Nutrition and Cancer

Fisetin Inhibits the Activities of Cyclin-Dependent Kinases Leading to Cell Cycle Arrest in HT-29 Human Colon Cancer Cells[1]

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ABSTRACT Fisetin, a natural flavonol present in edible vegetables, fruits, and wine, was reported to exert anticarcinogenic effects. The objective of the current study was to examine the effect of fisetin on the cell cycle progression of the human colon cancer cell line HT-29. HT-29 cells were cultured in serum-free medium with 0, 20, 40, or 60 μmol/L fisetin. Fisetin dose dependently inhibited both cell growth and DNA synthesis ($P < 0.05$), with a 79 ± 1% decrease in cell number observed 72 h after the addition of 60 μmol/L fisetin. Perturbed cell cycle progression from the G_1 to S phase was observed at 8 h with 60 μmol/L fisetin treatment, whereas a G_2/M phase arrest was observed after 24 h ($P < 0.05$). The phosphorylation state of the retinoblastoma proteins shifted from hyperphosphorylated to hypophosphorylated in cells treated with 40 μmol/L fisetin. ($P < 0.05$). Fisetin decreased the activities of cyclin-dependent kinases (CDK)2 and CDK4; these effects were likely attributable to decreases in the levels of cyclin E and D1 and an increase in $p21^{CIP1/WAF1}$ levels ($P < 0.05$). However, fisetin also inhibited CDk4 activity in a cell-free system ($P < 0.05$), indicating that it may directly inhibit CDk4 activity. The protein levels of cell division cycles (CDC)2 and CDC25C and the activity of CDC2 were also decreased in fisetin-treated cells ($P < 0.05$). These results indicate that inhibition of cell cycle progression in HT-29 cells after treatment with fisetin can be explained, at least in part, by modification of CDK activities. J. Nutr. 135: 2884–2890, 2005.

KEY WORDS: • *G_1 phase arrest* • *G_2/M phase arrest* • *cell division cycle 2* • *$p21^{CIP1/WAF1}$* • *retinoblastoma proteins*

Colon cancer remains one of the leading causes of cancer death in Western countries, and modifications of diet and life style offer measures for reducing the risk of developing colon cancer. Epidemiologic studies indicate that a diet containing plentiful vegetables and fruits can reduce the risk of several cancers, including colon cancer (1). Flavonoids are a group of naturally occurring polyphenolic compounds present in fruits, vegetables, and other edible plants. Some of these flavonoids possess chemopreventive properties, and numerous studies were performed recently to assess the mechanisms whereby each flavonoid prevents cancer, including induction of cell cycle arrest, apoptosis, and antiproliferation [reviewed in (2)].

The mammalian cell cycle is divided into 4 distinct phases; the G_1, S, G_2, and M phases. Cyclin-dependent protein kinases (CDKs)[3] are important regulators of the eukaryotic cell cycle progression, and the activities of these kinases require the binding of a positive regulatory subunit, known as a cyclin. The protein levels of cyclins (e.g., cyclins D, E, and A) vary during the G_1/S cell cycle transition (3), leading to activation of CDK2 and CDK4/6. A main function of these cyclin/CDK complexes is to phosphorylate members of the retinoblastoma protein (Rb) family. This event, in turn, promotes cellular proliferation and S-phase progression through the release of a family of transcriptional regulators, collectively named the E2Fs, which are normally bound to hypophosphorylated Rb (4). Released E2F elicits the expression of various genes associated with S-phase progression (5). On the other hand, CDK inhibitors (CDKIs), such as $p21^{WAF/CIP1}$ and $p27^{KIP1}$, play important roles in cell cycle regulation by coordinating internal and external signals that inhibit cell cycle progression at important checkpoints (6).

The cell division cycle (CDC)2 kinase is required for the onset of mitosis, and its activity is subject to multiple levels of regulation. CDC2 is active only at the G_2/M border, and turned off as the cells enter the anaphase stage of mitosis. The first step in generating active CDC2 is its association with a cyclin. In animal cells, CDC2 associates with an A- or B-type cyclin. The CDC2-cyclin B complex must enter the nucleus and be phosphorylated by cyclin-dependent kinase-activating kinase at threonine 161 to be active (7,8). In addition, it must be activated by the dephosphorylation of tyrosine 15 and threonine 14 of CDC2 at the G_2/M boundary by CDC25 (9).

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[3] Abbreviations used: BSA, bovine serum albumin; CDC, cell division cycle; CDK, cyclin-dependent kinase; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HRP, horse-radish peroxidase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PCNA, proliferating cell nuclear antigen; Rb, retinoblastoma protein.

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Fisetin, 3,3′,4′,7-tetrahydroxyflavone, is found in fruits and vegetables, such as strawberry, apple, persimmon, grape, onion, and cucumber at concentrations of 2–160 μg/g (10). Fisetin was reported to inhibit proliferation (11) and induce apoptosis (12,13) of various tumor cells at concentrations of 1–100 μmol/L in cell culture models. To our knowledge, the effects of fisetin on colon cancer have not been studied in detail, with the exception of a study that reported the decreased viability of HT-29 and Caco-2 colon cancer cells in the presence of fisetin (14). The initial stage of colon cancer development is often characterized by an increased proliferation of the epithelium leading to the formation of adenomas. This is primarily a result of dysregulated cell cycle control and/or decreased apoptosis, as is usually observed in colorectal cancers (15,16). In the present study, we investigated the mechanisms of fisetin inhibition of HT-29 cell growth by examining cell cycle regulatory proteins.

MATERIALS AND METHODS

Materials. The following reagents and chemicals were obtained from the respective suppliers: fisetin, anti-β-actin, RIA-grade bovine serum albumin (BSA), and transferrin (Sigma); antibodies against CDC25C, phsopho-CDC2 (Tyr15), cyclin B1, and phospho-Rb (Ser807/811) (Cell Signaling); horse-radish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG (Amersham); [γ-^{32}P]ATP (NEN-Life Sciences); anti-cyclin D1 antibody (Neomarkers); and antibodies against p21$^{CIP1/WAF1}$ (c-19), p27^{KIP1}, cyclin A (c-19), cyclin E (M-20), CDK2 (M-2), CDK4 (c-22), p34CDC2, E2F-1 (C-20), Rb (c-15), and proliferating cell nuclear antigen (PCNA, PC10) (Santa Cruz Biotechnology).

Cell culture. The HT-29 cell line was obtained from the American Type Culture Collection and maintained in DMEM/F-12, containing 100 mL/L of fetal bovine serum (FBS), with 100,000 U/L of penicillin and 100 mg/L of streptomycin. HT-29 cells between passages 139 and 150 were used in these studies. To examine the effect of fisetin, cells were plated with DMEM/F-12 containing 10% FBS. Before the fisetin treatment, the cell monolayers were rinsed and serum-starved for 24 h, with DMEM/F-12 supplemented with 5 mg/L transferrin, 1 g/L BSA, and 5 μg/L selenium (serum-free medium). After serum starvation, fresh serum-free medium, with or without the indicated concentrations of fisetin, was replaced. The medium was changed every 2 d. Viable cell numbers were estimated 24, 48, and 72 h after the cells were exposed to fisetin using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (17). Fisetin was dissolved in dimethyl sulfoxide (DMSO), and all cells received DMSO to a final concentration of 0.1%.

To determine [^{3}H]thymidine incorporation, HT-29 cells were plated in 96-well plates, at a density of 6000 cells/well, serum-starved, and treated with fisetin for 5 h, as described above. At this time, 0.5 μCi [^{3}H]thymidine was added to each well, and the incubation continued for an additional 3 h at 37°C. The [^{3}H]thymidine incorporation into the DNA of HT-29 cells was determined, as described previously (17).

Cell cycle analysis by flow cytometry. Cells were plated in 24-well plates, at 50,000 cells/well, in DMEM/F-12 containing 10% FBS. Cells were serum-starved and treated with 60 μmol/L fisetin, and were trypsinized. The nuclei were stained with propidium iodide as described previously (18) and subjected to fluorescence-activated cell sorting analysis utilizing FACScan (Becton Dickinson). The data were analyzed using Modfit version 1.2 software.

Immunoprecipitation and immunoblot analyses. Cell lysates were prepared as described previously (18), and the protein content determined using the bicinchoninic acid protein assay kit (Pierce). Cell lysates (750 μg protein) were precleared with 1.5 μg of normal rabbit IgG and 100 μL of protein A-Sepharose bead slurry (Amersham) on a rotating platform for 1 h and centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatants were incubated with 1 μg of anti-CDK2, anti-CDK4, or anti-CDC2 antibody for 1 h at 4°C. The protein A-Sepharose was added and incubated for another hour at 4°C; the beads were then washed 4 times with lysis buffer by centrifugation at 600 × *g* for 5 min at 4°C. Total cell lysates (50 μg protein) or immunoprecipitated proteins were resolved on a SDS-PAGE (4–20% or 10–20%) and transferred onto a polyvinylidene fluoride membrane (Millipore). The blots were incubated for 1 h with anti-cyclin D1 (1:200), anti-cyclin A (1:1000), anti-cyclin E (1:1000), anti-CDC2 (1:1000), anti-CDK2 (1:1000), anti-CDK4 (1:1000), anti-p21$^{CIP1/WAF1}$ (1:500), anti-p27^{KIP1}, anti-phospho-Rb (1:1000), anti-Rb (1:1000), anti-PCNA (1:1000), or anti-β-actin (1:2000) antibody. The blots were then incubated with anti-mouse or rabbit HRP-conjugated antibody. Signals were detected based on an enhanced chemiluminescence method, using the SuperSignal West Dura Extended Duration Substrate (Pierce). Densitometric analysis was carried out using the Bio-profile Bio-ID application (Vilber-Lourmat). Expression levels were normalized to β-actin and the control levels were set at 100%.

RT-PCR. Total RNA was isolated using Tri reagent (Sigma), and cDNA synthesized using 2 μg of total RNA with SuperScript™ II reverse transcriptase (Invitrogen), as described previously (19). For amplification of cDNA, primers for human p21$^{CIP1/WAF1}$ (upstream primer; 5′-AAT AAG GAA GCG ACC TGC AA-3′; downstream primer, 5′-CCA ACG CTT TTA GAG GCA GA-3′, annealing at 55°C for 1 min with 38 cycles) were used. The expressions of human β-actin transcripts were examined as an internal control, as described previously (20). For each combination of primers, the kinetics of the PCR amplification was studied, the number of cycles corresponding to the plateau determined, and the PCR performed within the exponential range. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide. Bands corresponding to each specific PCR product were quantified by densitometric scanning of the exposed film using the Bio-profile Bio-ID application (Vilber-Lourmat).

CDK kinase activity in fisetin-treated HT-29 cells. Cell lysate (750 μg protein) was immunoprecipitated with polyclonal antibody against CDK2, CDC2, or CDK4, washed with either 20 mmol/L Tris-HCl, pH 7.5, and 4 mmol/L $MgCl_2$ (CDK2 and CDC2 kinase buffer) or 50 mmol/L HEPES, 10 mmol/L β-glycerophosphate, 1 mmol/L NaF, 0.1 mmol/L sodium orthovanadate, and 10 mmol/L $MgCl_2$ (CDK4 kinase buffer). After washing, the beads were incubated in either 15 μL kinase buffer, containing 2 μg histone H1 (Roche) and 3 μCi [γ-^{32}P]ATP, at 37°C for 30 min (CDK2 and CDC2 kinase assay) or 30 μL kinase buffer with 1 μg Rb (769, Santa Cruz) and 10 μCi [γ-^{32}P]ATP at 30°C for 30 min (CDK4 kinase assay). The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The ^{32}P-labeled histone H1 or Rb was resolved by SDS-PAGE; the gel was dried and subjected to autoradiography. Signals were quantified by densitometric scanning of the exposed film.

Assessment of fisetin effect on CDK4 activity in cell-free system. Serum-starved HT-29 cells were lysed, and 750 μg total cellular protein used to immunoprecipitate the active CDK4 complexes using the anti CDK4 antibody. After capturing with protein A-Sepharose and subsequent washes, CDK4 activities of the active immune complexes were estimated in the presence of increasing concentrations of fisetin in CDK4 kinase buffer containing 10 μCi [γ-^{32}P]ATP and 1 μg Rb. Reactions were incubated at 37°C for 30 min, terminated by the addition of SDS loading dye, resolved on SDS-PAGE, and the dried gels subjected to autoradiography.

Statistical analysis. For all studies, 3–6 independent experiments were performed with separate batches of cells. The results are expressed as means ± SEM. They were analyzed by 1-way, 2-way, or 2-factor repeated-measures ANOVA. Differences between the treatment groups were analyzed using Duncan's multiple range or *t* tests. Different were considered significant at $P < 0.05$. Pearson's correlation coefficients were calculated between CDK2 activity and p21 binding to CDK2. All statistical analyses were performed using the SAS System for Windows V8.1 (SAS Institute).

RESULTS

Effect of fisetin on growth and cell cycle progression in HT-29 cells. Fisetin decreased the viable HT-29 cell numbers, in a dose-dependent manner, with a 79 ± 1% decrease in

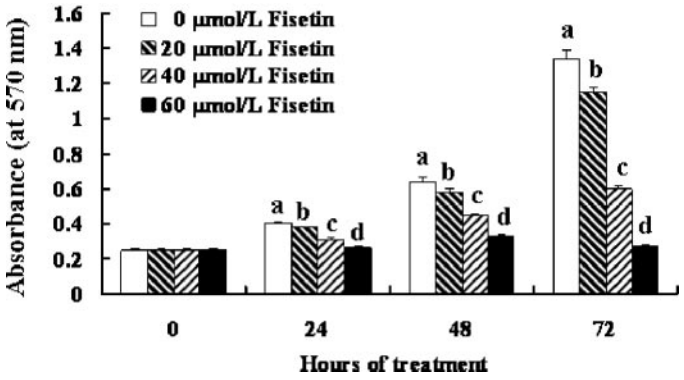

FIGURE 1 Fisetin decreases viable HT-29 cell numbers. Each bar represents the mean ± SEM, $n = 6$. Means at a time without a common letter differ, $P < 0.05$.

cell numbers within 72 h of the addition of 60 μmol/L fisetin (**Fig. 1**). This decrease in cell numbers was attributable to the induction of cell cycle arrest. At 8 h after the addition of fisetin, the percentage of cells in the G_1 phase increased, with a concomitant decline in the G_2/M phase fraction. However, after 24 h, there was an accumulation of cells in the G_2/M phase, with an equivalent decline in the G_1 phase (**Fig. 2**). Consistent with the occurrence of cell cycle G_1 arrest at 8 h, fisetin decreased the [^{3}Hthymidine incorporation into the DNA of HT-29 cells (**Fig. 3**).

Fisetin inhibits CDK2 and CDK4 activity leading to decreased Rb phosphorylation. We first examined the effects of fisetin on proteins controlling the G_1/S cell cycle transition.

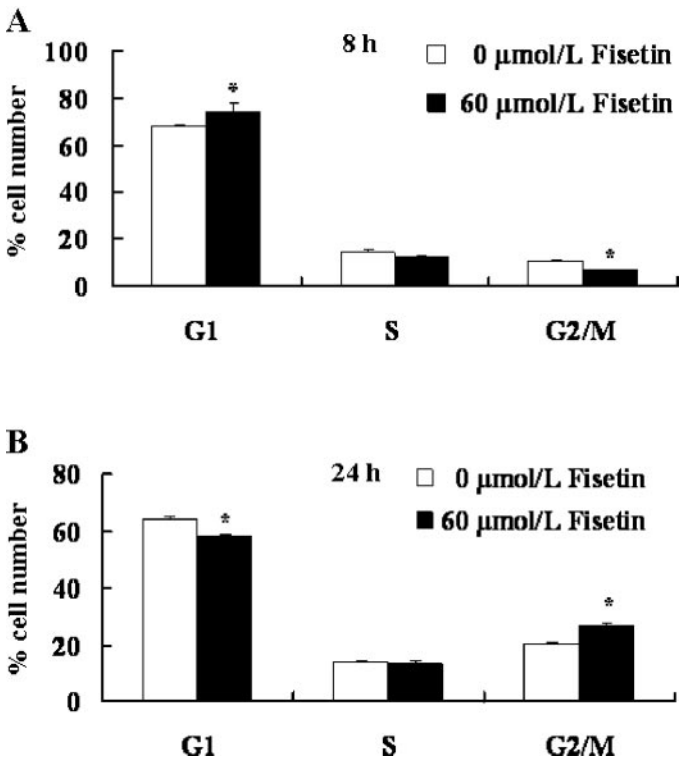

FIGURE 2 Fisetin induces cell cycle arrest in HT-29 cells. HT-29 cells were treated with 0 or 60 μmol/L fisetin for 8 h (*A*) or 24 h (*B*). Each bar represents the mean ± SEM, $n = 6$. *Different from 0 μmol/L fisetin, $P < 0.05$.

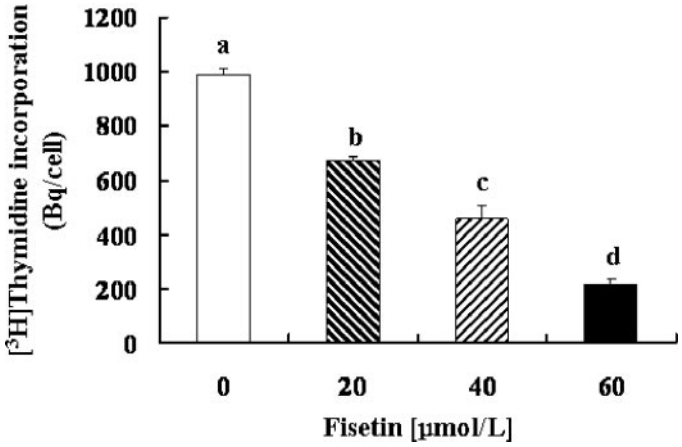

FIGURE 3 Fisetin decreases [^{3}H]thymidine incorporation in HT-29 cells. The incorporation of [^{3}H]thymidine into DNA was normalized to the viable cell number determined by the MTT assay. Each bar represents the mean ± SEM, $n = 6$. Means without a common letter differ, $P < 0.05$.

Fisetin did not affect CDK2 protein levels (**Fig. 4***A*). Fisetin did not change the cyclin A level, whereas that of cyclin E was decreased in HT-29 cells within 2 h after treatment with 60 μmol/L fisetin. At 8 h, cyclin E was decreased in cells treated with 40 μmol/L fisetin. Fisetin increased the p21 levels at 8 and 12 h and the increased p21 at 8 h was dependent on the dose of fisetin (Fig. 4*A*). However, the p27 level was not affected by fisetin treatment. To determine whether fisetin regulates the expression of $p21^{CIP1/WAF1}$ at the RNA level, RT-PCR analyses were performed. The treatment of HT-29 cells with increasing concentrations of fisetin led to a concentration-dependent increase in $p21^{CIP1/WAF1}$ mRNA levels, with a 300% increase in $p21^{CIP1/WAF1}$ transcripts 8 h after the addition of 60 μmol/L fisetin (Fig. 4*B*). To determine whether the decreased cyclin E and increased p21 expression induced by fisetin treatment led to inhibition of the CDK2 activity, total cell lysates were immunoprecipitated with the CDK2 antibody followed by in vitro kinase assays, using histone H1 as the substrate. A decrease in the CDK2 activity was detected within 2 h after 60 μmol/L fisetin treatment, with a significant difference evident during 12 h of treatment ($P < 0.05$). A decrease in CDK2 activity dependent on the fisetin dose was detected at 8 h. The decrease in the CDK2 activity correlated ($r = -0.79$, $P = 0.003$) with the enhanced binding of p21 to CDK2 (**Fig. 5**). Thus, the p21 induced after fisetin treatment of colon cancer cells binds to CDK2 and inhibits its activity.

We next examined fisetin regulation of the cyclin D1/CDK4 complex. The protein levels of CDK4 decreased after fisetin treatment ($P < 0.05$). The cyclin D1 levels were also lower at 4, 8, and 12 h in cells treated with 60 μmol/L fisetin; at 8 h, there were decreases in the CDK4 and cyclin D1 levels in cells treated with 40 μmol/L fisetin ($P < 0.05$, **Fig. 6***A*). CDK4 activity was next examined using an in vitro kinase assay employing the GST-Rb C-terminus as a substrate after CDK4 immunoprecipitation with the CDK4 antibody (Fig. 6*B*). CDK4 activity was drastically decreased within 30 min of fisetin treatment, which was consistently lower in fisetin-treated cells throughout the 12-h incubation period ($P < 0.05$). Because fisetin markedly decreased CDK4 activity within 0.5 h, with a moderate decrease in the CDK4 and increase in the cyclin D1 levels during this time period, we next examined whether fisetin directly inhibits CDK4 activity in a cell-free system. Fisetin at 20 μmol/L concentration

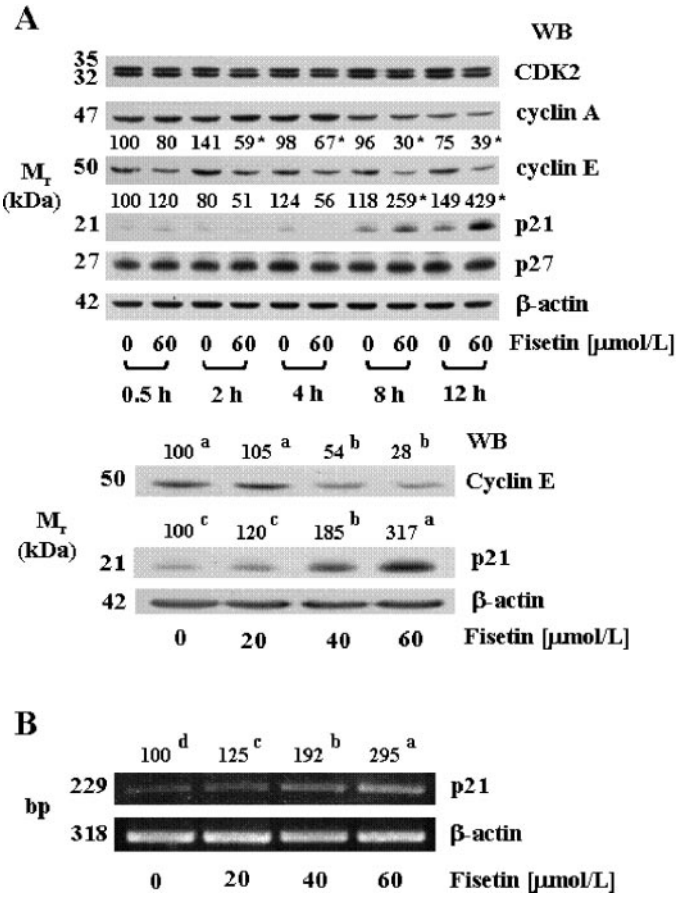

FIGURE 4 Fisetin decreases cyclin E levels and increases p21 levels in HT-29 cells. HT-29 cells were treated with 0 or 60 μmol/L fisetin for the indicated periods or for 8 h with various concentrations of fisetin. (*A*) Immunoblot analysis. (*B*) RT-PCR. Photographs of chemiluminescent detection of the immunoblots or photographs of the ethidium bromide–stained gel (p21 transcripts), which are representative of 3 independent experiments, are shown. The relative abundance of cyclin E and p21 to their own β-actin was quantified and the control levels were set at 100%. The adjusted mean level ($n = 3$) of each band is shown above each blot. The pooled SEMs for cyclin E and p21 data for the time study were 11.9 and 44.6, respectively. The pooled SEMs for cyclin E and p21 data for the dose-response study were 17.9 and 24.5, respectively. The pooled SEM for the p21 transcripts was 17.8. *Different from 0 μmol/L fisetin at a time, $P < 0.05$. Means without a common letter differ, $P < 0.05$.

decreased CDK4 activity in the cell-free system (Fig. 6C). These results indicate that the CDK4 inhibition was due to both the direct and indirect actions of fisetin on this kinase.

Because CDK2 and CDK4 activities were decreased by fisetin, we wanted to determine whether the decreased CDK activity induced by fisetin led to decreased phosphorylation of Rb proteins. Western blot analysis with the phospho-Rb antibody revealed decreased phospho-Rb levels in cells treated with 40 or 60 μmol/L fisetin (**Fig. 7***A* and *B*). When the immunoblot was probed using total Rb antibody, 2 bands were detected, with an increase in the intensity of the lower band (hypophosphorylated Rb) and the disappearance of the upper band (hyperphosphorylated Rb) in fisetin-treated cells (Fig. 7). Phosphorylation of the Rb family allows the accumulation of the E2F transcription factor activities (21,22), which are essential for regulation of the cell cycle (23,24). Fisetin decreased the E2F-1 protein levels within 30 min (**Fig. 8**).

PCNA, a processivity factor for DNA polymerase δ and ϵ, plays essential roles in DNA synthesis and repair (25). Western blot analysis revealed that fisetin did not affect PCNA protein levels (Fig. 8).

Fisetin inhibits CDC2 activity. Because fisetin induced G_2/M phase arrest at 24 h, the levels of G_2/M regulatory proteins were examined in HT-29 cells treated with various concentrations of fisetin for 24 h. The protein levels of CDC2 and CDC25C decreased in cells treated with 40 μmol/L fisetin, whereas that of p-CDC2 (Tyr15) increased in cells treated with 20 μmol/L fisetin ($P < 0.05$). Fisetin had no effect on the cyclin B1 protein level (**Fig. 9***A*). We next examined whether fisetin inhibited CDC2 activity. Cell lysates were incubated with the CDC2 antibody, and the immune complexes precipitated with protein A Sepharose. The results from an in vitro kinase assay using histone H1 as the substrate revealed that CDC2 activity decreased in cells treated with 40 and 60 μmol/L fisetin (Fig. 9*B*).

DISCUSSION

One of the most common incidents required for human cancer development is deregulation of the cell cycle mechanism (26). Cell cycle regulatory proteins are the potential molecular targets for cancer therapy/prevention because their functions are well regulated in normal cells, but they are regulated differently in tumor cells. In recent years, considerable attention has been paid to the ability of dietary flavonoids to inhibit cell cycle progression. However, the effects of fisetin, which is present in fruits and vegetables, have not been studied extensively. In the present study, fisetin induced G_1

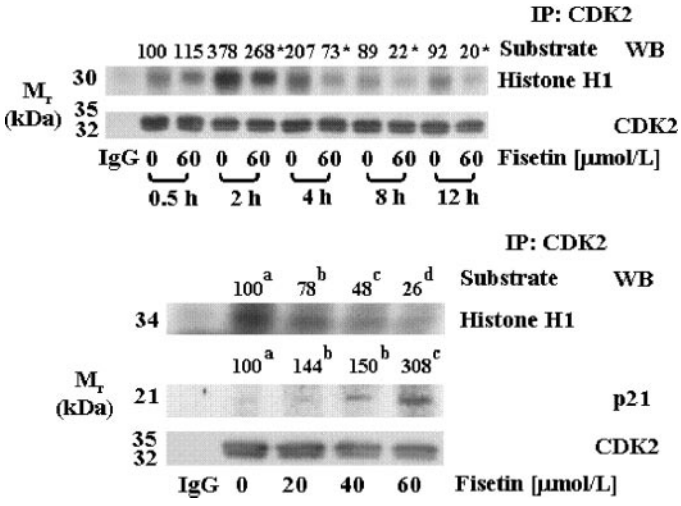

FIGURE 5 Fisetin decreases CDK2 activity in HT-29 cells. HT-29 cells were treated with 0 or 60 μmol/L fisetin for the indicated periods or for 8 h with various concentrations of fisetin. Total cell lysates were immunoprecipitated with anti-CDK2 antibody. The immune complexes were analyzed by Western blotting (CDK2 or p21) or by an in vitro kinase assay using histone H1 as a substrate. Photographs of chemiluminescent detection of the immunoblots or an autoradiograph of the dried gel (CDK2 activity), which are representative of 3 independent experiments, are shown. The relative abundance of each band to its own CDK2 protein was quantified and the control levels were set at 100%. The adjusted mean level ($n = 3$) of each band is shown above each blot. The pooled SEM for Histone H1 data for the time study was 27.1. The pooled SEMs for Histone H1 and p21 data for the dose-response study were 5.1 and 15.9, respectively. *Different from 0 μmol/L fisetin at a time, $P < 0.05$. Means without a common letter differ, $P < 0.05$.

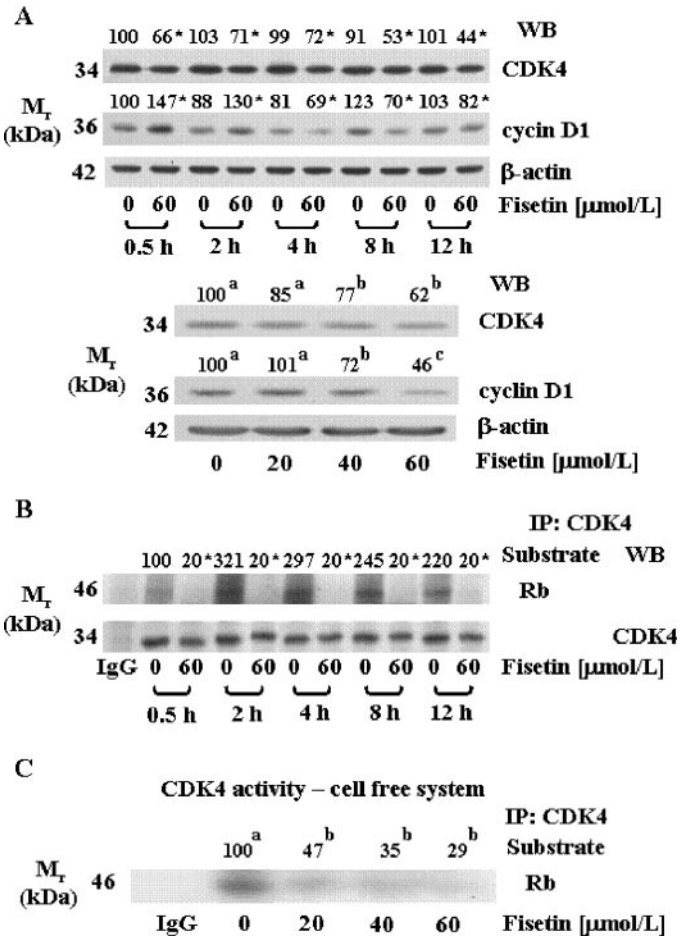

FIGURE 6 Fisetin decreases CDK4 activity in HT-29 cells. (*A*) Immunoblot analysis. HT-29 cells were treated with 0 or 60 μmol/L fisetin for the indicated periods or for 8 h with various concentrations of fisetin. Photographs of chemiluminescent detection of immunoblots, which are representative of 3 independent experiments, are shown. The relative abundance of each band to its own β-actin was quantified and the control levels were set at 100%. The adjusted mean level ($n = 3$) of each band is shown above each blot. The pooled SEMs for CDK4 and cyclin D1 for the time study were 16.4 and 23.2, respectively. The pooled SEMs for CDK4 and cyclin D1 for the dose-response study were 9.7 and 12.6, respectively. (*B*) Immunoprecipitation and in vitro kinase assay. HT-29 cells were treated with 0 or 60 μmol/L fisetin for the indicated periods. Total cell lysates were immunoprecipitated with an anti-CDK4 antibody. The immune complexes were analyzed by Western blotting (CDK4) or by an in vitro kinase assay using glutathione *S*-transferase-Rb as a substrate. Photographs of chemiluminescent detection of immunoblots or an autoradiograph of the dried gel (CDK4 activity), which are representative of 3 independent experiments, are shown. The relative percentage change in radioactive Rb signal to its own CDK4 protein band was quantified and the control levels were set at 100%. The adjusted mean level of each band ($n = 3$) is shown above each blot (the pooled SEM = 16.7). (*C*) CDK4 activity in cell-free system. CDK4 immune complex was prepared from serum-starved HT-29 cells and used for in vitro kinase assay in the absence or presence of various concentrations of fisetin. An autoradiograph of the dried gel (CDK4 activity), representative of 3 independent experiments, is shown. The relative mean changes ($n = 3$) in the level of radioactive Rb signal are shown above each blot (the pooled SEM = 7.6). *Different from 0 μmol/L fisetin at a time, $P < 0.05$. Means without a common letter differ, $P < 0.05$.

and G_2 arrests within 8 and 24 h, respectively, in HT-29 human colon cancer cells, and led to decreased CDK2, CDK4, and CDC2 activities. Because modulation of CDK activity is a very attractive target for the treatment and prevention of human cancers (27,28), future studies should investigate whether fisetin inhibits colon cancer development in animal models and human studies.

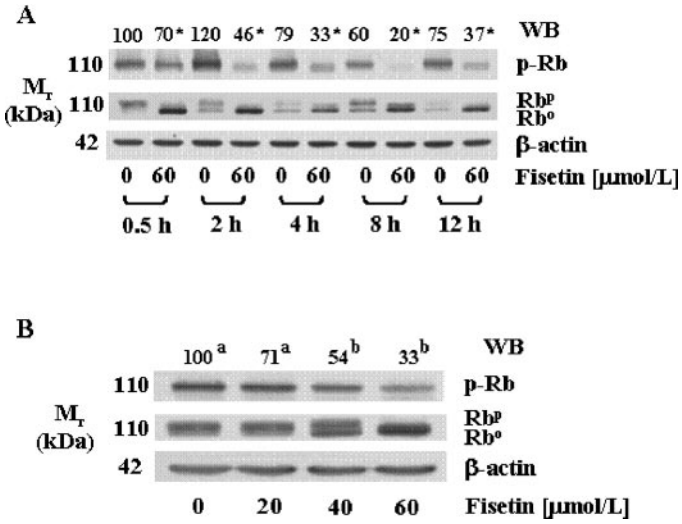

FIGURE 7 Fisetin decreases hyperphosphoylated Rb and increases hypophosphorylated Rb in HT-29 cells. HT-29 cells were treated with 0 or 60 μmol/L fisetin for the indicated periods (*A*) or for 8 h with various concentrations of fisetin (*B*). Total cell lysates were subjected to immunoblotting with antibodies against phospho-Rb (p-Rb), Rb or β-actin. Two bands detected with total Rb antibody were Rb^P, hyperphosphorylated Rb and Rb^O, hypophosphorylated Rb. Photographs of chemiluminescent detection of the blots, which are representative of 3 independent experiments, are shown. The relative abundance of p-Rb to its own β-actin was quantified and the control levels were set at 100%. The adjusted mean level ($n = 3$) of p-Rb is shown above each blot. The pooled SEMs for time study and the dose-response study were 14.8 and 16.3, respectively. *Different from 0 μmol/L fisetin at a time, $P < 0.05$. Means without a common letter differ, $P < 0.05$.

The levels of the cyclin proteins fluctuate temporally during the cell cycle, leading to activation of their respective CDKs. Treatment of cells with fisetin decreased cyclin E, but did not greatly affect CDK2 and cyclin A protein levels, suggesting that the decreased cyclin E contributed to the decreased CDK2 activities observed in fisetin-treated cells. Fisetin decreased the cyclin D1 protein levels, which might contribute to the decrease in CDK4 activity.

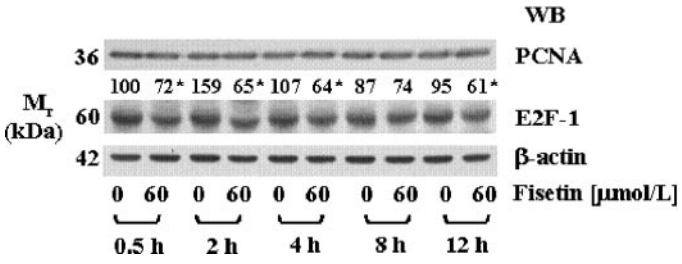

FIGURE 8 Fisetin decreases E2F-1 protein levels in HT-29 cells. HT-29 cells were treated with 0 or 60 μmol/L fisetin for the indicated periods. Photographs of chemiluminescent detection of the immunoblots, which are representative of 3 independent experiments, are shown. The relative abundance of E2F-1 to its own β-actin was quantified and the control levels were set at 100%. The adjusted mean level ($n = 3$) of E2F-1 is shown above each blot (the pooled SEM = 7.8). *Different from 0 μmol/L fisetin at a time, $P < 0.05$.

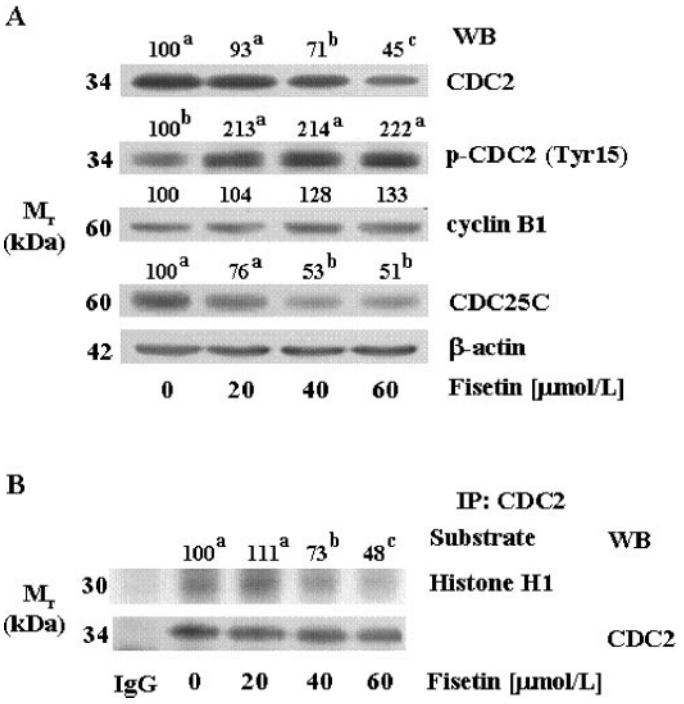

FIGURE 9 Fisetin decreases CDC2 activity in HT-29 cells. HT-29 cells were treated for 24 h with various concentrations of fisetin. (*A*) Immunoblot analysis. The relative abundance of each band to its own β-actin was quantified and the control levels were set at 100%. The adjusted mean level ($n = 3$) of each band is shown above each blot. The pooled SEMs for CDC2, p-CDC2, cyclin B1, and CDC25C were 14.1, 34.4, 24.3, and 20.2, respectively. (*B*) Immunoprecipitation and in vitro kinase assay. Total cell lysates were immunoprecipitated with an anti-CDC2 antibody. The immune complexes were analyzed by Western blotting (CDC2) or by an in vitro kinase assay using histone H1 as a substrate. Photographs of chemiluminescent detection of immunoblots or an autoradiograph of the dried gel (CDC2 activity), which are representative of 3 independent experiments, are shown. The relative percentage change in radioactive histone H1 signal to its own CDC2 protein band was quantified and the control levels were set at 100%. The adjusted mean level ($n = 3$) of each band is shown above each blot (the pooled SEM = 9.8). Means without a common letter differ, $P < 0.05$.

The CDK inhibitor $p21^{CIP1/WAF1}$ binds and inhibits the cyclin D-, E- and A-dependent kinases, regulating the G_1 to S phase transition of the cell cycle (29–31). $p21^{CIP1/WAF1}$ also contributes to the maintenance of cell cycle arrest in the G_2 phase (32). Overexpression of $p21^{CIP1/WAF1}$ inhibits the proliferation of mammalian cells (30,33). In the present study, fisetin dose dependently increased the p21 protein levels and p21 binding to CDK2, indicating that the increased $p21^{CIP1/WAF1}$ contributes to the fisetin-induced decrease in CDK activities due to binding with these enzymes. Transcription of the p21 gene is regulated by both p53-dependent and -independent mechanisms (34). We observed that fisetin induced p21 mRNA levels in a p53-independent manner in HT-29 cells because these cells lack functional p53 (35). Our results suggest that the increased $p21^{CIP1/WAF1}$ contributes to fisetin-induced G_1 arrest by binding to and inhibiting the activities of cyclin-dependent kinases.

Our in vitro kinase assay revealed that CDK4 activity was markedly decreased in fisetin-treated cells within 0.5 h, whereas CDK4 protein levels were moderately decreased and the cyclin D1 levels were not affected at this time point. In addition, when fisetin was added with immunoprecipitated CDK4 in a cell-free system, CDK4 activity was directly inhibited, indicating that fisetin directly inhibits the function of this enzyme. Indeed, it was shown recently that fisetin binds to the active form of the CDK4/6 subfamily, forming hydrogen bonds with the side chains of residues in the binding pocket, which go through large conformational changes during CDK activation by cyclin binding (36). These results indicate that the CDK4 inhibition was due to both direct and indirect actions of fisetin on this kinase.

CDKs phosphorylate members of the Rb family, which, in turn, promotes cell cycle progression ([reviewed in (37)]. Cyclin D-dependent kinases initiate Rb phosphorylation in the mid-G_1 phase, after which cyclin E-CDK2 becomes active and completes the process by phosphorylating the Rb at additional sites (21,22,38). In quiescent cells, hypophosphorylated Rb associates with the E2F transcription factors (21,39,40). Phosphorylation of Rb proteins liberates the E2F proteins, allowing them to act as transcription activators. The E2F target genes that are upregulated include those necessary for the completion of the G_1/S phase transition, as well as for DNA replication (41). In the present study, fisetin decreased the levels of phospho-Rb and increased hypophosphorylated Rb. These findings suggest that decreased phosphorylated Rb (or increased hypophosphorylated Rb), due to the decrease in CDK activity, may explain, at least in part, the effects of fisetin on cell cycle progression. In addition, the expression of E2F-1, a member of the E2F transcription factor family, was decreased in fisetin-treated HT-29 cells, suggesting that the decrease in E2F-1 levels may also contribute to cell cycle arrest at the G_1 phase.

The G_2 checkpoint prevents cells from entering mitosis when DNA is damaged, providing an opportunity for repair and stopping the proliferation of damaged cells. CDC2 is an important target of the pathways that mediate rapid G_2 arrest in response to DNA damage. Inhibition of CDC2 activity takes place rapidly after DNA damage by blocking the dephosphorylation of CDC2 at tyrosine 15 and threonine 14 (42,43). In the present work, the treatment of cells with fisetin led to a decrease in CDC2 activity, which may be responsible for the G_2/M arrest observed in fisetin-treated cells. Fisetin decreased CDC2 protein levels, but increased that of phospho-CDC2 (Tyr15), the inactive form of CDC2. Our results suggest that fisetin downregulates CDC25C, leading to decreased dephosphorylation of CDC2.

E2Fs regulate the expressions of genes required for the G_1/S transition, including those encoding DNA replication proteins, the enzymes involved in nucleotide synthesis, and components of the origin recognition complex [reviewed in (44,45)]. In addition to this role of E2F, recent work showed that E2F induces genes that are normally regulated at the G_2 phase of the cell cycle; they encode proteins such as cyclin B1 and CDC2 that function in mitosis (5,46–48). In the present study, fisetin downregulated the E2F-1 and CDC2 protein levels. It remains to be determined whether the decreased CDC2 levels in fisetin-treated cells were due to the decreased activity of E2F caused by the increased Rb hypophosphorylation.

To date, the absorption, metabolism, and blood concentrations of fisetin have not been well characterized. The present study utilized fisetin at concentrations of 20–60 μmol/L; other investigators (12–14) examined the effects of fisetin at slightly higher concentrations than those used in our studies. To determine whether the concentrations used in the cell culture studies are nutritionally relevant, the concentrations of fisetin in human serum as well as in a variety of foods should be determined in the future.

In summary, we showed that treating HT-29 cells with fisetin resulted in the arrest of the cell cycle at the G_1 and G_2 phases, which was due, at least in part, to the inhibition of CDKs. CDKs are promising anticancer drug targets because inhibitors of CDKs are cytotoxic to cancer cells in vitro and suppress tumor growth in animals (49–51). Future studies are warranted to investigate the effect of fisetin on colon cancer development in animal cancer models.

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