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# A polyphenol mixture from cinnamon targets p38 MAP kinase-regulated signaling pathways to produce G2/M arrest

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**Abstract**

We recently demonstrated that treatment of three leukemic cell lines with an aqueous extract of cinnamon (CE) for 24 h produced dose-dependent arrests in the G2/M phase of the cell cycle. To accomplish the goal of understanding underlying mechanisms, we selected the cell line most responsive to the CE treatment to study the effects of the extract on signaling molecules regulating cell cycle progression. Cell cycle analyses were conducted on treated versus nontreated cells from 0–6 h. The percentages of cells in G2/M in CE-treated cells increased significantly from 11.0±1.0 to 23.6±1.4 after 6 h, while the percentage for nontreated cells remained unchanged (12.3±0.8). Multiparametric flow cytometric analyses were used to associate activation of p38 mitogen-activated protein kinase (MAPK) with cells arrested in G2/M, the size of these cells, and the presence or absence of cyclin B1. After 4 h, there was a 26% increase in the activated phosphorylated form of p38 MAPK in CE-treated cells compared with the nontreated control cells, with larger cells showing the greater increases. Although the proportion of CE-treated cells in G2/M was higher than controls, this population was shown to be less positive for cyclin B1 than the control G2/M population. Our results demonstrate that CE significantly modulated two signaling proteins, p38 MAPK and cyclin B, that regulate progression through G2/M. Overall, the data provide evidence that CE affects proliferation in a leukemic cell line by disrupting critical phosphorylating/dephosphorylating signaling events that propel cells through the G2/M phase.


*Keywords:* Cinnamon; Polyphenols; G2/M; p38 MAPK; Cyclin B1

## 1. Introduction

Chemopreventive properties have long been attributed to polyphenolic compounds present in the human diet [1,2]. These natural substances are of interest as they are potential sources of anticancer compounds with minimal debilitating side effects and toxicity. Investigations at molecular and cellular levels have revealed multiple mechanisms to explain the chemoprotective action of these dietary constituents [3]. We recently demonstrated that a mixture of polyphenols from an aqueous extract of cinnamon (CE) possessed anticancer properties by blocking cell cycle progression of leukemic cell lines at the G2/M phase [4]. Progression through the phases of the cell cycle is controlled by a multiplicity of signaling networks linked by appropriately timed actions of kinases, phosphatases, and formation of regulatory protein complexes [5]. In addition to the G2/M arrest with CE, we also demonstrated that the extract reduced total phosphatase activity in the cell lines [4]. This observation is in accord with work in our laboratory characterizing both insulin mimetic and phosphatase-inhibitory properties of CE in insulin responsive cells [6]. By inhibiting phosphatase activity, CE contributes to a shift in the balance of kinases/phosphatases with the end result of an increased sensitivity to the action of insulin in both cells and in human subjects [7,8].

To further understand how CE affects signaling to produce the G2/M block, we have extended our investigations to look at consequences of short term CE treatment on cell cycle progression in a CD45 negative Jurkat clone [also known as the Wurzburg cell line (W)]. We have shown that W demonstrated an enhanced sensitivity to the CE-induced G2/M compared to the other cell lines used in our initial study [4]. Taking advantage of this characteristic, we

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designed experiments using W to identify specific CE molecular targets in signaling networks that control cell cycle progression through the G2/M phase. Two proteins, p38 mitogen-activated protein kinase (MAPK) and cyclin B1 were selected based upon published results linking them to G2/M regulation [9–12]. In this report, we show that W treated with CE had increased p38 MAPK activity that was followed by a decrease in cyclin B1 — a protein component of the complex regulating progress through the G2 checkpoint [5]. Such results would be expected if regulatory phosphatase activities are being inhibited by CE. In addition, we demonstrated the successful use of flow cytometry and multiparametric cell analyses to show relationships among cell cycle phase, cell size and the presence of intracellular signaling proteins.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Unless otherwise indicated, chemical materials were obtained from Sigma Chemical (St. Louis, MO, USA) and were of the highest purity available. The aqueous cinnamon extract was prepared as described [13].

Antibodies and staining buffer (554656) for intracellular staining were obtained from BD Biosciences (San Jose, CA, USA): phycoerythrin (PE)-conjugated anti-phospho-p38 MAPK (T180/Y182), fluorescein (FITC)-conjugated mouse antihuman cyclin B1 and isotype control, FITC-conjugated mouse IgG1 monoclonal antibody (Kit 554108). Additional products to verify the antibody binding specificity and presence of phosphop38 MAPK were obtained from Cell Signaling Technology (Beverly, MA, USA): Alexa Fluor 488-conjugated mouse anti-phospho-p38 MAPK (T180/Y182), Alexa Fluor 647-conjugated mouse anti-p38 MAPK and phospho-p38 MAPK (T180/Y182) blocking peptide (1170). SB 203580, the p38 MAPK inhibitor, was purchased from Upstate Biotechnology (Charlottesville, VA, USA).

### 2.2. Cells and culture conditions

The CD45 Jurkat clone, Wurzburg, was purchased from Dr. Lester Packer (University of California, Berkeley, CA, USA). Cells were either grown in 75 $cm^2$ flasks or in six-well plates in a humidified atmosphere with 5% $CO_2$ at 37°C. The following were added to the growth medium, RPMI1640 containing 10% fetal calf serum (Gibco/Invitrogen, Grand Island, NY, USA): 5 mM HEPES [*N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid)], 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin (Quality Biological, Gaithersburg, MD, USA). The W were periodically checked for phenotypic expression of CD45 which was consistently found to be <4% [4].

Exponentially, asynchronously growing cells were used in all experiments. Cell numbers at initiation of the cell cycle experiments were $2\times10^5$/ml and were treated or not with 0.10 mg/ml CE for the times indicated. This concentration was selected as our previous dose-range results had demonstrated this one to be effective in producing significant G2/M arrest in 24 h [4]. For experiments with the p38 MAPK inhibitor SB203580, cells were preincubated with 20 $\mu$M of the compound for 1 h before the addition of CE. Cell counts and volumes were determined electronically with a cell counter (Model Z2, Beckman-Coulter, Hialeah, FL, USA).

### 2.3. Cell cycle analysis

DNA distribution throughout the cell cycle was assayed using a FACScalibur flow cytometer (BD Biosciences). At the selected time points, $1-1.5\times10^6$ cells were washed with phosphate-buffered saline (PBS) (Ca, Mg-free), fixed in chilled 70% ethanol and stored overnight at 4°C [4]. 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 Pro software (BD Biosciences). Cell cycle distribution percentages of stained nuclei (2N and 4N) were calculated using Modfit LT software (v 3.1, Verity Software House, Topsham, ME, USA). For verification of instrument performance calibration standards, LinearFlow Green/Orange and DNA QC Particle Kit were purchased from Molecular Probes (Eugene, OR, USA) and BD Biosciences, respectively.

### 2.4. Detection of intracellular proteins — p38 MAPK and cyclin B1

Intracellular staining for both activated p38 MAPK and Cyclin B1 required that cells be sequentially fixed and permeabilized [14–16]. Using a modification of a protocol described by Krutzik et al. [14], cells were fixed by directly adding 16% formaldehyde (Polysciences, Warrington, PA, USA) to the culture media to give a final concentration of 1.5% formaldehyde. After standing at room temperature for 15 min, cells were centrifuged, the supernatant removed, and remaining pellet chilled on ice for 5 min. Cold BD staining buffer (50 μl) was added to pellets. After a preliminary loosening of these pellets by vortexing, a minimum of 500 μl of cold methanol per $10^6$ cells was added while still vortexing to permeabilize the cells. Methanolic cellular suspensions were stored at −20°C (1–7 days) before staining with antibodies. Prior to staining with the appropriate antibodies, a volume of methanol containing approximately $10^6$ cells was transferred to a polypropylene tube, centrifuged and washed 1× with staining buffer. Antibodies to activated p38 MAPK , p38 MAPK and cyclin B1 were added as recommended by the vendors. Recommendation of the vendor was also followed using the blocking peptide for the antibody to activated p38 MAPK. Cells that were fixed and permeabilized were also characterized for forward-angle

light scattering properties (FSC) and stained with propidium iodide as described above [17,18]. Cells thus treated allowed multiparametric analyses linking cell size and phase with presence of the intracellular proteins of interest [15,16].

### 2.5. Statistics

All data were expressed as means±S.D., $n$=3 or 4 as indicated. One-way analysis of variance and Student–Newman–Keuls tests were used to detect differences of means among treatments with significance defined as $P$<.05.

## 3. Results

### 3.1. CE and cell cycle analysis

Asynchronously growing W were treated with 0.1 mg/ml CE for 2, 4 and 6 h and then immediately fixed with ethanol as described in Materials and methods. Untreated control cells were run in parallel and treated in the same manner. CE treatment produced a time-dependent increase in the percentage of cells in G2/M, more than doubling after 6 h (Table 1). This was accompanied by expected reciprocal decreases in G0/G1 and increases in S. Even as soon as 2 and 4 h after addition of CE, this shift in phase distribution for the treated cells was found to be significant compared with untreated cells. As the distribution of control cells did not change at the several time points selected for these experiments, only the values for 0 and 6 h are included in Table 1. Histograms depicting the phase distribution for 0- and 6-h control cells and for 6-h CE-treated cells are shown in Fig. 1 (A, B and C, respectively). To establish that p38 MAPK activation was indeed necessary to produce the arrest, cells were preincubated with activation inhibitor SB 203580 (20 μM) for 1 h [19]. Distribution of the cell cycle phase for the SB 203580 pretreated cells was similar to that for control cells (compare DNA distribution in Fig. 1D with distributions for 0- and 6-h controls cells, Fig. 1A and B) showing that the inhibitor completely reversed the ability of CE treatment to produce G2/M arrest (Fig. 1D).

### 3.2. CE, cell volume/size and relation to G2/M arrest

With such large percentages of cells in the G2/M phase because of CE-treatment, we next determined if cell size parameters could be used to monitor and reflect this arrest. Two physical measurements that have been shown to be

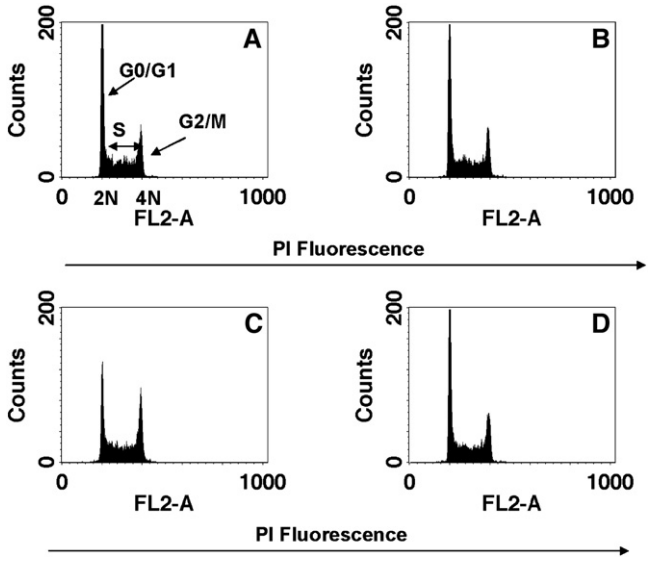


Fig. 1. Representative histograms showing increased number of cells in G2/M phase after 6 h of CE and reversal by SB203580, an inhibitor of p38 MAPK. (A and B) Phase distributions for control cells at 0 and 6 h, respectively. (C) Increase in the percentage of cells in G2/M after CE treatment for 6 h. (D) Cells pretreated for 1 h with SB203580 (20 μM) followed by CE treatment for 6 h. The phase distribution is similar to that for control cells indicating that the inhibitor has blocked the effect of CE-activated p38 MAPK on G2/M arrest.

correlated, the electronic volume and the FSC parameter, were determined in this study to evaluate cell size [17,18]. Data for these two measurements are shown in Table 2. CE treatment caused significant and corresponding increases in both size parameters after 6 h and 24 h, compared with the untreated controls cells. The increases seen in size measurements paralleled increases observed in percentages of cells in G2/M. Conversely, populations of exponentially growing control cells maintained similar values for size and percentages of G2/M, independent of sampling time.

### 3.3. CE and p38 MAPK activation

As activation of p38 MAPK has been linked to arrest in the G2/M phase arrest [9–12], the presence of this activated kinase in CE-treated cells compared with control cells was determined by intracellular staining with a specific antibody for this protein in fixed, permeabilized cells in combination with flow cytometry. Fig. 2A illustrates a typical population of cells by size and granularity parameters [FSC vs. side-angle light scatter (SSC)] with accompanying histograms

Table 1
Percent phase distribution of cells treated with CE from 0 to 6 h (0.1 mg/ml)

| Time (h) | 0 | 2 | 4 | 6 | 6 (Control) |
|---|---|---|---|---|---|
| G1 (%) | 43.7±0.6 | 35.7±0.8* | 29.0±2.7* | 24.4±1.9* | 43.4±0.7 |
| S (%) | 45.4±1.3 | 48.6±0.5 | 51.3±1.7* | 51.5±1.7* | 43.8±0.8 |
| G2/M (%) | 11.0±1.0 | 15.8±0.7* | 19.7±2.0* | 23.6±1.4* | 12.3±0.8 |

Values are means±S.D., $n$=3.
* Different from control values, 0 and 6 h ($P$<.05).

Table 2
Cell size parameters increased by CE treatment (0.1 mg/ml)

| Time (h) | 0 | 6 | 24 | 24 (Control) |
|---|---|---|---|---|
| MCV (FL) | 866±36.2 | 1021±34.3* | 1352±90.7* | 871±24.8 |
| FSC (MPC) | 397±6.2 | 428±15.2* | 560±2.5* | 398±6.0 |

Values are means±S.D., $n$=3.
MCV, mean cell volume; MPC, mean peak channel value, arbitrary units for FSC.
* Different from control values, 0 and 24 h ($P$<.05).

illustrating the presence of activated p38 MAPK. Fig. 2B shows that the CE-treated cells (24 h, 0.1 mg/ml) have increased FL-2 compared with untreated control cells when looking at the entire population of cells. This increase in FL-2 is due to the increased binding of the PE-conjugated antibody specific for the phosphorylated (activated) p38 MAPK. For comparison purposes, the autofluorescence of unstained cells are included. When gates are drawn to focus on larger cells with increased FSC, representative of cells in the G2/M phase, it is apparent that CE-treated cells are larger, more abundant and have increased FL-2 compared to control cells (Fig. 2D). Our earlier report showed that this level of treatment for 24 h produced a 36% increase in G2/M [4]. Smaller cells show less binding of the antibody to activated p38 MAPK (less FL-2) for all three cell populations (Fig. 2C). The presence of activated p38 MAPK in the entire populations of cells was determined by measuring increases in FL-2 indicative of increased binding of the PE-labeled antibody to this form of the kinase. Treated cells showed percentage increases in FL-2 of 26.8±3.10 at 4 h and 47.6±1.9 at 24 h. These increases were significantly different from values for control cells and correlated with the increases in the percentage of cells in G2/M arrest. Such increases in p38 MAPK activation were not observed for the untreated control cells. Correspondingly, these latter cells maintained

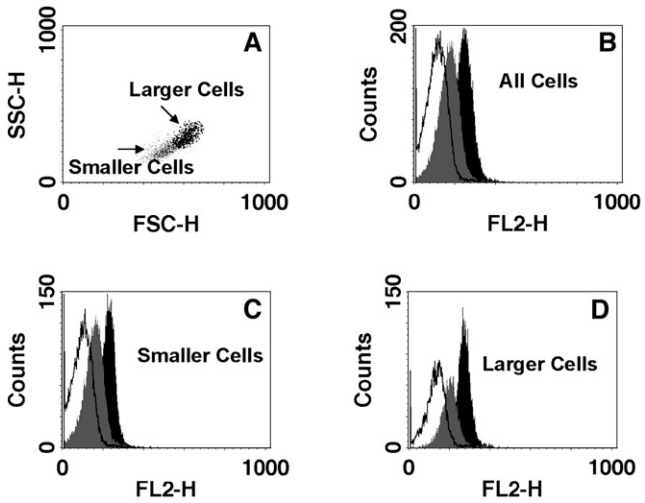


Fig. 2. Increased activated p38 MAPK in larger cells after CE treatment (24 h). Figures are representative of three independent experiments. FL-2, fluorescence due to binding of PE-labeled antibody to activated (phosphorylated) p38 MAPK. (A) FSC height (FSC-H) vs. SSC height (SSC-H) dot plot illustrates two populations designated as containing either larger cells (black region) or smaller cells (gray region). (B) Histograms depicting activated p38 MAPK in entire populations of untreated cells (gray) and CE-treated (black) cells compared to unstained control cells (white). (C) Activated p38 MAPK in population of smaller cells shown as gray in A. Histogram shades as for B. (D) Activated p38 MAPK in population of larger cells shown as black in A. Histogram shades as for B. Increases in FL-2 are indicative of an increase in activated p38MAPK. CE-treated cell populations showed significantly greater percentage increases in FL-2 compared to that for untreated control cells (26.8±3.10 at 4 h and 47.6±1.9 at 24 h).

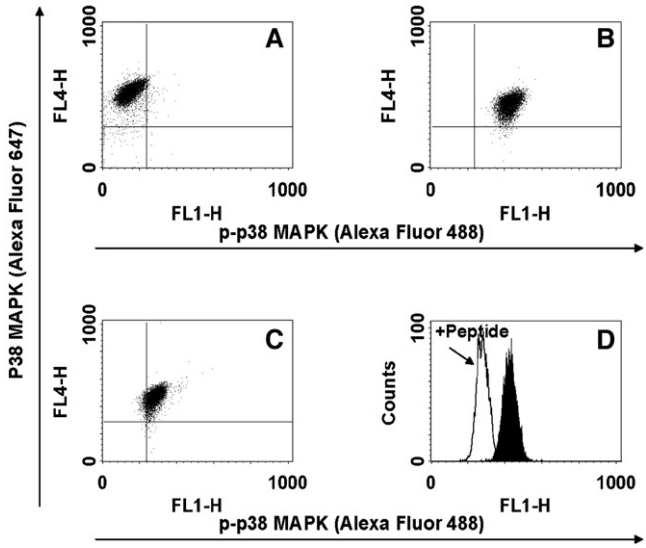


Fig. 3. Antibody specificity for activated p38 MAPK. CE-treated W (0.1 mg/ml for 24 h) were stained with antibodies to p38 MAPK and activated p38 MAPK (separately and combined). (A) Binding of the antibody to p38 MAPK was detected in larger cells in G2/M as shown by the increase in fluorescence emitted by the Alexa Fluor 647 probe (FL-4). (B) Double-staining of these larger cells was observed in the presence of both antibodies as demonstrated by increases in both FL-4 and FL-1 (Alexa Fluor 488 probe detecting activated p38 MAPK). (C) Preincubation with blocking peptide to the antibody to activated p38 MAPK decreased FL-1 but not FL-4. (D) Decreases in FL-1 were observed in cells stained with blocked anti-phospho-p38 MAPK (white histogram) compared to cells stained with unblocked antibody (black histogram). Similar results were seen with the PE-conjugated antibody. Panels A–D are representative of three separate experiments.

similar percentage of cells in the G2/M phase (11–12%, Table 1) at all measured time points.

To verify that intracellular staining with anti-phospho-p38 MAPK was detecting this protein, an additional set of experiments were conducted with fixed and permeabilized CE-treated cells (0.1 mg/ml for 24 h, $n$=3). Antibodies conjugated to different fluorescent probes were used to stain the cells for both activated and un-activated forms of the kinase (see Fig. 3A–D). As shown in Fig. 3A, antibody to p38 MAPK was detected in the larger cells, as shown by the increase in fluorescence emitted by the Alexa Fluor 647 probe (FL-4). Double-staining of the CE-treated cells was observed by an increase in FL-1 upon addition of the antibody to the activated form (Alexa Fluor 488) to samples prestained for anti-p38 MAPK (Fig. 3B). Preincubation with the blocking peptide produced a decrease in the binding of the antibody to activated p38 MAPK (FL-1) but did not decrease the binding of the antibody to p38 MAPK (FL-4), as shown in Fig. 3C. Staining only with blocked and unblocked antibody to activated p38 MAPK produced the data illustrated in the histogram overlay, which showed the decrease in FL-1 caused by the blocking peptide (Fig. 3D). Similar results were obtained when blocking experiments were conducted with the PE-conjugated antibody (data not shown).

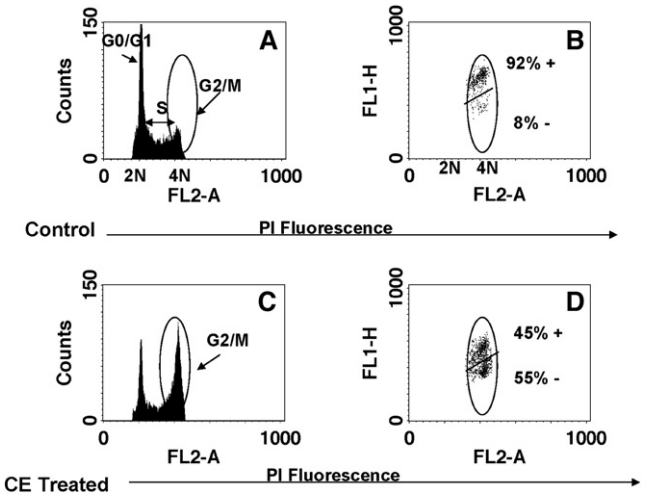


Fig. 4. CE-treatment effects on cyclin B1 in cells in G2/M phase after 24 h. (A and C) Representative histograms showing phase distribution of untreated and CE-treated cells after 24 h, respectively. Circled area represents cells in G2/M (4N). (B and D) Dot plots of cells in G2/M (circled areas from A and C). DNA content (FL2-A or PI fluorescence) versus cyclin B1 (FL1-H). Negative cells defined as those falling below the drawn line determined from cells stained with isotype control. The G2/M population of untreated cells (B) are 92% positive for cyclin B1 compared to a value of 45% for CE-treated cells (D).

### 3.4. CE and cyclin B1

The cyclin B1/cyclin-dependent (cdk)2 complex is the major regulator of progression through G2 and mitosis [5]. We next examined the consequence of increased activation of p38 MAPK on the presence of cyclin B1 in the G2/M population of cells. Cells that had been fixed and permeabilized were stained with the antibody specific for cyclin B1. This antibody is labeled with FITC, and binding is indicated by an increase in FL-1. Fig. 4 depicts the presence of the protein in the G2/M phase in both untreated and CE treated cells (24 h). Even though there were greater numbers of cells present in the G2/M phase (analysis gate represented by 4N DNA), it is evident that these cells were less positive for cyclin B1 (less FL-1). Cells were stained with the isotype control antibody to determine the line placement for positive and negative expression of cyclin B1. The increase in cells negative for cyclin B1 increased with CE treatment over time (Fig. 5), while untreated control cells did not demonstrate this effect.

## 4. Discussion

In our previous experiment, we demonstrated that treatment of three different myeloid cell lines for 24 h with an aqueous extract from cinnamon resulted in dose-dependent arrest in the G2/M phase of the cell cycle. In this report, we have focused on determining the effects of CE on signaling events rapidly affected by the presence of CE in the media. In our model of asynchronously growing cells and within hours of treatment, CE produced an increase in the percentage of cells in G2/M that coincided with an increase in activated p38 MAPK. Activation of p38 MAPK to produce the increase in G2/M phase was completely reversed by pretreatment with an inhibitor to this MAPK. This inhibitor has been used by other investigators to demonstrate the importance of p38 MAPK activation in initiating G2/M arrest in tumor cell lines [19,20].

The population of CE-blocked cells had increased indices of size measured by two independent methods along with increased numbers of cells in G2/M. The correspondence between size and cells in G2/M are in agreement with previous investigations [15,16]. Placing gates on the population of larger cells (4N DNA) demonstrated that increased size was correlated with the percentage of cells in G2/M along with increased numbers of cells positive for activated p38 MAPK. Conversely, these larger cells produced by treatment with CE were less positive for cyclin B1, a critical member of the complex that regulates progression into mitosis [5]. Activated p38 MAPK has been linked to inhibition of the formation of the mitotic promoting cyclin B1–cdk2 complex [5], suggesting that activation of this MAPK regulates the phosphorylation status of phosphatases and/or kinases required for formation of the complex. Our results are in agreement with Li et al. [21] who demonstrated that a compound isolated from *Rhizoma zedoariae*, a Chinese herb, produced both activation of p38 MAPK kinase and a decrease in cyclin B1 protein. In this latter investigation, proteins were determined by immunoblot analyses of cell lysates in contrast to our flow cytometric approach using fixed and permeabilized cells stained with appropriate antibodies to determine intracellular expression of activated p38 MAPK and cyclin B1. Both approaches have been shown to produce similar results, but it is well to note an important advantage of multiparametric analyses by flow cytometry. Protein levels can be related to cell cycle

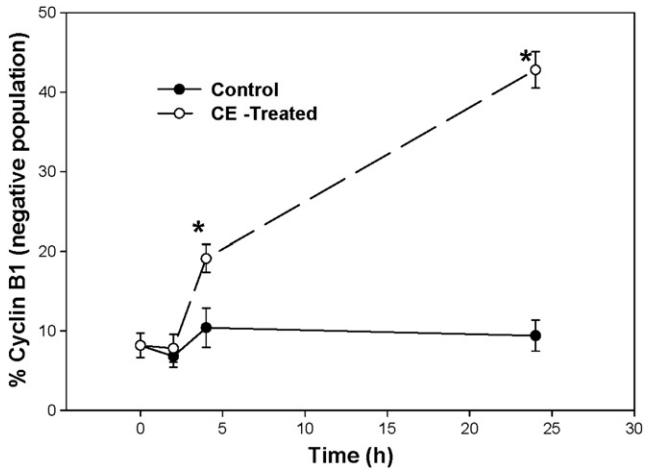


Fig. 5. Comparison of the percentage of cyclin B1 in the negative G2/M populations of untreated and CE-treated cells (0–24 h). Values are means±S.D., $n$=3. *Significantly different from untreated control cells, $P$<.05.

phase position (DNA content), as the flow cytometer can measure responses of individual cells. Our results are consistent with work reported by Sherwood et al. [22]. These investigators used the flow cytometric approach to show the requirement for a transient increase in cyclin B1 levels for proper progression through G2/M. The specificity of the antibody used to detect cyclin B1 in these seminal experiments was verified and is the same clone (GNS1) that we used in our experiments described here.

In our initial report describing CE effects on cell cycle progression, we showed that there was a decrease in total phosphatase activity in lysates from cells treated with CE compared with controls [4]. As phosphorylation/dephosphorylation signaling events regulate progression through the cell cycle, the ability to inhibit phosphatases may partially explain the results reported here. CE treatment produces imbalances in signaling molecules that depend on temporal and spatial phosphorylation/dephosphorylation events that propel the cell cycle through the duplicative process.

A diverse array of compounds with polyphenolic structures found in plant products have been shown to possess anticancer and anti-inflammatory properties [1–3]. It is reasonable to assume that such compounds, when present in the diet, have the potential to affect multiple targets with different outcomes on cell physiology. Well-studied polyphenols such as resveratrol, curcumin and tea compounds can have differential effects on cell cycle progression. Resveratrol can produce arrests in the cell cycle at both G0/G1 and G2/M [23] depending on the context of cell type and dose. Curcumin [24,25] and tea components [3,26], on the other hand, commonly induce G2/M arrests. It is not entirely clear how the various individual polyphenol compounds bring about changes in cell cycle regulation. These compounds have been linked with such a multiplicity of signal transduction pathways that underlying mechanisms of chemoprevention remain unclear at present [1–3]. Of note, one polyphenol produces effects similar to those seen with CE. The isoflavone from soy, genistein, has been shown to cause both a block at G2/M and activation of p38 MAPK [10,12,27]. Tumor cells that are arrested at a particular cell phase can be eliminated by more effective and less toxic strategies than when growth is completely out of control. Associated toxicities accompanying radiation and chemotherapy modalities have been reduced because of the genistein-induced arrest at the G2/M phase [27,28].

Although the CE effects reported here resemble to some extent those produced by genistein, to the best of our knowledge the complex chemical constituents of the cinnamon extract possess no isoflavone-type structures [13]. In addition, a cautionary note is warranted, as it may not be just one individual compound in CE that is producing the results that we observed in our experiments. Mixtures of polyphenols have been shown to have synergistic effects on cellular processes compared with less robust effects seen when individual compounds are assessed for their effects on the same processes [2,29].

In summary, the results of our experiments studying the effects of CE on signaling networks in a tumor cell line can be used to advance understanding of the underlying mechanism(s) that cause arrest at the G2/M phase of the cell cycle. Measured outcomes of CE treatment on cell cycle regulatory processes in W produced rapid and correlative increases in cell size, greater percentage of cells in G2/M, increased activated p38 MAPK and decreased cyclin B1.

The results of these multiparametric analyses strongly suggest that CE treatment inhibited entry into mitosis by altering key signaling components driven by kinase/phosphatase activities [5].

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