JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Isothiocyanates on Nuclear Accumulation of NF-κB, Nrf2, and Thioredoxin in Caco-2 Cells

JANA JAKUBÍKOVÁ,[†,‡] JÁN SEDLÁK,[†] JURAJ BOD'O,[†] AND YONGPING BAO*[,‡,§]

Laboratory of Tumor Immunology, Cancer Research Institute, Vlárska 7, Bratislava, Slovak Republic, Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, U.K., and BMRC, School of Medicine, Health Policy and Practice, University of East Anglia, Norwich NR4 7TJ, U.K.

Early effects (only 1 h of exposure) of three isothiocyanates (benzyl, phenylethyl, and sulforaphane) on nuclear accumulation of thioredoxin, APE/Ref-1, and transcription factors NF-κB and Nrf2, as well as production of reactive oxygen species (ROS) and reduced glutathione levels were examined in human adenocarcinoma Caco-2 cells. Nuclear increase of NF-κB, Nrf2, and thioredoxin contents was observed in all isothiocyanate-treated cells, whereas the nuclear Ref-1 and cytoplasmic Keap1 contents were not changed. Sulforaphane was the most potent inducer of Nrf2 nuclear accumulation (10 μM, 1.9-fold) and NF-κB nuclear accumulation at higher concentration (25 μM, 6.3-fold). In contrast, benzyl isothiocyanate induced more thioredoxin nuclear accumulation (10 μM, 2.9-fold), increased production of ROS, and gave the greatest induction of thioredoxin reductase 1 mRNA (10 μM, 10.2-fold), whereas phenylethyl isothiocyanate was more potent in the depletion of reduced glutathione levels. These results show that different individual isothiocyanates may possess some different activities in nuclear accumulation of thioredoxin, NF-κB, Nrf2, and production of ROS.

KEYWORDS: Isothiocyanate; NF-κB; Nrf2; thioredoxin; redox; nuclear translocation

1. INTRODUCTION

Dietary isothiocyanates (ITCs) from cruciferous vegetables are potentially important phytochemicals in cancer chemoprevention. The cellular targets of ITCs include phase I activating and phase II detoxification enzymes, MAPK kinases, histone deacetylase, cdc2 kinase, cdc25 phosphatase, and tubulin polymerization (*1*−*8*). Other activities such as cell cycle checkpoint activation, apoptosis induction, and mitochondrial depolarization were also observed in ITC-treated cells (*1*, *4*, *9*, *10*). These cellular manifestations of ITC effects are the results of interaction with the intracellular signaling network, whose role is to integrate and distribute regulatory information. It is directed from upstream inputs recognition of external stimuli to downstream activation of nuclear effectors, mainly transcription factors (*11*−*13*).

Glutathione (GSH) is the major thiol−disulfide redox buffer of the cell as its millimolar cytosol concentration is far higher than those of Trx and glutaredoxin (*14*). Correlation of the half-cell reduction potential of the GSSG/2GSH couple with markers of biological status such as proliferation, differentiation, and apoptosis was suggested (*15*). Thiol balance may be critical for the regulation of cholesterol homeostasis in intestinal cells influencing the lipid transport throughout the intestinal barrier and enhanced tight junction permeability (*16*). Redox-sensitive thiol groups of proteins such as NF-κB, SP1, or Keap1, a cytosolic repressor of Nrf2, are all involved in the regulation of cellular redox responses (*17*−*19*). Increased expression, nuclear translocation, and enhanced DNA binding of those transcription factors was induced in rat duodenal mucosa during cysteamine-induced duodenal ulceration (*20*). Additionally, protein tyrosine phosphatases are redox regulated and thus can attenuate the phosphorylation signaling pathways (*21*). Thus, PP5 phosphatase regulation of the ASK-1/MKK-4/JNK signaling (*22*) and Trx modulation of ASK-1-induced apoptosis (*23*, *24*) may represent a crosstalk of two redox-dependent signaling pathways.

Trx is one of the principal reducing factors in the nucleus in addition to APE/Ref-1 (*25*). Trx may be responsible for the redox cycling of APE/Ref-1, which has been shown to enhance the DNA-binding activity of AP-1 complex (*26*). Cytoplasmic/nuclear Trx-1 and mitochondrial Trx-2 are important during embryonic development, and embryonic lethality in -/- knockout mouse was documented (*27*, *28*). The redox state and activity of Trx are maintained by thioredoxin reductase (TR1), a selenocysteine-containing flavoprotein, that catalyses the NADPH-dependent reduction of Trx as well as numerous other oxidized cellular proteins (*29*). It has been shown that ITCs are potent inducers for TR1 expression at both transcriptional and translational levels (*30*, *31*).

ITCs activate another redox-sensitive pathway, Keap1−Nrf2, that induces expression of genes containing antioxidant responsive element (ARE) in their promoter regions (*32*). Under normal conditions, Nrf2 exists in an inactive cytoplasm-located

* Corresponding author. Fax: 44 1603 593752. E-mail: y.bao@uea.ac.uk.
† Cancer Research Institute.
‡ Institute of Food Research.
§ University of East Anglia.

10.1021/jf052717h CCC: $33.50
Published on Web 02/09/2006

state bound to the cytoskeleton-associated protein Keap1 (*33*, *34*). Sulforaphane (SFN) can modify Keap1 most readily in the Kelch domain form thionoacyl adducts (*35*), and Nrf2 is therefore released from Keap1 and translocated to the nucleus, resulting in the activation of ARE-regulated genes (*36*). Recently, we have shown that combined treatment of HepG2 cells with sulforaphane and selenium synergistically induced TR1 mRNA expression (*37*). The present study was to determine the short-term effect of three different ITCs, benzyl ITC (BITC, derived from garden cress), phenylethyl ITC (PEITC, derived from watercress), and SFN (from broccoli), on the nuclear contents of some transcriptional factors associated with the redox status in Caco-2 cells. 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.

2. MATERIALS AND METHODS

2.1. Reagents. BITC, PEITC, and SFN (purity 97%) were purchased from Aldrich (Milwaukee, WI) and ICN Biomedicals (Basingstoke, U.K.), respectively. Rabbit polyclonal antibodies against Nrf2 (C-20), NF-κB p65, Ref-1 (C-20), actin (H-300), goat polyclonal antibody against Keap1 (N-19), and mouse monoclonal antibody against thioredoxin (Thio-probe) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies antirabbit IgG-HRP (horseradish peroxidase), antigoat IgG-HRP, and antimouse IgG-HRP were from Santa Cruz Biotechnology (Santa Cruz, CA). Reagents for electrophoresis and Western blotting supplies were obtained from Invitrogen (Paisley, U.K.) and Amersham Bioscience (Little Chalfont, U.K.), respectively. Bradford reagent for protein quantification, dimethyl sulfoxide (DMSO), phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), hydrogen peroxide (H_2O_2), d,L-buthionine (*S*, *R*)-sulfoximine (BSO), and secondary antibody antimouse IgG-FITC were obtained from Sigma (Dorset, U.K.). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) and monochlorobimane (MCB) were purchased from Molecular Probes (Eugene, OR).

2.2. Cell Culture. The human colon adenocarcinoma cell line, Caco-2, was obtained from the European Collection of Cell Culture (Wiltshire, U.K.). Stock cells were routinely cultured in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 100 μg/mL penicillin, and 50 μg/mL streptomycin in 5% CO_2 atmosphere at 37 °C. The cultures were maintained for 4−5 days prior to experimental treatment.

2.3. Treatment with ITCs. Caco-2 cells were seeded in 60 mm dishes and treated when cultures achieved about 50−60% of confluence. Cells were exposed to various concentrations of ITC for 1 and 6 h. Stock solutions of ITCs were originally dissolved in DMSO, and an equal volume of DMSO (final concentration ≤0.1%) was added to the control cells.

2.4. Real-Time RT-PCR. Total RNA from Caco-2 cells was isolated using a GenElute Total Mammalian RNA kit (Sigma, U.K.) according to the manufacturer's instructions. The RNA concentration and the 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, Warrington, U.K.). Forward and reverse primers and the fluorogenic TaqMan probes were designed using the ABI PRISM Primer Express software. Primer and probe sequences for the assays performed were as follows: TR1 forward primer 5′- CCACTG-GTGAAAGACCACGTT-3′, reverse primer 5′-AGGAGAAAAGAT-CATCACTGC TGAT-3′, probe 5′-CAGTATTCTTTGTCACCAGG-GATGCCCA-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 primer, and 300 nM reverse primer to amplify TR1. 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 annealing/extension at 60 °C for 1 min. Reactions were carried out in triplicate. The data were analyzed 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′-GAAGGTGAAG-GTCGGAGTC-3′, reverse primer 5′-GAAGATGGTGATGGGATTTC-3′, probe 5′- CAAGCTTCCCGTTCTCAGCC-3′).

2.5. Preparation of Nuclear and Cytoplasmic Extracts. To obtain cytoplasmic proteins, cells were washed with cold PBS, resuspended in buffer containing 10 mM HEPES pH 7.6, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 50 mM sodium fluoride, 50 mM β-glycerophosphate, 5% glycerol, and 1× protease inhibitor mixture (Roche Molecular Biochemicals) and incubated on ice for 15 min. At the end of incubation, 0.05 volumes of 10% Nonidet P-40 were added. Cells were vortexed and pelletted by centrifugation for 30 s. Supernatants were collected as cytoplasmic extracts. Nuclei from Caco-2 cells were resuspended in buffer containing 20 mM HEPES pH 7.6, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol, and 1× protease inhibitor mixture and extracted on ice for 30 min followed by centrifugation at 12 000*g* for 10 min. The supernatants were collected as nuclear extracts. Protein concentrations of cytoplasmic and nuclear extracts were determined by the Bradford method using reagents from Bio-Rad.

2.6. Western Blot Analysis. Equivalent amounts of protein (20 μg) were mixed with 4× SDS−PAGE sample buffer and DTT reducing agent and then subjected to 10% SDS−polyacrylamide gel electrophoresis. The resolved proteins were transferred to nitrocellulose membranes (Bio-Rad) using a semidry transfer system. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, pH 7.4, containing 1% Tween-20 for 1 h at room temperature, followed by incubation with 1 μg/mL primary antibodies in Tris-buffered saline overnight at 4 °C. The membranes were washed three times with Tris-buffered saline and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Then, the membranes were washed five times with Tris-buffered saline, and the protein of interest was visualized with an enhanced chemiluminescent (ECL) system (Amersham, Arlington Heights, IL) as described by the manufacturer. The membranes were then exposed to Kodak film for various times.

2.7. GSH Assay. The intracellular content of reduced GSH was measured according to ref *38*. Briefly, Caco-2 cells (5×10^3 per well in 200 μL of medium) were seeded in a 96 black well with clear bottom culture plate and left to adhere and reach 50−60% confluence before being exposed to ITCs. BSO treatment (500 μM, 24 h) was used as a positive control. The cells were exposed to test compounds for 1 h, and each dose of ITCs was tested in quadruplicate. At the end of incubation period, Caco-2 cells monolayers were washed twice with PBS and incubated with monochlorobimane (MCB, 40 μM) in the dark for 20 min at room temperature. After washing twice with PBS, the plate was measured at 405/510 nm (excitation/emission) using a Fluoroskan Ascent FL fluorimeter.

2.8. Intracellular ROS Determination. Briefly, the Caco-2 cells at 50−60% of confluency were incubated with H_2DCFDA (1 μM, final concentration) in PBS for 30 min at 37 °C in a 96-well culture plate (*38*, *39*). After washing twice with PBS, the ITCs was added to complete medium and incubated for 1 h, each dose of ITCs tested in quadruplicate. At the end of incubation period, the cells were washed twice with PBS and fluorescence at 530 nm (excitation at 485 nm) was measured using a Fluoroskan Ascent FL fluorimeter.

3. RESULTS

3.1. ITCs Induce TR1 mRNA. Caco-2 cells were treated with various concentrations (1, 2.5, 5, 10, and 25 μM) of BITC, PEITC, and SFN. Real-time RT-PCR indicated that the three ITCs up-regulated TR1 mRNA levels in a dose-dependent manner. As shown in **Figure 1**, the TR1 mRNA level in Caco-2 cells exposed to 10 μM BITC for 6 h was increased 10.2-fold.

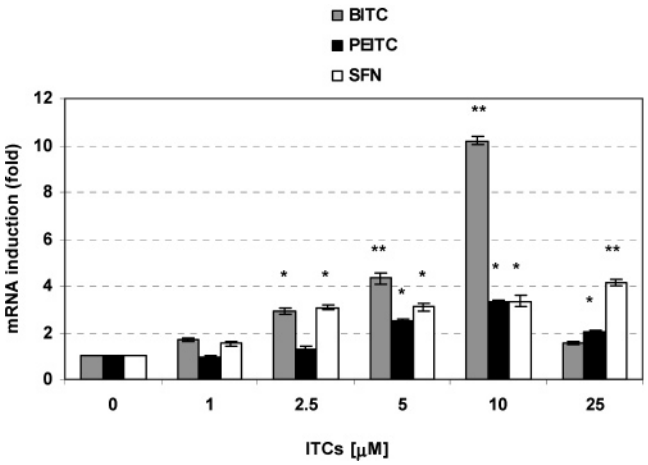

Figure 1. Concentration-dependent effect of ITCs on TR1 mRNA transcription. The Caco-2 cells were incubated with various concentrations (1, 2.5, 5, 10, and 25 μM) of ITCs for 6 h. An equal volume of DMSO was added to the controls. The effect of BITC, PEITC, and SFN on TR1 mRNA was analyzed by real-time PCR. GAPDH was used as an internal reference (data not shown). The data were normalized against the control. Experiments were performed in six replicates; data represent the mean ± SD. Statistical significance from the control was analyzed using the Student *t*-test; ∗ $p < 0.05$, ∗∗ $p < 0.01$.

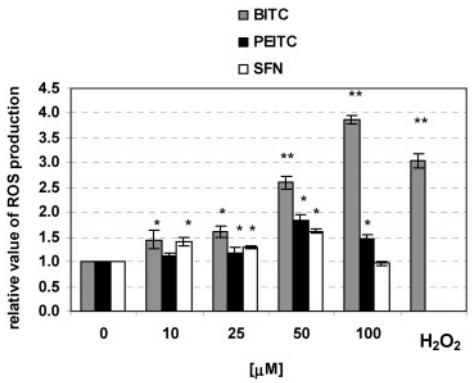

Figure 2. Effect of ITCs on the intracellular ROS production. The cells were preincubated with H_2DCFDA (100 μM) for 30 min and then treated with different concentrations (10, 25, 50, and 100 μM) of ITCs. After 1 h, the cells were washed twice with PBS and resuspended in PBS. The cells incubated with 100 μM H_2O_2 for 1 h were used as a positive control. The results shown are the average of eight replicates. The data were normalized against the control and represent the mean ± SD. Significant difference compared with controls was determined using the Student *t*-test; ∗ $p < 0.05$, ∗∗ $p < 0.01$.

The same concentrations of PEITC and SFN increased the TR1 mRNA level 3.3- and 3.4-fold, respectively. These results showed that BITC is a more potent inducer than SFN and PEITC for TR1 mRNA in Caco-2 cells.

3.2. ITCs Induce ROS Production. To assess the effect of ITCs on redox changes, the intracellular level of ROS was determined using H_2DCFDA as an intracellular fluorescence probe. Nonspecific cellular esterases cleave H_2DCFDA into dichlorofluorescein (DCF) that becomes fluorescent after oxidation with ROS. The marked fluorescent signal was generated in BITC-treated cells within the whole range of concentrations (10−100 μM) (**Figure 2**). The ROS production in PEITC- and SFN-treated cells peaked at 50 μM and remained increased above background in 100 μM PEITC-treated cells, while no ROS production was found at 100 μM of SFN.

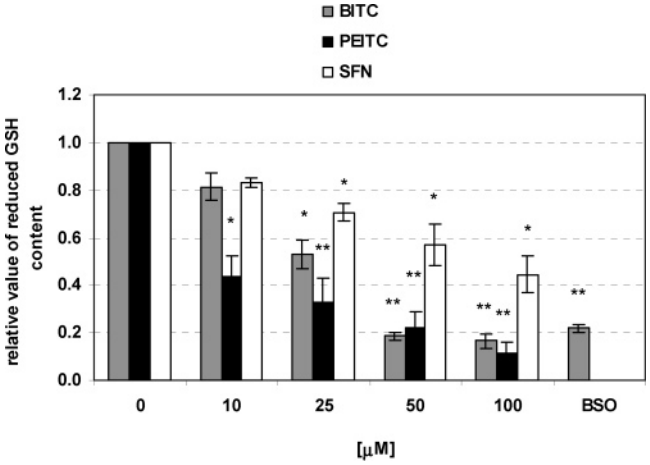

Figure 3. Effect of ITCs on the intracellular level of GSH. Caco-2 cells were treated with various concentrations (10, 25, 50, and 100 μM) of BITC, PEITC, and SFN for 1 h. An equal volume of DMSO was added to the controls. In addition, the cells incubated with 500 μM BSO for 24 h were used as a positive control. The intracellular reduced GSH content was measured as described in the Materials and Methods. The results shown are the average of eight replicates. The data were normalized against the control and represent the mean ± SD. Significant difference from controls was determined using the Student *t*-test; ∗ $p < 0.05$, ∗∗ $p < 0.01$.

3.3. ITCs Decrease Reduced GSH Content. The redox state of the cells can be altered due to a depletion of reduced GSH, the important antioxidant defense mechanism in the cell. MCB is a versatile, membrane-permeable nonfluorescent probe that binds irreversibly to sulfhydryl groups with high reactivity to reduced GSH yielding a fluorescent product (*40*). Significantly decreased GSH levels at all ITC concentrations tested were observed (**Figure 3**). The most profound GSH decrease was achieved in PEITC-treated cells, followed by BITC-treated cells, while SFN was the least potent in the depletion of GSH. BSO at 500 μM decreased GSH by 80%.

3.4. ITCs Induce Nrf2 Nuclear Translocation. ITCs can modulate the Nrf2−Keap1−ARE signaling pathway (*41*). Since Nrf2 translocation is a one of the key events required in this regulation, it is important to determine the dose response of ITCs on this translocation. Band intensities of Nrf2 in cytoplasmic lysate were very faint (data not shown), and there were no significant changes of Keap1 protein in the cytoplasmic fraction (**Figure 4**). The amounts of nuclear-localized Nrf2 protein increased after ITC treatment for 1 h in Caco-2 cells. At the 10 μM level, only SFN induced significant Nrf2 translocation in comparison to BITC and PEITC. Higher concentrations of all ITC treatments were more efficient, and unequivocal increases of nuclear Nrf2 content were observed. This is in accordance with the hypothesis that attenuated cytoplasmic sequestration of Nrf2 by Keap1 is essential for Nrf2 nuclear translocation.

3.5. ITCs Induce Trx and NF-κB Nuclear Translocation. Contradictory data on NF-κB activation in ITC-treated cells and its possible connection with the cell redox status motivated us to analyze the effect of BITC, PEITC, and SFN on NF-κB and Trx translocation in Caco-2 cells. Treatment for 1 h with 10 μM ITCs induced a marginal increase of nuclear NF-κB content, but a more significant increase was observed following higher treatment concentrations (**Figure 5**). SFN was the most potent inducer of NF-κB translocation at 25 and 50 μM. Both BITC (10 μM) and PEITC (25 μM) induced approximately 3-fold increases of nuclear Trx content. The concentration dependence

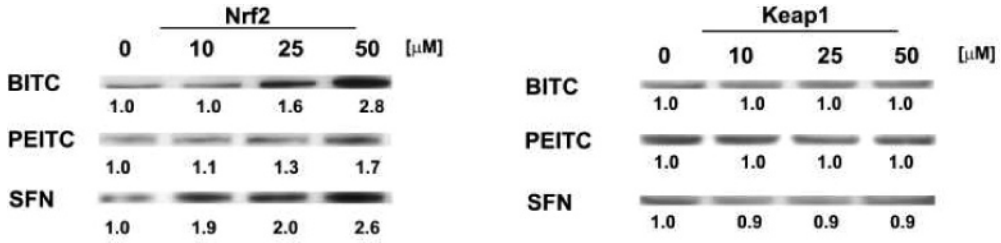

Figure 4. Effect of ITCs on the Keap1/Nrf2 complex. Caco-2 cells were treated with various concentrations (10, 25, and 50 μM) of ITCs for 1 h. After cell washing in PBS, cytoplasmic and nuclear fractions were prepared as described in the Materials and Methods, separated by SDS–PAGE, blotted, and probed with specific antibodies. The nuclear fraction was probed with rabbit polyclonal anti-Nrf2 antibody, and the amount of Keap1 in the cytoplasmic fraction was detected using goat polyclonal antibody. The protein was quantified by Bradford methods, and the staining of membranes was checked for the quality and efficiency of blotting (data not shown).

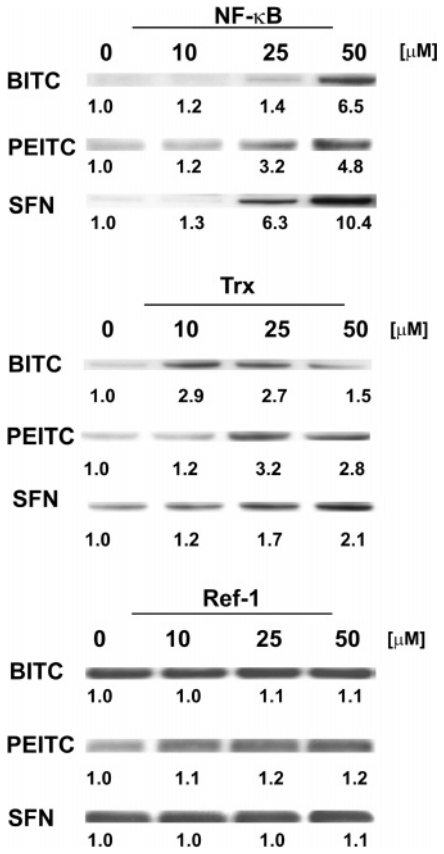

Figure 5. Effect of ITCs on the nuclear amount of NF-κB, Trx, and Ref-1. Caco-2 cells were treated with various concentrations (10, 25, and 50 μM) of BITC, PEITC, and SFN for 1 h. The nuclear fraction was prepared, electrophoresed, and transferred on nitrocellulose membrane as described in the Materials and Methods. Rabbit polyclonal antibodies for NF-κB and Ref-1 and mouse monoclonal antibody for Trx detection were used. The protein was quantified by Bradford methods, and the staining of membranes was checked for the quality and efficiency of blotting (data not shown).

of the Trx increase does not parallel the NF-κB profile. Nuclear amounts of Trx peaked at different concentrations of ITCs which are under subtoxicity levels (IC_{50} for BITC, PEITC, and SFN are 4, 9, and 23 μM after 72 h of treatment) (*42*). It is known that Trx-dependent Ref-1 activity is required for redox-sensitive DNA binding of NF-κB or AP-1. Ref-1 nuclear content was found to not change under 10−50 μM ITC treatments (**Figure 5**); this may indicate that Ref-1 might not be a direct target of ITCs in Caco-2 cells. It can be concluded that 1 h ITC treatment induced significant nuclear accumulation of NF-κB and Trx, with the highest dynamic change in the former.

4. DISCUSSION

Redox homeostasis in cells has to maintain a balanced redox potential using the major intracellular thiol buffering system, GSH and Trx (*28*). Conjugation of ITCs with GSH either spontaneously or mediated by activity of glutathione transferases leads to rapid decrease of reduced GSH concentration and subsequent intracellular redox stress (*14*, *43*). In addition, production of ROS was described in ITC-treated cells strengthening the shift to the more oxidative state (*44*). In contrast, ITCs inhibited TPA-induced superoxide generation in differentiated HL-60 cells (*45*), peritoneal macrophages (*46*), and granulocytes (*47*).

In the present study, BITC, PEITC, and SFN stimulated production of ROS in the short-term (1 h) treatment of Caco-2 cells. In parallel, a marked decrease of reduced GSH content was observed. BITC was the more effective inducer of ROS in comparison to PEITC but decreased cellular contents of reduced GSH less efficiently. These findings indicate that the actions of BITC and PEITC differ in the early induced intracellular events. The concentration of reduced GSH, measured using a monochlorobimane fluoromentric method (*40*), decreased to 20% after 1 h of treatment with both 100 μM BITC and PEITC. This finding is in agreement with recently published results measured using an enzymic assay (*48*). In contrast, however, we found a significant difference between BITC and PEITC in the extent of GSH decrease in Caco-2 cells at lower concentrations. This may be a cell-dependent effect as they presented concentration-dependent decrease of GSH using PANC-1 cells. SFN was less potent in induction of ROS and in the depletion of GSH. Moreover, SFN-induced ROS production peaked at 50 μM and decreased at higher concentrations. Higher concentration of ITCs induced both apoptosis and necrosis in Caco-2 cells; IC_{50} for SFN, BITC, and PEITC were 83, 46, and 40 μM, respectively (*9*, *49*). Thus, ITC treatment caused cellular redox changes in Caco-2 cells. Recently, the up-regulation of TR1 by SFN and selenium in hepatoma cells has been reported (*37*, *50*). This study extends those findings and also confirms the increase of TR1 mRNA in ITCs-treated Caco-2 cells. The effectiveness is similar to ROS production (BITC > PEITC > SFN), while up-regulation of TR1 by BITC and PEITC peaked at 10 μM and decreased at 25 μM. The highest concentration of SFN induced a 4-fold TR1 mRNA increase that represents the highest induction within the whole concentration range of SFN. SFN is also a direct inducer for Trx expression (data not shown). The ITC concentrations that give maximum potency of Trx nuclear translocation confer the effectiveness ranking BITC > PEITC > SFN. Similar nuclear localization of Trx during oxidative stress in ionizing radiation treated cells was observed (*26*). It has been proposed that Trx is reduced by TR and NADPH prior to translocation to the nucleus. Reduced Trx

but not oxidized Trx inhibits PTEN activity inducing an increase of Akt/PKB phosphorylation (*51*) and inhibits both ASK and p38 kinase activation (*52*−*54*). Activation of Akt and ERKs was induced by GSH-depleting agent DEM (*55*). An analogous GSH decrease and comparable ERK and Akt kinases activation in ITC-treated Caco-2 cells were observed (*49*, *56*). Interestingly, activation of ARE was signaled by ERK and JNK pathways modulated by the H_2O_2-dependent Ras pathway that were distinct from global oxidative stress (*39*). In addition to Trx translocation, Nrf2 nuclear accumulation was induced in ITC-treated Caco-2 cells. The half-life of Nrf2 is about 0.5 h, and marginal immunoblotting signals in cytoplasmic fraction of Hepa cells were found (*57*). In this study, only weak signals of cytoplasmic Nrf2 band in Caco-2 cells were obtained. Phosphorylation of Nrf2 is required for its release from Keap1, but phosphorylation is insufficient for nuclear accumulation (*58*). JNK and Akt were shown to be upstream activators of Nrf2 in induced expression of ARE-containing genes (*59*, *60*). The ARE core sequence contains an embedded sequence that shows similarity with the AP-1 binding site (*61*). Nrf2 can bind and activate ARE by complexing with AP-1-related proteins such as c-Jun or c-Fos (*62*). Our results showed an increase of Nrf2 nuclear content in ITC-treated Caco-2 cells, together with up-regulation of TR1, that was shown to regulate AP-1 activity (*26*).

Activation of NF-κB through degradation of IκB is required for induced transcription of certain proinflammatory genes. Recently, BITC was found to inhibit NF-κB activation by suppression of IκB degradation and by reduced nuclear translocation of NF-κB (*63*, *64*). Conversely, it has been shown that SFN reduced DNA binding of NF-κB without interfering with either LPS-induced nuclear translocation or induced degradation of IκB (*65*). Using Caco-2 cells, we have observed an increase of nuclear NF-κB content at higher concentrations of ITC after 1 h of treatment. This is in accord with our previous findings that NF-κB P65 was increased after 8 h of treatment with SFN and a suppression was observed after 24−72 h of treatment (*66*). Interestingly, SFN, the least cytotoxic ITC in the present study, was the most potent inducer of NF-κB nuclear localization after 1 h of treatment. One could speculate that in the case of 50 μM BITC treatment there was a lack of available nuclear Trx required for Ref-1-mediated activation of NF-κB binding to DNA (**Figure 5**). However, Trx translocation into the nucleus may be an argument for the DNA-binding activity of transcriptional factors including NF-κB (*67*). It was shown that free radical scavengers failed to prevent PEITC-induced redox stress accompanied by a burst of superoxide anion in HepG2 cells (*68*). It has been speculated that PEITC-mediated apoptosis and cell cycle arrest and apoptosis induction were not related to ROS production (*68*). In addition, the genetic background of cells may modulate the crosstalk of signaling pathways and the final cellular response to the altered endogenous oxidant state (*69*).

This study suggests that all three ITCs in short-term exposure induced significant production of ROS and decreased reduced GSH level in Caco-2 cells. In addition, up-regulation of TR1 mRNA and increased nuclear content of Trx, NF-κB, and Nrf2 were observed. GSH and ROS changes as well as NF-κB and Nrf2 changes were ITC concentration-dependent. Whether nuclear content differences of these proteins or covalent modification of redox-sensitive proteins can account for treatment effectiveness of ITCs is under further study.

ABBREVIATIONS USED

ITCs, isothiocyanates; BITC, benzyl ITC; PEITC, phenylethyl ITC; SFN, sulforaphane; NF-κB, nuclear factor-kappa B; Nrf2, nuclear factor E2-related factor 2; Trx, thioredoxin; TR1, thioredoxin reductase 1; Keap1, Kelch-like ECH associating protein 1; APE/Ref-1, apurinic/apyrimidinic endonuclease-1/redox factor-1; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate.

ACKNOWLEDGMENT

This work was supported in part by an EC Marie Curie Traning Site Fellowship (QLKF-1999-50510) to JJ, Slovakia Governmental Research and Development sub-programme (Food quality and safety, No. 2003SP270280E010280E01) and Slovak Grant Agency VEGA (No. 2/2094). We appreciate Ms. Margita Šuliková, Mr. Jim Bacon and Mr. Geoff Plumb for technical assistance.

LITERATURE CITED

(1) Hu, R.; Kim, B. R.; Chen, C.; Hebbar, V.; Kong, A. N. The roles of JNK and apoptotic signaling pathways in PEITC-mediated responses in human HT-29 colon adenocarcinoma cells. *Carcinogenesis* **2003**, *24*, 1361−1367.

(2) Jackson, S. J.; Singletary, K. W. Sulforaphane: a naturally occurring mammary carcinoma mitotic inhibitor, which disrupts tubulin polymerization. *Carcinogenesis* **2004**, *25*, 219−227.

(3) Pullar, J. M.; Thomson, S. J.; King, M. J.; Turnbull, C. I.; Midwinter, R. G.; Hampton, M. B. The chemopreventive agent phenethyl isothiocyanate sensitizes cells to Fas-mediated apoptosis. *Carcinogenesis* **2004**, *25*, 765−772.

(4) Singh, S. V.; Herman-Antosiewicz, A.; Singh, A. V.; Lew, K. L.; Srivastava, S. K.; Kamath, R.; Brown, K. D.; Zhang, L.; Baskaran, R. Sulforaphane-induced G2/M phase cell cycle arrest involves checkpoint kinase 2 mediated phosphorylation of Cdc25C. *J. Biol. Chem.* **2004**, *279*, 25813−25822.

(5) Bao, Y. P. Bioactivities of dietary isothiocyanates. *Agro—Food—Ind. Hi—Tech.* **2005**, *16*, 24−26.

(6) Xiao, D.; Srivastava, S. K.; Lew, K. L.; Zeng, Y.; Hershberger, P.; Johnson, C. S.; Trump, D. L.; Singh, S. V. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G2/M arrest and inducing apoptosis. *Carcinogenesis* **2003**, *24*, 891−897.

(7) Yu, R.; Lei, W.; Mandlekar, S.; Weber, M. J.; Der, C. J.; Wu, J.; Kong, A. T. Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. *J. Biol. Chem.* **1999**, *274*, 27545−27552.

(8) Myzak, M. C.; Karplus, P. A.; Chung, F. L.; Dashwood, R. H. A novel mechanism of chemoprotection by sulforaphane: Inhibition of histone deacetylase. *Cancer Res.* **2004**, *64*, 5767−5774.

(9) Jakubikova, J.; Bao, Y.; Sedlak, J. Isothiocyanates induce cell cycle arrest, apoptosis and mitochondrial potential depolarization in HL-60 and multidrug-resistant cell lines. *Anticancer Res.* **2005**, *25*, 3375−3386.

(10) Rose, P.; Armstrong, J. S.; Chua, Y. L.; Ong, C. N.; Whiteman, M. β-Phenylethyl isothiocyanate mediated apoptosis; contribution of Bax and the mitochondrial death pathway. *Int. J. Biochem. Cell Biol.* **2005**, *37*, 100−119.

(11) Avruch, J.; Khokhlatchev, A.; Kyriakis, J. M.; Luo, Z.; Tzivion, G.; Vavvas, D.; Zhang, X. F. Ras activation of the Raf kinase: tyrosine kinase recruitment of the MAP kinase cascade. *Recent Prog. Horm. Res.* **2001**, *56*, 127−155.

(12) Bode, A. M.; Dong, Z. Targeting signal transduction pathways by chemopreventive agents. *Mutat. Res.* **2004**, *555*, 33−51.

(13) Keum, Y. S.; Jeong, W. S.; Kong A. N. Chemoprevention by isothiocyanates and their underlying molecular signaling mechanisms. *Mutat. Res.* **2004**, *555*, 191−202.

(14) Zhang, Y. Role of glutathione in the accumulation of anticarcinogenic isothiocyanates and their glutathione conjugates by murine hepatoma cells. *Carcinogenesis* **2000**, *21*, 1175−1182.

(15) Schafer, F. Q.; Buettner, G. R. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biol. Med.* **2001**, *30*, 1191−1212.

(16) Napolitano, M.; Rainaldi, G.; Bravo, E.; Rivabene, R. Influence of thiol balance on micellar cholesterol handling by polarized Caco-2 intestinal cells. *FEBS Lett.* **2003**, *551*, 165−170.

(17) Meyskens, F. L., Jr.; Buckmeier, J. A.; McNulty, S. E.; Tohidian, N. B. Activation of nuclear factor-κ B in human metastatic melanomacells and the effect of oxidative stress. *Clin. Cancer Res.* **1999**, *5*, 1197−1202.

(18) Hansen, J. M.; Watson, W. H.; Jones, D. P. Compartmentation of nrf-2 redox control: regulation of cytoplasmic activation by glutathione and DNA binding by thioredoxin-1. *Toxicol. Sci.* **2004**, *82*, 308−317.

(19) Nguyen, T.; Sherratt, P. J.; Nioi, P.; Yang, C. S.; Pickett, C. B. Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1. *J. Biol. Chem.* **2005**. *280*, 32485−32492.

(20) Khomenko, T.; Deng, X.; Jadus, M. R.; Szabo, S. Effect of cysteamine on redox-sensitive thiol-containing proteins in the duodenal mucosa. *Biochem. Biophys. Res. Commun.* **2003**, *309*, 910−916.

(21) Chiarugi, P.; Cirri, P. Redox regulation of protein tyrosine phosphatases during receptor tyrosine kinase signal transduction. *Trends Biochem. Sci.* **2003**, *28*, 509−514.

(22) Zhou, G.; Golden, T.; Aragon, I. V.; Honkanen, R. E. Ser/Thr protein phosphatase 5 inactivates hypoxia-induced activation of an apoptosis signal-regulating kinase 1/MKK-4/JNK signaling cascade. *J. Biol. Chem.* **2004**, *279*, 46595−46605.

(23) Hodges, N. J.; Smart, D.; Lee, A. J.; Lewis, N. A.; Chipman, J. K. Activation of c-Jun N-terminal kinase in A549 lung carcinoma cells by sodium dichromate: role of dissociation of apoptosis signal regulating kinase-1 from its physiological inhibitor thioredoxin. *Toxicology* **2004**, *197*, 101−112.

(24) Ueda, S.; Masutani, H.; Nakamura, H.; Tanaka, T.; Ueno, M.; Yodoi, J. Redox control of cell death. *Antioxid. Redox Signaling* **2002**, *4*, 405−414.

(25) Arner, E. S.; Holmgren, A. Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.* **2000**, *267*, 6102−6109.

(26) Karimpour, S.; Lou, J.; Lin, L. L.; Rene, L. M.; Lagunas, L.; Ma, X.; Karra, S.; Bradbury, C. M.; Markovina, S.; Goswami, P. C.; Spitz, D. R.; Hirota, K.; Kalvakolanu, D. V.; Yodoi, J.; Gius, D. Thioredoxin reductase regulates AP-1 activity as well as thioredoxin nuclear localization via active cysteines in response to ionizing radiation. *Oncogene* **2002**, *21*, 6317−6327.

(27) Guo, Y.; Einhorn, L.; Kelley, M.; Hirota, K.; Yodoi, J.; Reinbold, R.; Scholer, H.; Ramsey, H.; Hromas, R. Redox regulation of the embryonic stem cell transcription factor oct-4 by thioredoxin. *Stem Cells* **2004**, *22*, 259−264.

(28) Watson, W. H.; Yang, X.; Choi, Y. E.; Jones, D. P.; Kehrer, J. P. Thioredoxin and its role in toxicology. *Toxicol. Sci.* **2004**, *78*, 3−14.

(29) Becker, K.; Gromer, S.; Schirmer, R. H.; Muller, S. Thioredoxin reductase as a pathophysiological factor and drug target. *Eur. J. Biochem.* **2000**, *267*, 6118−6125.

(30) Wang, W.; Wang, S.; Howie, A. F.; Beckett, G. J.; Mithen, R.; Bao, Y.; Sulforaphane, erucin, and iberin up-regulate thioredoxin reductase 1 expression in human MCF-7 cells. *J. Agric. Food Chem.* **2005**, *53*, 1417−1421.

(31) Hintze, K. J.; Wald, K.; Finley, J. W.; Phytochemicals in broccoli transcriptionally induce thioredoxin reductase. *J. Agric. Food Chem.* **2005**, *53*, 5535−5540.

(32) Zhang, D. D.; Hannink, M. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol. Cell. Biol.* **2003**, *23*, 8137−8151.

(33) Itoh, K.; Wakabayashi, N.; Katoh, Y.; Ishii, T.; Igarashi, K.; Engel, J. D.; Yamamoto, M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* **1999**, *13*, 76−86.

(34) Kobayashi, M.; Itoh, K.; Suzuki, T.; Osanai, H.; Nishikawa, K.; Katoh, Y.; Takagi, Y.; Yamamoto, M. Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. *Genes Cells* **2002**, *7*, 807−820.

(35) Hong, F.; Freeman, M. L.; Liebler, D. C. Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. *Chem. Res. Toxicol.* **2005**, *18*, 1917−1926.

(36) Katoh, Y.; Iida, K.; Kang, M. I.; Kobayashi, A.; Mizukami, M.; Tong, K. I.; McMahon, M.; Hayes, J. D.; Itoh, K.; Yamamoto, M. Evolutionary conserved N-terminal domain of Nrf2 is essential for the Keap1-mediated degradation of the protein by proteasome. *Arch. Biochem. Biophys.* **2005**, *433*, 342−350.

(37) Zhang, J.; Svehlikova, V.; Bao, Y.; Howie, A. F.; Beckett, G. J.; Williamson, G. Synergy between sulforaphane and selenium in the induction of thioredoxin reductase 1 requires both transcriptional and translational modulation. *Carcinogenesis* **2003**, *24*, 497−503.

(38) Troyano, A.; Fernandez, C.; Sancho, P.; de Blas, E.; Aller, P. Effect of glutathione depletion on antitumor drug toxicity (apoptosis and necrosis) in U-937 human promonocytic cells. The role of intracellular oxidation. *J. Biol. Chem.* **2001**, *276*, 47107−47115.

(39) Go, Y. M.; Gipp, J. J.; Mulcahy, R. T.; Jones, D. P. H_2O_2-dependent activation of GCLC-ARE4 reporter occurs by mitogen-activated protein kinase pathways without oxidation of cellular glutathione or thioredoxin-1. *J. Biol. Chem.* **2004**, *279*, 5837−5845.

(40) Kamencic, H.; Lyon, A.; Paterson, P. G.; Juurlink, B. H. Monochlorobimane fluorometric method to measure tissue glutathione. *Anal. Biochem.* **2000**, *286*, 35−37.

(41) Lee, J. S.; Surh, Y. J. Nrf2 as a novel molecular target for chemoprevention. *Cancer Lett.* **2005**, *224*, 171−184.

(42) Jakubikova, J.; Sedlak, J.; Bacon, J.; Goldson, A.; Bao, Y. Effects of MEK1 and PI3K inhibitors on allyl-, benzyl- and phenylethyl-isothiocyanate induced G2/M arrest and cell death in Caco-2 cells. *Int. J. Oncol.* **2005**, *27*, 1449−1458.

(43) Meyer, D. J.; Crease, D. J.; Ketterer, B. Forward and reverse catalysis and product sequestration by human glutathione S-transferases in the reaction of GSH with dietary aralkyl isothiocyanates. *Biochem. J.* **1995**, *306*, 565−569.

(44) Nakamura, Y.; Kawakami, M.; Yoshihiro, A.; Miyoshi, N.; Ohigashi, H.; Kawai, K.; Osawa, T.; Uchida, K. Involvement of the mitochondrial death pathway in chemopreventive benzyl isothiocyanate-induced apoptosis. *J. Biol. Chem.* **2002**, *277*, 8492−8499.

(45) Miyoshi, N.; Takabayashi, S.; Osawa, T.; Nakamura, Y. Benzyl isothiocyanate inhibits excessive superoxide generation in inflammatory leukocytes: implication for prevention against inflammation-related carcinogenesis. *Carcinogenesis* **2004**, *25*, 567−575.

(46) Manesh, C.; Kuttan, G. Anti-tumour and anti-oxidant activity of naturally occurring isothiocyanates. *J. Exp. Clin. Cancer Res.* **2003**, *22*, 193−199.

(47) Gerhauser, C.; Klimo, K.; Heiss, E.; Neumann, I.; Gamal-Eldeen, A.; Knauft, J.; Liu, G. Y.; Sitthimonchai, S.; Frank, N. Mechanism-based in vitro screening of potential cancer chemopreventive agents. *Mutat. Res.* **2003**, *523*−*524*, 163−172.

(48) Hu, K.; Morris, M. E. Effects of benzyl-, phenethyl-, and α-naphthyl isothiocyanates on P-glycoprotein- and MRP1-mediated transport. *J. Pharm. Sci.* **2004**, *93*, 1901−1911.

(49) Jakubikova, J.; Sedlak, J.; Mithen, R.; Bao, Y. Role of PI3K/Akt and MEK/ERK signaling pathways in sulforaphane- and erucin-induced phase II enzymes and MRP2 transcription, G2/M arrest and cell death in Caco-2 cells. *Biochem. Pharmacol.* **2005**, *69*, 1543−1552.

(50) Hintze, K. J.; Keck, A. S.; Finley, J. W.; Jeffery, E. H. Induction of hepatic thioredoxin reductase activity by sulforaphane, both in Hepa1c1c7 cells and in male Fisher 344 rats. *J. Nutr. Biochem.* **2003**, *14*, 173−179.

(51) Meuillet, E. J.; Mahadevan, D.; Berggren, M.; Coon, A.; Powis, G. Thioredoxin-1 binds to the C2 domain of PTEN inhibiting PTEN's lipid phosphatase activity and membrane binding: a mechanism for the functional loss of PTEN's tumor suppressor activity. *Arch. Biochem. Biophys.* **2004**, *429*, 123−133.

(52) Saitoh, M.; Nishitoh, H.; Fujii, M.; Takeda, K.; Tobiume, K.; Sawada, Y.; Kawabata, M.; Miyazono, K.; Ichijo, H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J.* **1998**, *17*, 2596−2606.

(53) Hashimoto, S.; Matsumoto, K.; Gon, Y.; Furuichi, S.; Maruoka, S.; Takeshita, I.; Hirota, K.; Yodoi, J.; Horie, T. Thioredoxin negatively regulates p38 MAP kinase activation and IL-6 production by tumor necrosis factor-α. *Biochem. Biophys. Res. Commun.* **1999**, *258*, 443−447.

(54) Nakashima, I.; Takeda, K.; Kawamoto, Y.; Okuno, Y.; Kato, M.; Suzuki, H. Redox control of catalytic activities of membrane-associated protein tyrosine kinases. *Arch. Biochem. Biophys.* **2005**, *434*, 3−10.

(55) Esposito, F.; Chirico, G.; Montesano, G. N.; Posadas, I.; Ammendola, R.; Russo, T.; Cirino, G.; Cimino, F. Protein kinase B activation by reactive oxygen species is independent of tyrosine kinase receptor phosphorylation and requires SRC activity. *J. Biol. Chem.* **2003**, *278*, 20828−20834.

(56) Xu, K.; Thornalley, P. J. Involvement of glutathione metabolism in the cytotoxicity of the phenethyl isothiocyanate and its cysteine conjugate to human leukaemia cells in vitro. *Biochem. Pharmacol.* **2001**, *61*, 165−177.

(57) Stewart, D.; Killeen, E.; Naquin, R.; Alam, S.; Alam, J. Degradation of transcription factor Nrf2 via the ubiquitin−proteasome pathway and stabilization by cadmium. *J. Biol. Chem.* **2003**, *278*, 2396−2402.

(58) Bloom, D. A.; Jaiswal, A. K. Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H: quinone oxidoreductase-1 gene expression. *J. Biol. Chem.* **2003**, *278*, 44675−44682.

(59) Keum, Y. S.; Owuor, E. D.; Kim, B. R.; Hu, R.; Kong, A. N. Involvement of Nrf2 and JNK1 in the activation of antioxidant responsive element (ARE) by chemopreventive agent phenethyl isothiocyanate (PEITC). *Pharm. Res.* **2003**, *20*, 1351−1356.

(60) Kraft, A. D.; Johnson, D. A.; Johnson, J. A. Nuclear factor E2-related factor 2-dependent antioxidant response element activation by *tert*-butylhydroquinone and sulforaphane occurring preferentially in astrocytes conditions neurons against oxidative insult. *J. Neurosci.* **2004**, *24*, 1101−1112.

(61) Moinova, H. R.; Mulcahy, R. T. An electrophile responsive element (EpRE) regulates β-naphthoflavone induction of the human γ-glutamylcysteine synthetase regulatory subunit gene. Constitutive expression is mediated by an adjacent AP-1 site. *J. Biol. Chem.* **1998**, *273*, 14683−14689.

(62) Venugopal, R.; Jaiswal, A. K. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H: quinone oxidoreductase1 gene. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 14960−14965.

(63) Murakami, A.; Matsumoto, K.; Koshimizu, K.; Ohigashi, H. Effects of selected food factors with chemopreventive properties on combined lipopolysaccharide- and interferon-γ-induced IκB degradation in RAW264.7 macrophages. *Cancer Lett.* **2003**, *195*, 17−25.

(64) Srivastava, S. K.; Singh, S. V. Cell cycle arrest, apoptosis induction and inhibition of nuclear factor κ B activation in anti-proliferative activity of benzyl isothiocyanate against human pancreatic cancer cells. *Carcinogenesis* **2004**, *25*, 1701−1709.

(65) Heiss, E.; Herhaus, C.; Klimo, K.; Bartsch, H.; Gerhauser, C. Nuclear factor κ B is a molecular target for sulforaphane-mediated antiinflammatory mechanisms. *J. Biol. Chem.* **2001**, *276*, 32008−32015.

(66) Svehlikova, V.; Wang, S.; Jakubikova, J.; Williamson, G.; Mithen, R.; Bao, Y. Interactions between sulforaphane and apigenin in the induction of UGT1A1 and GSTA1 in CaCo-2 cells. *Carcinogenesis* **2004**, *25*, 1629−1637.

(67) Nakamura, H. Thioredoxin and its related molecules: update 2005. *Antioxid. Redox Signaling* **2005**, *7*, 823−828.

(68) Rose, P.; Whiteman, M.; Huang, S. H.; Halliwell, B.; Ong, C, N. β-Phenylethyl isothiocyanate-mediated apoptosis in hepatoma HepG2 cells. *Cell. Mol. Life Sci.* **2003**, *60*, 1489−1503.

(69) Taylor, J. M.; Crack, P. J.; Gould, J. A.; Ali, U.; Hertzog, P. J.; Iannello, R. C. Akt phosphorylation and NFκB activation are counterregulated under conditions of oxidative stress. *Exp. Cell Res.* **2004**, *300*, 463−475.

Received for review November 2, 2005. Revised manuscript received January 12, 2006. Accepted January 17, 2006. This work was supported in part by an EC Marie Curie Training Site Fellowship (QLK5-1999-50510) to J.J., the Slovakia Governmental Research and Development Sub-program (Food Quality and Safety, No. 2003SP270280E010280E01), and the Slovak Grant Agency VEGA (No. 2/2094).

JF052717H