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Water-soluble polymeric polyphenols from cinnamon inhibit proliferation and alter cell cycle distribution patterns of hematologic tumor cell lines

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Abstract

To explore possible anti-cancer properties of water-soluble, polymeric polyphenols from cinnamon, three myeloid cell lines (Jurkat, Wurzburg, and U937) were exposed to increasing concentrations of an aqueous extract prepared from cinnamon (CE) for 24 h. Cell growth and cell cycle distribution patterns responded in a dose-dependent manner to CE. That is, an increase in the percentage of cells distributed in G2/M was observed in all three cell lines as the amount of CE increased. At the highest dose of CE, the percentage of Wurzburg cells in G2/M was 1.5- and 2.0-fold higher than those observed for Jurkat and U937 cells, respectively. Wurzburg cells lack the CD45 phosphatase and may be more sensitive to imbalances in signaling through kinase/phosphatase networks that promote growth. The results suggest the potential of CE to interact with phosphorylation/dephosphorylation signaling activities to reduce cellular proliferation in tandem with a block at the G2/M phase of the cell cycle.
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Keywords: Cell cycle; G2/M phase; Jurkat; U937; Phosphatase; Cinnamon; Polyphenols; Cancer

1. Introduction

The potential of the health-promoting and disease-preventing properties of plant-derived compounds has received increased attention from researchers in recent years [1]. Work in our laboratory has focused on characterizing compounds found in aqueous extracts of cinnamon and tea for their ability to improve insulin signaling and reduce the complications produced by resistance to this hormone [2–6]. Mechanistic studies to understand how such a diverse group can improve insulin-signaling point to the importance of the balance in kinase/phosphatase activities that are regulated by insulin binding to its receptor. For example, we have demonstrated that the cinnamon extract (CE) contains a mixture of polymeric polyphenols that can inhibit the activity of a purified phosphatase [4,5]. This extract was also

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shown to mimic the action of insulin-induced signaling via its receptor. Addition of the cinnamon extract to 3T3-L1 adipocytes increased the phosphorylated, activated state of the insulin receptor and stimulated downstream events characteristic of the insulin response in adipocytes [6]. When CE supplements were given to subjects with type 2 diabetes, improvements in health status ensued as evidenced by decreases in plasma glucose, cholesterol, and triglycerides [3]. Taken together, these findings support a role for the extract in improving insulin sensitivity by regulating phosphatase activity and thus insulin-initiated signaling cascades in cells vital to the metabolic balance maintained by this hormone.

Hyperinsulinemia has long been associated with obesity and diabetes, and interest has recently turned to its participation in the development of cancer [11]. Dietary components that decrease insulin and restore sensitivity to this hormone are receiving increased attention. Results from both epidemiological and experimental studies suggest that polyphenolic compounds found in plants have protective effects against the development of various forms of cancer [7,8]. The chemo-preventive properties have been ascribed to the ability of polyphenols to function as antioxidants. However, this ability cannot entirely explain their anti-cancer effects. Other potential mechanisms that have been invoked are the inhibition/stimulation of enzymes that direct the signals for proliferation, differentiation and death. Transient and sequential phosphorylation and dephosphorylation of signaling proteins are defining events in all cellular functions ranging in diversity from insulin-stimulated glucose metabolism to growth-factor stimulated cellular proliferation [9,10]. Indeed, aberrant proliferative signaling has been associated with hyperinsulinemia as increased risk for cancer has been associated with diabetes and obesity ([11] for review). To further our understanding of the mechanism of the polymeric polyphenols from cinnamon on cellular signaling cascades, we treated leukemic and lymphoma cell lines with increasing amounts of CE and determined its effects on growth parameters and cell cycle progression. One of the cell lines, the Jurkat/Wurzburg CD 45^- clone, was included because of its lack of the cell-surface protein tryosine phosphatase (PTP) ([12] and references therein). The lack of this enzyme provides a cellular model to study the impact of CE polyphenols on the balance in the phosphorylation–dephosphorylation reactions that regulate cellular proliferation.

2. Materials and methods

2.1. Chemicals and reagents

Sources of materials not included in the following descriptions were obtained from Sigma Chemical Co. (St Louis, MO) and were of the highest purity available. The aqueous cinnamon extract (CE) used in the experiments was prepared as described [5].

2.2. Cells

Jurkat (clone E6-1) and U937 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The $CD45^-$ Jurkat clone, Wurzburg, was purchased from Dr Lester Packer (University of California, Berkeley, CA). Cells were grown in 75 cm^2 flasks in a humidified atmosphere with 5% CO_2 at 37 °C. The following were added to the growth medium, RPMI1640: 5 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)], 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 100 U/mL penicillin, 100 ug/mL streptomycin (Quality Biological, Inc., Gaithersburg, MD, USA). To this mixture was added fetal calf serum at 10% vol/vol (Hyclone Laboratories, Logan, UT, USA). Cell lines were checked by flow cytometry for phenotypic expression of CD45 [Fluorescein isothiocyanate (FITC)-conjugated CD45, clone BRA-55, FITC-isotype contol, clone MOPC-210] [12]. Wurzburg cells were routinely shown to express $<4\%$ positivity for CD45. Both Jurkat and U937 cell lines expressed $>95\%$ positivity for this marker.

2.3. Cell growth

All experiments were conducted with exponentially, asynchronously growing cells. Cell numbers at initiation of the experiments were approximately 2×10^5/mL and were treated with 0.0, 0.05, 0.075, 0.10, and 0.20 mg/mL CE for 24 h. The 24 h time period was selected to encompass one population

doubling time for the cells [12]. For the vanadate studies, sodium metavandate was added at 0, 10, 25, 50, 100 and 200 μM. Cell counts were determined either electronically (Cell Counter, Model ZBI, Beckman-Coulter, Hialeah, FL, USA) or with a hemocytometer. Viability was assessed by trypan blue dye exclusion. This method showed that treated cells had viabilities $\geq 90\%$.

2.4. Cell cycle analyses

DNA distribution throughout the cell cycle was assayed using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA). Cells were grown in media with and without the cinnamon extract or sodium metavanadate (SMV) for the times indicated. After counting, $1–1.5 \times 10^6$ cells/mL were washed with PBS (Ca, Mg-free), fixed in chilled 70% ethanol and stored overnight at 4 °C [13]. Cells were centrifuged and washed again with PBS, then stained for DNA content (1 μg propidium iodide and 25 mg ribonuclease A in 1 mL PBS) for 30 min at room temperature and promptly analyzed by flow cytometry. Cytometric data were collected for 10,000 cellular events per sample and analyzed using CELLQuest software (Becton Dickinson). Cell cycle distribution percentages of stained nuclei (2N and 4N) were calculated using Modfit LT software (Verity Software House, Inc., Topsham, ME). Calibration standards, LinearFlow Green/Orange and DNA QC Particle Kit, for verification of instrument performance, were purchased from Molecular Probes (Eugene, OR) and BD Biosciences, respectively.

2.5. Phosphatase assay

Total phosphatase activity in cell lysates was determined using a fluorescence-based assay [14]. Cell lysates were prepared by freeze-thawing (3×) and protein concentrations were determined by the NanoOrange method (Molecular Probes). The phosphatase assay uses as substrate the non-fluorescent 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP, Molecular Probes) which, upon loss of the phosphate, exhibits excitation/emission maxima of 358/452 nm. The buffer for the phosphatase determination contained 25 mM MOPS [3-(*N*-morpholino)propanesulfonic acid], 50 mM NaCl and 1 mM dithiothreitol (DTT) and reactions were run at room temperature for 60 min in 96-well microplates. Enzyme units are defined as nanomoles phosphate equivalents released per min per mg protein.

2.6. Statistics

ANOVA and Student–Newman–Keuls tests were used to detect differences of means within a cell line with significance defined as $P < 0.05$. Data are expressed as means ± SD, $n = 4$, unless otherwise stated.

3. Results

3.1. Decreasing cell proliferation

The three cell lines were treated with increasing concentrations of the aqueous cinnamon extract for 24 h and cell counts were used as a marker of proliferative activity. Fig. 1 illustrates the dose-dependent decreases in percentage of cell number compared to the non-treated control for each of the three cell lines as the amount of CE increased from 0.05 to 0.2 mg/mL. The anti-proliferative property of CE resulted in similar declines in percentages of cell numbers for the three cell lines as the amount of the extract was increased. At the highest dose of CE (0.2 mg/mL), each cell line demonstrated

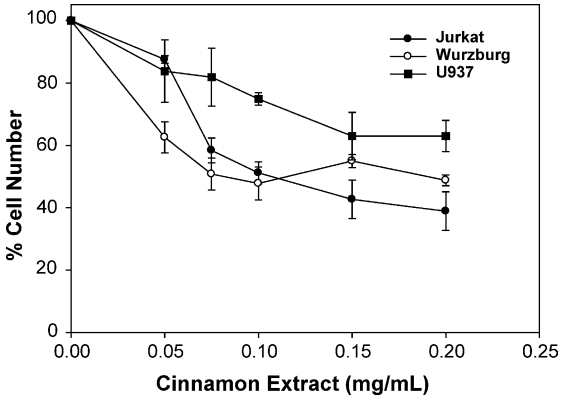

Fig. 1. Dose-dependent growth inhibition induced by CE after 24 h in the three cell lines-Jurkat ●, Wurzburg ○, and U937 ■—is represented by the percent decrease in cell number normalized to untreated control (100%). Data plotted as means ± SD, $n = 4$.

a significant decrease in the percentage of cells remaining as a result of exposure to the extract.

3.2. Cell cycle and cinnamon extract

Fig. 2A–C depict the effects of increasing concentration of the cinnamon extract on the progression of Jurkat, Wurzburg, and U937 cell lines through the three cell cycle phases, G0/G1, S, and G2/M. A decreasing pattern in the percentage of cells in G0/G1 was observed for the three cell lines as the amount of CE was increased from 0.0 to 0.2 mg/mL. Percentages in this phase for the three cell lines ranged from 44–48% for untreated controls to 7–18% for the highest CE amount tested. Treatment with CE had a more variable effect on the S phase distribution patterns among the three cell lines. Only modest changes were noted for percentage of cells in S phase for Jurkat and U937 cells. However, percentages of $CD45^-$ Wurzburg associated with this phase declined over 4-fold as CE ranged in amounts from 0 to 0.2 mg/mL (Control, 46.6 ± 2.3; 0.2 mg/mL CE, 10.4 ± 0.8, see Fig. 2B). At the three highest CE treatments, the percentage distributions of cells in S phase were significantly different compared to no treatment control for the Wurzburg cell line. All three cell lines exhibited dramatic increases in the percentage of cells in G2/M in parallel with exposure to increasing amounts of CE. The G2/M block was particularly pronounced in the Wurzburg cells,

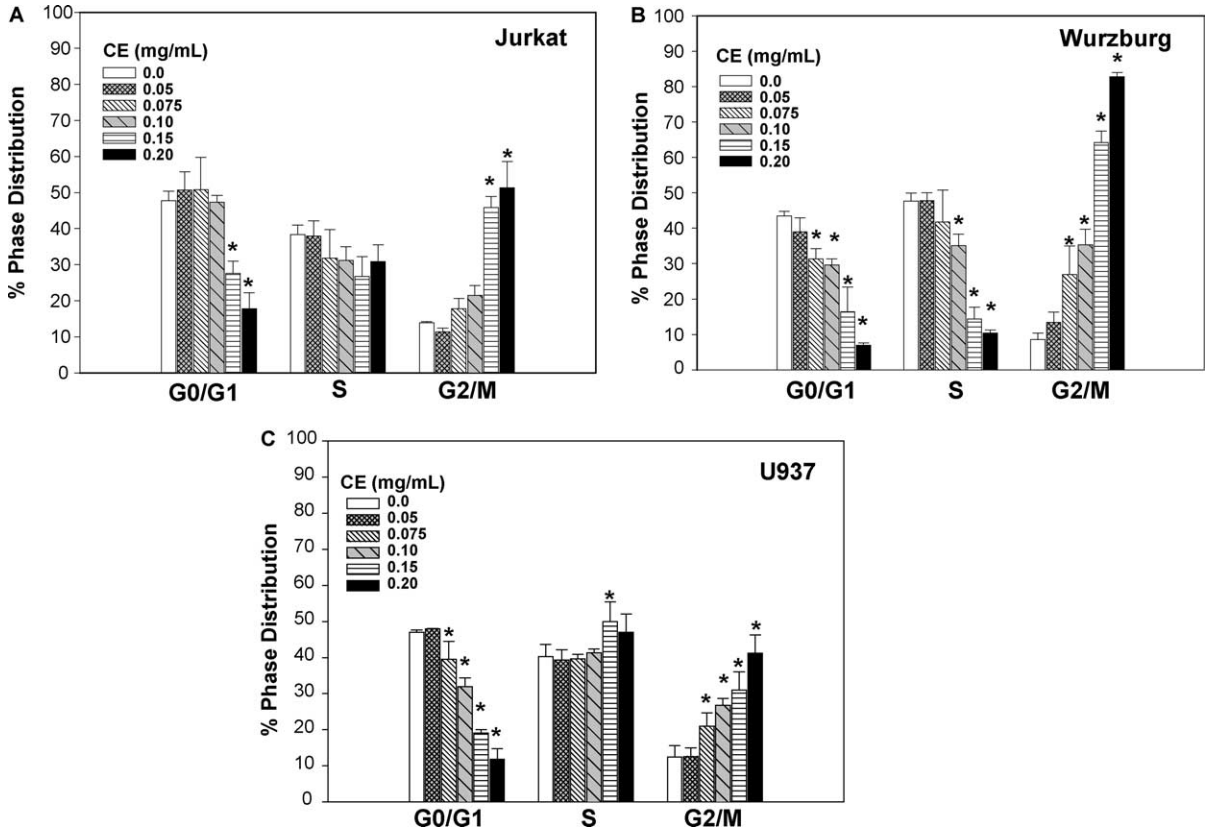

Fig. 2. (A) Effect of increasing concentration of CE on the percentage of DNA distributed in the three phases of the cell cycle in Jurkat cells. *Denotes a significant difference from the non-treated control value within the phase. Data plotted as means $\pm$ SD, $n=4$, $P<0.05$. (B) Effect of increasing concentration of CE on the percentage of DNA distributed in the three phases of the cell cycle in Wurzburg cells. *Denotes a significant difference from the non-treated control value within the phase. Data plotted as means $\pm$ SD, $n=4$ $P<0.05$. (C) Effect of increasing concentration of CE on the percentage of DNA distributed in the three phases of the cell cycle in U937 cells. *Denotes a significant difference from the non-treated control value within the phase. Data plotted as means $\pm$ SD, $n=4$, $P<0.05$.

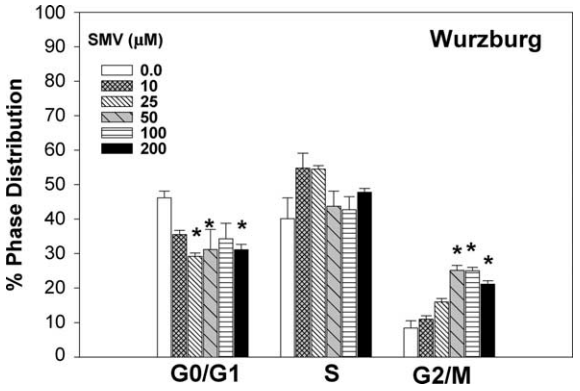

Fig. 3. Effect of increasing concentrations of sodium metavanadate (SMV) on percentage distribution among G0/G1, S, and G2/M phases of the cell cycle in Wurzburg cells. The maximum percentage block in G2/M occurs with 50 μM SMV and no further increases are obtained with 100 and 200 μM SMV. See Fig. 2A–C, for the response to increasing CE for the three cell lines, especially for Wurzburg cells, which displays no indication of reaching a maximum in the percent block at G2/M.

where the percentage of cells increased nearly 10-fold (8.6 ± 1.8–82.8 ± 1.1). Percentages for both Jurkat and U937 cells increased approximately 3.5-fold over the range of treatment with the extract.

3.3. Cell cycle and sodium metavanadate

To compare the ability of CE to inhibit cell cycle progression with a compound that is both phosphatase inhibitor and insulin mimetic [15,16], Wurzburg cells, which showed the greatest changes in G2/M distribution with CE, were treated with increasing concentrations of sodium metavandate (SMV, 0–200 μM) for 24 h. Cells were counted electronically followed by fixation of approximately 1×10^6 cells for subsequent cell cycle analyses as described above. Data for these experiments are shown in Fig. 3. At the highest dose of SMV, the cell counts were approximately 50% compared to the untreated controls, a comparable anti-proliferative effect to that seen with CE. The percentage of Wurzburg cells in G2/M reached a maximum of 25% with 50 μM SMV (approximately a 3-fold change) and did not increase further with the higher amounts of SMV added. These results observed with SMV are in striking contrast to the ever increasing percentage of cells in G2/M as the amount of CE is increased (Fig. 2B).

3.4. Phosphatase activity

Total phosphatase activities in lysates prepared from the three cell lines were determined using a fluorescence-based assay. Specific total phosphatase of the Wurzburg, Jurkat and U937 cells were 1.1 ± 0.4, 2.7 ± 1.1, and 3.7 ± 1.0 (nmoles phosphate released per min per mg of protein, mean $\pm$ SD, $n = 4$), respectively. The results of the newly established fluorescence-based assay agree with our results obtained in the past using *p*-nitrophenyl phosphate as substrate. With the latter colorimetric assay, Wurzburg cells were shown to have approximately 50% of the phosphatase activity compared to that found for the Jurkat cells [12]. A preliminary experiment was conducted using the cell lines incubated with CE (0.15 mg/mL) or not at 37 °C for 4 h. Specific activities for Wurzburg, Jurkat and U937 cells in the presence of CE were found to be 0.7 (37% inhibition compared to control value), 0.9 (50% inhibition), and 1.3 (57% inhibition), respectively.

4. Discussion

In these experiments, we have demonstrated that a water-soluble fraction, high in polymeric polyphenols from cinnamon [5], reduced cellular growth in a dose-dependent manner in both a lymphoma and two leukemic cell lines. Sensitivity of growth to the extract was similar among the cell lines. The decrease in proliferation of the tumor cells observed with CE is in agreement with growth arrest that is typically seen when cultured cells are treated with polyphenolic compounds, such as curcumin [17], tea catechins [18], and genistein [19]. Analyses of DNA distribution across cell cycle phases demonstrated a differential effect on the regulation of progression by the CE polyphenolic compounds. Cell cycle blockages observed depend on the type of cell and polyphenolic compounds [8]. In these experiments, the dissimilarities may be in part due to the expression of the CD45 phosphatase. The $CD45^-$ Wurzburg cells displayed increasingly greater percentages of cells at G2/M with increasing CE compared to the other two lines which are $CD45^+$. Similarly, a report appeared a decade ago demonstrating that CD45 activity was elevated in the late G2/M phase in a lymphoma and a T cell line [20].

Progress has been slow in defining the function of the CD45 phosphatase in leukocytes and it is still unclear how this phosphatase contributes to cell growth and death in either normal or leukemic cells. An imbalance in kinase/phosphatase regulation of proliferative signaling networks created by a paucity of CD45 in conjunction with a phosphatase inhibitor may contribute to this increased sensitivity to CE for Wurzburg cells observed in these experiments, but further studies are needed.

We demonstrated in earlier studies that CE inhibited purified PTP1B [4], stimulated phosphorylation of the insulin receptor in 3T3-L1 adipocytes [6] and, as reported here in preliminary experiments, inhibited total phosphatase activity in the three cell lines. The phosphatase assays for total activity showed that the activity in the three cell lines increased in the order Wurzburg < Jurkat < U937. Total phosphatase activity in cells provides a basis for relating changes in dephosphorylating processes with shifts in the balance of kinase/phosphatase regulated signaling. A complex array of phosphatase activities by different enzymes helps to regulate diverse kinases and progression through G0/G1, S and G2/M phases of the cell cycle [21]. Mitotic phosphatases, termed cdc25 (B and C), are possible targets of CE in our experiments. The action of these cdc25 enzymes are necessary for mitosis to occur [21,22]. Two widely diverse compounds that produce G2/M blocks, vanadate and genistein, have been shown to reduce the expression of the cdc25C mitotic phosphatase protein in breast and epithelial tumor cell lines [19,23]. We have not yet tested CE for its ability to inhibit expression of these phosphatases. However, along with a decrease in total phosphatase activity, an increase in percentage of G2/M is seen in Wurzburg cells as soon as 4 h after the addition of CE (unpublished observations), indicating rapid effects on cell cycle progression processes.

Wurzburg cells were treated with the well known non-specific phosphatase inhibitor and insulin mimetic, sodium metavanadate (SMV) [14,15], to compare its actions with that of CE. We have demonstrated that the CE is also a phosphatase inhibitor and insulin mimetic [4,6]. At the highest doses for both CE and SMV, the cell counts were approximately 50% compared to the untreated controls. A G2/M block was observed and the percentage of cells in G2/M reached a maximum of 25% with 50 μM SMV and did not increase further with higher amounts. These results with SMV are similar to those reported by Zhang et al [23]. This group demonstrated that 100 μM SMV treatment of an epithelial cell line resulted in a 30% distribution of cells in the G2/M phase. Although the amounts of CE and SMV used are not directly comparable, it is of note that the percentage of Wurzburg cells in G2/M phase continued to demonstrate a dose–response increase over the range of CE additions while the percentage in G2/M reached a plateau value nearly 3-fold less with the higher doses of SMV (See Figs. 2B and 3 for comparison). This difference underscores the complexity of effects that the two compounds can have on proliferative signals within cells and makes it difficult to pinpoint with certainty which signaling pathway(s) are being altered to change cellular responses.

Maintaining the appropriate balance between kinase and phosphatase activities is vital in regulating cellular proliferation in both normal and cancerous cells [9,10]. The results of our experiments with the cinnamon extract, which has been shown to alter insulin signaling [4,6], provide evidence that this polymeric polyphenol has the potential to alter proliferative signals regulating progression through the cell cycles. Future investigations are needed to determine if this CE inhibitory property is related to specific phosphatases and if this property sufficiently explains the mechanisms of action of CE on cell cycle progression and proliferation.

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