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Mechanism of Sulforaphane-Induced Cell Cycle Arrest and Apoptosis in Human Colon Cancer Cells

Géraldine Parnaud, PengFei Li, Georges Cassar, Patrick Rouimi, Jacques Tulliez, Lydie Combaret, and Laurence Gamet-Payrastre

Abstract: *Sulforaphane (SFN) is a natural micronutrient found in cruciferous vegetables that has been shown to possess antitumoral properties in carcinogen-treated rats. In vitro, SFN regulates phase II enzymes, cell cycle, and apoptosis. In the present study, we investigated the relationship between SFN induction of apoptosis and cell cycle arrest in HT29 human colon carcinoma cells.*

In previously published data, a significant increase in the G2/M phase of the cell cycle has been observed in SFN-treated cells that was associated with increased cyclin B1 protein levels. In the present study, our results show that SFN induced p21 expression. Moreover, preincubation of HT29 cells with roscovitine, a specific cdc2 kinase inhibitor, blocked the G2/M phase accumulation of HT29 cells treated with SFN and abolished its apoptotic effect (22.2 ± 4 of floating cells in SFN-treated cells vs. 6.55 ± 2 in cells treated with both SFN and roscovitine). These results suggest that the cdc2 kinase could be a key target for SFN in the regulation of G2/M block and apoptosis. Moreover, in SFN-treated cells the retinoblastoma tumor suppressor protein (Rb) is highly phosphorylated. Inhibition of the cdc2 kinase by roscovitine did not change the phosphorylation status of Rb in SFN-treated cells, suggesting that this cyclin-dependent kinase may not be involved. In our study, we did not observe any significant change in the proteasomal activity between control and SFN-treated cells. Moreover, inhibition of proteasomal activity through the use of MG132 diminished SFN-induced HT29 cell death, suggesting that the apoptotic effect of SFN requires a functional proteasome-dependent degradation system. In summary, we have elucidated part of the mechanism of action of SFN in the concomitant regulation of intestinal cell growth and death.

Introduction

Sulforaphane (SFN), a naturally occurring isothiocyanate (ITC), is produced from glucoraphanine, a glucosinolate mainly present in intact cruciferous vegetables (1,2). It has been shown to be a naturally occurring cancer chemopreventive agent in animal models. Indeed, previous studies using animals have demonstrated the protective effect of SFN with regard to chemically induced carcinogenesis in various organs, including lung, esophagus, liver, mammary gland, colon (3), and stomach (4). The chemopreventive activity of SFN has been associated with various effects. Talalay and Fahey (5) identified SFN as the main inducer of phase II detoxifying enzymes (namely, glutathione *S*-transferase, EC 2.5.1.18) in broccoli extracts. In addition, SFN was found to inhibit the phase I cytochrome P450 isozyme 2E1, which is responsible for the activation of various genotoxic chemicals in hepatocytes. The anticarcinogenic effect of SFN has also been attributed to its ability to induce the expression of multidrug resistance-associated protein 2 in primary rat and human hepatocytes (6,7). SFN has also been shown to possess an anti-inflammatory activity in macrophages, which could contribute to its cancer chemopreventive properties (8). In addition to the action of ITCs on phase I and II enzymes, these compounds could also lead to other anticarcinogenic mechanisms such as apoptosis induction and cell cycle arrest (9–13). We have previously shown that induction of apoptosis in HT29 human colon carcinoma cells is associated with cytochrome c release, Bax suppression, and caspase activation. Moreover, we also demonstrated that, in these cells, SFN-induced cell death was associated with cell cycle arrest at the G2/M phase with a sustained increase in the expression of cyclin B1 (9).

The molecular components governing the progression through the cell cycle have been described and studied in detail over the past few years (14). Cyclins appear to have a major role in this progression as their association with cyclin-dependent kinases (CDKs) leads to the subsequent activation of cyclin–CDK complexes. The activity of cyclin–CDK complexes is tightly regulated by a variety of mechanisms such as periodic cyclin accumulation, degradation, nuclear localization, phosphorylation of CDKs, and association with a number of different CDK inhibitors. Two families of CDK inhibitors have been identified; one of these

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families contains p21, p27, and p57, which have broad specificity for CDKs. Cyclin B types are the major activators of the cdc2 kinase that controls the G2/M transition. In addition to the well-characterized role of p21 as an inhibitor of cell cycle progression, recent findings suggest a role for p21 in G2 and mitotic checkpoints (15).

Recently, apoptosis has been hypothesized to be the result of abnormal cell cycle control (16). Cyclin overexpression and activation of CDKs have been shown to correlate with the onset of apoptosis in many experimental systems. Choi et al. (17) have shown that, in FaO hepatoma cells, cdc2 and cdk2 kinase activity phosphorylates retinoblastoma protein (Rb) and suggests that the hyperphosphorylation of Rb may trigger abrupt cell cycle progression leading to irreversible cell death.

Our previously published data demonstrated a sustained expression of cyclin B1 in SFN-treated human colon cancer cells (HT29). Here we show that SFN induces an increase in p21 protein expression and a sustained cdc2 kinase activity in comparison with control cells. Moreover, inhibition of cdc2 kinase activity through the use of roscovitine, a specific inhibitor of this enzyme, leads to an important decrease in SFN-induced apoptosis and G2/M arrest in HT29 cells, suggesting that, in our model, this kinase could play a role in the regulation of cell growth and death. Our results also show a hyperphosphorylation of Rb in SFN-treated cells. However, it appears that the cdc2 kinase is not involved in the phosphorylation of Rb in SFN-treated cells. The role of proteasome-dependent proteolysis of cell cycle regulatory proteins was also investigated.

Materials and Methods

Drugs and Chemicals

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Life Technologies (Invitrogen, Cergy-Pontoise cedex, France). Monoclonal antibodies raised against p21, retinoblastoma protein (Rb), and actin were obtained from Pharmingen (le Pont de Claix, France), and the cdc2 monoclonal antibody was purchased from Santa Cruz Technology (Heidelberg, Germany). Polyclonal antibody against phosphorylated Rb on ser-780 was purchased from Tebu (Le Perray en Yvelines, France). Phospho-cdc2 (Tyr15) polyclonal antibody was obtained from New England Biolabs, Inc. (Beverly, MA). Monoclonal α-tubulin antibody and HRP-labeled rabbit anti-mouse and goat anti-rabbit immunoglobulins were purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA) was obtained from Roche (Meylan, France). SFN was obtained from LKT Laboratories (St. Paul, MN). All other chemicals were purchased from Sigma or from Merck (Darmstadt, Germany) and were of the highest purity available.

Cell Culture

The HT29 cell line was established in permanent culture from a human colon carcinoma by Dr. Fogh (Sloan-Kettering Institute for Cancer Research, Rye, NY). HT29 cells were purchased from the European Collection of Cell Culture (ECACC, Salisbury, UK). Routinely, cells were cultured in DMEM containing 25 mM glucose, 43 mM bicarbonate, 60 μM/ml penicillin, and 100 μg/ml streptomycin at 37°C under an air/CO_2 (9/1) atmosphere supplemented with 5% heat-inactivated FCS.

For experiments, HT29 cells were seeded at a density of 5×10^4 cells per milliliter in 120-mm diameter Primaria dishes in standard medium containing 5% FCS. Twenty hours after seeding, 70% of cells were in DNA synthesis phase, and cells were then treated with SFN (15 μM). An equivalent amount (0.2%) of solvent (ethanol or DMSO) was added to control cells. At indicated times, cells were either prepared for flow cytometry analysis or harvested for Western blot analysis.

Estimation of Apoptotic Cell Number

As we have shown in our previously published data, SFN induces cell death through an apoptotic mechanism that is correlated with a loss of cell adhesion to the dish (9). In the present study, we have estimated the number of apoptotic cells by counting dead cells floating in the culture medium and calculating the percentage of total cell number. At the indicated times, floating cells were retrieved from the culture medium by centrifugation at 2,500 rpm for 5 min. The pellet was then resuspended, homogenized in 100 μl of PBS, and diluted in 10 ml of an isotonic solution (Isoton, Beckman Coulter, France), and cells were counted using a Coulter counter. Attached cells were rinsed with PBS and incubated with 500 μl of trypsin at 37°C for 5 min. Trypsin was inhibited by addition of 1 ml of 5% FCS-containing medium, and the cell suspension was centrifuged at 2,500 rpm for 5 min. Supernatant was discarded, and the pellet was resuspended in 1 ml PBS. After homogenization, an aliquot of 100 μl of the cell suspension was diluted in 10 ml of the isotonic solution for counting.

Flow Cytometry Analysis

The drug effect on cell proliferation was evaluated by measuring the distribution of cells in the different phases of the cell cycle using flow cytometry. This was determined by measuring the DNA content in nuclei stained with propidium iodide according to the method of Vindelov and Christensen (18), which was slightly modified. Cells from either control or treated cultures were obtained by trypsinization and added to the pool of floating cells. They were then washed twice with 0.9% NaCl. Cells (1×10^6) were suspended in 220 μl of solution A (trisodium citrate 3.4 mM, pH 7.6; Nonidet P40, 0.1%; spermine tetrahydrochloride, 1.5 mM; and Tris base, 0.5 mM) containing trypsin (30 mg/l) for 10 min at room temperature. Trypsin was then inhibited by the addition of 180 μl of solution A containing trypsin inhibitor (0.5 g/l) and RNase A (100 mg/l) for an additional 10 min. Finally, nuclei were labeled by addition of 180 μl of solution A containing propidium iodide (416 mg/l) and additional spermine

tetrahydrochloride (1,160 mg/l). The suspension was incubated overnight at 4°C to allow maximum labeling of DNA. Cell cycle analysis was performed on a Coulter ELITE flow cytometer through a 630-nm LP filter. Debris and doublets were eliminated by gating on peak versus integrated signals, and 1.5×10^4 cells were collected per sample. Calculations were performed using MultiCycle AV Software (Phoenix Flow System).

Western Blot Analysis

Whole cell extracts were prepared for Western blot analysis. Briefly, control and SFN-treated cells were collected, washed in PBS, and lysed in ice-cold lysis buffer (62.5 mM Tris at pH 6.8, containing 2% SDS, 5% glycerol, and 2.5% 2-mercaptoethanol and protease inhibitor cocktail, Biorad). The protein concentration of cell extracts was determined using the Shaffner and Weissmann method (19). Aliquots from extracts of indicated treatments and time points (30–120 µg protein per lane) were mixed with loading buffer (250 mM Tris at pH 8.8, 4% SDS, 16% glycerol, 8% 2-mercaptoethanol, and 0.1% bromophenol blue), fractionated by SDS-PAGE (acrylamide 7.5% for Rb, 10% for cdc2 and Pcdc2,12.5% for p-Rb ser 780, and 14% for p21), and transferred to nitrocellulose membranes (Schleicher & Schuell, Ecquevilly, France) by semidry blotting. After transfer, membranes were blocked using 1% skimmed dried milk and 1% BSA in TBS (20 mM Tris-HCl at pH 7.4 containing 0.05% Tween 20) for 1 h at room temperature or overnight at 4°C. Immunogenicity was detected by incubating the membranes for 90 min at room temperature with the appropriate primary antibody. After washing with saturating buffer, the membranes were incubated with diluted HRP-labeled secondary antibody for 60 min. The proteins were then visualized using the ECL+ detection system (Amersham, Sarclay, France) according to manufacturer's instructions. The blots were probed with α-tubulin antibody as an internal control for protein loading.

Assay for Proteasome Activity

HT29 cells were scraped in ice-cold buffer (Tris-HCl 50 mM at pH 7.5, 1 mM DTT, 10 mM ATP, 10 mM $MgCl_2$, 10 µg/l antipaïn, 10 µg/l aprotinin, 10 µg/l pepstatin A, 10 µg/l leupeptin, 200 µM PMSF, and 10% glycerol) and sonicated 5 s. Each lysate was centrifuged at 13, 000 *g* at 4°C for 10 min to remove insoluble material. The high-speed supernatants were assayed for proteasome activity. Briefly, supernatant (50 µg) was placed in assay buffer (50 mM Tris-HCl at pH 8, 1 mM DTT, 10 mM $MgCl_2$, and10 mM ATP) containing 100 µM of peptide substrate Suc-Leu-Leu-Val-Tyr AMC (Affiniti Research Products, UK). After incubation for 30 min at 37°C, the reaction was stopped by the addition of stop mix (sodium acetate/monochloroacetic acid). Proteasome activity was monitored by measuring the fluorescence of released AMC at excitation wavelength (370 nm) and emission wavelength (430 nm) by using a luminescence spectrometer. Specificity of the reaction was determined by performing the assay with MG132 (40 µM), a specific proteasome inhibitor.

Results

Effect of Sulforaphane on P34cdc2 Expression and Activity

Our previously published data (9) showed an increased expression of cyclin B1 in HT29 cells after SFN treatment. Cyclin B1 synthesis and accumulation are known to regulate P34cdc2 kinase activity from the G2 to the M phase of the cell cycle. Activation of P34cdc2 kinase is also controlled by its phosphorylation and subsequent dephosphorylation. Here, we have investigated the effect of SFN on P34cdc2 expression and activity through the use of an antibody that specifically detects the catalytically inactivated form (phosphorylated at tyr 15) of cdc2. Figure 1A represents a Western blot showing the expression and activity of cdc2 in SFN-treated cells (+) for 48 h compared with control cells (–). The phosphorylated inactive form of cdc2 kinase appears to be more expressed in control cells (–) than in SFN-treated cells (+). In Fig. 1B, the RS lane represents HT29 cells pretreated with 20 µM roscovitine, a specific cdc2 kinase activity inhibitor, and subsequently exposed to 15 µM SFN for 48 h. The RE lane represents cells exposed to roscovitine alone. In roscovitine-treated cells, cdc2 appeared under its phosphorylated inactive form (lanes RE and RS). Figure 1B also shows that the expression of phospho-cdc2 was only slightly different between control (DE) and SFN-treated (DS) cells. On the other hand, phospho-cdc2 was less expressed in SFN-treated cells compared with roscovitine-treated cells. This suggests that, in SFN-treated cells, the cdc2 kinase is maintained in its active dephosphorylated form.

Sulforaphane-Induced Cell Death and G2/M Arrest at Least in Part via the Activation of cdc2 Kinase

To determine whether the activation of cdc2 kinase was required for SFN-induced apoptosis and G2/M arrest, we examined the effect of roscovitine, a specific inhibitor of cdc2 kinase activity, on the effects of SFN. HT29 cells were pretreated for 1 h with roscovitine (20 µM) prior to a 24- or 48-h exposure to 15 µM SFN. The effect of SFN was estimated by counting dead cells floating in the culture medium and by cell cycle analysis. In Fig. 2A, the amount of dead cells is expressed as a percentage of total cell number (adherent and floating cells). As shown in Fig. 2A, SFN-induced cell death is diminished by roscovitine treatment. This effect was potent after 24 h and was maintained for 48 h. Cell cycle analysis of HT29 cells was performed on floating and attached cells as a whole after 24 (Fig. 2B) and 48 h (Fig. 2C) of treatment with SFN alone or in combination with roscovitine. Figure 2B shows that the inhibition of cdc2

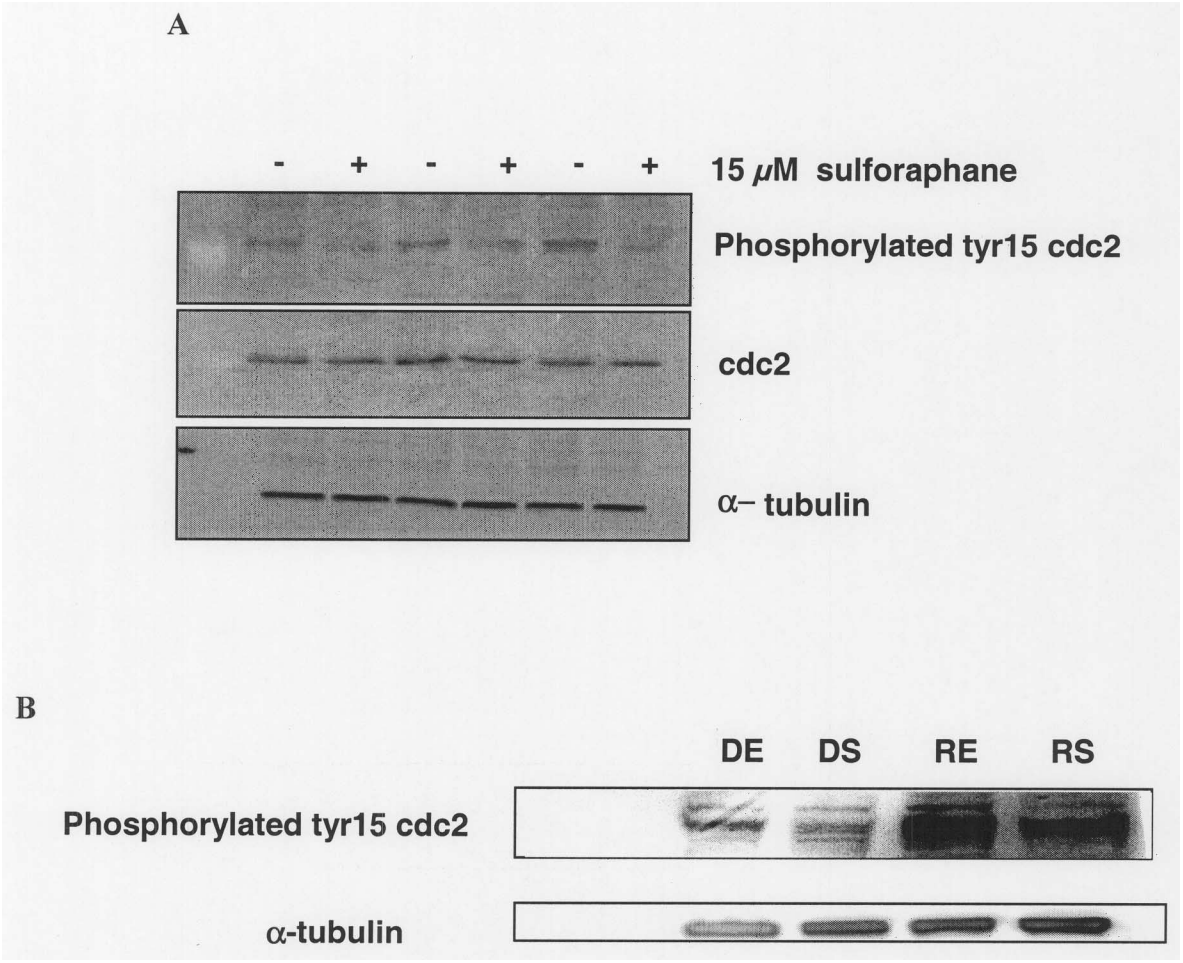


Figure 1. Western blot analysis of cdc2 kinase activity. HT29 cells were treated with 20 μM of roscovitine (R), a specific inhibitor of cdc2 kinase activity, or with DMSO (D) for 1 h and then treated for 48 h with 15 μM SFN (S) or ethanol (E; 2 μl/ml of medium). Equal volumes of whole cell extracts containing 30 μg of protein were separated on SDS-PAGE (12.5%) and electrophoretically blotted. The influence of SFN treatment on cdc2 expression and activity was investigated using a phosphospecific antibody; a representative immunoblot is shown ($n = 3$). DE, DMSO- and ethanol-treated cells; DS, DMSO- and SFN-treated cells; RE, roscovitine- and ethanol-treated cells; and RS, roscovitine- and SFN-treated cells.

kinase (RS) also leads to a decrease in the G2/M block induced by 24 h of incubation with SFN compared with control cells (DS). Figure 2C clearly shows the presence of apoptotic cells (subG1 peak) after 48 h of SFN treatment and absence of such cells following pretreatment with roscovitine (22.2 ± 4 of apoptotic cells in SFN-treated cells vs. 6.5% in HT29 cells treated with both SFN and roscovitine). This result suggests that the activation of cdc2 kinase could be correlated with the onset of apoptosis in SFN-treated cells.

Effect of Sulforaphane on P21 Expression

CDK inhibitors can negatively regulate cell cycle progression in response to antiproliferative signals. P21, the first CDK inhibitor to be identified, preferentially associates with the cyclin–CDK complex. Although a major effect of p21 is considered to be exerted during the G1 phase of the cell cycle, recent data demonstrate that this protein is also required for cell cycle arrest at G2 in some cancer cells (15). Treatment of HT29 cells for 48 h with 15 μM SFN led to a slight increase in p21 expression (Fig. 3). This was observed 8 h after the beginning of the experiment and appeared to be maintained until 48 h. Therefore, in our model SFN-induced G2/M arrest and apoptosis are associated with a slight induction of p21 expression.

Retinoblastoma Tumor Suppressor Protein Is Highly Phosphorylated in SFN-Treated Cells

Various studies have shown that, in some cellular models, Rb protein phosphorylation status can vary during the apoptotic cascade. Moreover, recent findings have demonstrated that the activated cdc2 kinase was responsible for a massive hyperphosphorylation of Rb in FaO cells (17). Because the active form of the cdc2 kinase appears to be maintained in SFN-treated HT29 cells, we investigated the phosphorylation status of endogenous Rb in response to SFN. The cells were treated during the S phase with 15 μM SFN for 48 h. Whole cell extracts were prepared and sub-

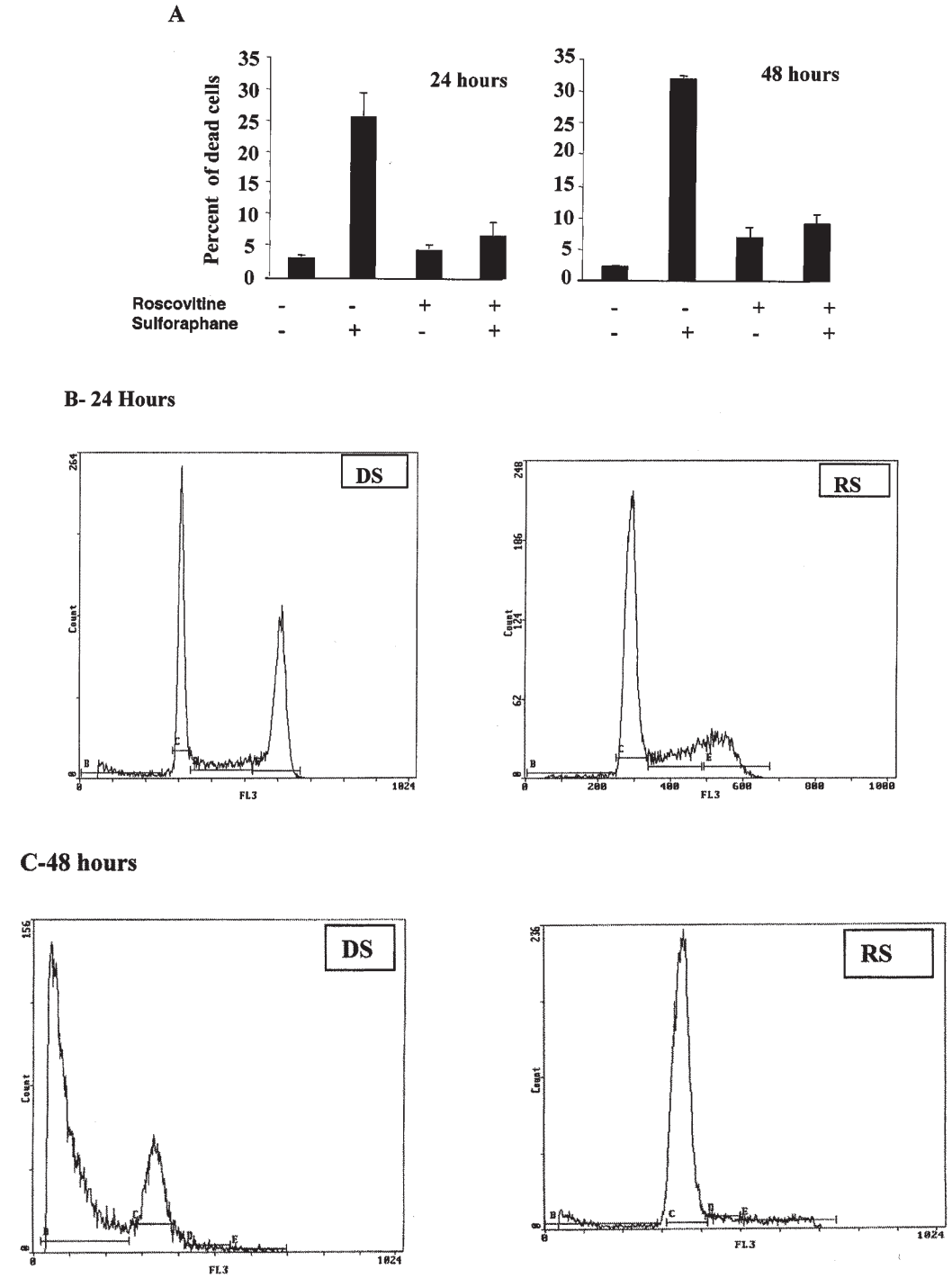


Figure 2. Effect of SFN on HT29 (A) cellular viability and (B and C) cell cycle in the presence of roscovitine. (B and C) HT29 cells were treated with 20 μM of roscovitine before exposure to SFN for 24 or 48 h. (A) Dead cells floating in the medium were counted and expressed as a percentage of total cells. (B) Cell cycle analysis of total cells (floating and attached) after 24 h of incubation with SFN in the presence (RS) or absence (DS) of roscovitine. (C) Cell cycle analysis of total cells (floating and attached) after 48 h of incubation with SFN in the presence (RS) or absence (DS) of roscovitine. Controls (DE) received corresponding amounts of ethanol and DMSO. Experiments have been done in triplicate, and one representative experiment is shown.

jected to immunoblotting using either a monoclonal antibody that recognized both the hypo- and hyperphosphorylated form of Rb (Fig. 4A and B) or a polyclonal antibody recommended for the detection of Rb phosphorylated at ser-780 (Fig. 4C). From 24 to 48 h, exposure to SFN markedly induced a stable level of Rb protein expression in comparison with the control (Fig. 4A). Moreover, Western blot analysis of cellular extracts showed an increase in Rb phosphorylation (Fig. 4B) following 48 h of SFN treatment coinciding with the activation of cdc2 (Fig. 1). However, Fig. 4C shows that inhibition of the cdc2 kinase activity by roscovitine in SFN-treated cells did not modify the induced

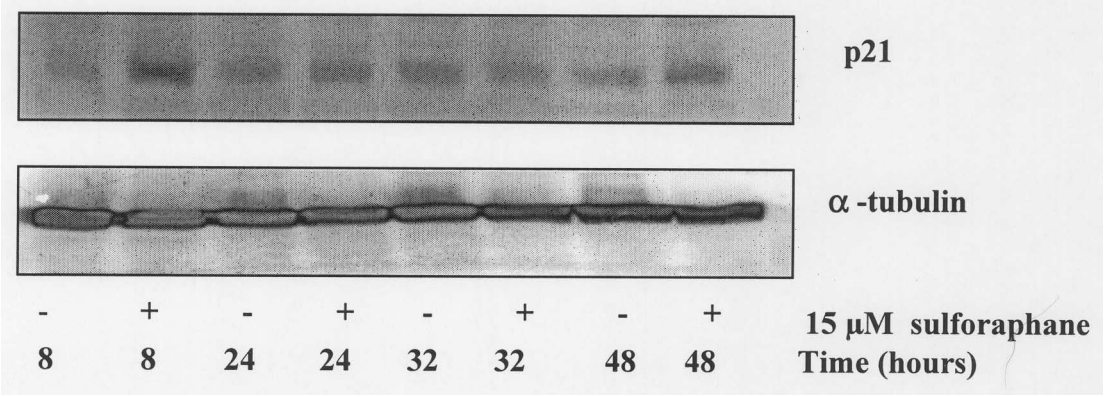


Figure 3. Western blot analysis of cyclin-dependent kinase inhibitor expression (p21) in SFN-treated cells. Protein (120 μg) was separated on SDS-PAGE (14%) and then electrophoretically blotted onto a nitrocellulose membrane as described in **Materials and Methods.**

expression of pRb. This last result suggests that the cdc2 kinase may not be involved in the phosphorylation status of Rb in SFN-treated HT29 cells.

Apoptotic Effect of SFN May Require a Functional Proteasome-Dependent Degradation System

Several proteins involved in cell cycle regulation are degraded via ubiquitin-mediated proteolysis (20). Ubiquitinized proteins are recognized by the 26S proteasome and degraded. In our model, we did not observe significant change in the proteasome activity between control and SFN-treated cells (Fig. 5A). Moreover, blocking proteasomal activity through the use of MG132 diminished SFN-induced cell death (Fig. 5B), suggesting that the apoptotic effect of SFN requires a functional proteasome-dependent degradation system.

Discussion

The results obtained from several studies have shown that fruit and vegetable consumption is inversely associated with colorectal cancer risk. Several natural plant micronutrients have been studied as key mediators of the cancer chemopreventive activity associated with vegetable-rich diets. ITCs have been investigated for use as chemopreventive agents (that is, benzyl-, phenylethyl- and allyl-ITCs, SFN) in animal models. Their mechanism of action has been described in vitro and seems to be mediated mainly by the regulation of detoxifying enzyme activities and signaling pathways regulating cell growth and death (9,21,22). In our previously published data (9), SFN, an ITC from broccoli, was shown to induce apoptosis and cell cycle arrest at the G2/M phase in HT29 human colon cancer cells. These effects were associated with overexpression of cyclin A and B.

Here we further investigate the relationship between induction of apoptosis and cell cycle arrest induced by SFN in this model. We show that ITC induces the expression of specific proteins involved in the concomitant regulation of cell growth and death. Our results clearly show that SFN treatment led to an increased expression of p21 and of the hyperphosphorylated form of Rb. Moreover, treatment with roscovitine, an inhibitor of cdc2 kinase activity, blocked SFN-induced apoptosis and G2/M arrest, suggesting a key role for this kinase in the signal transduction pathway triggered by SFN.

There is now evidence that cell cycle control and apoptosis are closely linked (16). Apoptosis has been hypothesized to result from abnormal cell cycle control. Indeed, the overexpression of cyclins and activation of CDKs have been shown to be correlated with the onset of apoptosis in many experimental systems (17,23,24). Specific inhibition of cdc2 kinase activity in SFN-treated cells led to a great decrease of the apoptotic effect and G2 block induced by this ITC. Indeed, one can suggest that activation of a cyclin B–dependent kinase by this ITC may be critical for both the G2 block and apoptosis in our model. Our results also suggest that the G2 block and cell death are linked. It will be interesting to determine whether the G2 block could be a cause of cell death in our model.

P21 is one of the universal inhibitors of CDKs and is required for cell cycle arrest at the G1 and G2 checkpoints after DNA damage. Overexpression of p21 in SFN-treated cells was not correlated with the inhibition of cdc2 kinase activity. As p21 induction is a good indicator of growth arrest in different cell types, including colorectal cancer cells, p21 may play a regulatory role in the maintenance of cell cycle arrest at G2 in our model. This has been shown by several investigators using HT29 cells treated with another natural anticancer agent, that is, butyrate (15,23–28). An increased expression of p21 in SFN-treated cells may also be linked to apoptosis. Indeed, several studies have examined the role of p21 in the apoptotic process (29–31). The use of a cell line with a targeted knockout p21 gene ($p21^{-/-}$ HCT116 cells) should be interesting to demonstrate the involvement of p21 in these pathways. Although p21 has often been shown to be expressed in a p53-dependent fashion in response to DNA damage (32), its expression could also be upregulated in a p53-in-

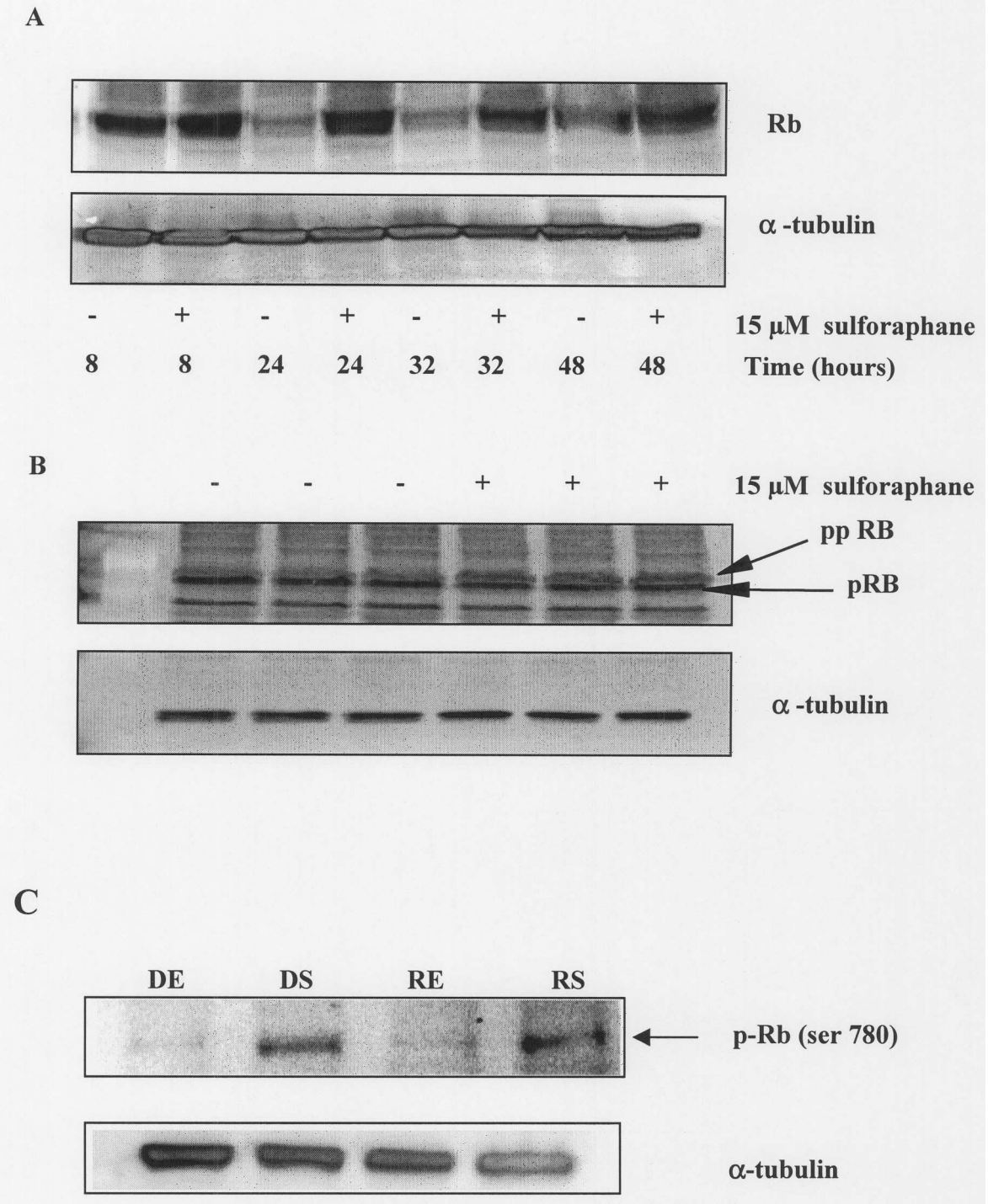
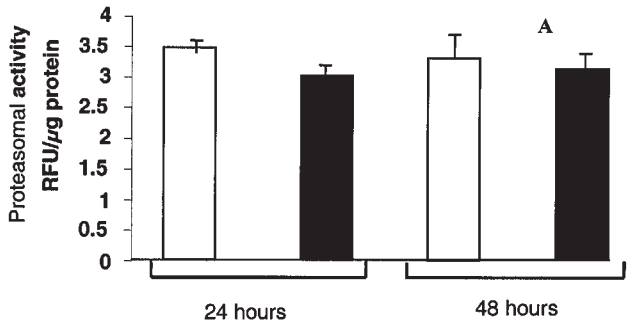


Figure 4. Western blot analysis of Rb expression in SFN-treated cells. Proteins (120 μg) were separated on SDS-PAGE (7.5%) and then electrophoretically blotted onto a nitrocellulose membrane as described in **Materials and Methods**. (A) Kinetic analysis of Rb expression in cells treated with SFN compared with control cells. (B) In 48-h SFN-treated cells, Rb appeared under its hyperphosphorylated form (ppRb). (C) Western blot analysis of Rb phosphorylated at ser-780 in SFN-treated cells. Proteins (30 μg) were separated on SDS-PAGE (12.5%) and then electrophoretically blotted onto a nitrocellulose membrane as described in **Materials and Methods.**

dependent manner by various stimuli (33). The activation of p21 by SFN appears to be p53 independent as HT29 cells express mutant forms of p53. P21 induction might also be dependent on the extracellular signal-regulated kinase (ERK) pathway (34–36). In Caco2 cells, we have shown (Rouimi et al., submitted data, personal communication) that SFN induced apoptosis through the induction of the ERKs and p38 pathway. Indeed, we are now investigating whether a specific inhibitor of these MAP kinases can modulate the expression of p21 and cell cycle arrest in HT29 cells.

Rb is an important tumor suppressor and cell cycle regulator. Recent studies suggest that Rb also plays a regulatory



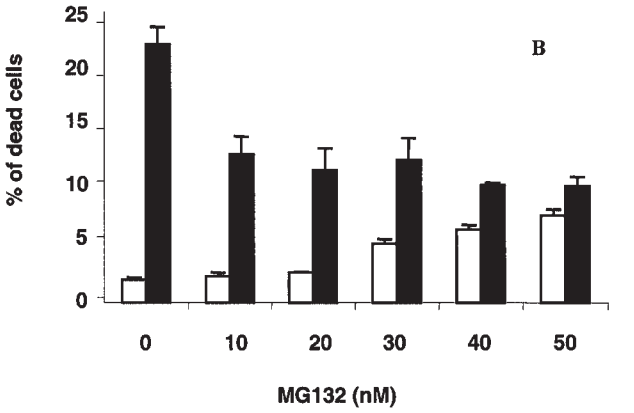


Figure 5. Lack of anti-proteasome activity of SFN. (A) Proteasome activity was assayed using a highly sensitive fluorogenic substrate in 24- and 48-h SFN-treated cells (black columns) compared with control cells (empty columns). Results are the mean ± SE of three separate experiments. (B) Blocking proteasomal activity through the use of MG132 diminished SFN-induced cell death. Cells were treated with various concentrations of MG132, an inhibitor of proteasomal activity in the presence (black columns) or absence (empty columns) of SFN. After 48 h of incubation, total cells (attached and floating) were counted as described in **Materials and Methods**. Percentage of dead cells was calculated as cells floating in the culture medium versus total cell number. Results are the mean ± SE of three separate experiments. Statistical parameters were obtained from raw data.

role in the apoptotic process (37,38). Various data support the hypothesis that unphosphorylated Rb may function as an apoptotic inhibitory factor (37). The kinase responsible for Rb phosphorylation has been reported to be a member of the CDK family (39). In our model, the cdc2 kinase does not seem to be involved in the regulation of the phosphorylation status of Rb. However, one can suggest that, in our model, phosphorylation occurring at specific Rb sites might be sufficient to trigger apoptosis through downstream signaling events such as the release of E2F (17).

Cyclin B1 expression is regulated at various levels, and its degradation appears to be necessary for the cell to exit from mitosis. (40). Cyclin B1 is degraded by the ubiquitin-mediated pathway (41–43). Ubiquitinized proteins are recognized by the 26S proteasome and degraded. In our study, blocking proteasomal activity through the use of MG132 diminished SFN-induced cell death, suggesting that the apoptotic effect of SFN requires a functional proteasome-dependent degradation system. We are further investigating the specific cyclin B ubiquitination in a time-course study to determine whether cyclin B accumulation is due to a ubiquitination defect.

In conclusion, our results show the relationship between induction of apoptosis and cell cycle arrest in a human colon cancer cell line by SFN, a natural food component. That the intestinal epithelium could be exposed to high concentrations of dietary micronutrients implies that SFN as well as other anticancer food components could have a role in the prevention of colon cancer through both inhibition of cell cycle and stimulation of apoptosis.

Acknowledgments and Notes

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