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Role of PI3K/Akt and MEK/ERK signaling pathways in sulforaphane- and erucin-induced phase II enzymes and MRP2 transcription, G_2/M arrest and cell death in Caco-2 cells

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Abstract

Isothiocyanate sulforaphane is an extensively studied cancer chemopreventive agent in human diet. In this study, the effects of sulforaphane (SFN) and its sulfide analog, erucin (ERN), have been examined on the induction of the phase II enzymes, quinine oxidoreductase (NQO1) and UDP-glucuronosyl transferase (UGT1A1), multidrug transporter (MRP2), cell cycle arrest and cell death in human colon adenocarcinoma Caco-2 cells. Additionally, the roles of PI3K/Akt and MEK/ERK signaling pathways have been assessed in these sulforaphane- and erucin-induced events. Although erucin and sulforaphane have similar IC_{50} values (21 and 23 μM after 72 h treatment), erucin was more effective in the induction of G_2/M accumulation, depletion of mitochondrial potential, induction of cell death and mRNA induction of phase II enzymes and MRP2. Erucin (20 μM) induced the mRNAs of NQO1, UGT1A1 and MRP2 by 11.1-, 11.6- and 6.7-fold, whereas sulforaphane (20 μM) induced 3.3-, 5.3- and 2.2-fold, respectively. Both erucin and sulforaphane induced activation (phosphorylation) of ERK1/2 and Akt kinases but had no effect on JNK and p38 activation. Erucin-induced phase II enzyme transcriptions were decreased by PI3K and MEK1 inhibitors (LY294002 and PD98059), but the decreases in sulforaphane-induced transcription were less marked. Erucin induced a large increase in G_2/M cell number than sulforaphane. The ability of kinase inhibitors to overcome G_2/M block was low with the exception of PD98059 in sulforaphane-treated cells. Both, sulforaphane and erucin at high concentrations induced accumulation of sub-G_1 cells, cell death and dissipation of mitochondrial membrane potential. Taken together, these results demonstrate that PI3K/Akt and MEK/ERK signals are important intracellular mediators in erucin- and sulforaphane-mediated phase II enzyme transcription and cell cycle arrest in Caco-2 cells.

Keywords: Isothiocyanate; Signaling pathway; Cell cycle; Apoptosis; Chemotherapeutics

1. Introduction

Isothiocyanates (ITCs) can inhibit many types of tumor formation in animal models and their consumption is inversely correlated with the risk of human cancers including lung, breast and colon [1–3]. Protective mechanisms of ITCs have been proposed including the induction of phase II detoxification enzymes and inhibition of phase I carcinogen-activating enzymes [4–5]. Recent studies showed that other mechanisms, such as inhibition of cell growth by cell cycle arrest, activation of apoptosis [6–8] and disruption of normal tubulin polymerization may be also involved [9]. Sulforaphane (SFN), a product of hydrolytic conversion of 4-methysulphinylbutyl glucosinolate by myrosinase [10], has been a focus in many studies and recently we have shown that SFN at physiological levels can decrease PhIP-induced DNA adduct formation in human hepatocytes [11]. Induction of phase II enzymes is largely mediated by a Nrf2-antioxidant responsive element (ARE) pathway, whereas different signaling kinase pathways (MAPK, PI3K and PKC) regulate Nrf2 [12]. The intracellular signaling network has

Abbreviations: Akt, protein kinase B; ERKs, extracellular signal-regulated kinases; ITCs, isothiocyanates; JNK, c-Jun N-terminal kinase; MAPKs, mitogen-activated protein kinases; MRP2, multidrug resistance-associated protein 2; NF-κB, nuclear factor-kappa B; NQO1, NADP(H):-quinine oxidoreductase; Nrf2, nuclear factor E2-related factor 2; PBS, phosphate-buffered saline; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; UGTs, UDP-glucuronosyl transferases; TBS, Tris-buffered saline

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to integrate and distribute regulatory information; recently, a class of signaling pathways affecting both the cell cycle and apoptosis has been suggested [13]. PI3K/Akt and MAPK cascades represent the cell cycle–apoptosis link, typical for signals possessing both proliferative and survival characteristics, i.e., apoptosis-inhibiting activity. The PI3K/Akt signaling pathway is required for normal G_2/M transition. Inhibition of PI3K by a specific inhibitor, LY294002, induces G_2 cell cycle arrest that can be alleviated/overcome by activated Akt [14]. During apoptosis, the death domain-containing proteins promote caspase-dependent cleavage of Akt and diminish its cell survival signaling [15]. MAPK plays a central role in transduction of various extracellular signals into the nuclei. The most extensively studied MAPK pathway is the Raf–MEK–ERK cascade, activated in response to upstream receptor tyrosine kinases and/or cytokine receptors [16]. The p38 subgroup is involved in inflammation, cell growth, cell cycle progression and cell death. Both the MEK/ERK and PI3K/Akt pathways are required for the survival of differentiated enterocytes, whereas PI3K is sufficient in the survival of undifferentiated enterocytes [17] and is, moreover, able to compensate for the inhibition of the MEK/ERK pathway.

Several studies have demonstrated that certain ITCs induce cell cycle arrest and apoptosis in several types of cultured human cancer cells [8,18–20]. A number of independent studies have also shown that apoptosis caused by ITCs was associated with sustained JNK activation in various cell types [21,22]. The ERK2 kinase pathway was shown to be involved in the ARE-mediated induction of phase II detoxifying enzymes by tBHQ and SFN [23]. The multidrug transporters, P-glycoprotein (P-gp) and MRPs, confer upon cancer cells the ability to resist lethal doses of certain cytotoxic drugs by actively pumping the drugs out of the cells [24,25]. SFN has been shown to increase MRP2 expression in human and rat primary hepatocytes through a mechanism that is likely to involve reactive oxygen species production [26].

SFN and its sulfide analog, erucin (ERN), are naturally occurring ITCs present in cruciferous vegetables, such as broccoli and rocket (*Eruca sativa*) [27,28]. There are few studies on ERN in comparison to SFN. In a very recent study, ERN (termed as MTBITC) has been shown to arrest cell cycle on Jurkat T-leukemia cells and holds a promise for future development as a chemopreventive agent [29]. ERN has also been identified as an in vivo metabolite of SFN in rat [30]. The human intestinal epithelium is under the continuous influence of a wide range of food components that are able to modulate its function and life cycle; Caco-2 cells display features of small intestinal epithelial cells. In the present study, the biological effects of SFN- and ERN-induced transcription of phase II enzymes, cell cycle arrest, mitochondrial membrane potential, MRP2 mRNA and cell death have been compared in Caco-2 cells and the role of two kinase inhibitors of PI3K/Akt and MEK/ERK signaling pathways have also been investigated.

2. Materials and methods

2.1. Reagents

SFN (1-isothiocyanato-4-methylsulphinylbutane) and ERN (1-isothiocyanato-4-methylthiobutane) were purchased from ICN Biomedicals and LKT Laboratories, respectively. Inhibitors LY294002 and PD98059 were purchased from Calbiochem Corp. Rabbit polyclonal antibodies against ERK1/2 (K-23), phospho-ERK1/2 (Tyr-204), p38 (H-147), phospho-p38 (Tyr-182), JNK1/2/3 (FL), phospho-JNK1 (Thr-183/185), Akt1/2 (H-136), phospho-Akt, actin (H-300) and horseradish peroxidase-conjugated anti-rabbit antibody were obtained from Santa Cruz Biotechnology. Reagents for electrophoresis and Western blotting were obtained from Invitrogen and Amersham Bioscience, respectively. The Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences Pharmingen. Bradford reagent for protein quantification, dimethyl sulfoxide (DMSO), phenylmethylsulphonylfluoride (PMSF), dithiothreitol (DTT), RNA-se A, propidium iodide (PI) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma. JC-1 (5,5′, 6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolylcarbocyanine iodide) was purchased from Molecular Probes.

2.2. Cell culture

The human colon adenocarcinoma cell line Caco-2, obtained from the European Collection of Cell Cultures, was routinely cultured in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin and 50 μg/ml streptomycin under 5% CO_2 in the atmosphere at 37 °C. The cultures were maintained for 4–5 days prior to experimental treatment.

2.3. Treatment with ITCs and inhibitors

Caco-2 cells were treated in 60-mm dishes. When cultures achieved 50–60% of confluence, they were exposed to various concentrations of ITC for 1 or 24 h. When inhibitors were used, LY294002 (10 μM) and PD98059 (25 μM) were added to the medium 1 h prior to the addition of ITCs. As stock solutions of ITCs were dissolved in DMSO, an equal volume of DMSO (final concentration $\leq$0.1%) was added to the control cells.

2.4. Cell survival assay

The effect of ITCs on survival of Caco-2 cells was determined using a MTT assay [31]. Caco-2 cells (5×10^3 per well in 200 μl of medium) were seeded in a 96-well culture plate and left to adhere to the plastic plates to reach 50–60% confluence before being exposed to ITCs. Each dose of ITCs was tested in quadruplicate, and

the cytotoxicity curve was constructed from at least six (5, 10, 20, 40, 80 and 160 μM) different concentrations of ITCs. After 24, 48 and 72 h, the cells were incubated with 50 μl of MTT (1 mg/ml) and left in the dark at 37 °C for an additional 4 h. Thereafter, medium was removed, the formazan crystals dissolved in 200 μl of DMSO and the absorbance measured at 540 and 690 nm in a Microplate reader (Dynatech Lab Inc.). The concentration of drug that inhibited cell survival to 50% (IC_{50}) was determined using Calcusyn software.

2.5. Western blotting for MAPK kinases

After treatment with ITCs, cells were washed twice with ice-cold phosphate-buffered saline (PBS), collected by scraping, resuspended in 100 μl of ice-cold cell lysis buffer containing 1% Nonidet P-40, 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM PMSF, 1 mM sodium vanadate, 1 mM sodium fluoride and 1× protease mixture and incubated on ice for 20 min. The lysates were, thereafter, centrifuged for 10 min at 10,000 × *g* and the supernatants collected. Protein concentrations were measured using a Bradford protein assay kit. Equivalent amounts of protein (20 μg) were mixed with 4× SDS-PAGE sample buffer and DTT reducing agent and subjected to 10% SDS-polyacrylamide gel electrophoresis. The resolved proteins were transferred to nitrocellulose membrane (Bio-Rad) using a semi-dry transfer system. The membranes were blocked with 5% non-fat dry milk in Tris–buffered saline (TBS), pH 7.4, containing 1% Tween 20 for 1 h at room temperature, followed by incubation with 1 μg/ml primary antibodies in TBS overnight at 4 °C. The membranes were washed three times with TBS, incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, washed five times with TBS and the protein of interest visualized with enhanced chemiluminescent (ECL) system (Amersham) according to the manufacturer's instructions. The membranes were then exposed to Kodak film for various times.

2.6. Real-time RT-PCR for phase II enzymes and multidrug transporter

Total RNA from Caco-2 cells was isolated using a GenElute™ Total Mammalian RNA kit (Sigma) as specified in the manufacturer's instructions. The RNA concentration and purity were determined by measurement of the absorbance at 260 and 280 nm. The target mRNA was quantified by real-time RT-PCR (TaqMan®) using an ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems). Forward and reverse primers and the fluorogenic TaqMan® probes were designed using ABI PRISM Primer Express Software. Primer and probe sequences for the assays performed were: UGT1A1 forward primer 5′-GGTGACTGTCCAGGACCTATTGA-3′, reverse primer 5′-TAGTGGATTTTGGTGAAGGCAGTT-3′, probe 5′-ATTACCCTAGGCCCATCATGCCCAATATG-3′; NQO1 forward primer 5′-TGGACCGAGCTGGAAAACC-3′, reverse primer 5′-GCCGTCAGCTATTGTGGATATG-3′, probe 5′-CCTTTACCAGATGCTGACTGGCACTGG-3′; MRP2 forward primer 5′-TTGTGGCCAGCCTGCAA-3′, reverse primer 5′-CCTCTGGCCTATGCTCAGGTT-3′, probe 5′-CACCAGCCTCTGTCACTTCGTGGGA-3′.

The probes were labeled with a 5′ reporter dye FAM (6-carboxyfluorescein) and 3′ quencher dye TAMRA (6-carboxytetramethylrhodamine). RT-PCR reactions were carried out in a 96-well plate in a total volume of 25 μl per well consisting of TaqMan® one-step RT-PCR master mix reagent (Applied Biosystems), 10 ng of total RNA, 100 nM probe, 200 nM forward and reverse primers to amplify UGT1A1, NQO1 and MRP2. A reverse transcription was performed for 30 min at 48 °C, then an AmpliTaq™ gold activation for 10 min at 95 °C, followed by 40 PCR cycles of denaturation at 95 °C for 15 s and finally annealing/extension at 60 °C for 1 min. Reactions were carried out in triplicates. The data were analysed by TaqMan® software using a standard curve method as described in User Bulletin No. 2 (ABI PRISM™ 7700 Sequence Detection System) to quantify the mRNA. Standard curves were constructed for each amplified gene sequence using 1, 5, 10, 20 and 40 ng of total RNA per reaction in triplicates. GAPDH was used as an internal reference gene (forward primer 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse primer 5′-GAAGATGGTGATGGGATTTC-3′, probe 5′-CAAGCTTCCCGTTCTCAGCC-3′).

2.7. Annexin V-FITC staining

Apoptotic cells were quantified using the Annexin V-FITC Apoptosis assay, which measures the phosphatidylserine translocation to the outer side of the plasma membrane in apoptotic cells. Propidium iodide (PI), a standard flow cytometric viability probe that stains only cells with damaged plasma membrane was used to differentiate between apoptotic (Annexin V^+/PI^-) necrotic cells (Annexin V^+/PI^+ double positive). Briefly, both floating and adherent cells were collected. The floating cells were collected by centrifugation at 700 × *g* for 3 min, whereas adherent cells were trypsinized and collected by centrifugation at 700 × *g* for 3 min. Pooled cells were washed twice with cold PBS. Approximately, 5×10^5 cells were resuspended in 100 μl of manufacturer-supplied 1× binding buffer, and mixed with 5 μl of Annexin V-FITC and 5 μl of PI. After 15 min incubation in the dark at room temperature, the cells were analysed using a Coulter Epics Altra flow cytometer.

2.8. Cytofluorimetric analysis of mitochondrial potential

Variations of mitochondrial membrane potential in ITC-treated Caco-2 cells were studied using a JC-1 fluorescent

probe. JC-1 is a mitochondria-selective probe that forms aggregates in normal polarized mitochondria resulting in an orange emission; its monomeric form, present in cells with depolarized mitochondrial membranes, emits only green fluorescence. For analyses of mitochondrial membrane potential, the cells were collected by trypsinization and pooled together with floating cells. Briefly, 5×10^5 cells were incubated in 400 μl of PBS/0.2% BSA containing 4 μM of JC-1 for 30 min at 37 °C. After 30 min incubation in the dark at 37 °C, the cells were analysed using a Coulter Epics Altra flow cytometer.

2.9. Cell cycle analysis

This determination was based on the measurement of the DNA content of nuclei labeled with PI. For flow cytometry analyses of the DNA cell cycle profile, approximately 5×10^5 cells were collected by trypsinization and pooled together with floating cells. The cells were washed twice with cold PBS and resuspended in 0.05% Triton X-100 and 15 μl RNA-se A (10 mg/ml) for 20 min at 37 °C. Then, cells were cooled and incubated on ice for at least 10 min before PI (50 μg/ml) was added. Finally, after 15 min the stained cells were analysed using a Coulter Epics Altra flow cytometer.

2.10. Flow cytometry measurements and data analysis

Coulter Epics Altra flow cytometer was equipped with 488 nm excitation laser and fluorescence emission was measured using a bandpass filter set 525, 575, 610 and 675 nm with respective photomultipliers FL1–FL4 required for fluorochrome used as follows: Annexin V-FITC and PI (FL1, FL2), JC-1 (FL1, FL2, ratio FL2/FL1), cell cycle (log FL2, sub G_1; lin FL3, DNA cell cycle histogram; FL4 peak versus integral for doublets discrimination). Forward/side light scatter characteristic was used to exclude the cell debris from the analysis. For each analysis, 1×10^4 cells were acquired for analysis. Data were analysed with WinMDI software (version 2.7, J. Trotter, Scripps Research Institute, CA, USA). The cell cycle calculations were performed with Multi-cycle Software (Phoenix Flow System).

3. Results

3.1. Effect of ERN and SFN on cell survival

The Caco-2 cell viability after treatment with ERN and SFN (from 24 to 72 h) was determined using MTT assay. The survival of Caco-2 cells was significantly reduced in a dose-dependent manner by 24 h exposure to ERN and SFN (IC_{50} = 72 and 83 μM, respectively). A previous study reported an IC_{50} value for SFN in Caco-2 cells of 55 μM (24 h treatment) [32]. The difference may be due to the percentage of cell confluence or passage numbers. Similar toxic effects of both ITCs on cell viability were observed after 72 h treatment (IC_{50} = 21 μM (ERN) and 23 μM (SFN)).

3.2. Effect of ERN and SFN on activation of kinases

ERN- and SFN-mediated activation of ERK1/2 and Akt kinase were determined by Western blot analyses using specific antibodies (Fig. 1). Exposure of Caco-2 cells to ERN and SFN resulted in a rapid and sustained phosphorylation of ERK1/2. In comparison to control cells, the phosphorylation of ERK1/2 was increased after 1 h exposure to both ITCs. However, the amount of ERK1/2 protein was not affected by ERN or SFN. The expression of JNK1/2/3 and p38 protein was unchanged by ERN and SFN treatment in Caco-2 cells. In contrast to SFN-treated HepG2-C8 cells [33], we did not find JNK and p38 phosphorylation in Caco-2 cells at any concentration of ERN or SFN tested (data not shown). These results indicate that JNK and p38 pathways may not be involved in the ERN- and SFN-induced cell signaling that leads to cell

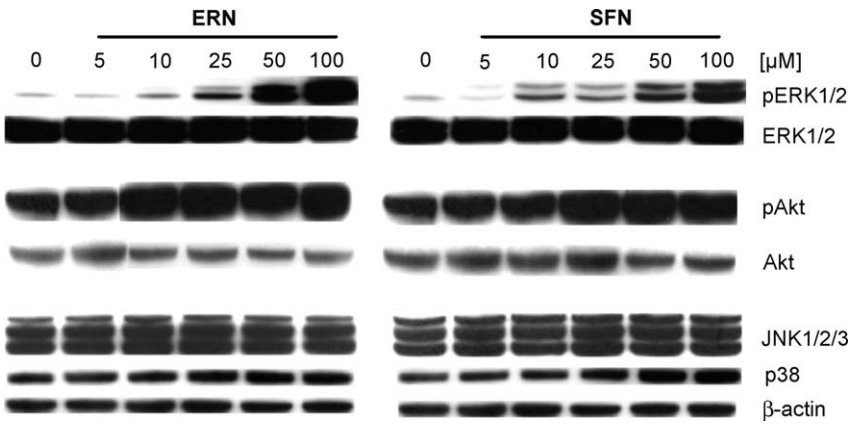

Fig. 1. Effect of ITCs on the activation of ERK1/2, JNK1/2/3, p38 and Akt1/2. The Caco-2 cells were treated with various concentrations (0–100 μM) of ERN and SFN for 1 h. Following treatments, the whole cell lysates were prepared and 20 μg of proteins/lane was applied for Western blotting using specific rabbit antibodies. The same blots were stripped and re-probed with antibody against β-actin.

death. Because of the possibility of cross-talk between Raf/MEK/ERK and PI3K/Akt pathways [34,35], the activation of Akt kinase has been analysed. Akt phosphorylation was increased after 1 h treatment with ERN (10–100 μM) or SFN (25–100 μM); however, the protein level was unchanged (Fig. 1).

3.3. Effect of PD98059 and LY294002 on ITC-induced phase II enzyme transcription

It has been shown that upstream signaling Raf/MEK kinases are involved in SFN-induced ERK2 activation in HepG2 cells [23]. The up-regulation of phase II detoxifying enzymes by ITCs is a well-documented effect [5,22,36]. As shown in Table 1, the induction of NQO1 and UGT1A1 mRNA by ERN (20 μM) was 11.1- and 11.6-fold, respectively, whereas the induction of NQO1 and UGT1A1 by SFN (20 μM) was 3.3- and 5.3-fold. The level of housekeeping gene GAPDH mRNA was unchanged after ERN or SFN treatments. LY294002 alone repressed UGT1A1 and NQO1 mRNA expression in comparison to the control cells, whereas PD98059 alone showed no significant effect on UGT1A1 but suppressed NQO1 expression. Caco-2 cells were pre-treated with either MEK1 inhibitor PD98059 or PI3K inhibitor LY294002 followed by either SFN or ERN treatment. ERN induced up-regulation of UGT1A1 and NQO1 mRNA and both inductions were significantly inhibited by LY294002 and PD98059. We have previously shown that SFN (10 μM) induced up-regulation of UGT1A1, which was also inhibited by pre-treatment of PD98059 [37]. However, there was only a 1/3 reduction of UGT1A1 induced by SFN (20 μM) by pre-treatment of PD98059. NQO1 mRNA induced by SFN was unaffected by LY294002. These results suggest that PI3K/Akt and MEK/ERK pathways may be affected more by ERN than by SFN in the induction of UGT1A1 and NQO1 transcription in Caco-2 cells.

Table 1
Effect of PD98059 and LY294002 on ERN- and SFN-induced UGT1A1 and NQO1 transcription

	Control	ERN	SFN
UGT1A1			
	1.0 ± 0.13	11.6 ± 0.15**	5.3 ± 0.13**
LY294002	0.5 ± 0.23*	7.8 ± 0.05#	5.5 ± 0.16
PD98059	1.3 ± 0.19	8.7 ± 0.23#	3.8 ± 0.15#
NQO1			
	1.0 ± 0.07	11.1 ± 0.11**	3.3 ± 0.15**
LY294002	0.4 ± 0.1*	8.0 ± 0.13#	3.1 ± 0.12
PD98059	0.6 ± 0.04*	6.9 ± 0.05#	3.1 ± 0.18
GAPDH			
	1.0 ± 0.16	1.0 ± 0.11	0.9 ± 0.13
LY294002	1.0 ± 0.10	0.9 ± 0.14	1.0 ± 0.04
PD98059	1.0 ± 0.16	0.9 ± 0.16	1.0 ± 0.08

Caco-2 cells were incubated with PD98059 (25 μM) or LY294002 (10 μM) for 1 h prior to exposure to 20 μM of ERN or SFN for 24 h. The effect of MEK1 inhibitor PD98059 or PI3K inhibitor LY294002 on UGT1A1 and NQO1 mRNA transcription was analysed by real-time RT-PCR. GAPDH was used as an internal reference. Data were normalized against control. Experiments were performed in triplicate; data represent mean ± S.D.

Statistical significant effect of combined ITC and inhibitor in comparison to ITC alone, $p < 0.05$.

* Significant difference from vehicle control, $p < 0.05$.

** Significant difference from vehicle control, $p < 0.01$.

3.4. Effect of PD98059 and LY294002 inhibitors on ITC-induced MRP2 transcription

Previous studies have shown that SFN increased MRP2 expression in human and rat primary hepatocytes [26]. Caco-2 cells were treated with ERN and SFN (5–40 μM) for 24 h. TaqMan® real-time RT-PCR analysis showed that ERN induced MRP2 mRNA in a dose-dependent manner at concentrations up to 20 μM (6.7-fold) (Fig. 2A), whereas SFN was less effective (2.2-fold at 20 μM). MRP2 mRNA expression declined to the control level at 40 μM of ERN and SFN treatment. Pre-treatment of Caco-2 cells with LY294002 inhibitor suppressed the increase in the level of MRP2 mRNA induced by ERN and SFN, whilst PD98059 pre-treatment showed no significant effect (Fig. 2B).

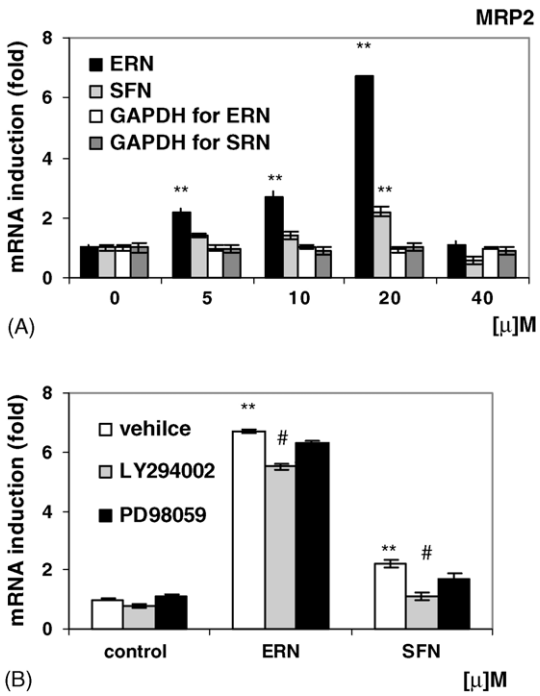

Fig. 2. Effects of ERN and SFN on MRP2 transcription and role of PD98059 and LY294002. (A) The effect of ERN and SFN on mRNA level of MRP2 in Caco-2 cells. Cells were incubated with ERN and SFN for 24 h. DMSO was added to the control cells. GAPDH was used as an internal reference. (B) Inhibition of ERN and SFN induced MRP2 gene expression by PD98059 and LY294002. Caco-2 cells were pre-treated with PD98059 (25 μM) or LY294002 (10 μM) for 1 h, prior to incubation with 20 μM ERN or 20 μM SFN for 24 h. The data presented are average of two experiments, each in triplicate, data represent mean ± S.D. Statistical significant effect of combined ITCs and inhibitors in comparison to ITCs alone, $^{\#}p < 0.05$; significant difference from controls, $^{**}p < 0.01$.

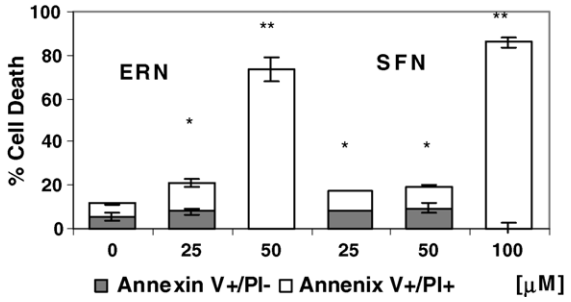

Fig. 3. Effect of ERN and SFN treatment on apoptosis induction in Caco-2 cells. Cells were exposed to either DMSO (control) or different concentration of ERN or SFN for 24 h. Both floating and adherent cells were collected and stained with Annexin V-FITC and PI as described in Section 2. After 15 min incubation, the cells were analysed using Coulter Altra flow cytometer. Percentage of apoptotic (Annexin V^+/PI^-) and necrotic (Annexin V^+/PI^+ double positive) cells are shown. The representative result presented is from three independent experiments. Significant difference from the controls, $^*p < 0.05$; $^{**}p < 0.01$.

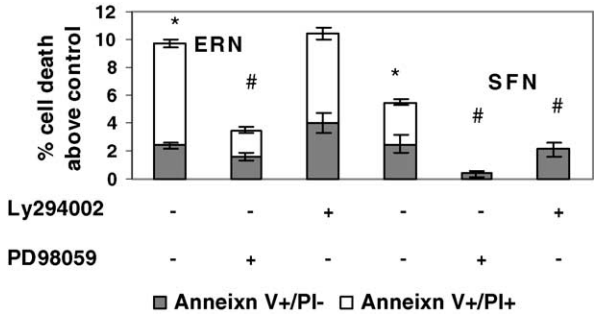

Fig. 4. Effect of PD98059 and LY294002 on ERN- and SFN-induced cell death. Cells were treated with 25 μM ERN or SFN for 24 h with or without PD98059 (25 μM) or LY294002 (10 μM). An equal volume of DMSO was added to the controls. Apoptotic cells were quantified by flow cytometry after staining with Annexin V-FITC and PI. After 15 min incubation, the cells were analysed using a Coulter Epics Altra flow cytometer. The data shown are representative of three independent experiments. Statistical significance of combined ITC and inhibitor in comparison to ITC alone, $^{\#}p < 0.05$; significant difference from controls, $^*p < 0.05$.

3.5. Apoptosis evaluation after ERN and SFN treatment

To determine whether reduced survival of Caco-2 cells in the presence of ITCs was associated with apoptosis induction, the apoptotic cells were quantified using FITC-conjugated Annexin V assay. Fig. 3 depicts the percentage of apoptotic cells and necrotic cells after treatment with ERN and SFN (24 h). Less than 6% of necrotic cells were present in the control sample and these represent later stages of apoptosis and cells undergoing rapid secondary necrosis in culture. In comparison with DMSO-treated control cells, treatment with ERN (25 μM) and SFN (50 μM) showed only a marginal increase of apoptotic (2.5 and 4%, respectively) and also increase of necrotic (7.3 and 4.3%, respectively) cells. Increasing concentration of ERN (50 μM) and SFN (100 μM) resulted in a significant increase in Annexin V^+/PI^+ double-positive cells (73 and 86%, respectively) in comparison with controls. Treatment of cells with 100 μM of ERN destroyed the cell membranes that became completely permeable to PI (data not shown). These results suggest that there is a narrow range of ITC concentrations required for induction of apoptosis and that at high concentration, the necrotic mode of cell death predominates.

3.6. Effect of PD98059 and LY294002 inhibitors on ITC-induced cell death

The possible involvement of PI3K and ERK signaling pathways in ERN- and SFN-induced cell death has been examined. Caco-2 cells were pre-treated with inhibitors and incubated in the presence of 25 μM ERN or SFN for 24 h. Inhibitors alone had no effect on numbers of Annexin V^+/PI^- (apoptotic) and Annexin V^+/PI^+ double-positive (necrotic) cells in comparison to control cells. The PD98059 inhibitor decreased mainly necrotic cell fraction in ERN-treated cells, while LY294002 had no effect on either cell population (Fig. 4). SFN-induced cell death was inhibited by both inhibitors and both, apoptotic and double-positive necrotic cell fractions were decreased. It is, thus, hypothesized that the inhibitors used can influence cell death by suppressing some of the signaling pathways activated by ITCs treatment.

3.7. Effect of ERN and SFN on mitochondrial membrane potential

To delineate the mechanism of the cytotoxicity of ITCs in the Caco-2 colon cell line, the lipophilic cation JC-1 was employed to determine whether ITC induced alterations in mitochondrial membrane potential. The changes in the

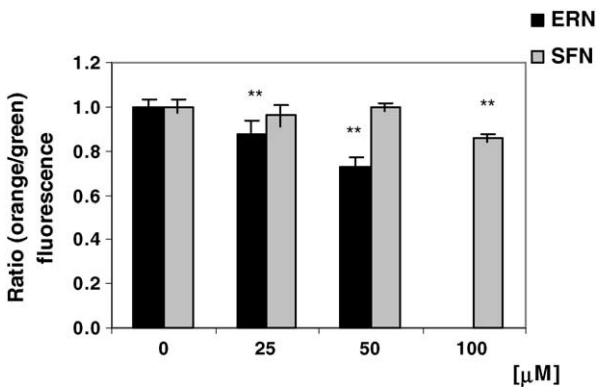

Fig. 5. SFN and ERN induce depolarization of mitochondrial membranes. Variations of the orange/green (FL2/FL1) fluorescence ratio as a function of the ITCs (SFN and ERN) concentration. The green fluorescence refers to the JC-1 monomers and the orange fluorescence corresponds to the formation of J-aggregates. The Caco-2 cells were exposed to either DMSO (control) cells or different concentrations of SFN and ERN (25, 50 and 100 μM) for 24 h and stained with mitochondrial-selective JC-1 dye as described in Section 2. After 30 min incubation, the cells were analysed using a coulter Epics Altra flow cytometer. Data normalized against control, and represent mean ± S.D. Significant difference from controls, $^{**}p < 0.01$.

ratio of orange/green fluorescence, therefore, reflect the variations of ψ_{mt}. The Caco-2 cells were treated with DMSO (control) and with different concentrations of ERN and SFN for 24 h, stained with JC-1 and analysed using flow cytometry. Fig. 5 shows the variations of the orange/green fluorescence ratio as a function of ITC concentration. Whereas the reduction of mitochondrial membrane potential was dose-dependent for ERN treatment, SFN decreased membrane potential only at 100 μM. The decrease of orange/green fluorescence ratio was mainly due to the dose-dependent increase of cells with apoptotic characteristics (lower forward and increased side scatter).

3.8. Effect of ERN and SFN on cell cycle

Effect of ITCs on cell proliferation was evaluated by measuring the distribution of the cells in the different phases of the cell cycle. Untreated control cells were primarily in the G_0/G_1 (56.4%) and in the S phase (25.4%), with a small percentage at G_2/M phase (15.1%). However, following ERN treatment (10 and 25 μM for 24 h), the percentage of cells in the G_2/M phase increased to 53.3 and 32.9% and following SFN treatment (25 and 50 μM), this percentage also increased to 29.3 and 51.7%, respectively (Fig. 6). The G_2/M phase accumulation was accompanied by a corresponding decrease in G_1 phase of the cell cycle. At higher ITC concentrations (50 μM ERN and 100 μM SFN), cell cycle distribution profiles reverted to those of control cells and a significant increase of the sub-G_1 apoptotic cells was observed. To test for the induction of apoptosis, flow cytometry was used to measure the appearance of cells with DNA content below that of G_1 cells, i.e., sub-G_1 cells (Fig. 6). After 24 h treatment with ERN (50 μM) and SFN (100 μM), 38.4 and 29.3% (approximately, 1/2 and 1/3 of Annexin V^+/PI^+ double-positive cells) of sub-G_1 cells were found. Cells in sub-G_1 fractions in control samples and in those treated with lower concentrations of ITCs were less than 5%, in good correlation with Annexin V-FITC staining. The appearance of sub-G_1 cells increased only after cells achieved marked G_2/M block.

3.9. Effect of PD98059 and LY294002 inhibitors on ITC-induced cell cycle arrest

In a separate experiment, both PD98059 and LY294002 inhibitors induced small but significant increases of cells in G_2/M phase. The concentrations used in combination with inhibitors for ERN and SFN were 10 and 25 μM, respectively. LY294002 marginally inhibited the increase in ERN-induced G_2/M block and had no effect on SFN-induced G_2/M population. However, PD98059 abolished the induction of G_2/M cells by SFN to the control level, but only decreased the G_2/M population from 83 to 67% in ERN-treated cells (Fig. 7).

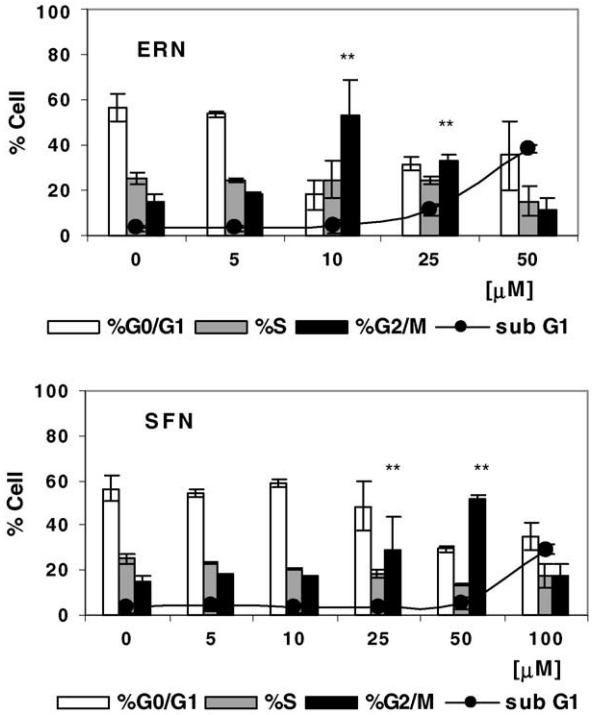

Fig. 6. Effect of ERN and SFN on cell cycle and sub-G_1 fraction generation. The Caco-2 cells were exposed to DMSO (control) or different concentrations of ERN or SFN for 24 h. Both floating and adherent cells were collected, detergent permeabilised and stained with 50 μg/ml concentration of PI in the presence of RNA-se. The distribution of cells in G_0/G_1, S and G_2/M phase was analysed by flow cytometry and Multi-cycle software. Percentage of sub-G_1 fraction was obtained from analysis of SSC-log FL2 dot plot using WinMDI software. Three independent experiments were performed and mean ± S.D. are presented. Significant difference from treated cells in comparison to controls, $^{**}p < 0.01$.

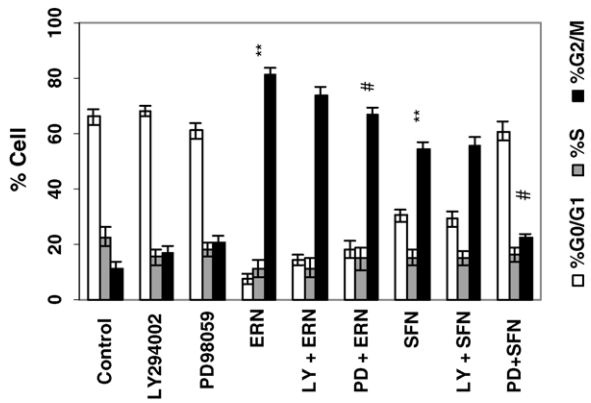

Fig. 7. Effect of PD98059 and LY294002 on ERN- and SFN-induced cell cycle arrest. The cells were treated with ERN (10 μM) or SFN (25 μM) for 24 h either in the absence or presence of PD98059 (25 μM) or LY294002 (10 μM). An equal volume of DMSO was added to the control cells. Distribution of the cells in G_0/G_1, S and G_2/M phase was analysed by flow cytometry. The representative result out of three independent experiments is presented. Statistical significance of combined ITCs and inhibitors in comparison to ITCs alone, $^{\#}p < 0.05$; significant difference from controls, $^{**}p < 0.01$.

4. Discussion

The molecular events affected by chemopreventive phytochemicals include induction of detoxifying enzymes, suppressor gene expression, activation of MAPK cascade, cell cycle regulation and activation of apoptotic machinery [38]. Induction of apoptosis is one of the potential biomarkers of a biologically effective dose of chemopreventive molecules [6]. Effects of ITCs depend on their concentrations and on cell types used. ITCs at low concentrations induce enzyme expression and at high concentrations induce apoptosis [3].

In this study, undifferentiated Caco-2 cells were treated with ITCs within a week of subculture. Undifferentiated Caco-2 cells, at a lower maturation status (as a colon carcinoma model), give a relatively consistent response to ITCs in comparison to differentiated Caco-2 cells (small intestine model). There are reports of significant changes in the mRNA expression profile of transporters and channels upon differentiation of Caco-2 cells and they are more resistant to treatments than undifferentiated Caco-2 cells. Genes expressed during spontaneous differentiation of post-confluent Caco-2 cells include several small-intestine-specific genes involved in nutrient transport/metabolism, indicating that this colonic adenocarcinoma cell line has a hybrid colonocyte/enterocyte phenotype [39].

The apoptotic effect of ERN and SFN on Caco-2 cells has been examined using annexin-V staining and analysis of the sub-G_1 population. Sub-G_1 analysis confirmed DNA fragmentation during ITC treatment, with ERN more effective than SFN. Mitochondria have been suggested as a target of benzyl-ITC (BITC) in experiments with rat liver epithelial cells [40]. In concordance with the increase in sub-G_1 fraction, a profound mitochondrial membrane potential decrease in ERN-treated cells was also found; SFN affected mitochondrial potential only at high concentration (100 μM). The percentage of cells with diminished orange fluorescence correlated to the percentage of sub-G_1 cells.

Antiproliferative effects of the ITCs studied have previously been achieved more easily in leukemia and myeloma cells than in epithelial cancer cell lines. The intestinal epithelium is under the continuous influence of a wide range of food components that can modulate its functions and life cycle. The plasma concentration of isothiocyanates has been reported as 1–2 μM [41], however, epithelial cells of gastrointestinal track will experience both topological exposure of ITCs of relatively high concentrations and followed by systemic exposure to lower concentration. Cells accumulated in both G_0/G_1 and G_2/M phases of cell cycle, depending on the ITC used [20]. Another study has shown SFN-induced cell cycle arrest and apoptosis in colon adenocarcinoma HT-29 cells (24 h after cell seeding) [42]. SFN induced G_0/G_1 arrest in PHA-stimulated human lymphocytes and G_2/M arrest in HT29 cell lines [18,42]. Both, ERN and SFN have been shown here to possess dose-dependent antiproliferative effects on Caco-2 cells. ERN was more effective in inducing G_2/M arrest than SFN. However, SFN-induced G_2/M blockage can be abolished by MEK1 inhibitor PD98059. The G_2/M block is a ''decision'' point where cells begin to lose viability and the observed decrease of G_2/M cells is due to appearance of sub-G_1 apoptotic cells. It is suggested that this necrotic cell death, when cells stop proliferation after treatment, occurs independently of cell cycle phase.

SFN induces phase II detoxification enzymes and antioxidant enzymes, such as GSTs, NQO1, UGTs and thioredoxin reductase (TrxR1) [43,44]. The induction of NQO1 and UGT1A1 mRNA by ERN and SFN has been demonstrated here with ERN being a more potent inducer than SFN. Abolition of SFN-induced GST mRNA up-regulation by LY294002 has recently been reported [37]. In this study, ERN-induced NQO1 and UGT1A1 mRNA expression were also decreased in the presence of LY294002 or PD98059 inhibitors, while SFN-induced up-regulation of UGT1A1 was inhibited by MEK inhibitor PD98059 only. Given that phase II detoxifying enzymes are induced by a variety of compounds, multiple signaling pathways may exist.

Inhibition of p38 by SB203580 inhibitor revealed that the p38 kinase pathway functions as a negative regulator of ARE-induced phase II detoxifying enzymes mediated by an upstream kinase, MKK3, in HepG2 cells. BHA induced p38 substrate ATF2 phosphorylation, while SFN had no effect [22,42]. In the present study, JNK and p38 activation by ITCs was not observed. One study showed that PEITC was involved in ERK-dependent induction of apoptosis in prostate cancer cell lines [19]. The mechanism was proposed to be through sustained JNK activation, even though the upstream kinases, MKK4 and MKK7, and JNK itself were not primary targets of PEITC. Apoptotic signaling was maintained via the proteosomal degradation pathway of JNK-specific dual-specificity phosphatase M3/6 [45]. The physiological role of JNK in the signaling of stress-induced apoptosis is still open to debate; and it has been suggested that JNK is activated primarily for inflammatory cytokine-induced gene expression and is not essential for mitochondria-dependent apoptosis [46]. A very recent study showed that BITC-induced cell cycle arrest and apoptosis in Jurkat cells were mainly through p38 MAPK pathway but not ERK [47]. In contrast, a marked increase of ERK kinase phosphorylation has been found in both ERN- and SFN-treated Caco-2 cells. ERK phosphorylation increased across the whole ITC concentration range tested. These results provoke a question about the role of ERK activation in cell death or cell survival. Taking into consideration the evident necrosis at high ITC concentrations, the gradual increase of *p*-ERK cannot be correlated to either apoptosis or necrosis.

Signaling pathway members Ras/Raf/MEK/ERK represent nodule proteins interacting with other pathways. Recent studies suggested a direct connection at the level

of Raf and Akt [34,35]. In the current study, protein levels of phospho-Akt increased in both ERN- and SFN-treated Caco-2 cells. Although this Akt activation was small in comparison with the ERK activation, PI3K/Akt inhibitor LY294002 influenced SFN-induced changes in cell death. One possibility for resistance to cellular xenobiotic compound attack is an increased expression of ABC protein family members. Cells acquire the ability to resist lethal doses of certain cytotoxic drugs by actively pumping them out of the cells, and thus, reducing their cytotoxicity [24,25]. Overexpression of these proteins results in increased efflux and, therefore, decreased intracellular concentrations of many chemotherapeutic agents. A recent study has demonstrated that SFN markedly increases the expression levels of both MRP2 mRNA and protein in human and rat primary hepatocytes [26]. In agreement with other studies, MRP2 mRNA induction in both ERN- and SFN-treated Caco-2 cells has been demonstrated in this study. Increases of MRP2 mRNA were dose-dependent (5–20 μM). PI3K/Akt inhibitor LY294002 downregulated ITC-induced MRP2 mRNA in Caco-2 cells. The pharmacological blocking of PI3K/Akt regulates Nrf2-dependent gene (UGT1A1, NQO1 and MRP2) expression and is in agreement with a very recent study showing that heme oxygenase-1 expression is regulated by increase of Nrf2 in a PI3K/Akt-dependent manner [48]. Inhibitors alone had no effect on cell death but G_2/M was induced by LY294002 (1.5-fold) and PD98059 (1.9-fold) compared to control cells. This observation is in line with earlier findings that Akt and MEK are required for cell cycle progression [14,49,50]. Despite the low concentrations (25 μM) of ERN and SFN required to induce cell death, the mainly necrotic cell fraction was inhibited by the inhibitors. This may be a consequence of either reduced progression through apoptosis to necrosis in cell culture or elimination of necrotic death if both apoptosis and necrosis occurred in parallel. The former hypothesis is supported by the observation of cell cycle changes during combined treatment of inhibitors and ITC. It is evident that the dynamics of G_2/M block appearance in Caco-2 cells is decreased in the presence of MEK1 inhibitor PD98059, and achievement of checkpoint was delayed. This observation is in accordance with recent findings that the signaling event in prometaphase may be a plausible target of ITCs [8,23].

In conclusion, ERN has more potent bioactivity than SFN in the induction of phase II detoxification enzymes and induction of G_2/M block in Caco-2 cells. Both ERN and SFN induced pronounced ERK and Akt activation, though they had no effect on JNK and p38 activity. Inhibitors of PI3K/Akt and Raf/MEK/ERK pathways decreased ERN-induced phase II enzyme mRNA to a greater extent than those induced by SFN. The ability of inhibitors to overcome G_2/M block was not significant with the exception of MAPK inhibitor PD98059 on SFN treatment. This study indicates that individual ITCs, such as erucin and sulforaphane, may modulate signaling pathways (PI3K and MAPK) differently and these may contribute to the complexity of chemopreventive and therapeutic mechanisms. Building of a knowledge database of molecular mechanisms, including cross-talk of signaling pathways in phase II enzyme expression, cell proliferation and apoptosis, may lead to a better understanding of the role of different ITCs in cancer chemotherapeutics.

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