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Chrysin induces G1 phase cell cycle arrest in C6 glioma cells through inducing p21$^{Waf1/Cip1}$ expression: Involvement of p38 mitogen-activated protein kinase

Meng-Shih Weng [a], Yuan-Soon Ho [b], Jen-Kun Lin [a,*]

[a] *Graduate Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei 10018, Taiwan*
[b] *Graduate Institute of Biomedical Technology, Taipei Medical University, Taipei, Taiwan*

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Abstract

Flavonoids are a broadly distributed class of plant pigments, universally present in plants. They are strong anti-oxidants that can inhibit carcinogenesis in rodents. Chrysin (5,7-dihydroxyflavone) is a natural and biologically active compound extracted from many plants, honey, and propolis. It possesses potent anti-inflammatory, anti-oxidant properties, promotes cell death, and perturbing cell cycle progression. However, the mechanism by which chrysin inhibits cancer cell growth remains poorly understood. Therefore, we developed an interest in the relationship between MAPK signaling pathways and cell growth inhibition after chrysin treatment in rat C6 glioma cells. Cell viability assay and flow cytometric analysis suggested that chrysin exhibited a dose-dependent and time-dependent ability to block rat C6 glioma cell line cell cycle progression at the G1 phase. Western blotting analysis showed that the levels of Rb phosphorylation in C6 glioma cells exposed to 30 μM chrysin for 24 h decreased significantly. We demonstrated the expression of cyclin-dependent kinase inhibitor, p21$^{Waf1/Cip1}$, to be significantly increased, but the p53 protein level did not change in chrysin-treated cells. Both cyclin-dependent kinase 2 (CDK2) and 4 (CDK4) kinase activities were reduced by chrysin in a dose-dependent manner. Furthermore, chrysin also inhibited proteasome activity. We further showed that chrysin induced p38-MAPK activation, and using a specific p38-MAPK inhibitor, SB203580, attenuated chrysin-induced p21$^{Waf1/Cip1}$ expression. These results suggest that chrysin exerts its growth-inhibitory effects either through activating p38-MAPK leading to the accumulation of p21$^{Waf1/Cip1}$ protein or mediating the inhibition of proteasome activity.

Keywords: Chrysin; Cell cycle; p21$^{Waf1/Cip1}$; p38-MAPK; Proteasome activity

1. Introduction

Flavonoids are dietary polyphenolic compounds present in many fruits, vegetables, and beverages [1]. Many experiments have strongly demonstrated flavonoids to be preventive in coronary heart disease [2], stroke [3] and cancers [4]. The most common nonmutagenic flavonoid, apigenin (4′,5,7-trihydroxyflavone), has shown remarkable effects in inhibiting cancer cell growth both in cell culture systems and in vivo tumor models [5,6]. Apigenin also possesses anti-inflammatory and free radical scavenging properties [7,8] and inhibits tumor cell invasion, metastasis [9], and mitogen-activated protein kinases (MAPKs) and downstream oncogenes [10]. These findings suggest that flavonoids possess strong cancer-preventative effects. Chrysin (5,7-dihydroxyflavone) (Fig. 1), an apigenin analog, present at high levels in honey and propolis has been shown recently to be a potent inhibitor of the enzyme aromatase [11], of human immunodeficiency virus activation in models of latent infection [12], and of modulation of $GABA_A$ and $GABA_C$ receptors through binding to the benzodiazepine site located on the $GABA_A$ receptor [13]. Chrysin also has anti-inflammatory [14], and anti-oxidant [15] effects, and it has been found to possess cancer chemopreventive activity through inhibiting malignant cell growth by down-regulated expression of PCNA in HeLa cells [16], and induced apoptosis through caspase activation and Akt inactivation in U937 leukemia cells [17], and induced cell cycle arrest in human colon carcinoma cells [18]. The molecular mechanisms of induced cell cycle arrest, however, remained to be elucidated.

* Corresponding author. Tel.: +886 2 2356 2213; fax: +886 2 2391 8944.
E-mail address: jklin@ha.mc.ntu.edu.tw (J.-K. Lin).

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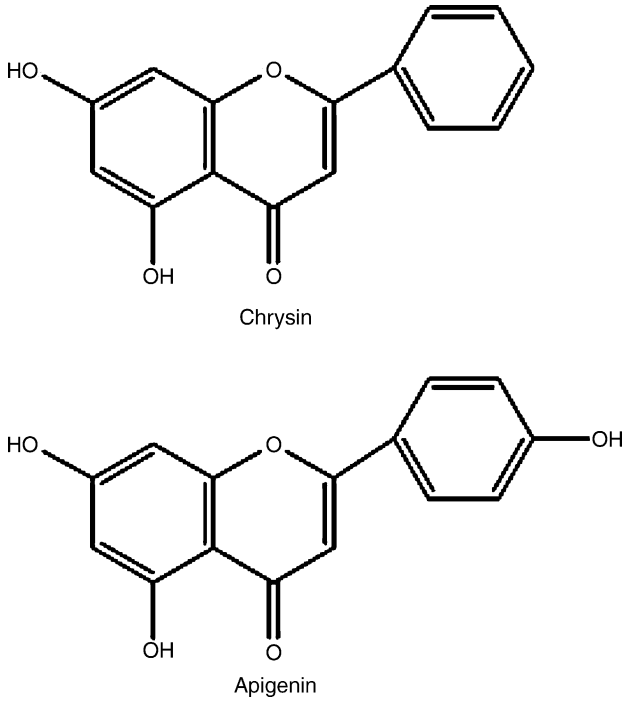

Fig. 1. Chemical structure of the flavonoid chrysin and apigenin.

Cancer cells that grow uncontrollably, such as brain glioma, are largely resistant to chemotherapy. One of the strategies of cancer management is to inhibit cell proliferation [19]. The eukaryotic cell cycle is regulated by activating or deactivating cyclin/cyclin-dependent kinase (CDK) through coordinating internal and external signals at several key checkpoints [20]. CDK activation requires cyclin binding and phosphorylation of conserved threonine residue by CDK-activating kinase (CAK), which leads to the phosphorylation of the Rb protein [21]. This phosphorylation releases a number of factors, including the E2F family of transcription factors. E2F has been shown to activate the transcription of several genes, the products of which are important for entering the S phase and for DNA replication [22]. Otherwise, CDK2, associated with either D- or E-type cyclins, and CDK4 and CDK6, associated with D-type cyclins, regulate G1 progression [23]. The activated CDK/cyclin complexes can be changed to an inactive state by phosphorylation of a conserved threonine–tyrosine pair or binding to CDK inhibitory subunits (CKIs) [24]. The CKIs fall into two classes: (1) p21 (Cip1/Waf1/Cap20/Sdi1/Pic1), p27 (Kip1), and p57 (Kip2), related proteins with a preference for Cdk2 and Cdk4/cyclin complexes; and (2) $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$, closely related CKIs specific for CDK4 and CDK6/cyclin complexes [25]. Most in vivo study suggests the $p21^{Waf1/Cip1}$ is a potent inhibitor protein of CDK/cyclin complexes, which play an important role in G1 and G2 arrest by inhibiting CDK2/cyclin E and CDC2/cyclin B activities, respectively. The expression of $p21^{Waf1/Cip1}$ appears to be regulated by both transcriptional and post-transcriptional mechanisms [26], and by the ubiquitin-proteasome system [27]. At the transcriptional level, $p21^{Waf1/Cip1}$ is induced either dependently or independently by p53 tumor suppressor protein and by the presence of DNA damaging agents [28–30]. Post-transcriptional regulation of $p21^{Waf1/Cip1}$ has been demonstrated during hematopoietic differentiation [31] or TNF-α treatment [32].

The mitogen-activated protein kinase family of serine/threonine protein kinases are involved in a wide range of cellular functions [33]. Upon stimulation, the MAPKs phosphorylate their specific substrates at serine and/or threonine residues. Thus, the MAPK signaling pathways modulate gene expression, mitosis, proliferation, and programmed cell death [34,35]. Three subfamilies of MAPKs have been identified: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38-MAPKs. The ERK pathway is primarily activated by growth factors and the regulation of Ras-regulated Raf-MEK-MAPK/ERK protein kinase cascade has been linked to cell proliferation, cell growth, and differentiation [36–39]. JNKs are ubiquitously expressed and control a spectrum of cellular processes, including cell growth, differentiation, transformation, or apoptosis [34,39]. Like JNK pathways, p38-MAPK signaling pathways are involved in a variety of cellular responses, including survival enhancement, cell growth, inflammation, and differentiation [40–43]. However, controversial evidence has indicated that more complex roles of these pathways exist to transmit more ultimately distinct cellular effect in different cell lineages. For example, the persistent activation of ERK mediates growth arrest or differentiation signals in muscle cells and leukemia cells [44–46]. In contrast, transient p38 and JNK induction could provide a survival signal, whereas persistent activation induces apoptosis [47,48].

Recent study has demonstrated that chrysin can induce malignant cell apoptosis and cell cycle arrest [17,18]. The mechanism of chrysin-induced apoptosis was through caspase activation and Akt inactivation [17], but the mechanism by which chrysin-induced cell cycle arrest in malignant cells remains poorly understood. Here, we developed an interest in the relationship between MAPK signaling pathways and cell growth inhibition after chrysin treatment. We have found that chrysin induced a dose- and time-dependent G1 cell cycle arrest in C6 glioma cells. Our data demonstrated that chrysin enhanced the protein level of $p21^{Waf1/Cip1}$ and concomitantly inhibited CDK4 and CDK2-mediated phosphorylation of Rb. Besides, p38-MAPK phosphorylation and the ubiquitin-proteasome system are also down-regulated in chrysin-treated cells, which may have resulted in the accumulation of $p21^{Waf1/Cip1}$ protein.

2. Materials and methods

2.1. Cell lines and reagents

The rat C6 glioma cell line was obtained from the American Type Culture Collection (Manassas, VA) and

cultured in Dulbecco's modified Eagle's medium (Hyclone Laboratories, Logan, UT) supplemented with 10% fetal calf serum (FCS, Hyclone Laboratories, Logan, UT) and 100U penicillin-streptomycin. Cells were maintained at 37 °C in a humidified atmosphere at 95% air and 5% CO_2. Chrysin, SB203580, PD98059, MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal), and bovine serum albumin were purchased from Sigma Chemical (St Louis, MO). Anti-cyclin D3, anti-CDK2, anti-p21, anti-p27, anti-ERK, anti-JNK, anti-p38-MAPK and anti-p53 were from Transduction Laboratories, (Lexington, KY, USA). Anti-pRb, anti-phospho-ERK, anti-phospho-JNK, and anti-phospho-p38-MAPK were from Cell Signal Technology (Beverly, MD) and anti-cyclin E was from Upstate Biotechnology (Lake Placid, NY). Anti-CDK4, anti-cyclin D1, Histone H1, and GST-Rb were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Radioisotopes were obtained from Amersham (Arlington Heights, IL, USA).

2.2. Determination of cell growth

Rat C6 glioma cells were plated 5×10^4/well in a six-well plate. After 24 h, the medium was changed and chrysin (10, 30, and 50 μM) was added. The incubation medium was changed everyday during the experiment. At the end of incubation, cells were harvested for cell counting with a hemocytometer. Control cells were treated with solvent, dimethyl sulfoxide (DMSO), in a final concentration of 0.05% (v/v).

2.3. Cell synchronization, drug treatment, and flow cytometric cell analysis

After 24 h of plating of cells, the medium was removed. Cells were washed three times with phosphate buffer saline and then incubated with serum-free medium for 24 h. Under these conditions, cells were arrested in the G0/G1 phase, as determined by flow cytometry analysis. The serum-free medium was removed and changed to the fresh medium containing 10% FCS. Chrysin solutions were prepared by dissolving this compound in a final concentration of 0.05% (v/v) DMSO. The cell cycle progression was measured by flow cytometry analysis.

2.4. Western blot analysis

Treated and untreated cells were rinsed twice with ice-cold phosphate buffer saline, then lysed in an appropriate extraction buffer (10 mM Tris–HCl, pH 7; 140 mM sodium chloride; 3 mM magnesium chloride; 0.5% [v/v] NP-40; 2 mM phenylmethylsulfonyl fluoride; 1% [w/v] aprotinin; and 5 mM dithiothreitol) for 30 min on ice. The extracts were centrifuged for 30 min at 12,000 × *g*. Proteins were loaded at 50 μg/lane on sodium dodesyl sulfate (SDS)–polyacrylamide gel, and then transferred to nitrocellulose membranes. The membranes were blocked for 30 min at room temperature in PBS plus 0.5% Tween 20 containing 1% bovine serum albumin, then incubated for 2 h at room temperature with a 1:1000 dilution of one of the mouse monoclonal antibodies against human cyclin D1, cyclin D3, CDK2, Rb, p21, p27, β-actin or rabbit polyclonal anti-human CDK4, phosphor-Rb (1:250 dilution), cyclin E, and p53 antibody. After washing, the membranes were incubated for 60 min at 25 °C with the 1:3000 dilution of an appropriate horseradish peroxidase-labeled secondary antibody, and a bound antibody visualized and quantified by chemiluminescence detection. β-Actin was used as the internal control. The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β-actin. The density of the band was then expressed as the relative density compared to that in untreated cells (control), which was taken as one-fold.

2.5. Immunoprecipitation and CDKs kinase assay

For CDK kinase assay, C6 glioma cells (3×10^5/dish) were pre-cultured in 100 mm culture dishes for 24 h and then starved for 24 h. After starvation, cells were changed to fresh, complete medium and treated with 0, 10, 30, and 50 μM of chrysin for 24 h. After 24 h of chrysin treatment, C6 glioma cells were rinsed twice with ice-cold phosphate buffer saline and lysed with Gold lysis buffer (10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris–HCl pH 7.9, 100 μM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin) for 30 min at 4 °C. The cell lysate was clarified by centrifugation at 12,000 × *g* for 30 min at 4 °C. A total of 250 μg of protein was incubated with anti-CDK2 or anti-CDK4 antibody and protein A/G plus agarose (Santa Cruz) for 18 h at 4 °C. The immunoprecipitate was washed thrice with immunoprecipitate buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.5% NP-40) and thrice with kinase buffer (50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM DTT for CDK4; 50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA, 1 mM DTT for Cdk2).

For the IP-Western analysis, the CDK2 and CDK4 immunoprecipitates were resuspended in 25 μL of lysis buffer, mixed with 5× Laemmli's loading buffer, and separated by SDS–PAGE.

For the kinase assay, the CDK2 and CDK4 immunoprecipitates were washed with one of two kinase buffers (50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA, 1 mM DTT for CDK2; or the same buffer but with 10 mM β-glycerophosphate and 1 mM NaF for CDK4) thrice. The kinase reactions were carried out in a final volume of 40 μl containing 2 μg histone H1 (Calbiochem, San Diego, CA) or 1 μg Gst-Rb (Santa Cruz), 20 μM cold ATP, and 5 μCi

[r-^{32}p] ATP (5000 Ci/mmol, Amersham) and incubated for 20 min at 25 °C. Each sample was mixed with 10 μl of Laemmli's loading buffer to stop the reaction, heated for 10 min at 100 °C, and subjected to SDS–PAGE. The gels were dried, visualized by autoradiography, and quantified by densitometry (IS-1000 Digital Imaging System). The kinase activities in each treatment were normalized with the levels of immunoprecipitation loading control as the relative activities compared to that in untreated cells (control), taken as 100%.

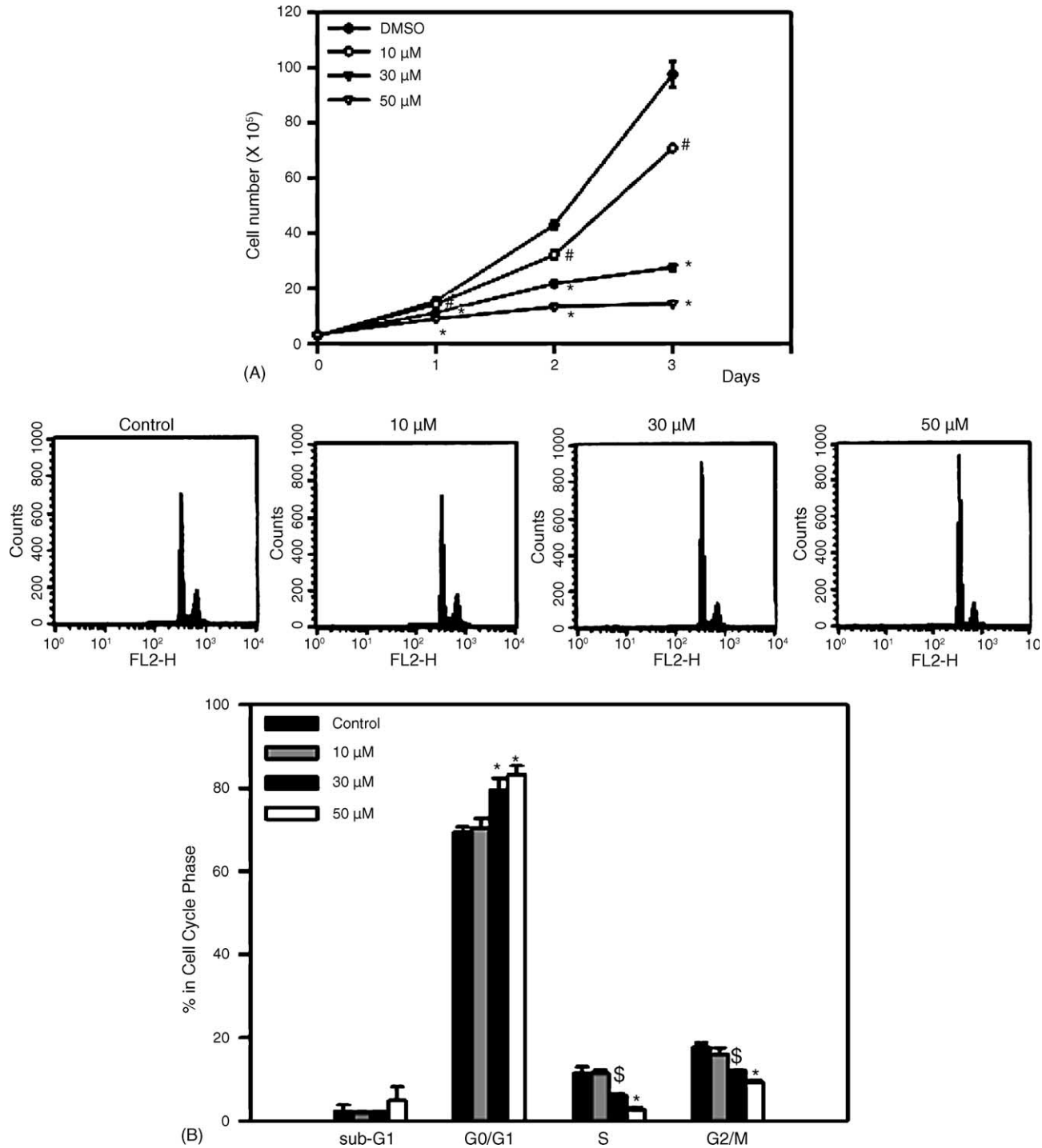

Fig. 2. Effects of chrysin on growth and viability of rat C6 glioma cells. (A) Dose-dependent inhibition of cell growth was observed in C6 cells treated with various concentrations of chrysin (10, 30, and 50 μM). The medium containing various doses of chrysin was renewed everyday, and the total cell number was counted at the indicated time points. (B) Effect of chrysin on DNA content in C6 glioma cells. Cells harvested after a 24-h treatment with chrysin (0, 10, 30, and 50 μM) were stained with propidium iodide. Cell cycle distribution was determined by FACs analysis. (C) Time-dependent response of chrysin-induced G1 phase arrest in C6 glioma cells. C6 glioma cells were synchronized with serum-free medium for 24 h as described under Section 2. After synchronization, cells were treated with 30 μM chrysin for 24, 48, and 72 h and harvested for cell cycle distribution analysis. Three samples were analyzed in each group, and results were presented as mean ± S.E. Significantly different from the DMSO-treated control: $^{\#}p < 0.05$, $^{\$}p < 0.01$, $^{*}p < 0.001$.

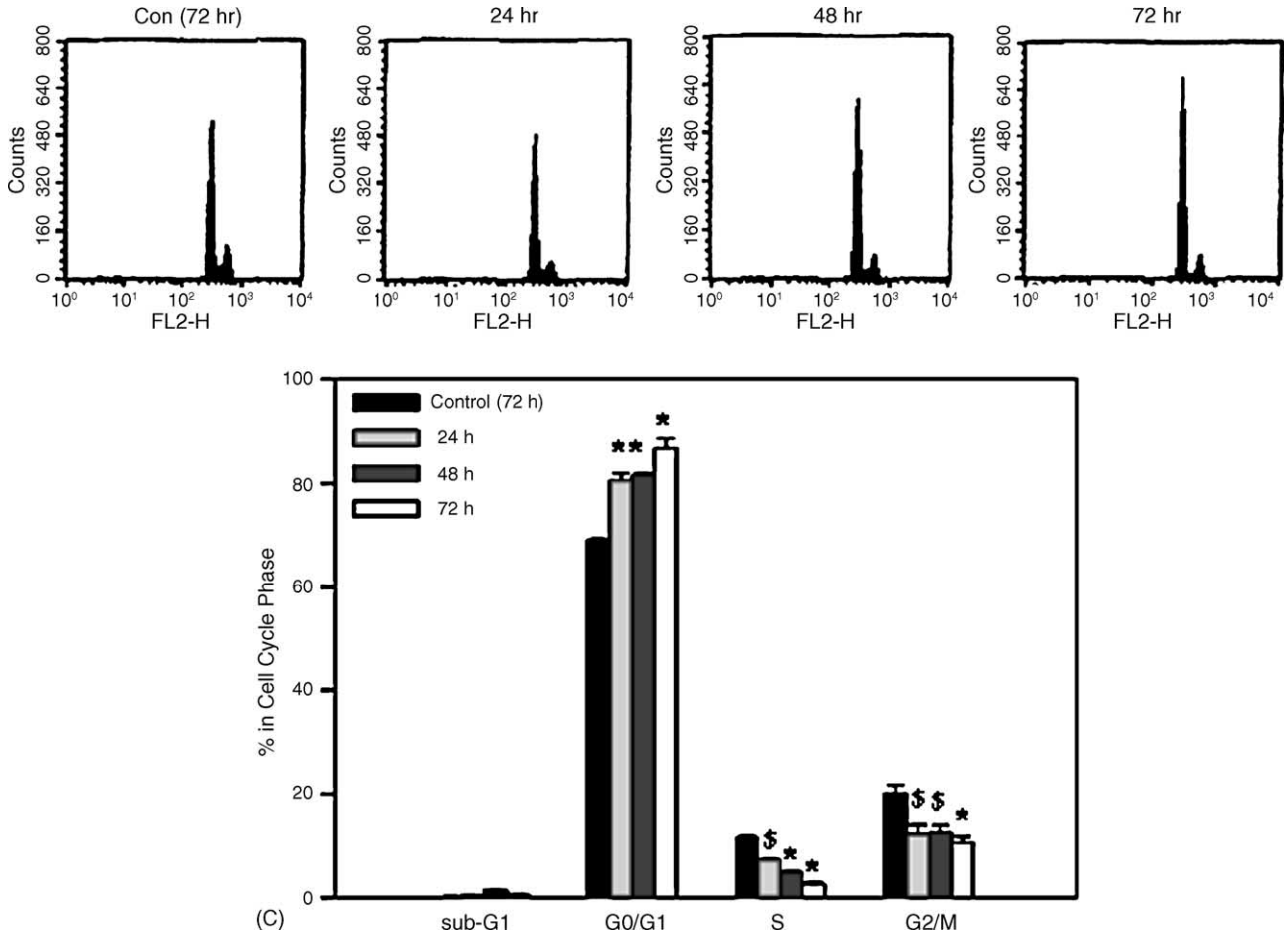

Fig. 2. (*Continued*).

2.6. RT-PCR

Following the drug treatment, the cells were washed in ice-cold PBS and total RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction according to the manufacturer's instructions (Nippon Gene, Japan). The cDNA was prepared from the total RNA (5.0 μg) with reverse transcriptase and oligo $(dT)_{18}$ primer at 42 °C for 60 min. The PCR was performed in a final volume of 25 μl containing dNTPs (each at 200 μM), 1.0× reaction buffer, 2.0 μl above cDNA product, and 50 U/ml pro Taq DNA polymerase (Promega, Madison, WI). PCR primers for $p21^{Waf1/Cip1}$ and G3PDH were synthesized according to the following oligonucleotide sequences: $p21^{Waf1/Cip1}$, forward primer 5′-TGGCCTTGTCGCTGTCTT-3′, reverse primer 5′-CTAAGGCAGAAGATGGGGAA-3′; G3PDH, forward primer 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, reverse primer 5′-CATGTAGGCCATGAGGTCCACCAC-3′. After an initial denaturation for 5 min at 95 °C for $p21^{Waf1/Cip1}$ PCR, 30 cycles of amplification (95 °C for 70 s, 55 °C for 70 s, and 72 °C for 90 s) were performed followed by a 10 min extension at 72 °C. The PCR products were separated by electrophoresis on 1.0% agarose gel and visualized by ethidium bromide staining.

2.7. Proteasome activity assay

C6 glioma cells were plated in a six-well tissue culture plate, and on the following day cells were treated with chrysin (0, 10, 30, and 50 μM) for 24 h. Cells were then isolated and suspended in 100 ml of proteasome assay buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 5 mM ATP, 5 mM dithiothreitol, and 20% [v/v] glycerol), lysed by sonication, and then centrifuged at 15,000 × *g* for 15 min at 4 °C. The supernatant (25 μg) was incubated in the proteasome activity assay buffer (50 mM Tris, pH 7.4, 0.5 mM EDTA, and 50 μM of each proteasome substrate) for different time periods to obtain linearity of the reaction. The substrates Suc-Leu-Leu-Val-Tyr-MCA, and Z-Leu-Leu-Glu-AMC were used to determine chymotrypsin-like and peptidylglutamyl-peptide hydrolytic-like (PGPH) proteolytic activities, respectively. Protease activities at a particular time point (30 min) within the linear range were used to calculate the data. The fluorescence intensity was measured at 380-nm excitation and 460-nm emissions using a Wallac multi-label counter.

2.8. Statistical analysis

The results were expressed as mean ± S.E. calculated from the specified numbers of determination. A Student's

t-test was used to compare individual data with control value. A probability of $p < 0.05$ was taken as denoting a significant difference from control data.

3. Results

3.1. Effect of chrysin on C6 glioma cell proliferation

To investigate the growth inhibitory effect of chrysin, we first assessed the anti-proliferation efficacy of chrysin on rat C6 glioma cells by trypan blue exclusion assay. It was observed that treatment of C6 glioma cells with 10, 30, and 50 μM doses for 24, 48, and 72 h showed a moderate cell growth inhibition by chrysin (Fig. 2A). After 72 h incubation, the cell numbers of 10, 30, and 50 μM chrysin-treated cultures fell approximately 30% ($p < 0.05$), 80% ($p < 0.001$), and 90% ($p < 0.001$), respectively. As Fig. 2A shows, a strong time- as well as dose-dependent cell growth inhibition occurred.

3.2. Chrysin induces G1 phase cell cycle arrest in rat C6 glioma cells

To assess whether chrysin-induced cell growth inhibition is mediated via alternations in cell cycle progression, rat C6 glioma cells were synchronized by serum-free medium for 24 h and then by serum-supplemented medium containing chrysin (10, 30, and 50 μM). To evaluate the effect of chrysin on cell cycle phase distribution, flow cytometry analysis was performed. Results shown in Fig. 2B indicate that by 30 and 50 μM treatment, chrysin increased the proportion of cells in the G1 phase of the cell cycle from 69 to 79% ($p < 0.01$) and from 69 to 83% ($p < 0.001$), respectively, and decreased the proportion of S phase cells from 11.4 to 6.1% ($p < 0.01$) and from 11.4 to 2.8% ($p < 0.001$) and the proportion of G2/M phase cells from 17.9 to 12.2% ($p < 0.01$) and from 17.9 to 9.2% ($p < 0.001$), respectively (Fig. 2B). Furthermore, treatment of C6 glioma cells by 30 μM chrysin sustained G1 arrest at time points ranging from 24 to 72 h. As shown in Fig. 2C, chrysin-treated (30 μM) C6 glioma cells underwent G1 arrest in a time-dependent manner, accounting for 69–80.6% ($p < 0.001$), 81.6% ($p < 0.001$), and 86.7% ($p < 0.001$) after 24, 48, and 72 h of treatment. Chrysin caused a roughly 17% ($p < 0.001$) increase in G1 arrest at 30 μM for 72 h of treatment in C6 glioma cells.

3.3. Cyclin D, cyclin E, and associated CDK expression in chrysin-treated C6 glioma cells

It is well known that cyclin D, cyclin E, CDK2, and CDK4/6 cooperate to promote G1 phase progression. We first determined whether chrysin regulated the expressive

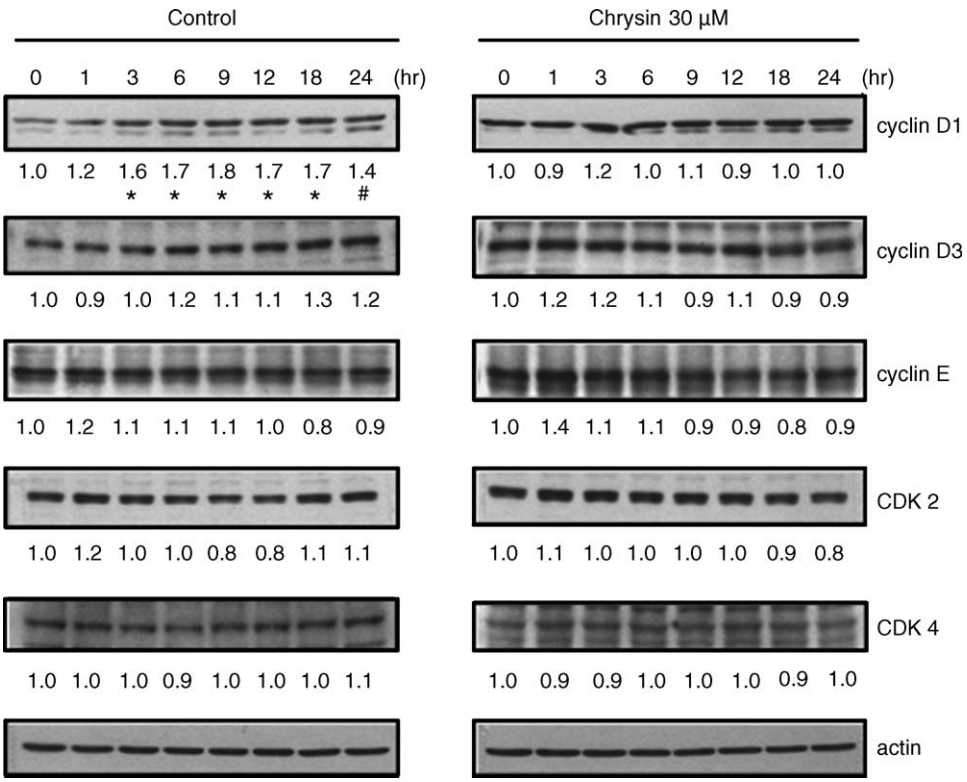

Fig. 3. Effects of chrysin on cyclin D/CDK4 and cyclin E/CDK2 protein expression in C6 glioma cells. C6 glioma cells were treated and Western blot analyses were done with anti-cyclin D, cyclin E, CDK4, CDK2 and β-actin antibodies as described in Section 2. The protein levels in each treatment after normalization with the levels of β-actin are shown in parentheses. Data shown are representative of at least three independent experiments. Significantly different from the DMSO-treated control: $^{\#}p < 0.05$, $^{*}p < 0.001$.

levels of these proteins in the G1 phase. C6 glioma cells were treated with chrysin (30 μM) for various time points and Western blot analyses were done with anti-cyclin D1, D3, E, CDK2 and CDK4 antibodies, respectively. As shown in Fig. 3, the protein levels of cyclin D1 increased in control cells (DMSO-treated) at 3 h, but they fell following treatment with 30 μM chrysin at the experimental time points ($p < 0.001$). Cyclin D3, E, and CDK4 protein levels underwent no change in control cells, in line with results of cells after 1–24 h of treatment with chrysin 30 μM in C6 glioma cells. Another cyclin-dependent kinase, CDK2, showed a slight decrease in control cells (DMSO-treated) at the time points ranging from 6 to 18 h ($p > 0.05$) (Fig. 3). CDK2 protein levels fell slightly after 18 h of chrysin treatment ($p > 0.05$).

3.4. Modulation of CDK2- and CDK4-associated kinase activities by chrysin

To verify whether modifications in cyclin D/CDK4 and cyclin E/CDK2 kinase activities contributed to G1 phase arrest, the effects of chrysin on cyclin D/CDK4 and cyclin E/CDK2 kinase activity were examined. C6 glioma cells were treated chrysin (10, 30, and 50 μM) for 24 h. Kinase activities of cyclin D/CDK4 and cyclin E/CDK2 were assayed as described in Section 2. As shown in Fig. 4A, chrysin inhibited cyclin E/CDK2 kinase activity in a dose-dependent manner. The percentages of inhibition exerted by chrysin 10, 30, and 50 μM were $71.8 \pm 3.9\%$ ($p < 0.01$), $61.6 \pm 5.7\%$ ($p < 0.001$), and $46.1 \pm 8.9\%$ ($p < 0.001$), respectively. In CDK4 kinase activity, chrysin also caused a marked decrease in a dose-dependent manner (Fig. 4B). The percentages of inhibition were $74.0 \pm 9.4\%$ ($p < 0.05$), $53.9 \pm 6.3\%$ ($p < 0.001$), and $42.9 \pm 5.7\%$ ($p < 0.001$) after 10, 30, and 50 μM of chrysin treatment, respectively. These data indicated that chrysin can inhibit G1-S transition-related CDKs kinase activities, which should contribute to G1 arrest.

3.5. Effect of chrysin on the phosphorylation status of the Rb protein

The phosphorylation of the Rb protein is mediated by both cyclin D/CDK4 and cyclin E/CDK2 and is required for cells to progress from the G1 into the S phase. To confirm that chrysin inhibits the kinase activities of CDK4 and CDK2, we examined its inhibitory effect in C6 glioma cells on the phosphorylation of Rb using anti-phospho-Rb antibody. As shown in Fig. 5, the ratio of p-Rb/Rb protein showed 2.1-fold increases at 3 h in the medium with serum-supplement, later increasing to 5.3 folds at 18 h. In contrast, this ratio declined after treatment with 30 μM chrysin for 24 h. These results indicate that the degree of Rb protein phosphorylation decreased within 24 h of chrysin treatment.

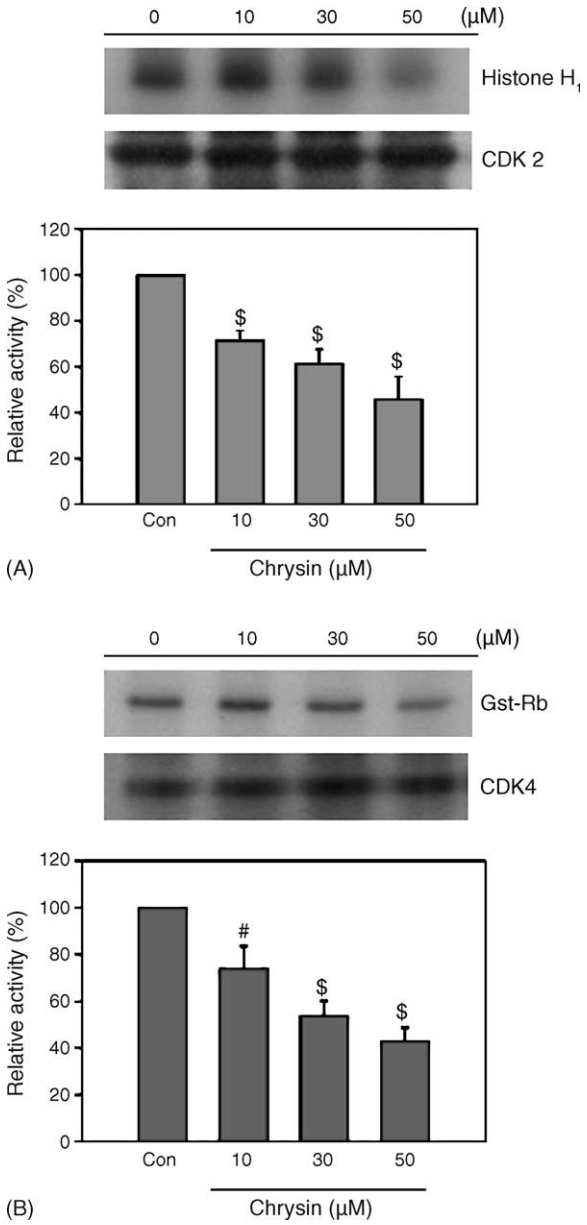

Fig. 4. Effects of chrysin on the activities of CDK2 and CDK4 kinases in cell-free system. C6 glioma cells were treated for immunoprecipitation and kinase activities as described in Section 2. The kinase activities association with (A) CDK2 immunocomplexes was analyzed with histone H1 and (B) CDK4 was analyzed with Gst-Rb as substrates. CDK2 and CDK4 levels were used as loading controls. The kinase activities in each treatment normalized with the levels of immunoprecipitation loading control are shown in parentheses. Data represent the mean ± S.E. of three samples, and ^{32}p-labeled histone H1 or Gst-Rb were shown. Significantly different from the DMSO-treated control: $^{\#}p < 0.05$, $^{\$}p < 0.01$.

3.6. Effects of chrysin on CDK inhibitors of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$

Since the CDK activity can be controlled by a group of CDKIs, we further examined the changes of protein levels of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ in the chrysin-treated C6 glioma cells. As Fig. 6A shows, the protein levels of $p21^{Waf1/Cip1}$ increased in the chrysin-treated C6 glioma

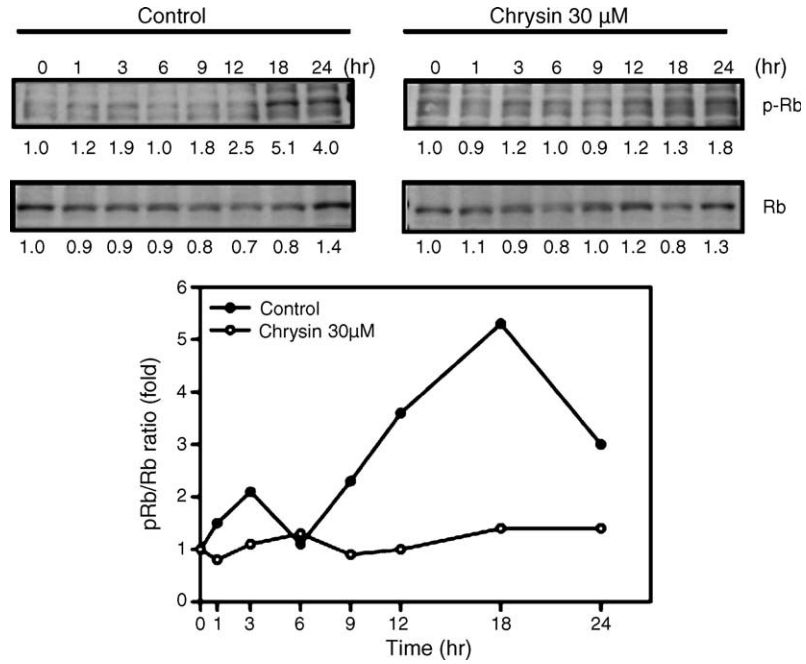

Fig. 5. Effects of chrysin treatment on the phosphorylation state of the Rb protein in C6 glioma cells. C6 glioma cells were treated and Western blot analyses were done with anti-phosphorylation forms of the Rb (p-Rb) protein as described in Section 2. Then, blots were stripped and re-probed with anti-Rb antibody. The p-Rb and Rb were quantified by PhosphoImage and the ratio of p-Rb/Rb was normalized to 0 h. Data shown are representative of at least three independent experiments.

cells as compared with the DMSO-treated cells in a time-dependent manner. The protein level of $p27^{Kip1}$ increased after 1 h of DMSO treatment, and was sustained for 24 h. By contrast, the protein level of $p27^{Kip1}$ increased slightly after chrysin treatment for 24 h. We next examined the change of p53 level under chrysin treatment in C6 glioma cells. The p53/$p21^{Waf1/Cip1}$ pathway plays a critical role in regulating the G1-S transition in response to a variety of cellular stresses. However, p53 levels changed only slightly in chrysin-treated cells (Fig. 6A). To determine whether the levels of $p21^{Waf1/Cip1}$ mRNA had changed, C6 glioma cells were treated with 30 μM chrysin for indicated time. Total RNA was harvested, and RT-PCR was conducted as described in Section 2. As shown in Fig. 6B, our data indicated that although $p21^{Waf1/Cip1}$ protein increased in a time-dependent manner, levels of mRNA remained unchanged after exposure to 30 μM chrysin.

3.7. Effect of chrysin on the activity of proteasome

The ubiquitin-proteasome pathway is highly complex in its degradation of cell proteins and plays an important role in regulation of basic cellular pathways and processes, such as cell cycling, apoptosis, and differentiation. It has been demonstrated that several cell cycle-regulatory proteins, such as cyclin [49], $p21^{Waf1/Cip1}$, and $p27^{Kip1}$ [50] are involved in the ubiquitin-proteasome degradation pathway. We analyzed the chymotrypsin-like and peptidylglutamyl-peptide hydrolytic-like (PGPH) proteolytic activities in chrysin-treated C6 glioma cells. As shown in Fig. 7, chymotrypsin-like and peptidylglutamyl-peptide hydrolytic-like (PGPH) proteolytic activities were inhibited in a dose-dependent manner (10, 30, 50 μM) by exposure to chrysin for 24 h. The chymotrypsin-like proteolytic activity decreased significantly after treatment with chrysin at 30 and 50 μM for 24 h ($p < 0.001$, compared with control) and peptidylglutamyl-peptide hydrolytic-like (PGPH) proteolytic activity also fell after treatment with chrysin at 50 μM for 24 h ($p < 0.05$, compared with control).

Our data showed that chrysin can elevate the levels of $p21^{Waf1/Cip1}$ and cause cell cycle arrest at the G1 phase. This CKI is known substrates of the proteasome, and its proteasomal degradation is responsible for cell cycle progression [50]. Thus, chrysin may increase the accumulation of $p21^{Waf1/Cip1}$ protein parallel to its inhibition of proteasome activity in C6 glioma cells. To confirm that the enhanced stabilities of proteasome-related substrate $p21^{Waf1/Cip1}$ in the presence of chrysin was due to proteasome inhibition, we examined the ability of proteasome inhibitor MG132 to induce up-regulation of $p21^{Waf1/Cip1}$ in C6 glioma cells. Consistent with chrysin treatment, exposure of C6 glioma cells to proteasome inhibitor MG132 for 24 h also caused an increase in $p21^{Waf1/Cip1}$ protein (Fig. 7B).

3.8. Effects of chrysin on activation of MAPK

Recent studies have indicated that MAPK pathways are involved in the regulation of the cell cycle [33,36–38]. To

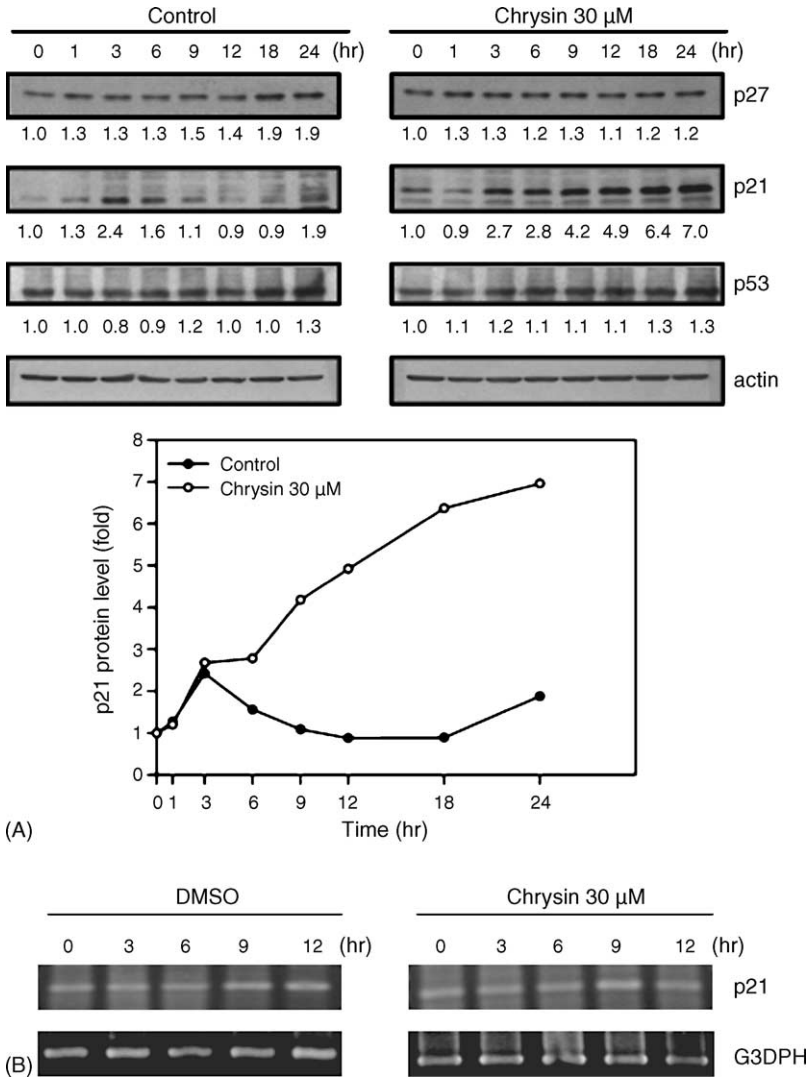

Fig. 6. Effects of chrysin on protein level of CDKI and p21$^{Waf1/Cip1}$ mRNA level. (A) Effect of chrysin on p21$^{Waf1/Cip1}$, p27^{Kip1}, and p53 proteins levels in C6 glioma cells. C6 glioma cells were treated and Western blot analyses were done with anti-p21$^{Waf1/Cip1}$, p27^{Kip1}, and p53 primary antibodies as described in Section 2. The values below the figure represent change in protein expression of the bans normalized to β-actin. (B) Effect of chrysin on p21$^{Waf1/Cip1}$ mRNA expression. C6 glioma cells were treated for RT-PCR as described in Section 2. Data shown are representative of at least three independent experiments.

further elucidate the molecular basis for the chrysin-induced cell cycle arrest, we examined the phosphorylation of these pathways in chrysin-treated C6 glioma cells. After treatment with 30 μM chrysin for the indicated time, cell extracts were prepared for to detect the activation of MAPKs using immunoblotting analysis with antibodies against ERK, JNK, and p38-MAPK. As shown in Fig. 8A, maximum stimulation of ERK phosphorylation after serum addition (containing DMSO) was reached at 0.5 h. In a similar experiment, we treated C6 glioma cells with chrysin at time points ranging from 0 to 24 h. The treatment under 30 μM chrysin was reflected in increased levels of phosphor-ERK at 24 h. In contrast, the activation of JNK was unaffected by chrysin treatment. To further investigate the effects of chrysin in these pathways, cells treated with chrysin or DMSO in the presence of serum were carried out at various time lengths. The studies showed that chrysin also sustained the activation of p38-MAPK within a 24-h treatment (Fig. 8A). Our data implicate chrysin in promoting the duration of ERK and p38-MAPK phosphorylation which may contribute to G1 arrest in C6 glioma cells.

3.9. *Chrysin-induced p21$^{Waf1/Cip1}$ is blocked by specific inhibitor of p38-MAPK*

The expression of p21$^{Waf1/Cip1}$ protein has been reported to be regulated by the MEKK1/ERK pathway [38] and to be stabilized by p38-MAPK and JNK phosphorylation [51]. To investigate the correlation between chrysin-induced p21$^{Waf1/Cip1}$ expression and MAPK activation, we utilized the specific inhibitors of ERK 1/2 and p38-MAPK, namely PD98059 and SB203580. C6 glioma cells were serum-starved and treated with 50 μM ERK 1/2 inhibitor, PD98059, or 1 μM p38-MAPK inhibitor,

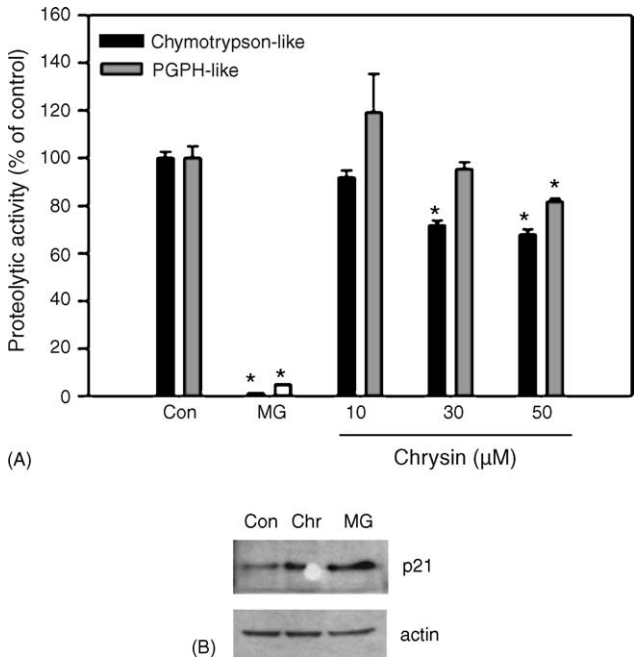

Fig. 7. Induction of $p21^{Waf1/Cip1}$ protein by inhibition of proteasome activity in chrysin-treated C6 glioma cells. (A) C6 glioma cells were treated for chymotrypsin-like and peptidylglutamyl-peptide hydrolyic-like (PGPH) proteolytic activity assays as described under Section 2. Data were presented as the mean ± S.E. from three experiments. Statistically different from control: $^{\#}p < 0.05$, $^{*}p < 0.001$ (Student's *t*-test). (B) C6 glioma cells were synchronized and then treated with 30 μM chrysin or 10 μM MG132 for 24 h. Western blot analyses were done with anti-$p21^{Waf1/Cip1}$ primary antibody as described in Section 2.

SB203580 for 30 min. Then, cells were treated with 30 μM chrysin for 12 h. Total cell lysates were prepared for immunoblotting analysis using anti-$p21^{Waf1/Cip1}$ antibody. Our data indicated that $p21^{Waf1/Cip1}$ protein expression was induced to about six to eight folds of control in chrysin-treated cells, and pre-treatment with the p38-MAPK inhibitor was able to inhibit chrysin-induced $p21^{Waf1/Cip1}$ expression (from 6.2 to 3.1 folds) (Fig. 8B). However, pre-treatment with ERK inhibitor, PD98059, for 30 min did not significantly decrease the protein level of $p21^{Waf1/Cip1}$. Based on these data, we conclude chrysin enhanced the protein levels of $p21^{Waf1/Cip1}$ regulated through the p38-MAPK pathway.

4. Discussion

In this study, we first observed that chrysin induced a dose- and time-dependent G1 phase arrest in C6 glioma cells. Chrysin-induced G1 arrest has been demonstrated to be caused by increased $p21^{Waf1/Cip1}$ protein levels and suppression of CDK4 and CDK2 kinase activities, which mediate the phosphorylation of Rb. However, no significant induction was observed in the levels of $p21^{Waf1/Cip1}$ mRNA in chrysin-treated C6 glioma cells. The proteasome activity assay indicated that chrysin may increase $p21^{Waf1/Cip1}$ protein through regulation of the ubiquitin-proteasome pathway. Additionally, our data showed that the p38-

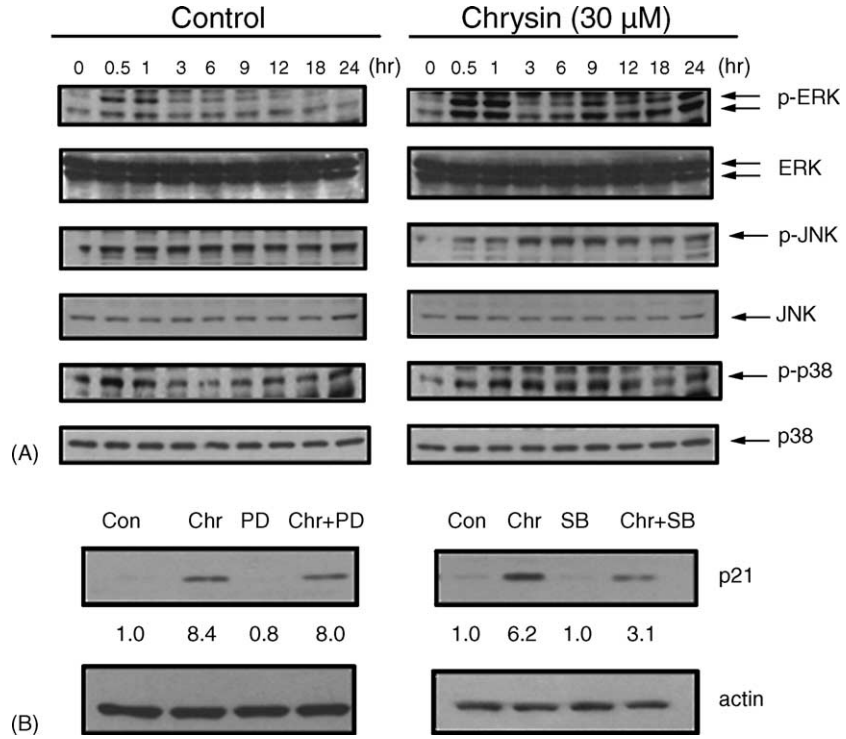

Fig. 8. Effects of chrysin on MAPK activation. (A) C6 glioma cells were treated, and Western blotting analyses were done as described under Section 2. Phosphorylation levels of ERK, JNK, and p38-MAPK were detected, and membranes were stripped and re-probed with non-phosphorylation form ERK, JNK, and p38-MAPK. (B) C6 glioma cells were treated with 50 μM PD98059 or 1 μM SB203580 for 30 min before stimulation with or without 30 μM chrysin at 12 h and Western blot analysis with anti-$p21^{Waf1/Cip1}$ primary antibody as described in Section 2. Data shown are representative of at least three independent experiments.

MAPK pathway also contributed to the stabilization of $p21^{Waf1/Cip1}$ protein in mediating the G1 arrest.

Cell cycle control is a highly regulated process that involves a complex cascade of events. Modulation of the expression and function of the cell cycle-regulatory proteins, including cyclins, CDKs, CDKIs, p53, and pRb, provides an important mechanism for the inhibition of growth [23,25]. Cyclin/CDKs complexes control cell cycle progression through their ordered activation and inactivation, and may play an important role in the pathogenesis of various human malignancies. In mammalian cells, the key G1 phase CDKs are CDK4 and CDK2. They are activated by the binding of D type cyclins (to CDK4) or cyclin E (to CDK2) [25]. The active form cyclin D1/CDK4 complex phosphorylated Rb protein then activates E2F target genes that trigger cell progression from the G1 to the S phase [20,22]. In the present studies, the protein level of cyclin D1 was inhibited, but cyclin D3 and E showed no significant difference after chrysin treatment for 24 h. In CDK4, the protein level was no different after DMSO or 30 μM chrysin treatments, but the protein level of CDK2 edged downward after 24 h of 30 μM chrysin treatment. Furthermore, we examined the effect of chrysin on CDK4 and CDK2 kinase activities and the Rb status. Chrysin inhibited CDK4 and CDK2 kinase activities in a dose-dependent manner (Fig. 4). The ratio of p-Rb/Rb decreased after chrysin treatment for 24 h. These data implicate chrysin in the induction of G1 phase cell cycle arrest through inhibition CDK4 and CDK2 kinase activities and then inhibition Rb hyper-phosphorylation.

During the progression of the cell cycle, the cyclin/CDK complexes are inhibited by the binding of CDKIs. CDKIs are tumor suppressor proteins that down regulate the cell cycle progression by binding with active cyclin/CDK complexes, thereby inhibiting their kinase activity [19–22]. The important CDKIs include $p21^{Waf1/Cip1}$, a universal inhibitor of CDKs, with expression mainly regulated by the p53 tumor suppressor protein, and $p27^{Kip1}$ that is also up regulated in response to anti-proliferative signals [21,22]. Our results showed that chrysin rapidly increased $p21^{Waf1/Cip1}$ protein expression after 6 h of 30 μM chrysin treatment but that the protein level of p53 made no significant difference. Consistent with $p21^{Waf1/Cip1}$ induction kinetic, phosphorylation of Rb protein was strongly reduced after 9 h of chrysin treatment. These results suggest that chrysin-mediated induction of $p21^{Waf1/Cip1}$ expression was through a p53-independent pathway and was responsible for the decrease of CDK4 and CDK2 activities and for the subsequent cell cycle arrest.

The ubiquitin-proteasome pathway is one of the major systems for nuclear and extra-lysosomal cytosolic protein degradation in eukaryotic cells. Several studies have demonstrated the relationship between ubiquitin-proteasome pathway and cell cycle, differentiation, and apoptosis [49,50]. The $p21^{Waf1/Cip1}$ protein is known to be regulated at several levels, including transcriptional activation and the ubiquitin-proteasome pathway [30,49,50]. Chen and Lin have reported that inhibitions of proteasome activities were a result of an accumulation of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, after which cells went to apoptosis [50]. We also examined the inhibition by chrysin of proteasome activities. Three distinct types of proteolytic activities for 20S proteasome—chymotrypsin-like, trypsin-like, and post-glutamyl peptidyl hydrolytic-like—were examined. Our data demonstrated that 50 μM chrysin inhibited chymotrypsin-like and post-glutamyl peptidyl hydrolytic-like proteolytic activities of 20S proteasome ($p < 0.01$) (Fig. 7A), but treatment with 30 μM of chrysin only inhibited chymotrypsin-like proteolytic activity. Besides, chrysin did not significantly change the trypsin-like proteolytic activity of 20S proteasome (data not shown). To examine the possibility of that $p21^{Waf1/Cip1}$ accumulation was due to proteasome inhibition, the specific proteasome inhibitor MG132 was employed. The data of Fig. 7B indicate that the $p21^{Waf1/Cip1}$ increased after MG132 and chrysin treatment for 24 h. Thus, chrysin-induced $p21^{Waf1/Cip1}$ accumulation may occur through inhibition of proteasome activities. However, the real role of proteasome in chrysin-treated C6 glioma cells remains to be examined.

Recently, it was revealed that the MAPK kinase super family plays a crucial role in cell growth, differentiation, or even programmed cell death in response to diverse extracellular stimuli in eukaryotic cells [34–38]. Pumiglia and Decker indicated that nerve growth factor (NGF)-induced NIH 3T3 cell growth arrest occurred through a MEK/MAPK pathway [37]. We examined the effect of chrysin on the signal transduction pathway using the following parameters: ERK, JNK, and p38-MAPK. In this report, we demonstrated that the ERK signaling pathway was enhanced by chrysin treatment. Meanwhile, JNK and p38-MAPK phosphorylation were also enhanced. Previous studies have shown that the ERK and p38-MAPK pathways were involved in cell growth inhibition and the regulation of the cell cycle [33,36–38,40–42]. Since $p21^{Waf1/Cip1}$ induction was observed with chrysin treatment, it was interesting to study the role of ERK and p38-MAPK in the regulation of $p21^{Waf1/Cip1}$ expression. To examine the correlation of the ERK and p38-MAPK pathways in the regulation of $p21^{Waf1/Cip1}$, we employed specific chemical inhibitors of ERK and p38-MAPK, PD98059 and SB203580, respectively. Using PD98059 had no effect on chrysin-induced $p21^{Waf1/Cip1}$ expression but its level was reversed after treatment with SB203580. Based on this, we demonstrated that chrysin-induced $p21^{Waf1/Cip1}$ expression may occur through activated p38-MAPK but not ERK. Just how, however, does p38-MAPK regulate $p21^{Waf1/Cip1}$? Kim et al. have reported that increase in the stress-activated protein kinases p38α and JNK1 in $p21^{Waf1/Cip1}$ protein were due to the increase of $p21^{Waf1/Cip1}$ protein stability resulting from phosphorylation [51]. Li et al. also indicated that $p21^{Waf1/Cip1}$ can be phosphorylated by Akt/PKB, and this phosphorylation enhanced $p21^{Waf1/}$

^{Cip1} stability [52]. In the present study, chrysin-induced p21$^{Waf1/Cip1}$ protein accumulation occurred through activation of p38-MAPK phosphorylation which enhanced p21 $^{Waf1/Cip1}$ stability and prevented its degradation.

Taken together, our data show that chrysin-induced C6 glioma cell growth arrest can be ascribed to the inhibition of CDK2 and CDK4 kinase activities by the increase of p21$^{Waf1/Cip1}$ protein. This chrysin-induced cell growth arrest appears due to the activation of p38-MAPK and proteasome activity inhibition and consequently to the stabilization of p21$^{Waf1/Cip1}$.

Acknowledgements

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