7,8-dihydroxyflavone induces G1 arrest of the cell cycle in U937 human monocytic leukemia cells via induction of the Cdk inhibitor p27 and downregulation of pRB phosphorylation

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Abstract. We investigated the mechanisms of the anti-proliferative action of 7,8-dihydroxyflavone (7,8-DHF), a member of the flavonoid family, in U937 human monocytic leukemia cells. We found that 7,8-DHF time-dependently inhibited the growth of U937 cells, arresting them in the G1 phase of their cell cycle and inducing apoptosis. 7,8-DHF-induced G1 arrest was correlated with downregulation of cyclin E, with a concomitant upregulation of cyclin-dependent kinase (Cdk) inhibitors including p27, and association of p27 with Cdk2 was markedly induced in 7,8-DHF-treated cells. We also observed that downregulation of the phosphorylation of retinoblastoma protein (pRB) by this flavonoid was associated with enhanced binding of pRB and the transcription factor E2F-1. Overall, our results demonstrate a combined mechanism for the anticancer effects of 7,8-DHF that involves the inhibition of pRB phosphorylation and induction of p27 as targets for 7,8-DHF.

Introduction

Cell cycle and apoptosis control mechanisms serve major regulatory functions of cell growth. In mammalian cells, the progression of the cell cycle is regulated by sequential activation and inactivation of cell cycle regulators, including cyclins, cyclin-dependent kinases (Cdks) and Cdk inhibitors (1,2). Early G1 phase is regulated by complexes of D-type cyclins and Cdk4/6, and the G1/S transition is controlled by a complex of cyclin E and Cdk2 (3). Cdk inhibitors, including p21 and p27, play a key role in negative regulation of cell cycle progression by binding to cyclin/Cdk complexes (3,4). Retinoblastoma protein (pRB) is also important for cell cycle progression during the G1 to S phase transition. Dephosphorylation of pRB inhibits cell cycle progression by interacting with transcription factors of the E2F family, whereas phosphorylation of pRB results in induction of cell cycle progression through the breaking of pRb/E2F complexes (2-4). Accumulating data have shown that many anti-cancer agents arrest the cell cycle and subsequently induce apoptotic cell death (5-8). Therefore, the induction of cell cycle arrest associated with apoptotic cell death is an important mechanism that is involved in the anti-cancer properties of many anti-cancer agents.

Flavonoids are polyphenolic compounds that are present in high concentrations in fruits and vegetables. These compounds display a remarkable spectrum of biological activities, including anti-allergic, anti-inflammatory, anti-oxidant, and anti-cancer effects, and immunomodulation and modulation of enzymatic activities (9-12). Among them, 7,8-dihydroxyflavone (7,8-DHF, Fig. 1) is a selective tyrosine kinase receptor B (TrkB) agonist that can pass the blood-brain barrier (13-15). Several studies have shown that 7,8-DHF has neurotrophic and anti-oxidant activity (13,15-17). Nevertheless, no studies have investigated the molecular mechanisms underlying its anti-cancer effects.

In the present study, we investigated the effects of 7,8-DHF on proliferation of U937 human monocytic leukemia cells to characterize the relationship between cell cycle arrest and related signaling pathways. The observed decrease in viability of U937 cells exposed to 7,8-DHF was mediated by the induction of G1 arrest in response to inhibition of cyclin E expression and the phosphorylation of pRB. Up-regulation of expression of

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the Cdk inhibitor p27 by the 7,8-DHF treatment was also associated with the increased binding to Cdk2.

Materials and methods

Cell culture, 7,8-DHF treatment and cell viability study. The human leukemia cell line U937 was purchased from the American Type Culture Collection (Rockville, MD), and maintained at 37˚C in a humidified condition of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. 7,8-DHF was obtained from Professor Jin Won Hyun of Jeju National University (Jeju, Korea), and dissolved in dimethyl sulfoxide (DMSO). Measurement of cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of MTT to MTT-formazan by mitochondria.

Flow cytometry analysis. After treatment with 7,8-DHF, cells were collected, washed with cold phosphate buffered saline (PBS), and fixed in 75% ethanol at 4˚C for 30 min. The DNA content of cells was measured using a DNA staining kit (CycleTest™ PLUS Kit, Becton-Dickinson, San Jose, CA). Propidium iodide (PI)-stained nuclear fractions were obtained by following the kit protocol. Fluorescence intensity was determined using a FACScan flow cytometer and analyzed by CellQuest software (Becton-Dickinson).

Detection of apoptosis by annexin-V FITC staining. Cells were washed with PBS and resuspended in an annexin-V binding buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$. Aliquots of the cells were incubated with annexin-V fluorescein isothiocyanate (FITC), mixed, and incubated for 15 min at room temperature in the dark. PI at a concentration of 5 μg/ml was added to distinguish necrotic cells. The apoptotic cells (V^+/PI^-) were measured by a fluorescence-activated cell sorter analysis in a FACScan flow cytometer (18).

Immunoprecipitation, gel electrophoresis and western blot analysis. The cells were harvested, lysed, and protein concentrations were quantified using the Bio-Rad protein assay (Bio-Rad Lab., Hercules, CA), following the procedure described by the manufacturer. For immunoprecipitation, cell extracts were incubated with an immunoprecipitating antibody in extraction buffer for 1 h at 4˚C. The immuno-complex was collected on protein G/A-Sepharose beads (Sigma-Aldrich, St. Louis, MO). Western blot analysis was performed as previously described (19). Briefly, the immunoprecipitated proteins and total proteins were subjected to electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) by electroblotting. Blots were probed with the desired antibodies for 1 h, incubated with diluted enzyme-linked secondary antibody, and then visualized by enhanced chemiluminescence (ECL) according to recommended procedures (Amersham Corp., Arlington Heights, IL). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham.

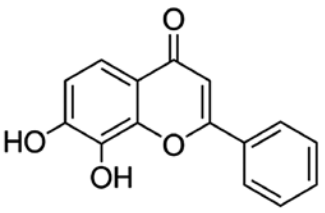

Figure 1. Chemical structure of 7,8-dihydroxyflavone (7,8-DHF).

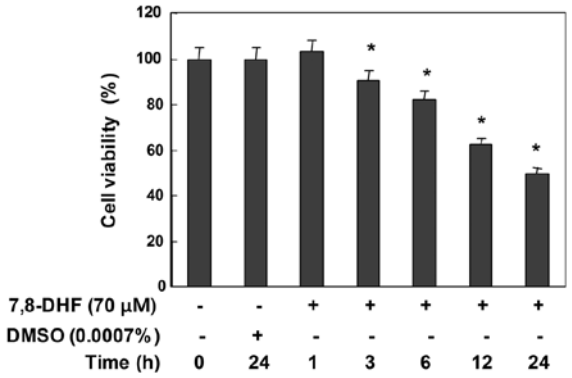

Figure 2. Growth inhibition in response to 7,8-DHF treatment of U937 cells. The cells were plated at $1x10^5$ cells per 35 mm dish. The cells were then treated with 70 mM 7,8-DHF for the indicated times, and the level of growth inhibition was measured using a metabolic-dye-based MTT assay. The data are reported as the mean ± SD of three independent experiments. The significance was determined by a Student's t-test (*$p<0.05$, compared with control).

Statistical analysis. Data are presented as the mean ± SD of at least three separate experiments. Comparisons between groups were analyzed using Student's t-test. P-values <0.05 were considered statistically significant.

Results

Inhibition of cell viability and induction of apoptosis by 7,8-DHF in U937 cells. To determine if 7,8-DHF influenced the cell viability of U937 cells, the cells were cultured in the presence of 70 μM 7,8-DHF for the indicated times and cell viability was determined by MTT assay. As shown in Fig. 2, 7,8-DHF time-dependently led to a reduced viability of U937 cells. Further experiments were carried out to determine if the inhibitory effect of 7,8-DHF on cell viability was the result of apoptotic cell death. For this study, flow cytometry analysis with annexin-V and PI staining was performed to determine the magnitude of the apoptosis elicited by 7,8-DHF. The results indicated that the number of annexin V-positive cells were increased in 7,8-DHF treated U937 cells in a time-dependent manner suggesting that the inhibition of cell viability observed in response to 7,8-DHF is associated with the induction of apoptotic cell death (Fig. 3).

Induction of G1 arrest by 7,8-DHF in U937 cells. To further characterize the inhibitory effect of 7,8-DHF on U937 cells, the cell cycle patterns of the cells were examined to determine whether 7,8-DHF treatment of cells resulted in alteration of cell cycle progression. Analyses of the cell cycle distribution of cells after exposure to 7,8-DHF showed that these cells were accumulated in the G1 phase of the cell cycle in a time-dependent

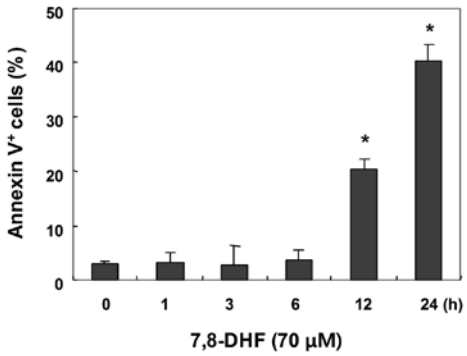

Figure 3. Induction of apoptosis by 7,8-DHF in U937 cells. Cells were incubated with 70 μM 7,8-DHF for the indicated times. The cells were collected and stained with FITC-conjugated annexin-V and PI for flow cytometry analysis. The apoptotic cells were determined by counting the percentage of annexin V(+), PI(-) cells and the percentage of annexin V(+), PI(+) cells. The results are expressed as the mean ± SE of three independent experiments. UT, untreated control; D, vehicle control (0.0007% DMSO).

Table I. Induction of G1 arrest of U937 cells by 7,8-DHF treatment.

	Cells (%)		
Time (h)	G1	S	G2/M
0	57.80	25.53	16.70
1	56.47	25.55	17.98
3	56.44	25.20	18.35
6	74.05	16.60	9.35
12	79.73	10.49	9.78
24	90.27	3.95	5.78
DMSO	56.95	24.96	18.09

The cells grown under the same conditions as described in Fig. 2 were collected, fixed, and stained with PI for flow cytometry analysis. The percentages of cells in the each phase are presented. The data represent the average of two independent experiments. DMSO, vehicle control (0.0007% DMSO).

fashion (Table I), which was accompanied by a decrease in the number of cells in S and G2/M phases when compared with untreated control cells. Taken together, these results suggest that the growth inhibitory and apoptotic effects of 7,8-DHF in U937 cells are the result of a block during G1 phase.

Effects of 7,8-DHF on the levels of G1 phase cell cycle regulators. Because 7,8-DHF arrested U937 cells in the G1 phase of the cell cycle, we determined the expression levels of the cell cycle regulating factors at the G1 boundary, such as cyclin D1, cyclin E, Cdk2, Cdk4 and Cdk6, by Western blot analysis. As shown in Fig. 4, protein levels of cyclin E were markedly inhibited after 12-24 h exposure to 7,8-DHF; however, the levels of cyclin D, Cdk2, Cdk4 and Cdk6 were not.

7,8-DHF induces association of p27 with Cdk2. Since Cdk activity is highly regulated by association with Cdk inhibitors, we next examined the possible up-regulation of these proteins

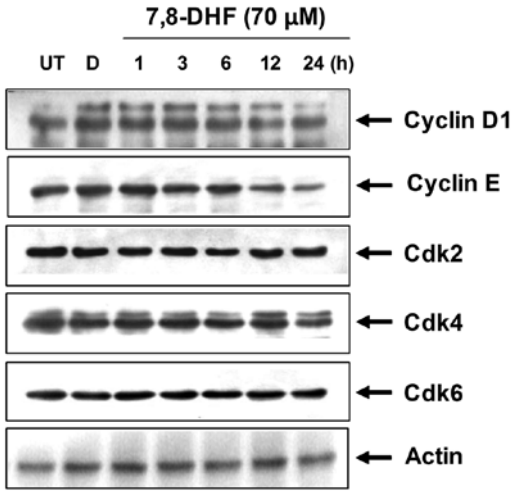

Figure 4. Effects of 7,8-DHF on the expression of G1 phase-associated cyclins and Cdks in U937 cells. U937 cells were treated with 70 μM 7,8-DHF for the indicated times. The cells were lysed and the cellular proteins were then separated by electrophoresis on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Next, the membranes were probed with the indicated antibodies and the proteins were visualized using an ECL detection system. Actin was used as an internal control. UT, untreated control; D, vehicle control (0.0007% DMSO).

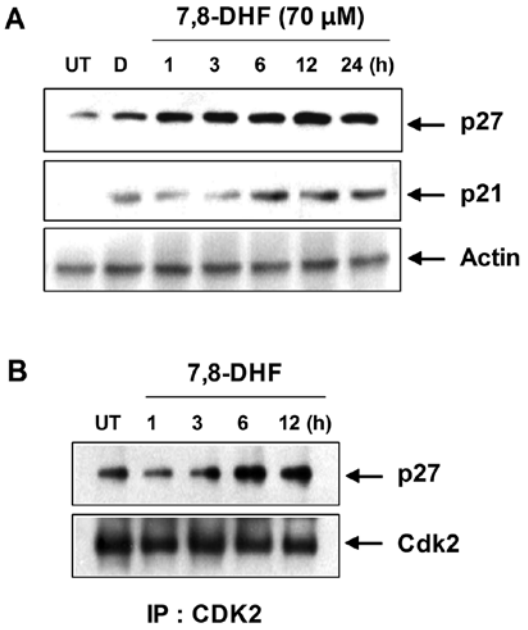

Figure 5. Effects of 7,8-DHF on the expression of Cdk inhibitors in U937 cells. (A) The cells grown under the same conditions as described in Fig. 3 were lysed and the cellular proteins were then separated by electrophoresis on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Next, the membranes were probed with the anti-p21 and anti-p27, and the proteins were visualized using an ECL detection system. Actin was used as an internal control. (B) Whole cell lysates (0.5 mg of protein) from control cells and cells treated with 70 μM 7,8-DHF for the indicated times were immunoprecipitated with anti-Cdk2 antibody. Immuno-complexes were separated on SDS-polyacrylamide gels, and then transferred to nitrocelluloses. p21 and Cdk2 levels were detected with anti-p21 antibody and ECL detection. UT, untreated control; D, vehicle control (0.0007% DMSO).

in U937 cells treated with 7,8-DHF. In untreated control cells, the levels of Cdk inhibitors such as p21 and p27 were undetectable by Western blot analysis (Fig. 5A). However, incubation of

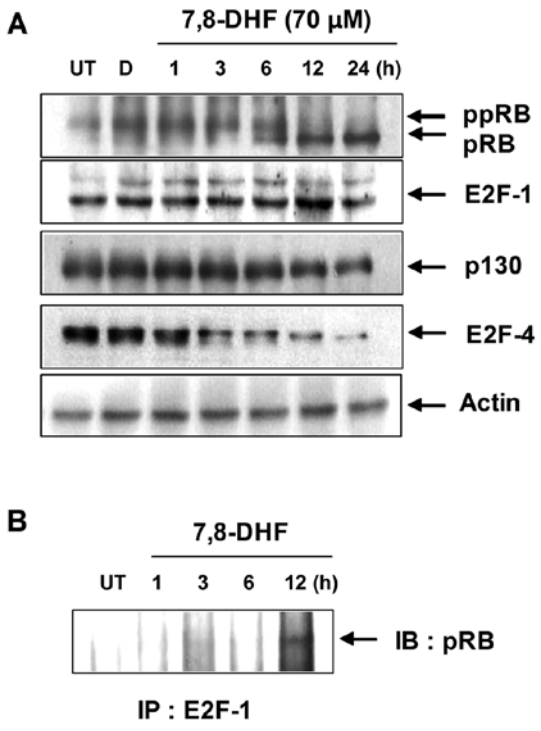

Figure 6. Hypophosphorylation of pRB and enhanced association of pRB and E2F-1 in U937 cells after exposure to 7,8-DHF. (A) Cells were treated with 70 μM 7,8-DHF for the indicated times and the total cell lysates were then prepared and separated by electrophoresis on an 8 or 10% SDS-polyacrylamide gel. Western blotting was then performed using anti-pRB, anti-p130, anti-E2F-1 and anti-E2F-4 antibodies. Actin was used as an internal control. (B) Whole cell lysates (0.5 mg of protein) from control cells and cells treated with 70 μM 7,8-DHF were immunoprecipitated with anti-E2F-1 antibody. Immunocomplexes were separated by 8% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with anti-pRB antibody. Proteins were detected by ECL detection. UT, untreated control; D, vehicle control (0.0007% DMSO).

U937 cells with 7,8-DHF resulted in a time-dependent increase in these proteins. In addition, co-immunoprecipitation analysis indicated that treatment of cells with 7,8-DHF time-dependently resulted in a significant increase in the binding of Cdk2 with p21 (Fig. 5B). However, the association of p21 with Cdk2 was almost undetectable by co-immunoprecipitation analysis of the untreated log phase cells and 7,8-DHF-treated U937 cells (data not shown).

Down-regulation of pRB phosphorylation by 7,8-DHF. Because pRB family proteins including pRB and p130 are important checkpoint proteins in the G1 phase of the cell cycle, we next determined the kinetics between phosphorylation of pRB and p130, and the transcription factors, E2F-1 and E2F-4. As shown in Fig. 6, the levels of pRB expression changed from the hyperphosphorylated form to the hypophosphorylated form after 7,8-DHF treatment, and these changes occurred in a time-dependent manner. Total protein levels of E2F-4 were time-dependently down-regulated in 7,8-DHF-treated cells, however, the levels of p130 and E2F-1 proteins remained relatively unchanged. In addition, co-immunoprecipitation analysis indicated that the association of pRB and E2F-1 was almost undetectable in the untreated cells. However, there was an increase in the association of pRB and E2F-1 in 7,8-DHF-treated cells (Fig. 6B), suggesting that 7,8-DHF inhibits the release of E2F-1 protein from pRB.

Discussion

Recent studies have reported that 7,8-DHF, a member of the flavonoid family, is a selective TrkB agonist that has neurotrophic effects in various neurological diseases such as stroke and Parkinson's disease (13-15). This compound has been reported to display significant neurotrophic and anti-oxidant activity (13,15-17). However, the precise mechanism of the anti-cancer effect of 7,8-DHF is unclear. In the present study, we found that 7,8-DHF causes G1 phase arrest with apoptosis induction in human leukemia U937 cells, which appeared to account for its anti-proliferative activity. These results provide the first evidence that 7,8-DHF can inhibit abnormal cancer cell cycle transitions that are associated with induction of apoptosis.

In terms of regulation of cell cycle progression, Cdks play a most critical role via binding with their catalytic subunit cyclins and Cdk inhibitors (1,2). Progression from G1 to S phase of the cell cycle is regulated by D-type cyclins and cyclin E, and their cognate kinases, Cdk2, 4 and 6, which act by phosphorylating and inactivating pRB family proteins prior to the restriction point at which cells commit to DNA synthesis. Cyclin E-Cdk2 acts later in G1 phase (3,4). Therefore, any factor affecting the activity of any of these kinases could abrogate the normal inactivation of pRB and cause cells to arrest in G1 phase. Under normal conditions, pRB binds to the members of the E2F transcription family proteins. However, growth factors induce phosphorylation and dissociation of pRB from E2F, which triggers G1 cell cycle progression (20). Thus, the complex between cyclin D/E and Cdks is an obvious candidate for control of pRB phosphorylation. If decreased levels of either protein or the association between respective binding partners were to be observed, a concomitant decrease in the degree of pRB phosphorylation would be expected (3,4). Based on these ideas, we investigated the effects of 7,8-DHF on the expression of G1 phase regulatory factors in U937 cells. The present results demonstrated that 7,8-DHF treatment did not affect the intracellular protein levels of cyclin D1, Cdk2, Cdk4 and Cdk6. However, immunoblotting analyses demonstrated that 7,8-DHF markedly inhibited the levels of cyclin E proteins (Fig. 4), and 7,8-DHF induced Cdk inhibitors such as p21 and p27 (Fig. 5A).

In general, Cdk inhibitors are well known to inhibit the activity of Cdks by direct binding with various cyclin/Cdk complexes, so that the complex formation of cyclins, Cdks, and Cdk inhibitors is increased in cells arrested by DNA damaging agents (21,22). Therefore, we further investigated whether 7,8-DHF might increase binding between Cdks and Cdk inhibitor proteins. The data generated in the present study demonstrated that 7,8-DHF treatment resulted in a selective increase in the binding of Cdk2 with p27 (Fig. 5B). Furthermore, 7,8-DHF also blocks pRB phosphorylation and decreases the level of transcription factor E2F-4 expression without marked alteration of p130 and E2F-1 proteins (Fig. 6A). Thus, we determined whether the ability of pRB to associate with E2F-1 was compromised in response to 7,8-DHF. Association of pRB with E2F-1 was almost undetectable by co-immunoprecipitation analysis of the untreated log phase cells (Fig. 6B). However, 12 h after addition of 7,8-DHF there was an increase in the association of pRB and E2F-1. These

results demonstrate that 7,8-DHF may induce G1 phase arrest of U937 cells through the down-regulation of Cdks kinase activity, via selective binding of Cdk2 with p27 and inhibition of pRB phosphorylation.

Although elucidation of the detailed molecular mechanism for induction of cell cycle arrest by 7,8-DHF is beyond the scope of this study, the present study demonstrates that: i) reduced survival of U937 cells after exposure to 7,8-DHF is associated with G1 phase cell cycle arrest; ii) 7,8-DHF can inhibit cell cycle progression at the G1 phase by decreasing cyclin E and pRB phosphorylation; iii) treatment with 7,8-DHF results in increased binding of Cdk2 with p27 and E2-F1 with pRB. These novel phenomena have not been previously described for 7,8-DHF and suggest that 7,8-DHF and related compounds may have significant potential as targets for cancer treatment.

Acknowledgements

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