Inhibition of Growth and Induction of Differentiation Markers by Polyphenolic Molecules and Histone Deacetylase Inhibitors in Colon Cancer Cells

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Abstract. *Previously we found that a fruit-derived polyphenol fraction caused an inhibition of proliferation and an induction of differentiation markers in Caco-2 human colon cancer cells. In the present work, we sought to determine if individual polyphenols would exert similar actions. Proliferation was inhibited by several polyphenolic molecules including gallic acid, ellagic acid, quercetin and resveratrol. In Caco-2 cells, growth inhibition was accompanied by increased specific activities of two differentiation markers, alkaline phosphatase and dipeptidyl peptidase, but not of aminopeptidase. Increased enzyme activities were not seen in HT29 and SW1116 colon cancer cells. In Caco-2 cells there were additive effects of butyrate or valproate and polyphenolic molecules. Histone acetylation was not greatly affected by the polyphenols. Cycloheximide inhibited protein synthesis in the 3 cell types examined but paradoxically, in Caco-2 cells it caused increased specific activities of alkaline phosphatase and dipeptidyl peptidase. Several plant polyphenols can inhibit the growth of colon cancer cells but increased specific activity of some differentiation markers seen in Caco-2 cells did not appear to be a general phenomenon in colon cancer cells.*

In earlier work, we observed that methanolic extracts of plum and peach varieties with a high anthocyanin content caused an inhibition of proliferation and an induction of differentiation markers in Caco-2 human colon cancer cells (1). Initial fractionation on C18 indicated that the activity of the extracts was associated with a fraction containing anthocyanins, but subsequent fractionation of this solution on LH20 revealed that much of the activity was associated with a fraction containing phenolic molecules other than anthocyanins. The action of the fruit extracts was additive with the action of an inhibitor of histone deacetylase, butyrate, and with an inhibitor of mitogen-activated protein kinase kinase (MEK) one and two, U0126. To determine if these effects are given by individual polyphenols, we have investigated the action of a number of phenolic molecules that are frequently found in fruit extracts, including caffeic, chlorogenic, ellagic, gallic and protocatechuic acids, quercetin and resveratrol (2-5). These polyphenolic molecules were examined as single agents and in combination with the established inhibitors of histone deacetylase activity, butyrate and valproate. Since some phenolic acids, including cinnamic acid (6) and caffeic acid (7), have been reported to exhibit some inhibition of histone deacetylase (HDAC) activity, we investigated the contribution of polyphenolic molecules to hyperacetylation of histones when combined with butyrate or valproate. Alkaline phosphatase and dipeptidyl peptidase activities were monitored as markers of differentiation in colon cancer cells. In an attempt to determine if increased specific activities of these enzymes represented increased synthesis, we used cycloheximide as an inhibitor of protein synthesis. However, in Caco-2 cells it was found that cycloheximide as a single agent could cause increases in the specific activity of alkaline phosphatase and dipeptidyl peptidase suggesting that cycloheximide, perhaps indirectly, might be affecting the turnover of the enzyme molecules.

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Materials and Methods

Cells and determination of cell proliferation. SW1116, HT29 and Caco-2 human colon cancer cells were obtained from American Type Culture Collection, Rockville, MD, USA, and were incubated at 37˚C in RPMI-1640 medium with 5% fetal calf serum and 25 mM *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) buffer. NCM460 human colon cells were obtained from INCELL Corp., San Antonio, TX, USA, and were maintained in M3:10 medium from INCELL Corp. The incorporation of [^{3}H]thymidine into DNA was measured after incubating cells for 2 hours with 2 microcuries [3H]thymidine as previously described (8). A greater number of studies were performed

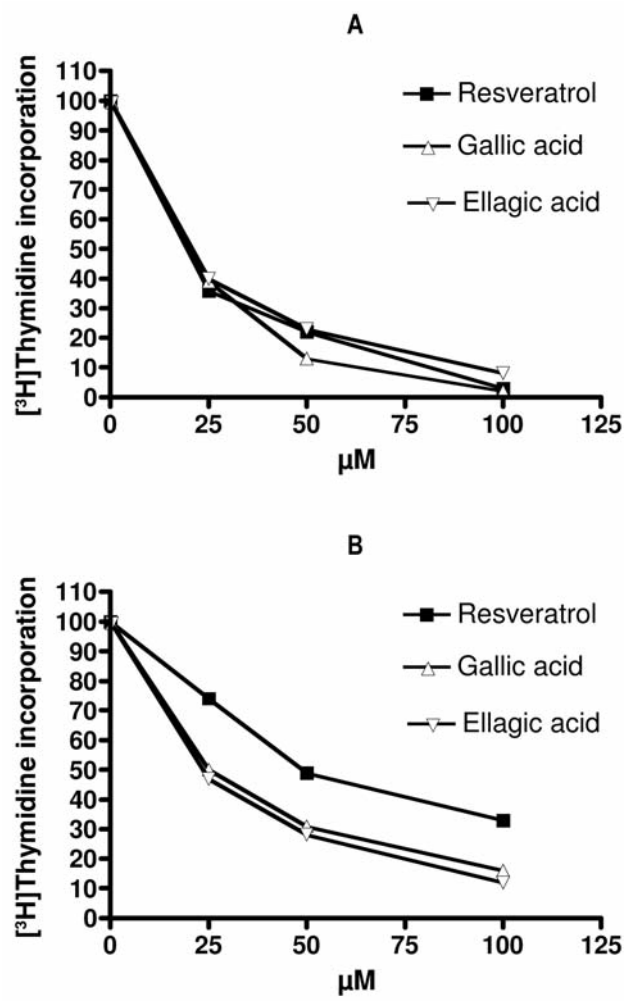

Figure 1. *Inhibition of the incorporation of [³H]thymidine into DNA of Caco-2 (A) and HT-29 (B) cells after a 72-hour incubation with polyphenols. The data are expressed as a percentage of control values and are given as means for three determinations.*

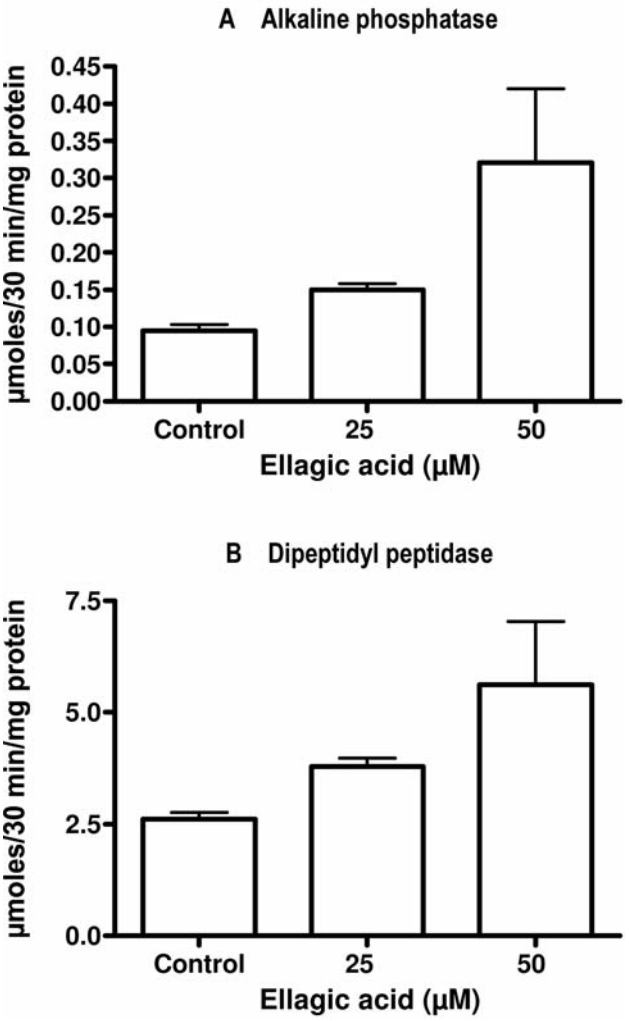

Figure 2. *Effects of incubation of Caco-2 cells for 72 hours with ellagic acid on the activities of alkaline phosphatase (A) and dipeptidyl peptidase (B). The data are given as the means and standard deviations for four determinations.*

with Caco-2 cells because that line was more responsive in previous studies with polyphenol-enriched fractions (1). The least number of studies was done with NCM460 cells because that cell line tends to show more rapid phenotypic changes when maintained in culture (9).

Enzyme assays. A total of 1.5×10^6 cells were incubated with 10 ml RPMI-1640 medium with 25 mM HEPES and 5% fetal calf serum. The medium was changed after 24 hours and unless stated otherwise the cells were incubated for 72 hours before harvesting. The cells were washed with phosphate-buffered saline and extracted with 0.5% NP40, 0.25 M NaCl, 5 mM EDTA and 50 mM Tris pH 8.0. The protein concentration of the extract was determined using the BCA Protein Assay Reagent from Pierce, Rockford, IL, USA. Enzymes were assayed at 37˚C as previously described (9). Alkaline phosphatase was assayed using *para*-nitrophenyl phosphate as substrate. Formation of product was monitored by the change in absorbance at 410 nm. Aminopeptidase and dipeptidyl peptidase were assayed using L-alanyl-4-nitroanilide or Gly-Pro-4-nitroanilide as substrates, respectively. Production of the product, 4-nitroaniline, was monitored at 405 nm.

Histone acetylation. The isolation of histones and electrophoresis on urea-acetic acid polyacrylamide gels was performed as previously described (10). Acetylation of H3 histone was visualized by immunoblots using a primary rabbit polyclonal antibody against acetyl H3 histone. The secondary antibody was a horse-radish peroxidase conjugate of goat anti-rabbit antibody. Antibodies were obtained from Millipore, Temecula, CA, USA.

Statistical evaluation. Statistical significance of the results was determined by a two-tailed Student's *t*-test or by Dunnett's test for multiple comparisons using the Instat program from GraphPad Software, Inc., La Jolla, CA, USA. A probability of less than 5% was considered significant.

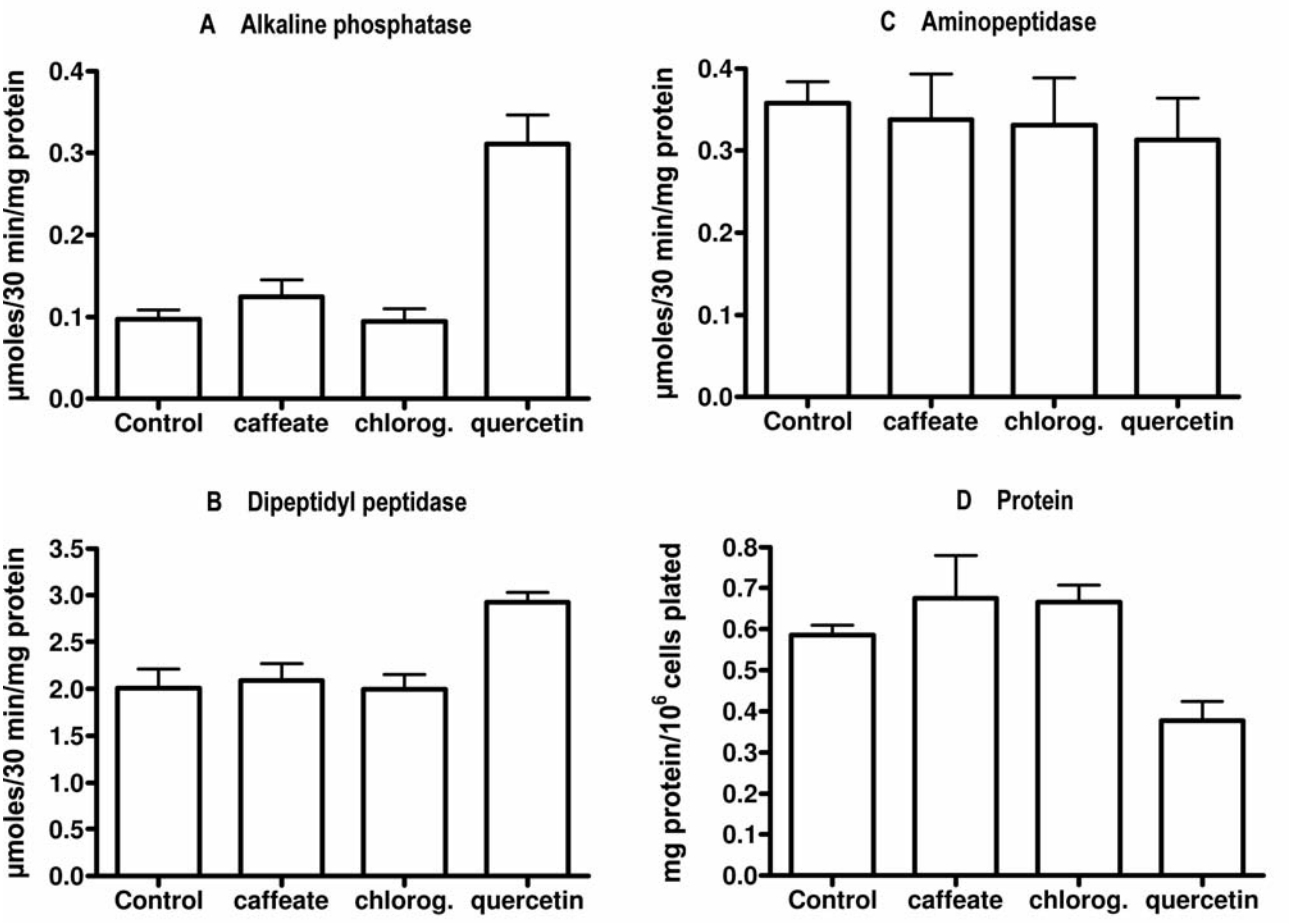

Figure 3. *Effects of incubation of Caco-2 cells for 72 hours with 50 µM caffeic acid, chlorogenic acid (chlorog.) and quercetin on enzyme activities (A-C) and protein yield (D). The data are given as the means and standard deviations for three determinations.*

Results

In preliminary studies, we investigated the action of a number of phenolic molecules that are frequently found in fruit extracts including caffeic acid, chlorogenic acid, ellagic acid, gallic acid, protocatechuic acid and resveratrol. As judged by effects on thymidine incorporation and protein synthesis, growth inhibition was similar with gallic acid, ellagic acid and resveratrol and was greater than with the other compounds. Inhibitory effects on the incorporation of [^{3}H]thymidine into DNA are illustrated for Caco-2 cells and for HT29 cells in Figure 1A and B, respectively.

In Caco-2, but not in the other cells examined, incubation with ellagic acid, and resveratrol resulted in an increase in the specific activity of alkaline phosphatase and generally to a lesser degree for the specific activity of dipeptidyl peptidase. This is illustrated for the effect of ellagic acid on alkaline phosphatase and dipeptidyl peptidase activities (Figure 2A and B). An increase in the specific activity of alkaline phosphatase in Caco-2 cells was greatest with ellagic acid and resveratrol, being 2.5- to 5-fold after incubation for 72 hours with 50 µM concentration of the polyphenolic molecule. Under the same conditions, the specific activity of dipeptidyl peptidase was increased approximately 2-fold and there was no significant effect on aminopeptidase activity. A similar pattern was seen when Caco-2 cells were incubated with 50 µM quercetin (Figure 3A-D). The increase in alkaline phosphatase was greater than the increase in dipeptidyl peptidase. There was no statistically significant change in aminopeptidase activity and the data indicated a significant decrease in protein synthesis with 50 µM quercetin. None of the observed parameters were significantly affected when the Caco-2 cells were incubated with 50 µM caffeic acid or chlorogenic acid. In similar studies with protocatechuic acid, concentrations greater than 100 µM were required to observe significant effects on Caco-2 and HT29 cells (data not shown).

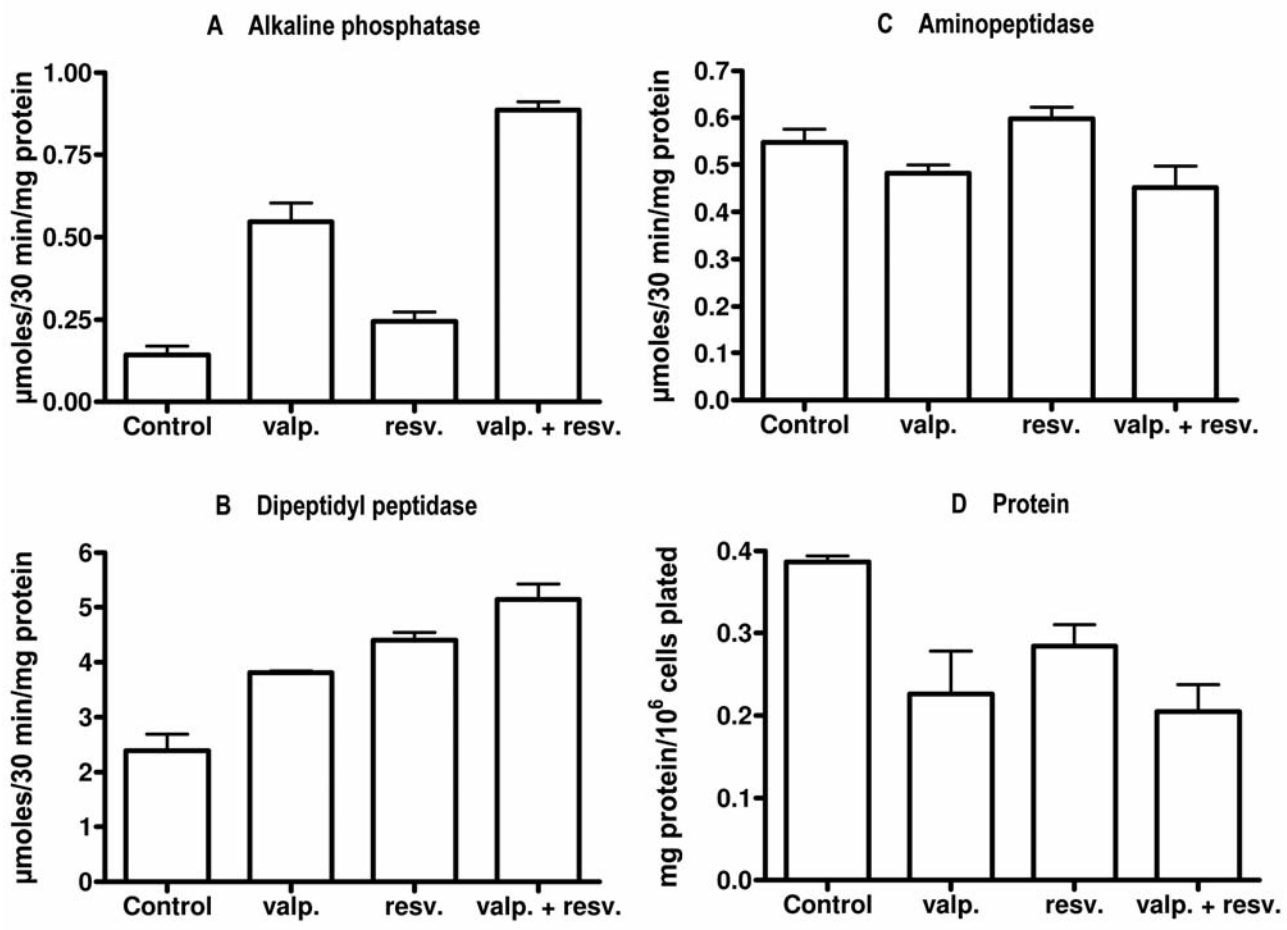

Figure 4. *Effects of incubation of Caco-2 cells for 72 hours with 1 mM valproate (valp.) and 25 µM resveratrol (resv.) as single agents and in combination on enzyme activities (A-C) and protein yield (D). The data are given as the means and standard deviations for three determinations.*

Gallic acid, ellagic acid and resveratrol had growth inhibitory effects on HT29 and SW1116 colon cancer cells but no increased activities of alkaline phosphatase or dipeptidyl peptidase were seen after incubation of these cells with the compounds for 72 hours (data not shown).

Since polyphenol-containing plum and peach extracts were found previously to act synergistically with butyrate or valproate to increase the activity of alkaline phosphatase in Caco-2 cells (1), we tested combinations of butyrate and valproate and the polyphenolic molecules ellagic acid, gallic acid and resveratrol. Additive effects were seen for the increase in alkaline phosphatase activity in Caco-2 cells but not with HT29 and SW1116 cells. This is illustrated for the action of 1 mM valproate and 25 µM resveratrol as single agents and in combination on enzyme activities and protein yield after 72 hour incubation with Caco-2 cells (Figure 4A-D). The effects were greater for the increase in alkaline phosphatase activity than the activity of dipeptidyl peptidase and no significant change was seen in aminopeptidase activity. The significant decrease in protein yields indicates growth inhibitory action and was greatest for the combined effect of valproate and resveratrol.

It was established in previous work that colon cancer cell lines differ in the induction of differentiation markers in response to HDAC inhibitors (9). We wished to determine if changes with polyphenols reflected differences in the effects on histone side-chain modification. Butyrate and valproate caused an increased acetylation of H3 histone in the four colon cancer cell lines that were examined (Caco-2, HT-29, SW1116, and NCM460). Increased acetylation was similar in the different cell lines and was prolonged. Thus in Caco-2 cells, acetylation of H3 histone was similar after 24- or 72-hour incubations with 1 mM valproate (Figure 5A and B). Under the conditions examined, histone side-chain modifications were not greatly affected by the polyphenols gallate, ellagate, and resveratrol, either as single agents or in combination with valproate, relative to valproate as a single agent. Although NCM460 cells do not show marker enzyme

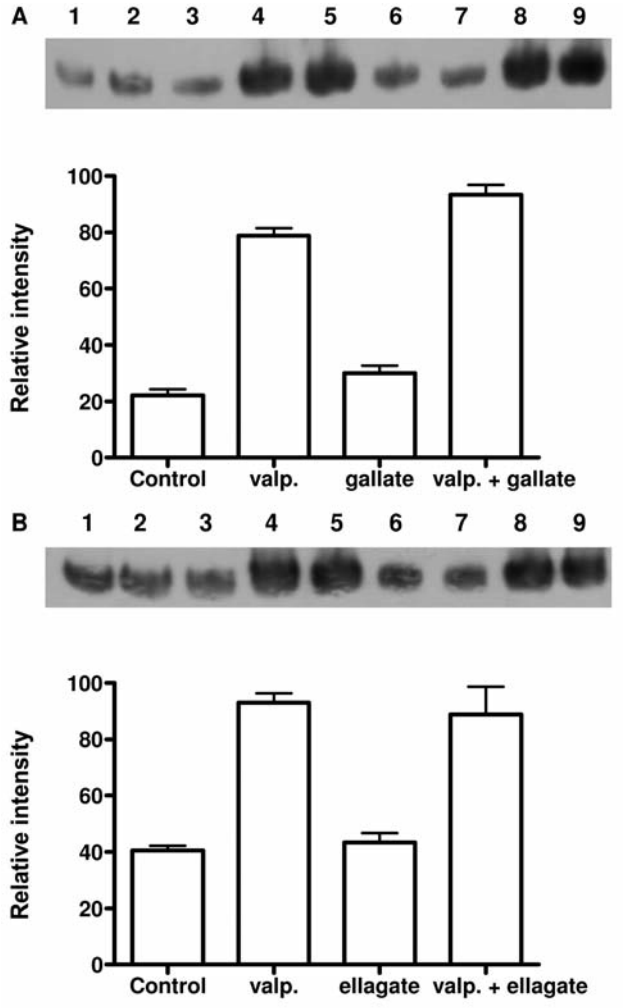

Figure 5. *Effects of incubation of Caco-2 cells for 24 hours with 1 mM valproate (valp.) and 25 μM gallate (A) and for 72 hours with 1 mM valproate and 25 μM ellagate (B) as single agents and in combination on acetylation of H3 histone. Lanes 1-3 represent histones from control cells. Lanes 4 and 5 represent histones from cells treated with 1 mM valproate; lanes 6 and 7 from cells treated with 25 μM gallate (A) or 25 μM ellagate (B); lanes 8 and 9 from cells treated with 1 mM valproate plus 25 μM gallate (A) or 25 μM ellagate (B). Densitometric data are given as the means and standard deviations for 2-3 determinations.*

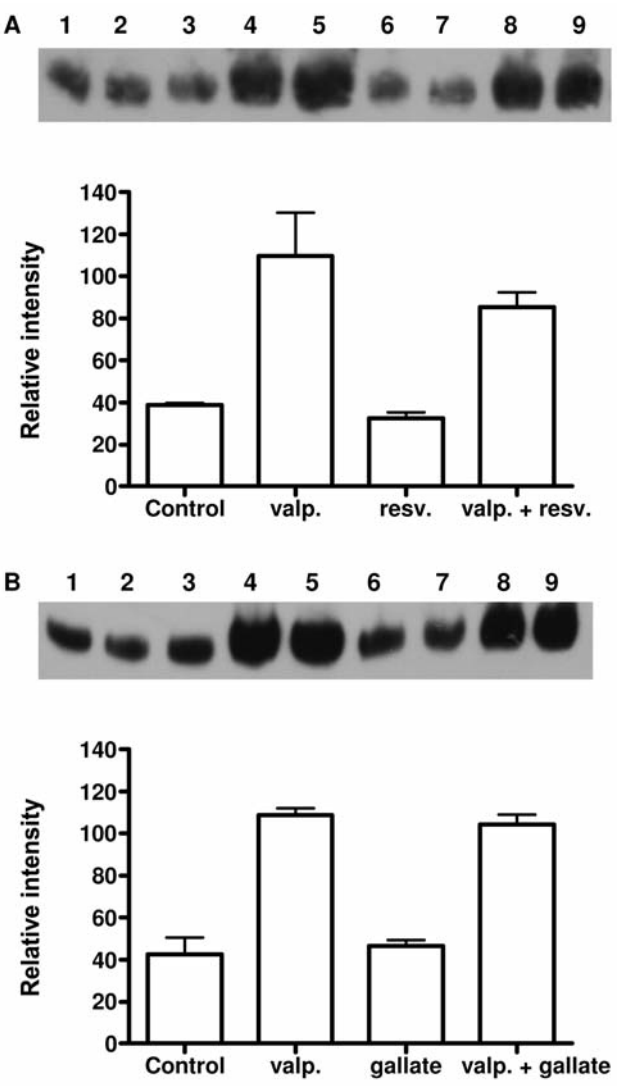

Figure 6. *Effects of incubation of NCM460 cells for 24 hours with 1 mM valproate (valp.) and 50 μM resveratrol (A) and for 72 hours with 1 mM valproate and 25 μM gallate (B) as single agents and in combination on acetylation of H3 histone. Lanes 1-3 represent histones from control cells; lanes 4 and 5 from cells treated with 1 mM valproate; lanes 6 and 7 from cells treated with 50 μM resveratrol (A) or 25 μM gallate (B); lanes 8 and 9 from cells treated with 1 mM valproate plus 50 μM resveratrol (A) or 25 μM gallate (B). Densitometric data are given as the means and standard deviations for 2-3 determinations.*

induction when treated with valproate, the data in Figure 6A and B indicate increased acetylation after incubation with valproate but there was little or no effect of the polyphenols examined. Figure 7 presents similar data for SW1116 cells.

The cells were also distinguished by their response to 1 μM cycloheximide. Protein synthesis was significantly inhibited in the three cell lines examined and, in HT29 and SW1116 cells, the induction of alkaline phosphatase by butyrate was significantly reduced by cycloheximide (data not shown). However, in Caco-2 cells, although cycloheximide caused a significant decrease in protein synthesis at all three concentrations (Figure 8A) and a significant decrease in the specific activity of aminopeptidase at 1.0 μM (Figure 8B), there were significant increases in the specific activities of alkaline phosphatase (Figure 8C) and dipeptidyl peptidase (Figure 8D) at 0.5 and 1.0 μM cycloheximide. The response to the combined effects of butyrate and cycloheximide are contrasted for alkaline phosphatase activity in Caco-2 and SW1116 cells in Figure 9A and B, respectively. In Caco-2

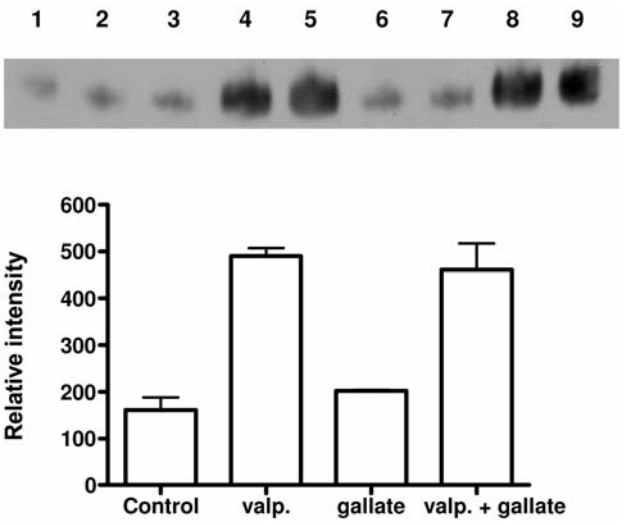

Figure 7. *Effects of incubation of SW1116 cells for 24 hours with valproate and gallate as single agents and in combination on acetylation of H3 histone. Lanes 1-3 represent histones from control cells; lanes 4 and 5 from cells treated with 1 mM valproate (valp.); lanes 6 and 7 from cells treated with 50 μM gallate; lanes 8 and 9 from cells treated with 1 mM valproate plus 50 μM gallate. Densitometric data are given as the means and standard deviations for 2-3 determinations.*

cells, there was an additive increase in alkaline phosphatase activity whereas in SW1116 there was no significant increase in activity relative to the level with butyrate as a single agent. For both Caco-2 and SW1116 cells there was a significant decrease in protein yield with 1.0 μM cycloheximide either alone or with 0.5 mM butyrate but not with 0.5 mM butyrate as a single agent.

Discussion

Many but not all studies have indicated that polyphenolic molecules or polyphenolic-enriched preparations may have chemopreventive potential against cancer (11-14). The relative apparent importance of anthocyanins *versus* other polyphenolics in the inhibition of colon cancer cell proliferation has varied in different studies (15, 16). In previous studies with Caco-2 colon cancer cells treated with extracts of anthocyanin-enriched plums and peaches, there were increased activities of alkaline phosphatase and dipeptidyl peptidase but only modest effects were seen with cyanidin or cyanidin glucoside at concentrations up to 100 μM (1). The activities of the fruit extracts appeared to be caused by a fraction containing polyphenolic molecules other than anthocyanins. The present work indicates that incubation of Caco-2 cells with a variety of polyphenolic molecules caused increases in the enzymic markers of colon cell differentiation. These polyphenolic molecules, which are widely distributed in fruits and vegetables, included ellagic acid, gallic acid, resveratrol and quercetin that had activity in the 25-50 μM concentration range, whereas caffeic acid, chlorogenic acid and protocatechuic acid had little if any activity at such concentrations. Structurally, the active compounds show considerable variability in the number of aromatic rings and hydroxyl groups.

Additive increases in alkaline phosphatase activity have been seen in Caco-2 cells after combined treatment with butyrate and inhibitors of mitogen-activated protein kinase (MAPK) signaling (9, 17, 18). Some polyphenolic molecules have been shown to be inhibitors of kinases and signal transduction pathways (13, 19-21). Several kinases may be inhibited, including phosphoinositide-3-kinase and some protein kinases. It seems possible that the polyphenolic molecules that we have studied have an additive effect with butyrate that is similar to that previously shown for U0126, a MEK1/2 inhibitor (9, 18).

Valproate caused an increased acetylation of H3 histones in the four colon cell lines that were examined (Caco-2, HT-29, SW1116, and NCM460). Increased acetylation was similar in the different cell lines and was prolonged, being similar after 24- or 72-hour incubations. Under the conditions examined, histone acetylation was not greatly affected by the polyphenols gallate, ellagate, and resveratrol, either as single agents or in combination with valproate. Although NCM460 cells do not show marker enzyme induction when treated with valproate, the increase in histone acetylation was similar to that in other cell lines in which there is a large increase in enzyme activity. The data suggested that although valproate caused increased histone acetylation in all cell lines examined, this effect may not be sufficient to cause an induction of differentiation markers in some cell lines.

Cycloheximide is widely used as an inhibitor of protein synthesis in mammalian cells. It has been observed to inhibit several actions of butyrate on human colorectal cancer cells including inhibiting the induction of alkaline phosphatase by butyrate in HT29 colon cancer cells (22), HRT-18 rectal cancer cells (23) and LoVo colon cancer cells (24). Cycloheximide after incubation for 72 hours caused a decrease in extractable protein in the three cell lines that were examined (Caco-2, HT29 and SW1116). This was accompanied by an increase in the specific activity of dipeptidyl peptidase in all three cell lines but an increase in the specific activity of alkaline phosphatase was only seen in Caco-2 cells and there was frequently a decrease in the specific activity of aminopeptidase in the three cell lines. An increase in the specific activity of an enzyme after treatment with cycloheximide might reflect a low turnover of the enzyme, or alternatively may reflect the loss of a protein involved in the enzyme degradation.

Caco-2 human colon cancer cells have been widely investigated as models for colonocyte transport mechanisms and in differentiation studies. The present work indicates that

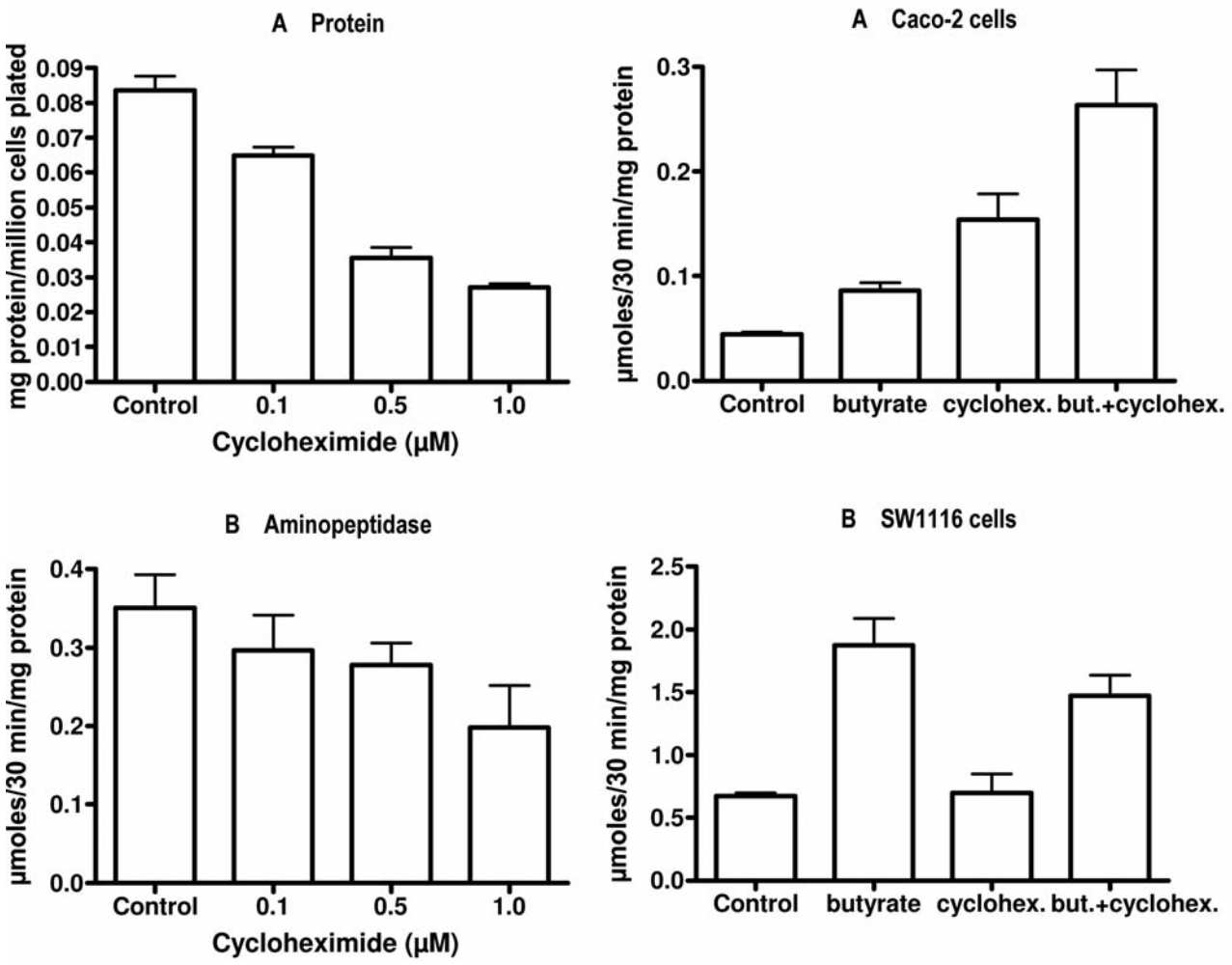

Figure 8. *Effects of incubation of Caco-2 cells for 72 hours with cycloheximide on protein yield (A) and enzyme activities (B-D). The data are given as the means and standard deviations for three determinations.*

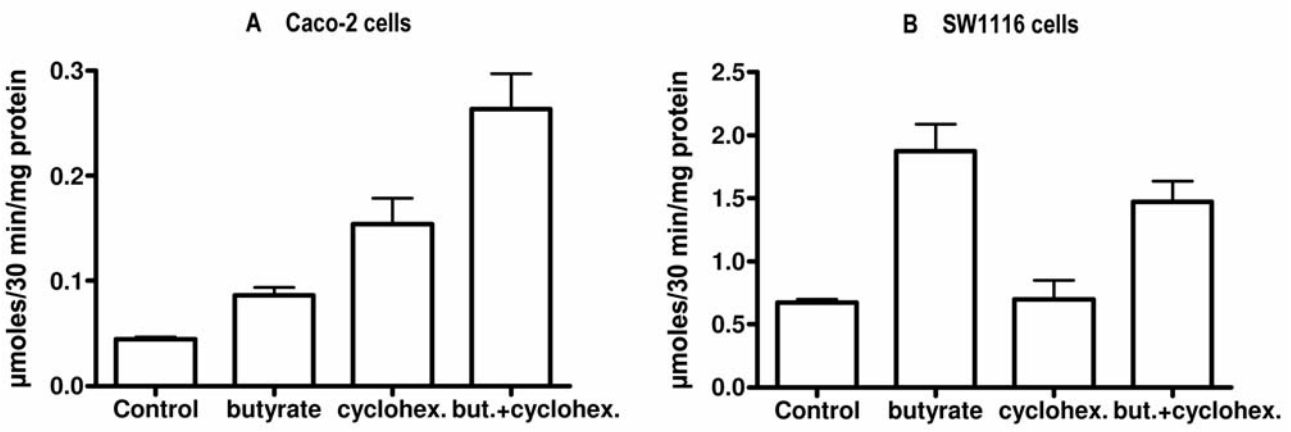

Figure 9. *Effects of incubation for 72 hours with 0.5 mM butyrate and 1 µM cycloheximide (cyclohex.) as single agents and in combination on alkaline phosphatase activity in Caco-2 (A) and SW1116 cells (B). The data are given as the means and standard deviations for three determinations.*

the properties of Caco-2 cells are not always a feature of other colon cancer cell lines. It is a point of interest for future studies to determine the extent to which induction of differentiation markers in Caco-2 cells represents a retention of properties of primary adenocarcinomas. In conclusion, a number of plant polyphenolic molecules can inhibit the growth of colon cancer cells and, particularly in Caco-2 cells, several polyphenols can increase the specific activity of some differentiation markers.

Acknowledgements

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