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ABSTRACT

Consensus has been building that diets rich in fruits and vegetables are associated with lower risks of developing various type of cancer. Increasing evidence suggest a link between prevention of carcinogenesis and natural isothiocyanate intakes provided by a normal consumption of cruciferous vegetables. Isothiocyanates occur in the vegetal as biologically inactive thioglucosides, which are processed by plant or intestinal microflora myrosinases, and have been described as effective inhibitors of chemically induced carcinogenesis in animal models. Indeed, sulforaphane, an isothiocyanate from broccoli, was shown to protect carcinogen-treated rats from tumorigenesis. Little is known about the mechanism by which sulforaphane operates, except that it involves -at least in part- phase I enzymes inhibition, phase II enzymes induction, cell cycle arrest and/or apoptosis. Therefore, we investigate possible intracellular targets of SFN using human colon carcinoma cell lines. In an earlier study, we showed that sulforaphane-induced apoptosis and cell cycle arrest at the G2/M phase in HT29 cells. We now investigate MAP kinase modulation, glutathione S-transferase expression and the tyrosine kinase phosphorylation status in the similar cell model, Caco2. We also compared some parameters from the well-described cell differentiation induced by sodium butyrate to sulforaphane effects. Our results show that sulforaphane induces cell death through ERK1/2 and transient P38 activation. Involvement of JNK is less clear. On an other hand, the strong induction of class alpha GSTs observed as well in confluent/differentiated untreated cells as in exponentially-growing Caco2 cells exposed to sulforaphane, appears independent of the MAP kinase pathway. In addition, sulforaphane properties do not seem to be related with a marked histone hyperacetylation in this cell model.

Key words: MAP Kinases, sulforaphane, GST, colon, cancer cells, apoptosis, cell cycle arrest, cell signaling pathway

INTRODUCTION

Various epidemiological and experimental evidence has suggested that dietary isothiocyanates (ITC), which occur as glucosinolates in a variety of cruciferous vegetables, could be key mediators of the cancer chemopreventive activity associated with a frequent consumption of these plants (1). Sulforaphane [1-isothiocyanato-4-methylsulphinylbutane] (SFN), a natural ITC abundantly represented in broccoli, has demonstrated efficacy in protecting from initiation and/or even promotion of carcinogenesis in various organs, including mammary gland (2), colon (3) and stomach (4). Various cellular modifications induced by SFN have been correlated to its cancer chemopreventive properties. Indeed, SFN was identified in broccoli extracts as the principal inducer of phase II detoxifying enzymes such as quinone reductase (5) or glutathione S-transferases (GST) (6). SFN was also described to inhibit phase I cytochrome p450 involved in multidrug resistance (7, 8), to induce apoptosis and cell cycle modifications in cancer cell lines (9-10), and to possess anti-inflammatory activity (11). Although the primary intracellular target of isothiocyanate is not known, recent studies suggested that Phase II gene inducers could interact with cytosolic proteins and activate the MAPK cascade (12).

Therefore, in this study we used human colon cancer Caco2 cells to investigate the involvement of some proteins regulating early events of cell growth and death (i.e., MAP kinase activity and tyrosine kinase expression) in the induction of cell death by SFN. In previous publications, we showed that 15 µM SFN exerted cytostatic and cytotoxic effects on the colon carcinoma cell line HT29 (13), and that it induced overexpression of class alpha GST (GSTA) in Caco2 cells, similar to the one observed in confluent/differentiated cells (6). Therefore, in this study we compared the effects of SFN with those induced by the typical differentiating and apoptotic agent sodium butyrate (NaBT). Moreover, we examined the status of active ERK, P38 and JNK MAP-kinases using western blotting or specific chemical inhibitions. Our results suggest the co-ordinate modulation of MAPK cascades by SFN is central in the induction of cell death without being involved in the typically described regulation of ARE-dependent phase II detoxifying enzymes (i.e., GSTs). The role of histone acetylation was also examined in sulforaphane-treated colon cancer cells and is discussed.

MATERIALS AND METHODS

Dubelco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and TRIzol reagent were obtained from GibcoBRL (Life Technologies sarl, Cergy-Pontoise, France). SFN was purchased from LKT Laboratories (St Paul, Minessota, USA) and stored in ethanol at -20°C. Antibodies to activated MAP kinases (JNK, ERKs1/2, and P38), PC12 cell extracts for positive controls were from Promega (Charbonnières, France. Anti-phosphotyrosine antibody (4G10) was a gift of Dr B. Payrastre (INSERM U563, Toulouse, France). Anti-acetyl histone H3 (lys 19) and anti–acetyl histone H4 (all sites and lys 8) rabbit polyclonal IgGs and anti-histone H1 mouse monoclonal IgG2a were purchased from Upstate (Lake Placid, NY, USA). Goat anti-rabbit horseradish peroxidase HRP-conjugated secondary antibodies were from Sigma Chemical Co. (Saint Quentin Fallavier, France) and Jackson Laboratories (Bar Harbor, Maine, USA) for histone characterization. Western blot Protran® 85 nitrocellulose was purchased from Schleicher & Schuell (Ecquevilly, France). SDS-PAGE molecular weight markers and protease inhibitor cocktail were from BioRad (Yvry sur Seine, France) and ECL reagents from Amersham (Saclay, France). Kinase-specific inhibitors SB 203580 (SB) and PD 098,059 (PD, a specific inhibitor of MEK1, an upstream kinase in the ERK1/2 signaling pathway), Curcumin (CUR) and sodium butyrate (NaBT) were purchased from Sigma. All other reagents were either from Sigma or from Merck (Nogent-sur-Marne, France).

In typical experiments, exponentially growing Caco2 cells (in DMEM, 1% FCS, 1% non essential amino acids) were treated 0-48 hours with 15 µM of sulforaphane or equivalent amount of ethanol (2µl/ml) at 37°C. Cell layer was either trypsinated for counting, or rapidly rinsed with cold PBS, lysed in Laemly buffer, boiled for 5 min and stored at –80°C. Detached cells were harvested in the culture medium by centrifugation. Samples were analysed for specific protein expression by western blot after SDS-PAGE (12.5 % acrylamide) and transfer to nitro-cellulose membrane and probing using appropriate anti active-MAPKs antibodies according to the manufacturer's instructions (Promega, Madison, WI, USA). Storm Imager scanning for quantification performed using ECL+ reagent (Amersham) followed. Band intensities were adjusted according to alpha-tubulin expression. Apoptotic effect was measured by counting (using a counter Coulter) floating and adherent cells after a 48 hours period of exposure to SFN. Cells exposed to sulforaphane (15 µM) and sodium butyrate NaBT (2 mM) were examined by flow cytometry analysis after

propidium iodide (PI) staining, in order to evaluate the distribution of the cells in the different phases of the cell cycle. In addition, chromatin condensation analysis was performed using double labelling with PI and Hoechst. MAPK inhibition experiments were performed on cells incubated one hour with specific inhibitors of ERK 1-2, P38 and JNK (i.e., 25µM PD, 5µM SB and 5µM CUR, respectively) before exposure to 15µM SFN for 48h. Additional examination of acetylated histone expression was performed by Western blotting using specific antibodies and purified histones (14) as positive controls. Analysis of serum-induced tyrosine phosphorylation was evaluated by western blotting using the 4G10 antiphosphotyrosine antibody on cells starved in serum-free medium for 18 hours before treatment and stimulated by addition of 1% FCS at different time points at 37°C.

RESULTS

Sulforaphane induces cell death and stops proliferation through cell cycle arrest at the G2/M phase in human colon carcinoma Caco2 cells.

Counting of adhesive cells after SFN exposure confirmed inhibition of cell proliferation and occurrence of cells floating in the culture medium expressed cell death in Caco2 cells exposed to SFN (table 1). Figures of apoptotic cells were observed in Hoechst-stained cultures after 48h of treatment (data not shown), which could support occurrence of an apoptotic process in Caco2 cells exposed to SFN. Cell cycle response of Caco2 cells to 15 µM SFN exposure was also examined. In addition, the properties of SFN on cell growth and death were compared to those of the well-known differentiating agent NaBT. As shown by flow cytometry analysis, the percentage of cells in the G2/M phase of the cell cycle was clearly increased as the number of cells in the S-phase decreased after 48h exposure to SFN. Untreated controls exhibited an expected pattern of continuously growing cells (table 2). These observations confirmed previous results on HT29 cells. Unlike SFN, NaBT was found to stop the cell cycle at the G0/G1 phase.

GSTA expression is strongly induced by sulforaphane in exponentially growing Caco2 cells.

In a previous work (6), Western-blot analysis revealed that exponentially-growing Caco2 cells exposed to various doses of SFN exhibited a slight increase (1.4 fold) in the constitutive-expression of class pi GST (GSTP) together with a marked dose-dependent induction of the class alpha (GSTA) by 4.0, 6.0 and 7.8 fold at concentration 5, 10 and 15µM, respectively (fig. 1A). We noticed that cell-confluence and differentiation was accompanied by a similar GSTA induction and that unlike SFN did, significant amount (2mM) of NaBT were not able to induce GSTA (fig. 1B). This seems to avoid a possible relationship between SFN properties and the differentiating process which characterizes this cell model.

Signal transduction enzymes involved in the regulation of cell death are not involved in GSTA induction.

Currently three distinct but parallel MAP kinase cascades (ERK, P38 and JNK) have been identified (12). Accordingly, we exposed exponentially growing Caco2 cells to 15 µM sulforaphane during different

time points and examined some active phosphorylated forms of ERK, JNK, and P38 by Western blot analysis using specific antibodies. As shown in figure 2, 15 µM sulforaphane induced the activity of ERK in Caco2 cells in a time-dependent manner and resulted in a rapid and sustained activation (phosphorylation) of ERK1/2. This activation was evident after a 20 minute-period of incubation and persisted for at least one hour. A small activation of P38 protein kinase occurred 5 minutes after treatment and persisted for at least 120 minutes (fig. 3A). Only a transient activation of the JNK1/2 was observed under SFN exposure (fig. 3B). On another hand, specific inhibition of the different MAPKs examined interfered with the cell-death process induced by SFN (table 3). Indeed, ERK1/2 and p38 inhibitors are found to significantly decrease the number of dead cells in treated cultures. Moreover, these inhibitions have small or no effect on SFN-induced GSTA overexpression (fig 4). It is noteworthy that curcumin caused an induction of GSTA in control cells. However, it had no effect on the GSTA induction by sulforaphane.

The present results suggest that, in human colon cancerous cells, the ERK and P38 pathways might be involved in SFN induced cell death. However, the MAP kinase pathway might not mediate the regulation of GSTA expression by this ITC in this cell model.

Examination of the tyrosine phosphorylation and the histone acetylation status under sulforaphane exposure.

The tyrosine phosphorylation status of total cytosolic proteins assessed by Western blotting using anti-phosphotyrosine 4G10 antibody was found almost unchanged during exposure to SFN (not shown).

Figure 5 shows Western blot analysis of histones isolated from control- and SFN-treated-cells using specific anti acetylated histone H1, H3 and H4 antibodies. Trichostatine A (TSA) which is known to inhibit histone deacetylase, a key enzyme in the modulation of histone acetylation, was used to generate positive controls from Caco2 cells. TSA-treated cells exhibit a marked increase in acetylation of histone H3 and H4 at 8h. After 48h exposure to SFN, a significant decrease in histone acetylation in SFN-treated versus untreated cells is noticed, instead of the hyperacetylation observed with TSA treatment. These results suggest that the initial mechanism by which SFN regulates cell growth and death in colon cancer cells is

distinct from the differentiation process initiated by NaBT.

DISCUSSION

Various data have shown that fruit and vegetable consumption was inversely associated with colorectal cancer risk. Several natural plant microcomponents have been studied as key mediators of the cancer chemopreventive activity associated with vegetal-rich diets. Isothiocyanates have been investigated for use as chemopreventive and potent therapeutic agents (i.e. benzyl-, phenylethyl-, methylthiobutyl- and allyl-isothiocyanates or sulforaphane). Their mechanism of action is poorly known even if it has been studied in various cells. It seems to be mediated mainly by a regulation of the detoxifying enzyme activities and the signaling pathways leading to apoptosis. It was anticipated that sulforaphane would regulate phase II detoxification enzymes through MAP kinase inductions (1). In this study, we investigated several aspects of the mechanism by which sulforaphane induced cell death, and how it could be related with alpha-class GST overexpression in undifferentiated human Caco2 colon cancer cells.

Our data show that sulforaphane induces cell death in Caco2 cells at least in part via ERK1/2 and P38 activation. Involvement of JNK is less clear as inhibition of this enzyme had no significant effect on cell death. On the other hand and in contrast to previous findings, the induction of GSTA by SFN is not clearly found to be mediated by the MAPK kinases family.

Among MAPK modulations examined in this study, ERK1/2 were the most clearly activated. This corroborates the findings by some authors that ERK2 was activated by phase II enzyme inducers (15). The P38 MAPK pathway has often been described as activated by various physiological and environmental stress signals such as cytokines, UV, heat shock, etc. (16). In colorectal cancer, the constitutive activation of P38 was associated with increased proliferative and apoptotic states (17, 18). Moreover, SB 203580 was shown to inhibit serum deprivation-induced apoptosis in the colonic adenocarcinoma cell line LST 174T (18). Some other data suggested that activation of P38 also played a determinant role in intestinal epithelium cell differentiation by enhancing the transduction capacity of the CDX2 homeobox gene product, which broad effect on enterocyte differentiation is well known (19). Since we were not able to detect specific markers previously described by other authors (20) in NaBT-induced differentiation (i.e., alkaline phosphatase -ALP- activity), and since SB 203580 was found powerful in inhibiting cell death in our model, we suggest that P38 more likely plays a role in an SFN-induced cell

death process in Caco-2 cells.

Previous studies on Hela cells have shown that apoptosis induced by some ITC like phenylethyl-ITC, involved clear activation of JNKs (21). Opposite results were obtained with PC3 prostate carcinoma cells (22) and HepG2 or Hepa1C1C7 cells (15). The transient and weak activation of JNK we observe in this study suggests that the level of recruitment of the JNK pathway in apoptosis may vary depending on the chemical nature of the apoptotic agent, and among isothiocyanates, on their structure.

Along the gastrointestinal tract in humans, GSTA isoforms are expressed at high level in the duodenum and small intestine while their expression in stomach and colon are much lower, which may be a factor in the greater susceptibility of stomach and colon to cancer development (23, 24). On another hand, the differentiating process occurring *in vivo* along the crypt-to-villous axis in the intestinal epithelium leads to apoptosis without enhancing GSTA expression (25, 26). It was previously found that the enterocyte-like differentiation induced by cell-confluence in the Caco2 cell line strongly enhances expression of GSTA (6,27). On another hand, we observe a similar strong induction of GSTA in undifferentiated Caco2 cells treated with SFN. Unlike in confluent/differentiated cells or after exposure to 2mM NaBT (28), the differentiation marker enzyme ALP activity could not be measured in subconfluent SFN-treated Caco2 cells. Moreover, NaBT typically induces GSTP (not shown) but not GSTA. In addition, SFN or NaBT stop the cell cycle at different phases (i.e., G2/M and G0/G1, respectively). Although the overexpression of GSTA suggests an intestinal-like differentiating effect of SFN on subconfluent Caco2 cells, our other data much likely show that SFN acts on cell death instead of on the particular cell differentiation process occurring in the Caco2 cell model.

A critical role for tyrosine phosphorylation in signal transduction and induction of apoptosis has been proposed (29). Wether such a mechanism would be involved in SFN properties is not clearly confirmed by our data.

Acetylation of histones seems to be an important mechanism for the regulation of gene transcription (30). There is extensive litterature suggesting that the extent of histone acetylation could influence growth and differentiation. At a first approximation it seems that differentiation of cancer cells is favoured by agents that increase histone acetylation (31). Moreover, it has been demonstrated that transient hyperacetylation of histones in colon cancer cells is sufficient to induce growth regulatory gene expression and produce a cell growth arrest (32). Nevertheless, a prolonged histone hyperacetylation was required

for the induction of differentiation or apoptosis programs. We show here that SFN-related gene modulation, cell cycle arrest or cell death are accompanied with a global slight decrease in histone acetylation. These results are in accordance with a recent study demonstrating that neither SFN nor its glutathione conjugate were effective inhibitors of histone deacetylase *in vitro*. This was not the case for an isothiocyanate from mustard oil (i.e., allyl-isthiocyanate) which has been demonstrated to increase acetylation of histones in mouse erythroleukemia cells (31). As already mentioned above it appears that some class-specific differences exist among isothiocyanates concerning their mechanism of action.

In conclusion, our results clearly show that activation of the MAP kinase pathway by SFN leads to cell death but seems neither involved in the regulation of GSTA expression nor in the differentiation process. Whether SFN could initiate an apoptotic pathway is likely but remains to be supported by further experiment using step-specific signaling enzyme inhibitors. We also show that protein expression modulated by SFN is not likely mediated via hyperacetylation of histones in our model. Further studies are needed to refine our understanding on the signaling pathway activated by SFN in order to support the efficacy and safety of this micronutrient as a potential therapeutic drug.

Table 2. Effect of sulforaphane under specific inhibition of MAPKs in Caco2 cells.

Inhibitors	Percent of apoptotic cells [a]	
	Ethanol	Sulforaphane
No	7.2 ± 3.9	26.2 ± 8
PD 098 059 (ERKs)	6.6 ± 1.0	13.6 ± 7 *
SB 203 580 (p38)	4.2 ± 3.6	10.0 ± 6 **
Curcumin (JNK)	5.6 ± 3.8	18.2 ± 10.4 [NS]

[a] Values are mean ± SD from 5 experiments.
(Student test : *, P<0.01; **, P<0.001; [NS], not significant)

FIGURE LEGEND

Figure 1. Western blot analysis of GSTA and GSTP expression in subconfluent SFN-treated and confluent/differentiated untreated Caco2 cells. **A**) Subconfluent cells were exposed either to ethanol (0), 5, 10 and 15 µM SFN or to 2mM sodium butyrate (NaBT) for 48h. **B**) Untreated cells were cultured for 3, 7 and 16 days before analysis. Each blot was obtained from 12.5% SDS-PAGE where 20µg total cell proteins were loaded per lane. Controls (C) were obtained using differentiated cell extracts. Other experimental conditions are given in the Materials and Methods section. Revelation by ECL and autoradiography. Expression of alpha-tubulin was used to estimate loading homogeneity and relative band intensity.

Figure 2. Western blot analysis of ERK1 and ERK2 MAPKs modulation by sulforaphane in subconfluent Caco2 cells. After a short time (minutes) exposure to sulforaphane (SR) or ethanol (control), cells were lysed and 20µg total proteins per lane was loaded on 12.5% SDS-PAGE. The primary antibody recognizes human active ERKs 1 and 2. Other information is given in the Material and Methods section. Revelation by ECL+ and Imager scanning. Expression of alpha-tubulin was used to estimate loading homogeneity and relative band intensity.

Figure 3. Western blot analysis of P38 and JNK MAPKs modulation by sulforaphane in subconfluent Caco2 cells. A) Active P38 expression B) Active JNK expression. Conditions are the same as in Figure 2 except PC12 cell extracts were used as positive controls. MW : molecular weight markers

Figure 4. Effect of specific inhibition of MAP kinases on GST A expression in exponentially growing Caco2 cells (Western blot analysis). Cells were incubated for 48 h at 37°C with ethanol (Control) or 15µM sulforaphane (SR) together with specific MAPK inhibitors: PD (PD 098,059, ERK inhibitor), SB (SB 203580 P38 inhibitor) and CUR (curcumin JNK inhibitor). Inhibitor concentrations were 25µM, 5µM and 5µM for PD, SB, and CUR, respectively. Cell samples were lysed and 20µg total proteins were loaded per lane on 12.5% SDS-PAGE. Post-confluent cells (16 days of culture) were used as positive controls of GST A expression (Confluent). The primary antibodies specifically recognized human class alpha GST isoforms

A1 or A2. More details are given in the Methods section. Revelation by ECL+ and Phosphorimager scanning. Expression of alpha-tubulin was used to estimate loading homogeneity and relative band intensity.

Figure 5. Histone acetylation during sulforaphane treatment of Caco2 cells (Western blot).

Proteins (5µg) were separated on 15% SDS-PAGE. Cells were cultured for 8 hours and 48 hours in presence (S) or absence (C) of 15µM sulforaphane. Protein loads were homogeneous as controlled by ponceau-red staining of the membrane after blotting (not shown). Antibodies are specific for each acetylated histone isoform mentioned. Other information is given in the Material and Methods section. Immunoreactive bands were revealed by ECL and autoradiography.

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