reaction), and these OH radicals cause DNA strand breakage. Therefore, we next investigated whether Gomisin J and GR-12 lignan compounds prevent the cytotoxicity caused by Fe^{2+} ions. Gomisin J and GR-12 both provided significant protection against the cell death induced by Fe^{2+} (Fig. 3B).

Among the 14 lignan compounds that were examined for their antioxidative effects, only two compounds, Gomisin J and GR-12, have two hydroxyl functional groups attached to an aromatic hydrocarbon group and are designated polyphenols (Fig. 1). In fact, use of the xanthine/xanthine oxidase-luminol chemiluminescence system previously revealed that Gomisin J is a strong hydroxyl radical scavenger [24], suggesting that the antioxidative effect of Gomisin J may result from hydroxyl radical trapping due to its polyphenolic property. Among the 14 lignans, Pregomisin also has phenolic functional groups, similar to Gomisin J and GR-12. However, Pregomisin did not show any detectable antioxidative activity in C2C12 cells (data not shown). H_2O_2 induces cell death in many types of cells, including mouse neuroblastoma Neuro2A cells [25], but neither Gomisin J nor GR-12 could prevent H_2O_2-induced cell death in Neuro2A cells (data not shown). If the protection in C2C12 cells results from the hydroxyl radical trapping property, both lignans should have prevented cell death in other cells to some extent. Overall, these data imply that GR-12 and Gomisin J may have other antioxidative properties, other than acting as hydroxyl radical scavengers.

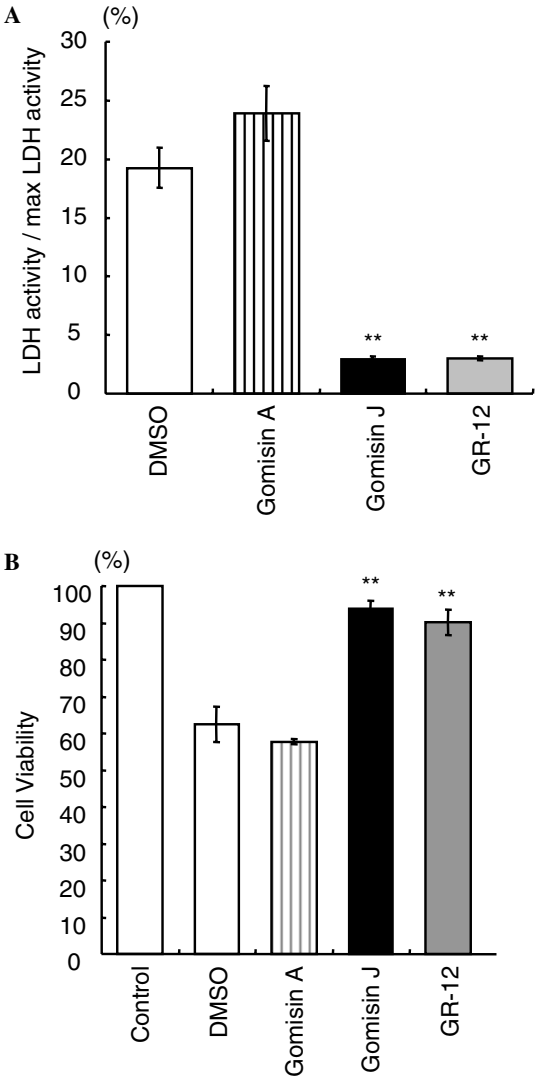

Fig. 3. Antioxidative effects of Gomisin J and GR-12. (A) LDH assay. C2C12 cells were treated with 200 μM H_2O_2 in the absence (0.1% DMSO) or presence of 10 μM of each lignan. (B) C2C12 cells were treated with 200 μM Fe^{2+} in the absence (0.1% DMSO) or presence of 10 μM Gomisin A, 10 μM Gomisin J or 10 μM GR-12. The data represent means ± SEM of three or four experiments performed in triplicate. Significant differences from DMSO treatment were detected. $^{**}P < 0.01$, one-way ANOVA followed by Dunnett's test.

Gomisin J and GR-12 are derived from natural plants and their structures are very complex (Fig. 1). As the next step, we searched for more simple compounds that show antioxidative activity toward C2C12 cells. We sought and investigated several compounds based on the structures of Gomisin J and GR-12, and finally found that 4,4′-dihydroxybiphenyl was the simplest structure displaying antioxidative activity in C2C12 cells (Fig. 1). The antioxidative activity of 4,4′-dihydroxybiphenyl was dose-dependent (Fig. 4). The IC_{50} values of 4,4′-dihydroxybiphenyl in the presence of H_2O_2 at 200, 400, and 600 μM were 3.17 ± 0.17, 6.26 ± 0.28, and 11.54 ± 0.13 μM, respectively, and nearly equivalent to those of GR-12.

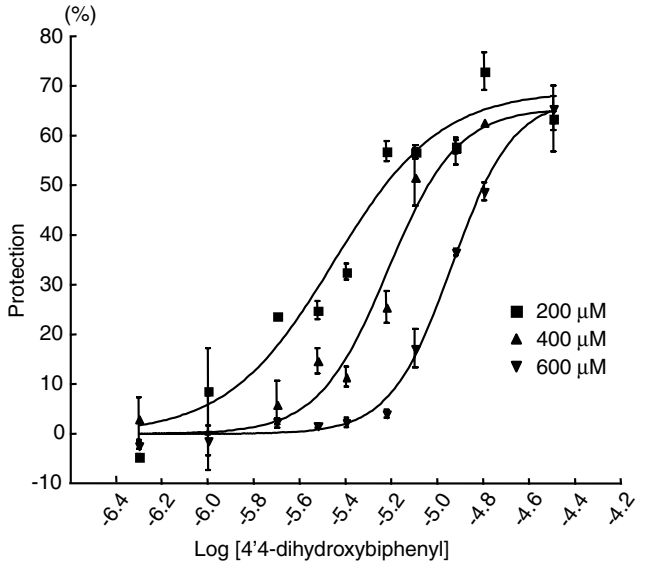

Fig. 4. Effects of 4,4′-dihydroxybiphenyl. C2C12 cells were treated with the indicated concentrations of H_2O_2 for 6 h in the absence (0.1% DMSO) or presence of 4,4′-dihydroxybiphenyl. Control cells were not treated with H_2O_2. 4,4′-Dihydroxybiphenyl by itself has no effects on the cell viability and proliferation. Cell viability was measured using the MTT assay. The data represent means ± SEM of three or four experiments performed in triplicate.

Many extracellular stimuli initiate intracellular signaling through protein phosphorylations, thereby leading to the activation of mitogen-activated protein kinases (MAPKs). The mammalian MAPK family includes extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK). As shown in Fig. 5A, treatment of C2C12 cells with 200 μM H_2O_2 decreased ERK phosphorylation and increased p38 phosphorylation. These results are consistent with widely accepted findings, indicating the involvement of ERK activity in cell proliferation and p38 activity in a variety of stresses. On the other hand, phosphorylation of JNK, which is stimulated in response to various stresses similarly to p38, could not be observed in C2C12 cells under either oxidative or non-oxidative stress conditions (Fig. 5A). Moreover, 10 μM 4,4′-dihydroxybiphenyl dramatically prevented the H_2O_2-induced increase in p38 phosphorylation (Fig. 5B), but had no effect on ERK phosphorylation. These findings suggest that H_2O_2-induced cell death in C2C12 cells is regulated by p38 activity and that the antioxidative effect of 4,4′-dihydroxybiphenyl is mediated upstream of p38.

3-*tert*-Butyl-hydroxyanisole is a well-known synthetic phenolic antioxidant that is frequently used as a food additive. It is metabolized to *tert*-butylhydroquinone, which induces the expression of phase II metabolizing enzymes with antioxidant functions via transcriptional activation of the genes [26]. On the other hand, the p38 kinase pathway was reported to function as a negative regulator of the antioxidative response element-mediated induction of phase II enzymes in hepatoma cells [27]. Thus, it can be

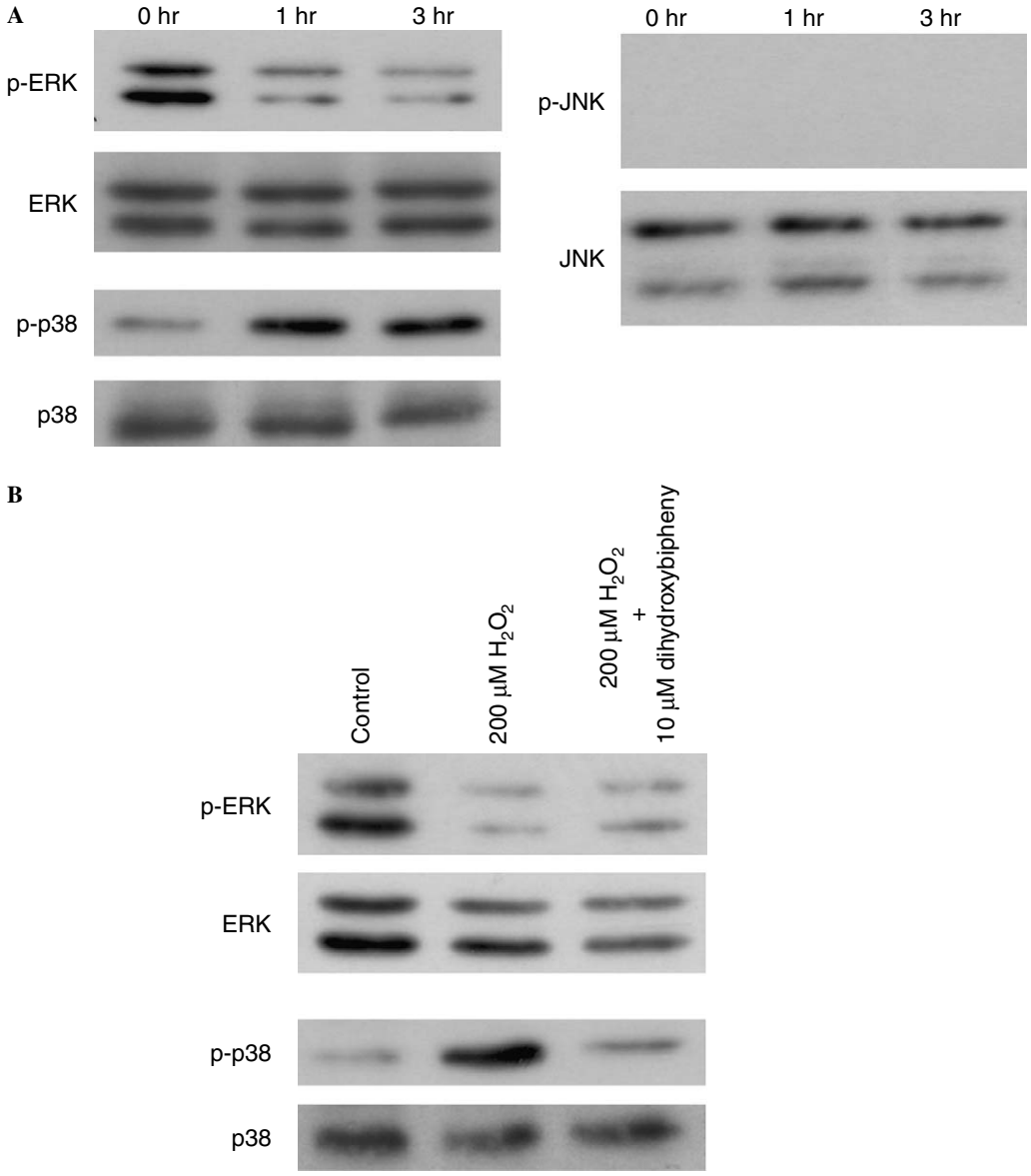

Fig. 5. Phosphorylation of MAPKs in C2C12 cells. (A) Time course of phosphorylation of ERK, p38, and JNK following treatment with 200 μM H_2O_2. (B) Effects of 4,4′-dihydroxybiphenyl on p38 and ERK phosphorylation after 3 h of treatment with 200 μM H_2O_2.

speculated that 4,4′-dihydroxybiphenyl may inhibit p38 phosphorylation and then regulate endogenous antioxidative enzymes in C2C12 cells, thereby preventing cell death.

In conclusion, we have provided the first evidence that treatment of C2C12 cells with 4,4′-dihydroxybiphenyl can protect against H_2O_2-induced cell death through inhibition of p38 phosphorylation. 4,4′-Dihydroxybiphenyl may become a valuable compound as a lead molecule for additional in vivo and clinical studies aimed at oxidative stress in skeletal muscle.

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