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Differential Effects of Ferulic Acid and *p*-Coumaric Acid on S Phase Distribution and Length of S Phase in the Human Colonic Cell Line Caco-2

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Ferulic acid (FA) and *para*-coumaric acid (*p*-CA) may mediate the protective effects of whole-grain cereals against colon cancer. Therefore, the effects of FA and *p*-CA on the metabolic activity, proliferation, cell cycle phase distribution, and kinetics of the colonic endothelial tumor cell line Caco-2 was studied. Both compounds at 1500 μM decreased the number of cells to 43−75% of control after 2−3 days of treatment. Cell cycle phase distribution and cell cycle kinetics were determined by flow cytometric analysis after bromodeoxyuridine labeling. Each compound at 1500 μM decreased the proportion of cells in the G_1 phase and increased the proportion of cells in the S and G_2 phases. Treatment with 1500 μM FA significantly increased the length of the S phase, while *p*-CA did not. It was concluded that FA and *p*-CA inhibited cell proliferation by presumably affecting different cell cycle phases, and this warrants further investigations because this inhibition may be one explanation for the diet-related protection against cancer.

KEYWORDS: Cell cycle; cereals; *p*-coumaric acid; ferulic acid; proliferation

INTRODUCTION

Colonic tumors are among the most common malignancies in the Western world. The Colon epithelial cells are exceptionally fast proliferating and are chronically exposed to potentially mutagenic and carcinogenic food residues and bacterial metabolites. Therefore, these cells are particularly vulnerable to mutational events. Diet is one important factor that influences the susceptibility to cancer, and many components and mechanisms of action have been proposed. For example, several components such as quercetin, curcumin, and ferulic acid (FA) have been described to modulate proliferation and apoptosis of intestinal epithelial cells, thereby increasing their genomic integrity to protect against carcinogenesis (*1*−*3*).

One food group that may influence colon cancer incidence is whole grain cereals. Several epidemiological case-control studies on the association of diet to colon cancer have been performed, and they revealed a significantly lower incidence of colon cancer in individuals with a high whole grain consumption than in individuals with intermediate and low whole grain consumption (*4*, *5*). Prospective investigations of dietary fiber in relation to colorectal cancers have, however, shown contradictory results. Whereas the EPIC study (*6*) found an inverse relation between dietary fiber intake and incidence of colorectal cancers, another study found no such relation regarding cereal fiber intake (*7*). Moreover, the intake of whole grain cereals was found to be inversely associated with total mortality and mortality from cardiovascular diseases (*8*). Also, human intervention studies with cereal products have shown beneficial effects on markers of several diseases such as LDL cholesterol levels in blood and systolic blood pressure (*9*, *10*). Several components, such as dietary fiber and phenolic compounds in the cereals, may be responsible for these beneficial effects.

The phenolic compounds are important constituents of cereals. In the plant, their function is to protect against photosynthetic stress, reactive oxygen species, wounds, and herbivores. Several phenolic compounds in cereals such as FA and *p*-coumaric acid (*p*-CA) belong to the hydroxycinnamic acid family and have shown anti-mutagenic and anti-tumor effects. FA is linked through ester bonds to hemicellulose of the cell wall and is found in almost all plant species (*11*, *12*). The richest dietary source of these cell wall bound cinnamates is the cereal bran. Servings of 10 g wheat bran or maize bran could supply up to 70 and 300 mg of esterified cinnamic acid, respectively (*13*). Free FA is mainly taken up in the small intestine where it is metabolized through phase I and phase II reactions (*14*), while FA bound to hemicellulose passes undegraded through the small intestine to the colon where it can be released due to bacterial fermentation. A high intake of cereal bran can increase the plasma concentration of FA up to 200 nM (*15*).

It has been hypothesized that the effect of the phenolic compounds on tumor cells is due to their antioxidant effect, by which the compound scavenges reactive oxygen species (ROS) and related compounds. Normal cells are highly sensitive

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to ROS, and FA has been demonstrated to reduce ROS-mediated damage in neuronal cell systems (*16*). Tumor cells may, however, be dependent on a certain amount of hydrogen peroxide, and deprivation of hydrogen peroxide could cause cell cycle arrest or apoptosis of these cells (*17*). In addition, many other cancer protection mechanisms beyond these ROS chemopreventive effects may be important in the mechanism of action of phenolic compounds (*1, 18*).

Several in vivo studies in animals have shown beneficial effects of dietary FA and *p*-CA. Feeding of rats with FA prevented the development of azoxymethane-induced abberrant crypt foci and suppressed the progression of preneoplasia to malignant neoplasia of the colon (*19*). Moreover, exposure to FA significantly increased the activities of the detoxification enzymes glutathione S-transferase and quinone reductase in rat liver and colonic mucosa (*3*).

Much attention has been given to the effects of flavonoids in fruits, berries, spices, wine, coffee, tea, and chocolate, while phenols in cereals have received less attention. Because many beneficial effects of cereals have been reported, it is necessary to further exploit the mechanisms of action of these grain phenols on a cellular and molecular level. We have investigated the effects of FA and *p*-CA on metabolic activity, proliferation, the cell cycle phase distribution and kinetics of the colon cancer cell line Caco-2, because this, to our knowledge, has not yet been done.

MATERIAL AND METHODS

Materials. RPMI 1640 growth medium, nonessential amino acids, penicillin/streptomycin, and fetal calf serum (FCS) were obtained from Biochrom KG, Berlin, Germany. Tissue culture plastics were purchased from Nunc, Roskilde, Denmark. AlamarBlue Reagent was acquired from BioSource Intl Inc., Camarillo, CA, and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide reagent (MTT) was from ICN Biomedicals Inc., Aurora, OH. Phosphate-buffered saline (PBS: 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, pH 7.3) was obtained from Invitrogen, Carlsbad, CA. Dimethyl sulfoxide (DMSO) was purchased from Merck KgaA, Darmstadt, Germany. Propidium iodide (PI), FA, and *p*-CA were purchased from Sigma Chemical Co., St. Louis, MO. Caco-2 cells, a human colon cell line, were obtained from ATCC-LGC promochem, Teddington, UK.

Cell Culture. Caco-2 cells were kept pre-confluent and sub-cultured twice a week. The cells were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum, 1% nonessential amino acids, penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were incubated at 37 °C in 5% CO_2 in water-saturated air. All experiments were performed at passages 8−30.

Assays of Metabolic Activity. The 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) and AlamarBlue reagents were used to determine the metabolic activity of the cells. MTT added to the medium of the cells is taken up via endocytosis and reduced to blue formazan crystals inside the cell via mitochondrial NADH-dependent dehydrogenases. The amount of formazan produced reflects the metabolic activity of the cells. The AlamarBlue solution, containing an oxidation−reduction indicator, was added to the culture medium of the cells. The oxidized resazurin (nonfluorescent) indicator is taken up by cells and is reduced to resorufin (fluorescent) by the action of the electron transport chain. Resazurin is assumed to be reduced at the end of the electron transport chain at the level of oxygen. The amount of resorufin produced reflects the metabolic activity of the cells (*20*, *21*). Both assays are assumed to be proportional to the cell numbers.

The cells were seeded into 96-well plates with 7500 cells in 150 μL medium per well, and they were left to adhere for 1 day prior to challenge with 50 μL of the test substance. Stock solutions of FA and *p*-CA of 6000 μM were made in complete medium. The solutions were sterile-filtered before use, and they were protected from light. The test concentrations used were 0, 0.1, 10, 50, 100, 250, 500, and 1500 μM.

The cells were exposed to the substance for 1−3 days prior to analysis. The AlamarBlue reagent was then added according to the manufacturer's instructions, and the 96-well plates were incubated for 3 h in the CO_2 incubator. The fluorescence was determined at 590 nm in a Millipore CytoFluor 2300 spectrophotofluorometer using the software CytoFluor 2300 v.3A1 (Millipore Co., Bedford, MA). For the MTT assay, 20 μL of the MTT reagent (5 mg/mL PBS) was added to each well, and the plates were incubated for 1 h in the CO_2 incubator. The medium was removed, and the cells were washed once with 200 μL of PBS. Next, 100 μL of DMSO was added to each well to dissolve the blue formazan crystals. The plates were incubated on a shaker for 10 min, and the absorbance was measured at 540 nm in a Labsystems iEMS Reader MF spectrophotometer (Labsystems Oy, Helsinki, Finland) using the software DeltaSoft II v. 4.14 (Biometallics Inc., Princeton, NJ).

Cell Proliferation. One million cells were seeded in 12 mL of growth medium in Petri dishes (diameter 9 cm). The cells were then incubated for 24 h before the addition of test substances to final concentrations of 0, 15, 150, or 1500 μM. The attached cells were harvested by a standard trypsination procedure, and they were pooled with the detached cells floating in the medium. The cells were stained with TrypanBlue and counted in a hemocytometer. The cell number was determined after 1−3 days of treatment.

Flow Cytometric Analysis of Cell Cycle Phase Distribution. Cells used for flow cytometric (FCM) analysis of the cell cycle phase distribution and detection of apoptotic/dead cells (sub-G_1 peak) were pelleted by centrifugation at 750*g* for 10 min at 4 °C. The medium was decanted, and 70% ethanol was added to the cells during vigorous vortexing (approximately 1 mL/2 × 10^6 cells). The cells were stored at −20 °C until further processing. Approximately 2 × 10^6 cells were transferred to new tubes and washed twice with 10 mL of PBS. The cells were then stained with the DNA intercalating fluorescent dye PI (100 μg/mL PI, 100 μg/mL Rnase, and 0.6% NP-40 in PBS). Immediately prior to FCM analysis, the nuclear suspension was suctioned three times through a 0.7 mm cannula and filtered through a 50 μm nylon mesh filter to minimize the number of nuclear aggregates. The DNA content was recorded as the fluorescence intensity at 620 nm after excitation of PI by the 488 nm band of a 200 mW argon ion laser beam using a Cytograph System 50-H flow cytometer (Ortho instruments, Westwood, MA) equipped with a 4 W argon-ion laser (Lexel Corp, Palo Alto, CA). The computational analysis was performed with MULTIPLUS software (Phoenix Flow Systems, San Diego, CA). Nuclear doublets were excluded before calculation of the distribution of cells in G_1, S, G_2, and sub-G_1 phases.

Flow Cytometric Analysis of Cell Cycle Kinetics. Cells (1 × 10^6) were seeded in 12 mL of growth medium in Petri dishes (diameter 9 cm) and were incubated for 1 day before the addition of the test substances (0, 150, and 1500 μM). After 1, 2, and 3 days of treatment, the cells were pulse-labeled with 5 μM BrdUrd for 30 min. Cells were then collected directly after labeling for the calculation of relative movement at time zero (RM_{zero}). RM_{zero} is a measure of the initial distribution of cells in S phase when harvested directly after BrdUrd labeling (*22*). In addition, to be able to calculate the length of the S phase, at 48 h of treatment, a number of parallel cultures were rinsed twice with medium containing 0.5% FCS after the 30 min BrdUrd labeling period. Next, 10 mL of complete medium (37 °C) with the appropriate substance was added. The cultures were then further incubated for 6 h at 37 °C in the CO_2 incubator and then trypsinized. The cells were pelleted at 750*g* for 10 min at 4 °C and resuspended in 70% ethanol and stored at −20 °C until further preparation. Before the analysis, the cells were pelleted at 750*g* for 10 min at 4 °C and washed in 5 mL of PBS. To isolate nuclei, the cells were incubated in 2 mL of 0.2 mg of pepsin in 0.1 M HCl for 20 min at 37 °C. To partly denature DNA (necessary for BrdUrd antibody binding), 2 M HCl was added, and the samples were incubated for 20 min at 37 °C in a shaking water bath. The DNA denaturation was terminated by immersing the tubes in an ice bath and adding 9 mL of 0.1 M $Na_2B_4O_7$ (pH 9.3). The cells were then pelleted at 750*g* for 10 min at 4 °C and washed in 5 mL of PBS. The primary anti-BrdUrd antibody (100 μL, diluted 1:10

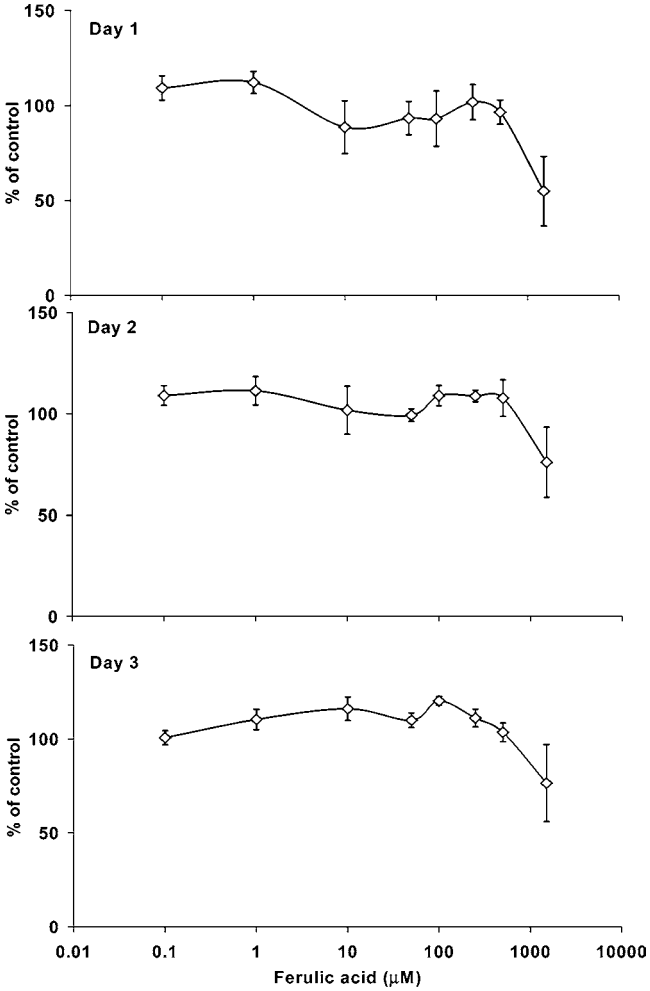

Figure 1. The metabolic activity of Caco-2 cells treated with ferulic acid (FA) as analyzed with the AlamarBlue assay. Cells were seeded in 96-well plates, and, 1 day after seeding, FA was added to the concentrations shown. Metabolic activity was determined after 1, 2, and 3 days of treatment. The symbols represent the mean, and the bars, SD of six samples. The diagrams represent one out of three experiments with similar results.

in PBT; PBS containing 0.5% Tween 20 and 1% bovine serum albumin) was added, and the samples were incubated for 1 h at 37 °C. The cells were washed once with PBS and were incubated with the secondary FITC-conjugated antibody (100 μL, diluted 1:20 in PBT) for 1 h at 37 °C. After resuspending the cells in 5 mL of PBS and pelleting them at 750*g* for 10 min at 4 °C, PI solution (20 μg/mL PI, 20 mg/mL Rnase, and 0.12% NP-40 in PBS) was added. Immediately prior to FCM analysis, the nuclear suspension was suctioned three times through a 0.7 mm cannula and filtered through a 50 μm nylon mesh filter. Both fluorochromes (PI and FITC) were excited at 488 nm using the flow cytometer mentioned above. The DNA content was recorded as the fluorescence intensity at 620 nm, and the BrdUrd content was recorded as the fluorescence intensity at 520 nm. The fluorescence signals were stored as list data files. For the computerized analysis, MULTI2D and MULTICYCLE software in the cytometer was used.

Data Analysis. Data analysis was performed as described previously (*23*). In short, BrdUrd-labeled and nonlabeled cells were analyzed regarding their DNA content using MULTICYCLE software. After the contribution of nuclear doublets by electronic threshold settings in the red area versus red peak bivariate distributions (*24*) was minimized, the nuclei were displayed in DNA versus BrdUrd cytograms. From these analyses, the length of the S phase was calculated, as well as the distribution of cells in the S phase (RM_{Zero}).

Statistical Calculations. For the statistical evaluation, one-way ANOVA followed by Tukey HSD and Bonferroni tests were used.

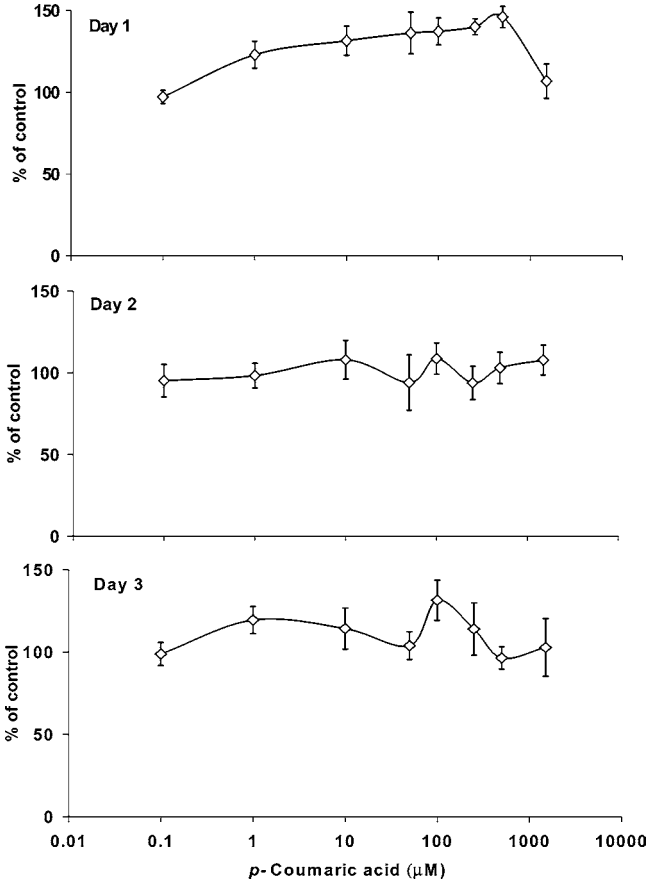

Figure 2. The metabolic activity of Caco-2 cells treated with *p*-coumaric acid (*p*-CA) as analyzed with the AlamarBlue assay. Cells were seeded in 96-well plates, and, 1 day after seeding, *p*-CA was added to the concentrations shown. Metabolic activity was determined after 1, 2, and 3 days of treatment. The symbols represent the mean, and the bars, SD of six samples. The diagrams represent one out of three experiments with similar results.

RESULTS

Assay of Metabolic Activity. The metabolic activity of cells treated with FA was not impaired except at the highest concentration (1500 μM) as determined by the AlamarBlue assay (**Figure 1**). *p*-CA did not have any remarkable effect on cell metabolism in cells treated for 2 or 3 days (**Figure 2**). However, 1 day of treatment with *p*-CA resulted in an increased reduction of resazurin in AlamarBlue at 1−500 μM concentrations. At 1500 μM *p*-CA, cell metabolism was at the level of that found in the control. The MTT assay gave similar results (results not shown), and neither substance showed any strong toxicity. On the basis of these results, we decided to use the concentrations 15, 150, and 1500 μM for further studies.

Cell Proliferation. Treatment with 15 or 150 μM FA or *p*-CA did not affect the proliferation of Caco-2 cells (**Figures 3** and **4**). When the cells were treated with 1500 μM FA, however, the cell number was reduced to 75% and 43% ($p < 0.05$ and $p < 0.001$) of control after 2 and 3 days of treatment, respectively. Similarly, when Caco-2 cells were treated with 1500 μM *p*-CA for 2 and 3 days, the cell number was 74% and 55% ($p < 0.05$ and $p < 0.001$) of control, respectively.

Flow Cytometric Analysis of the Cell Cycle Phase Distribution. Treatment with 15 and 150 μM FA for 1−3 days did not affect the cell cycle phase distribution (**Figure 5**). However, when the cells were treated with 1500 μM FA, the percentage of cells in the G_1 phase was substantially lowered as compared

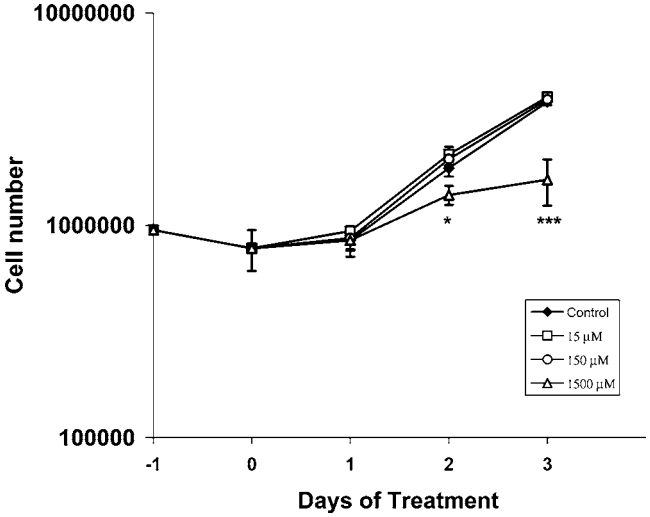

Figure 3. Effect of ferulic acid (FA) on the proliferation of Caco-2 cells. Cells were seeded 1 day before the addition of FA (day 0) at different concentrations. The symbols represent the mean, and the bars, SD of three samples. The data represent one out of two experiments with similar results. Data points denoted by * ($p < 0.05$) and *** ($p < 0.001$) were significantly different from controls.

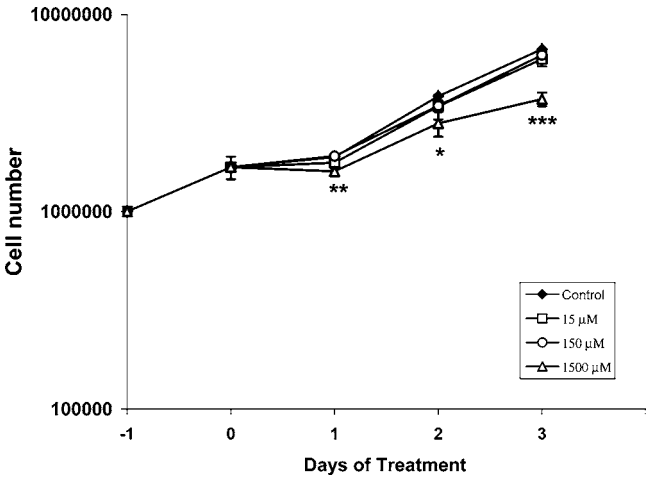

Figure 4. Effect of *p*-coumaric acid (*p*-CA) on the proliferation of Caco-2 cells. Cells were seeded 1 day before addition of *p*-CA (day 0) at different concentrations. The symbols represent the mean, and the bars, SD of three samples. The diagram represents one out of two experiments with similar results. Data points denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) were significantly different from controls.

to control at all days of treatment ($p < 0.001$ to $p < 0.05$). The decrease in the G_1 phase was reflected in an increased S phase population after 1 day of treatment and an increased G_2 phase population after 2 and 3 days of treatment ($p < 0.01$ and $p < 0.001$). At 2 days of treatment, when we determined cell cycle kinetics, the fractions of the cells in the G_1, S, and G_2 phases were 0.75, 1, and 2.25 times that of the control, respectively.

Treatment with 15 and 150 μM *p*-CA did not affect the cell cycle phase distribution (**Figure 6**). Similar to the results with FA, treatment with 1500 μM *p*-CA resulted in a decreased G_1 phase population as compared to control at all days of treatment ($p < 0.01$), which lead to an increased S phase population. In the 2 day samples, the fractions of the cells in the G_1, S, and G_2 phases were 0.75, 1.12, and 1.15 times that of the control, respectively. Neither FA nor *p*-CA treatment had any significant effect on the proportion of cells in the sub-G_1 peak except for

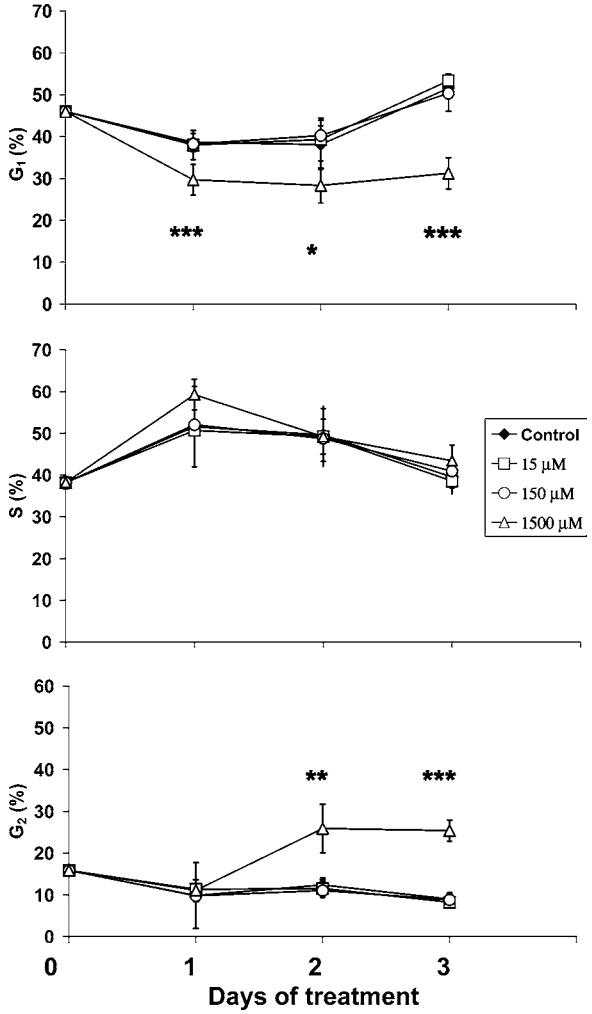

Figure 5. Effect of ferulic acid (FA) on the cell cycle phase distribution of Caco-2 cells. Cells were seeded 1 day before the addition of FA (day 0) at different concentrations. The cell cycle phase distribution was determined by flow cytometry. The symbols represent the mean, and the bars, SD of three samples. The data represent one out of two experiments with similar results. Data points denoted by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) were significantly different from controls.

a small increase at day 3 for the cells treated with 1500 μM FA or *p*-CA (**Figure 7**).

Flow Cytometric Analysis of Cell Cycle Kinetics. Treatment with 150 μM FA did not affect the fraction of DNA synthesizing cells (LI) (**Table 1**). Treatment with 1500 μM FA caused a pronounced increase of LI at all 3 days of treatment ($p < 0.001$ to $p < 0.01$). In the *p*-CA treated cells, the results were similar to those for FA treated cells. The S phase duration time (Ts), measured at 2 days of treatment, was slightly longer in cells treated with 150 μM FA as compared to the control but more than twice longer as compared to control in the cells treated with 1500 μM FA ($p < 0.001$). *p*-CA did not affect Ts at any concentration. The percentage of divided BrdUrd-labeled cells of the total number of BrdUrd-labeled cells (% divided) was slightly reduced in the cells treated with 150 μM FA or *p*-CA and further reduced in those treated with 1500 μM FA (~60%) or *p*-CA (~50%, $p < 0.05$).

The DNA histograms of BrdUrd-labeled cells are shown in **Figure 8**. The peak to the left in each histogram shows cells in the early S phase, while the peak to the right shows cells in the late S phase. Mid S phase cells are in the middle of the DNA histogram of the BrdUrd-labeled cells. In the control, most cells

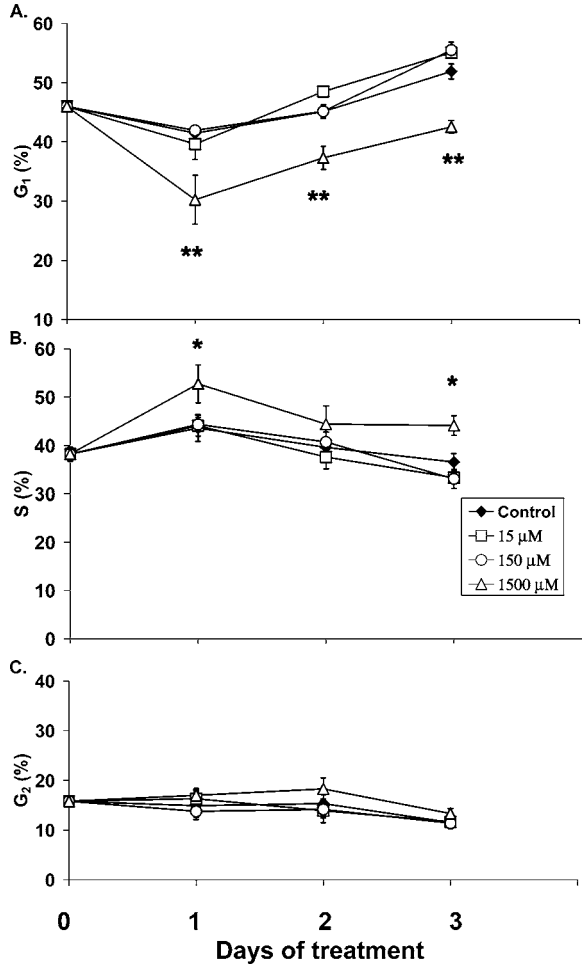

Figure 6. Effect of *p*-coumaric acid (*p*-CA) on the cell cycle phase distribution of Caco-2 cells. Cells were seeded 1 day before addition of *p*-CA (day 0) at different concentrations. The cell cycle phase distribution was determined by flow cytometry. The symbols represent the mean, and the bars, SD of three samples. The data represent one out of two experiments with similar results. Data points denoted by * ($p < 0.05$) and ** ($p < 0.01$) were significantly different from controls.

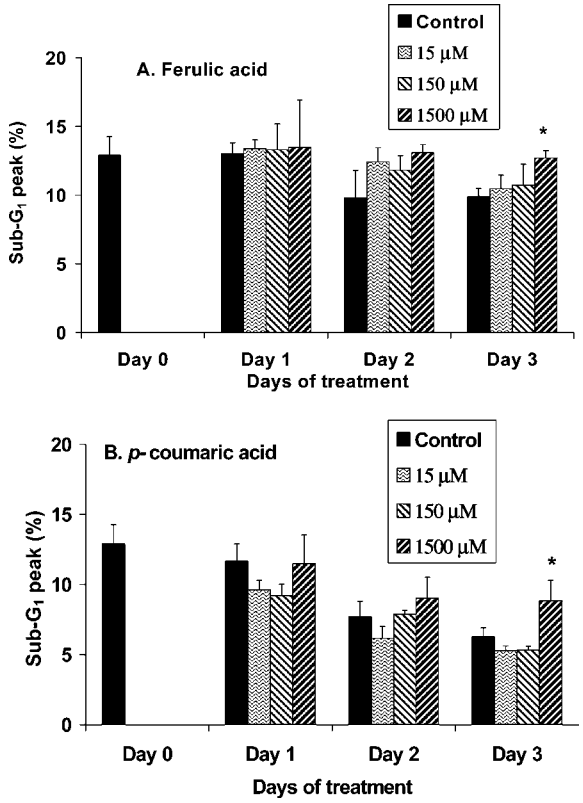

Figure 7. Effect of ferulic acid (**A**) and *p*-coumaric acid (**B**) on the appearance of a sub-G_1 peak. The experiments are described in Figures 5 and 6. All cells, that is, both adherent and detached cells, were collected and analyzed. The bars represent the mean value and SD of three samples. The diagrams represent one out of two experiments with similar results. Data points denoted by * ($p < 0.05$) were significantly different from controls.

were found in the beginning and at the end of the S phase, which is the normal S phase distribution of exponentially growing cells. Treatment with 1500 μM FA clearly resulted in a redistribution of the cells toward late S phase at all days of treatment. The numbers above each histogram are calculated RM_{zero} values, which mathematically reflect the S phase distribution. RM_{zero} is the mean DNA content of BrdUrd-labeled cells in relation to the DNA content of G_1 and G_2 cells (*23*). Treatment with 1500 μM FA resulted in a redistribution of the cells toward the end of the S phase as compared to control, and this was also reflected in the RM_{zero} values at all days of treatment ($p < 0.01$ days 1−2, $p < 0.001$ day 3). FA at a concentration of 150 μM did not significantly affect the distribution of cells in the S phase, although the values were somewhat higher than those of control after 2 and 3 days of treatment. Treatment with 150 μM *p*-CA did not affect the distribution of cells in the S phase. Treatment with 1500 μM *p*-CA for 2 and 3 days resulted in a shift toward lower RM_{zero} values as compared to control.

DISCUSSION

A number of possible cancer protective mechanisms for cereal bran have been described in the literature (*5*, *6*, *25*, *26*). We have studied the effects of two of the major phenolic components of cereal bran, FA and *p*-CA, on metabolic activity, cell proliferation, and cell cycle kinetics in Caco-2 cells, trying to explore the mechanisms whereby cereal bran may exert its effects on the consumer.

The absorbtivity, absorption site, and metabolism of FA and *p*-CA in the gastrointestinal tract depend much on the food matrix carrying the compounds. In bran, 10% exists as free FA, while the rest is esterified to polysaccharide chains (*27*). The bound phenolic components are released only after microbial attack, and the metabolites formed depend on the nature of the plant cell wall and the degree of cross linkage (*28*). After consumption of coffee, also rich in bound phenolic acids, free phenolic acids were found in plasma of test subjects, indicating that the bound phenolic acids are bioavailable to humans. The plasma concentration of caffeic acid ranged from 0.32 to 0.98 μM, 1 h after consumption of 1 cup of coffee containing 166 mg of bound caffeic acid (*29*). For comparison, wheat bran can supply up to 50 mg of FA per 10 g (*13*).

The concentrations of FA and *p*-CA that can be obtained in the colon are hard to predict because they depend on many factors. Most of the bound dietary FA enters the colon and may be released there by microbial action. As was mentioned above, 10 g of wheat bran can supply 50 mg of FA. In a colonic volume of 200 mL, this would yield a 1300 μM concentration of FA. On the basis of this calculation, we used 1500 μM of FA and *p*-CA as the highest concentration in the present study. However, important to note is that all FA presumably is not released at the same time, that absorption takes place continuously, and that the luminal content moves along the colon. Because the

Table 1. Effect of Ferulic Acid (FA) and *p*-Coumaric Acid (*p*-CA) on Cell Cycle Kinetics during 1–3 days after Seeding[a]

		control	150 μm FA	1500 μm FA	150 μm *p*-CA	1500 μm *p*-CA
day 1	LI[b]	44.5 (0.7)	44.2 (0.7)	52.1 (1.0)[c]	44.6 (1.3)	54.0 (1.0)[c]
day 2	LI	43.4 (3.3)	47.3 (1.0)	60.0 (0.1)[d]	48.5 (0.5)	57.0 (6.5)[e]
	Ts[f]	19.3 (1.2)	22.6 (2.7)	46.4 (6.0)[c]	18.8 (1.7)	20.6 (3.3)
	% divided[g]	38.5 (2.1)	36.8 (8.9)	23.7 (5.3)	32.6 (4.3)	20.8 (3.0)[e]
day 3	LI	43.2 (1.5)	44.9 (2.0)	53.7 (6.1)[d]	41.2 (1.4)	51.6 (1.8)[e]

[a] The cell cycle kinetics was determined by flow cytometry after labeling with bromodeoxyuridine. The data represent the mean values and SD from three independent cell cultures. The data set represents one of two experiments with similar results. [b] Labeling index (LI, %): the fraction of DNA synthesizing cells, calculated as the percentage of BrdUrd-labeled cells in relation to the total cell number. [c] Significantly different from control ($p < 0.001$). [d] Significantly different from control ($p < 0.01$). [e] Significantly different from control ($p < 0.05$). [f] S phase duration time (Ts, h). [g] Percentage of divided BrdUrd-labeled cells of total BrdUrd-labeled cells.

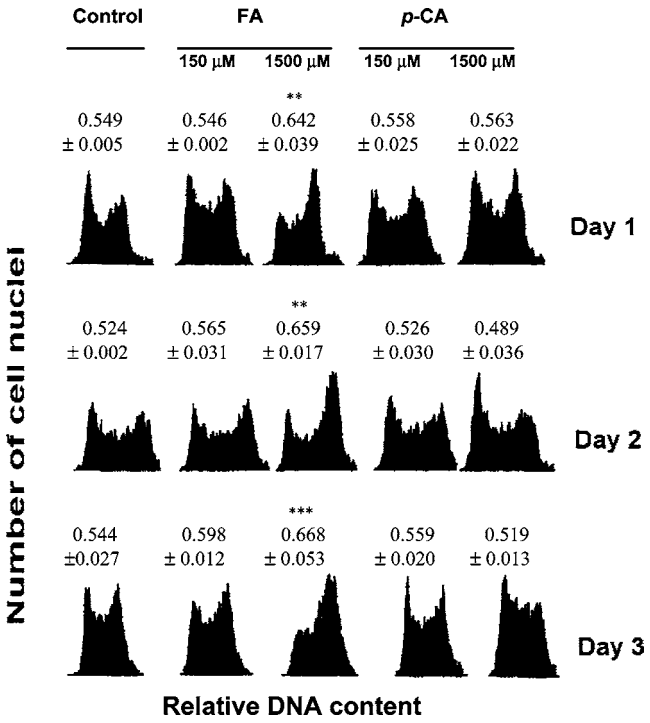

Figure 8. Effect of ferulic acid (FA) and *p*-coumaric acid (*p*-CA) on distribution of cells in S phase. Cells were seeded 1 day before the addition of FA or *p*-CA at different concentrations, and the cells were analyzed 1–3 days after addition of the substances. Cell cycle kinetics was determined by flow cytometry after labeling with bromodeoxyuridine. The DNA histograms of BrdUrd-labeled cells represent one out of six samples from two independent experiments with similar results. The left region of each histogram represents cells in early S phase, the middle region represents mid S phase cells, and the right region represents cells in the late S phase. The numbers above each histogram are the mean values ($n = 3$) and SD of the relative movement at the timepoint zero (RM_{zero}, mean DNA content of the cohort of BrdUrd-labeled cells in relation to G_1 and G_2 phase cells). Data points denoted by ** ($p < 0.01$) and *** ($p < 0.001$) were significantly different from control.

diet contains many components containing FA and *p*-CA other than wheat bran, the intake of these acids is usually higher than that from bran intake alone. Another important point is that additive and synergistic effects of different phenolic compounds probably have physiological relevance. Because also the bioavailability can differ between compounds and individuals, all of these factors contribute to make an estimation of intake and physiological concentrations of phenolic acids in body fluids a complex task. Most importantly though, we believe that a 1500 μM concentration of FA or *p*-CA is within a physiological range. However, although we believe 1500 μM concentrations are physiologically feasible, it should only occur in pulses related to food intake in the colon. Thus, the biggest discrepancy between the colon in a person and our cell culture experiments is the constant exposure time in the latter.

The assays monitoring metabolic activity showed that neither substance had any inhibitory effects at concentrations up to 500 μM. At 1500 μM, FA reduced the metabolic activity, while *p*-CA had no effect. The reduction in metabolic activity after FA treatment was reflected in a decreased rate of cell proliferation. Interestingly, *p*-CA increased the metabolic activity of cells treated for 24 h, but we have no explanation for this. Treatment with 1500 μM *p*-CA inhibited cell proliferation slightly less than treatment with 1500 μM FA. However, the decreased cell proliferation in cells treated with 1500 μM *p*-CA was in contrast to 1500 μM FA not accompanied by decreased metabolic activity.

Others have also found growth inhibition in cells treated with FA using the human breast cancer cell line T47D (*30*), primary smooth muscle cells, and the human endothelial cell line ECV304 (*31*). The latter cells were blocked in the G_0/G_1 phase. In the study using T47D cells, a FA concentration of 1 μM resulted in 60% reduction of cell proliferation. Thus, the T47D cells were substantially more sensitive to FA treatment than our Caco-2 cells. This may illustrate that Caco-2 cells are derived from an environment with very high concentration of dietary substances and thus are adapted to tolerate higher concentrations of various compounds. Moreover, the Caco-2 cells have an effective intrinsic transportation system via a monocarboxylic acid transporter that carries FA and *p*-CA across the cell monolayer, as part of a nutrient transportation mechanism, preventing the substance to accumulate in the cell (*32*). The higher sensitivity of the T47D cell line is interesting because breast cancer cells, via the blood, will be exposed to lower concentrations of FA and *p*-CA than colon cancer cells. The growth inhibitory properties of FA and *p*-CA are clearly interesting and warrant further study from a viewpoint of comparing different normal cells and cancer cells. Any further differences in sensitivity may have relevance in the treatment and prevention of proliferative diseases.

To the best of our knowledge, this is the first investigation of the effects of FA and *p*-CA on cell cycle kinetics, taking our knowledge of the substances beyond effects on cell proliferation only. Treatment with 1500 μM FA but not with *p*-CA for 48 h increased the length of the S phase more than twice as compared to the control as measured by a BrdUrd-DNA FCM method. There was a remarkable difference in LI obtained by the BrdUrd-DNA FCM method and the S phase population size estimated from DNA histograms using the Multicycle software. Both are different ways to determine the size of the S phase cell population, but the former method estimates the S phase more accurately because only S phase cells incorporate BrdUrd. The latter method can quite correctly discern a large G_1 phase

population, but there is a problem in distinguishing between late S phase cells and cells in a small G_2 phase population because of overlaps in DNA distribution among these populations. The software evaluating the DNA histograms has clearly overestimated the G_2 phase and underestimated the S phase fractions in FA-treated cells. Analysis of the DNA histograms of BrdUrd-labeled cells clearly showed that FA treatment resulted in an accumulation of cells in the late S phase, which was also reflected in an increased RM_{zero}. The cells accumulating at the end of the S phase have incorrectly been included in the G_2 phase fraction in the Multicycle evaluation of DNA histograms. For *p*-CA-treated cells, there was a greater resemblance between LI obtained by the BrdUrd-DNA FCM method and the S phase population size determined from the DNA histograms. Analysis of a sub-G_1 peak did not show any evidence that the compounds caused substantial cell death even at 1500 μM.

For FA, a lengthening of the S phase is part of the explanation for the inhibition of cell proliferation in Caco-2 cells. *p*-CA also inhibited cell proliferation; however, it is clear that it did not involve a lengthening of the S phase. The BrdUrd-DNA FCM method can be used in a more complex manner than used in this paper to determine the length of the G_1 phase, and the G_2 phase, besides the length of the S phase. In addition, it is also possible to determine the rates of G_1/S and S/G_2 transitions. Our present study clearly shows that the effect of FA and *p*-CA on cell cycle kinetics should be extended in a future study.

Other phenolic food constituents reported to act as inhibitors of cell proliferation are the catechins, abundant in cocoa beans, red wine, and green tea. Caco-2 cells, treated with cocoa powder, showed a 70% growth inhibition with a block of the cell cycle at the G_2/M phase (*33*). However, in comparison to FA treatment in our study, the BrdUrd-DNA FCM method may reveal that the cells actually are blocked in the late S phase. Epigallocatechin-3-gallate from green tea caused a block at the G_0/G_1 phase and induced apoptosis as determined by evaluation of DNA histograms in the human prostata carcinoma cell lines LNCaP and DU145 (*34*), but in Caco-2 cells there was also a block at the G_2/M phase (*35*). Resveratrol, a compound of red wine, has also been reported to inhibit cell cycle progression (*36*). Curcumin (diferuloylmethane) found in the yellow spice turmeric has received much attention lately due to its tumor prevention activities (*37*). It is easily decomposed into its constituents vanillin and FA, and its effects may actually be mediated by its constituents (*38*). Furthermore, many compounds undergo metabolism in the intestine and liver, and thus their biological effects may be exerted by different metabolites (*39*).

In conclusion, our paper shows that *p*-CA and FA have antiproliferative effects on Caco-2 human cancer cells. This antiproliferative effect was reflected in a lengthening of the S phase for FA. Further studies are, however, needed to reveal which cell cycle phases are prolonged by *p*-CA treatment. It is important to investigate these effects on the cell cycle further to identify the specific cell cycle proteins responsible for the delay of the cell cycle. Cereal bran and whole grain cereals have many documented health benefits, and their consumption has important public health implications as reflected in recent health claims (*40*). Because the phenolic constituents FA and *p*-CA delay cell proliferation as shown by us, this adds further support to the recommendation to the public to eat more whole grain cereals. This also supports the notion to use cereal bran as an ingredient of future functional foods for protection against colon cancer.

ABBREVIATIONS USED

BrdUrd, 5-bromo-2′-deoxyuridine; *p*-CA, *para*-coumaric acid; DMSO, dimethyl sulfoxide; FA, ferulic acid; FCM, flow cytometry; Caco-2, Human Caucasian colon adenocarcinoma, MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide; PBS, phosphate buffered saline; PI, propidium iodide; RM_{zero}, relative movement at timepoint zero.

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