Hydrogen sulfide protects colon cancer cells from chemopreventative agent β-phenylethyl isothiocyanate induced apoptosis

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Abstract

AIM: Hydrogen sulfide (H₂S) is a prominent gaseous constituent of the gastrointestinal (GI) tract with known cytotoxic properties. Endogenous concentrations of H₂S are reported to range between 0.2-3.4 mmol/L in the GI tract of mice and humans. Considering such high levels we speculate that, at non-toxic concentrations, H₂S may interact with chemical agents and alter the response of colonic epithelial cells to such compounds. The GI tract is a major site for the absorption of phytochemical constituents such as isothiocyanates, flavonoids, and carotenoids, with each group having a role in the prevention of human diseases such as colon cancer. The chemopreventive properties of the phytochemical agent β-phenylethyl isothiocyanate (PEITC) are well recognized. However, little is currently known about the physiological or biochemical factors present in the GI tract that may influence the biological properties of ITCs. The current study was undertaken to determine the effects of H₂S on PEITC mediated apoptosis in colon cancer cells.

METHODS: Induction of apoptosis by PEITC in human colon cancer HCT116 cells was assessed using classic apoptotic markers namely SubG1 population analysis, caspase-3 like activity and nuclear fragmentation and condensation coupled with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) viability assay and LDH leakage.

RESULTS: PEITC significantly induced apoptosis in HCT116 cells as assessed by SubG1 population formation, nuclear condensation, LDH leakage and caspase-3 activity after 24 h; these data being significant from control groups (P<0.01). In contrast, co-treatment of cells with physiological concentrations of H₂S (0.1-1 mmol/L) prevented PEITC mediated apoptosis as assessed using the parameters described.

CONCLUSION: PEITC effectively induced cell death in the human adenocarcinoma cell line HCT116 in vitro through classic apoptotic mechanisms. However, in the presence of H₂S, apoptosis was abolished. These data suggest that H₂S may play a significant role in the response of colonic epithelial cells to beneficial as well as toxic agents present within the GI tract.

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INTRODUCTION

Cruciferous vegetables such as broccoli, watercress and Brussels sprouts are an important food source of phytochemicals known as glucosinolates (GSLs). Following tissue disruption GSLs can be converted by plant and/or enteric gut bacterial myrosinase to the organosulfur compounds isothiocyanates (ITCs) [1,2]. Exposure to ITCs can reduce carcinogen induced tumor formation in a diverse range of organs such as lung, mammary gland, esophagus, liver and intestine[8-12]. Supporting these findings are recent epidemiological studies showing that consumption of cruciferous vegetables, the primary source of ITCs in the human diet, can reduce cancer incidence in humans[13,14]. Due to the promising chemopreventative properties of crucifers and ITCs, numerous reports have attempted to determine the mechanism(s) of action of ITC. ITCs can inhibit phase I enzymes[11] and induce phase II detoxification enzymes[15,16]. Moreover, the induction of apoptosis in target tissues and cancer cells has also been demonstrated. Previous investigations have shown that β-phenylethyl isothiocyanate (PEITC), benzyl isothiocyanate (BITC), allyl isothiocyanate (AITC) and sulforaphane (MSB) can induce apoptosis in both in vitro and in vivo situations[16-19].

Considering the beneficial effects of ITC exposure in the diet, surprisingly few studies have placed much emphasis on endogenous or exogenous factors that may alter the beneficial properties of these phytochemical agents. To date, storage and cooking regimes, plant and bacterial myrosinase
activity and altered conversion of GSLs to non-bioactive nitriles have been shown to influence the response of host tissues and cells to ITCs[28,29]. As ITCs are predominately absorbed along the gastrointestinal tract (GI) it is conceivable that the colonic environment may also contribute to the host’s response to ITCs.

Hydrogen sulfide (H\textsubscript{2}S) is a well known pungent gas and a prominent constituent present in the GI lumen. Exogenous sources show high toxicity towards the nervous, cardiovascular and respiratory systems in humans[30]. The toxic effect of H\textsubscript{2}S exposure are associated with its ability to bind reversibly to the heme site of cytochrome c aa3 site of cytchrome c oxidase thus inhibiting oxidative phosphorylation, although other mechanism(s) may also be involved. In addition, endogenous sites of H\textsubscript{2}S production also exist in mammals[31]. Catabolism of the amino acids L-cysteine and homeocystene by cystathionine-β-synthetase (CBS) generates appreciable levels of H\textsubscript{2}S in the brain. CBS is highly expressed in the hippocampus and cerebellum and CBS knockout mice have no detectable amounts of H\textsubscript{2}S in brain tissues. In contrast, H\textsubscript{2}S is also formed from the same precursors by cystathionine-β-lyase (CSE) which is the predominant enzyme in the vasculature. In addition, mammalian cells may also be exposed to high levels of H\textsubscript{2}S through the actions of enteric sulfate reducing bacteria in the GI tract[32]. Recent, studies have shown that H\textsubscript{2}S concentrations in the large intestine of mice ranges between 0.2-1 mmol/L and are comparable to the concentration of H\textsubscript{2}S in human feces (0.3-3.4 mmol/L)[33,34]. Considering the high in vivo levels of H\textsubscript{2}S it is plausible that such concentrations may directly affect the GI environment. Indeed, several clinical trials have shown a correlation between H\textsubscript{2}S levels and the development of colorectal cancer and ulcerative colitis, such studies highlighting a potential deleterious effect of H\textsubscript{2}S exposure[35]. However, it is not currently known whether H\textsubscript{2}S is the causative factor or a consequence of such diseases. In a more recent study Deplancke and Gaskins [36] demonstrated that H\textsubscript{2}S promotes cell cycle entry, MAPK kinase activation, and cell proliferation in non-transformed intestinal IEC-18 cell in vitro. This data suggests a link between H\textsubscript{2}S exposure and cell cycle dysregulation which perhaps contributes to colon cancer and ulcerative colitis development. However, considering the potential toxic effects of H\textsubscript{2}S the GI tract has developed mechanisms(s) to reduce the adverse effects of H\textsubscript{2}S such as increased rhodanese expression and activity, detoxification via oxidation to thiosulfate and bicarbonate secretion[32-35]. Therefore, H\textsubscript{2}S production in health subjects may not lead to disease formation due to adequate protective mechanisms. Recent studies have shown that H\textsubscript{2}S prevents glutamate induced oxidative stress in human neurons by stimulating glutathione biosynthesis[36]. H\textsubscript{2}S has also been shown to inhibit peroxynitrite, hydrogen peroxide and superoxide mediated cellular damage and death in neurons and myocytes by acting as an antioxidant[36,37]. Likewise, organisms like S. cerevisiae and deep sea hydrothermal vent Archea use H\textsubscript{2}S for the detoxification of methylmercury and heavy metal ion (Cu, Zn and Co) induced toxicity[38,39], thus physiological concentrations in the human GI tract may protect colon cancer cells from undergoing apoptosis.

In view of the large quantities of H\textsubscript{2}S in the GI tract we have systematically evaluated whether H\textsubscript{2}S can alter the biological response of human colon cancer HCT116 cells to the chemopreventative agent PEITC. Such data may provide a novel insight as to how H\textsubscript{2}S, at non-toxic concentrations, may act indirectly on colon cells apoptosis.

**MATERIALS AND METHODS**

**Cells and chemicals**

Human colon cancer HCT116, HT29, SW480 and LS174T cell lines were purchased from the American Culture Collection (Rockville, MD). HCA-7 cells were kindly supplied by Susan C. Kirkland, Royal Postgraduate Medical School, UK. β-Phenylethyl isothiocyanate, DMSO, ATP, SDS, penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA); MEM, trypsin and FBS from GIBCO BRL (Gaithersburg, MD, USA).

**Cell culture**

HCT116, HT29, HCA-7 and LS174T cells were cultured in complete DMEM (containing 100 mL/L FBS, 100 000 U/L penicillin, 100 mg/L streptomycin, pH 7.4) in 75-cm culture flasks at 37 °C in 50 mL/L CO\textsubscript{2}. Similarly, SW480 cells were grown in RPMI 1640 medium under the above cell culture conditions.

**Cell culture treatment**

NaSH was used to generate readily controlled amounts of H\textsubscript{2}S as it dissociates to Na\textsuperscript{+} and HS\textsuperscript{-} in solution, then HS-associates with H\textsuperscript{+} to produce H\textsubscript{2}S. We herein use the term H\textsubscript{2}S to reflect the sum of the species H\textsubscript{2}S, HS- or S\textsuperscript{2-} present at physiological pH. PEITC was dissolved in DMSO to a final concentration of 100 mmol/L and adjusted to the required concentration by dilution in cell culture medium. All reagents were prepared fresh for each individual experiment.

**Cell viability testing**

Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) viability assay as previously described[40]. Activity of lactate dehydrogenase (LDH) in the medium was measured using an Abbott VP Biochemical Analyser with the test kit (Abbott Laboratories, Irving, TX, USA). The total LDH activity was determined by ultrasonication and assessed by expressing as percentage LDH leakage (LDH in medium/total LDH activity ×100).

**Detection of nuclear morphological changes and apoptosis**

To assess apoptotic cell death morphologic changes in chromatin structure were detected by DAPI staining. Briefly, attached cells were harvested washed with PBS, and re-suspended in PBS prior to fixing with 700 mL/L ethanol for 15 min at room temperature. Cells were centrifuged, re-suspended in PBS and stained with 2 g/L DAPI in the dark for 5 min. Cell suspensions were mounted on glass slides and subjected to fluorescence microscopic examination using a Nikon photomicroscope (Thornwood, NY, USA). Apoptotic cells were identified by their morphology and by
the condensation and fragmentation of their nuclei.

DNA fragmentation in HCT116 cells was measured using propidium iodide staining followed by flow cytometry analysis as described. From each treatment 10,000 cells were analyzed using flow cytometry ( Coulter Epics Elite ESP, Miami, FL, USA). Data was analyzed using WinMDI 2.7 software (Scripトル Institute, La Jolla, CA, USA).

**Measurement of caspase activity**

Caspase-3 activity was determined spectrofluorometrically using the synthetic substrate Ac-DEVD-AFC respectively (BIOMOL Research Labs, Plymouth, PA, USA) as previously described. Cell homogenates were incubated with synthetic substrate 0.2 mmol/L at 37 °C/2 h. The fluorescence intensity of liberated AFC was monitored using a Tecan spectrofluor plus plate reader (USA) using an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

**Statistical analysis**

All data are represented by three separate experiments. All experimental data consists of mean±SD unless otherwise stated and were analyzed by one-way ANOVA.

**RESULTS**

**Evaluation of PEITC mediated reduction of colon cancer cell viability**

Accumulating evidence has shown that cancer cells treated with PEITC undergo apoptosis by inducing mitochondrial dysfunction, sequential caspase activation, and MAPKinase mediated apoptotic signaling. To evaluate the cytotoxic effects of PEITC on the viability of colon cancer cells, we preferentially screened several commonly available cell lines namely colorectal carcinoma HCT116, and colorectal adenocarcinoma cell lines HT29, SW480, HCA-7 and LS174T cells, following 24 h treatments with increased concentrations of PEITC. Cell viability was determined by MTT assay. All cells lines were responsive to PEITC treatment with viability decreasing in a concentration dependent manner (Figure 1).

**Induction of apoptosis by PEITC**

We next selected two cell lines for further study these being the HCT116 and HT29 cell lines. Several key parameters that are known to be involved in PEITC induced apoptosis in HCT116 cells and HT29 cells were evaluated. Previous investigations have shown both HCT116 and HT29 cells are responsive to PEITC treatment, which we needed to reconfirm under our laboratory conditions. As represented in Figure 2, PEITC mediated a time and concentration dependant induction of caspase-3 like activity in HCT116 and HT29 cells. Caspase activity was determined by measurement of AFC fluorescence intensity derived from the catalytic cleavage of the substrate Ac-DEVD-AFC by endogenous caspase. The fluorescence intensity of treated over control cells, was used to determine the fold increase in caspase activity. Supporting a role of caspases in PEITC induced apoptosis was the inhibitory effects of the pharmacological caspase inhibitors Z-VAD-FMK (pan-caspase inhibitor), Ac-LEHD-CHO (caspase 9 inhibitor), and Ac-DEVDECHO (caspase 3 inhibitor) on PEITC induced apoptosis. In both cell types tested all inhibitors reduced DNA fragmentation as determined by measurement of SubG1 populations by propidium iodide staining and LDH leakage (Figure 2).

**Inhibition of PEITC mediated apoptosis by low molecular weight sulfur compounds**

Because PEITC is absorbed along the GI tract we next evaluated whether hydrogen sulfide and other low molecular weight sulfur compounds could protect HCT116 cells from PEITC mediated apoptosis. Incubation of HCT116 cells with up to 1 mmol/L concentrations of hydrogen sulfide (H2S) and its metabolic by-products sulfate (SO42-), sulfite (SO32-) and thiosulfate (S2O32-) had no appreciable effects on cell viability as determined using the MTT assay and LDH leakage at 24 h (Figure 3). Co-treatment of individual sulfur compounds (SO4, SO3, S2O32-) at 1 mmol/L concentrations failed to prevent the apoptotic effects of PEITC (40 µmol/L). In contrast, H2S co-treatment but not pre-treatment inhibited PEITC induced apoptosis in a concentration dependant manner that was comparable to the low molecular weight thiol N-acetylcysteine (Figure 3). Associated with the inhibitory effects of both H2S and NAC was a significant reduction in caspase activity (Figure 3D).

**H2S and NAC protect against BSO mediated sensitization of HCT116 cells to PEITC induced apoptosis**

Intracellular depletion of glutathione (GSH) can be achieved using buthionine-S-sulfoximine (BSO), an inhibitor of the rate limiting enzyme required for GSH biosynthesis. BSO treatment sensitizes cells to apoptotic agents by reducing intracellular GSH levels. The sensitizing effects of combined treatment of PEITC and BSO in HCT116 cells were next investigated in the presence of H2S and NAC. BSO at the concentration used had no appreciable effects on cell viability. Pre-treatment with 0.5 mmol/L BSO/24 h increased the apoptotic response of HCT116 cells to PEITC (40 µmol/L) as determined by the MTT assay and LDH leakage (Figure 4A). Indeed, BSO pre-treatment decreased cell viability by 83% and increased LDH leakage to 84.9% (P<0.01) in PEITC treated cells. Cellular protection was afforded by co-treatment with either H2S or NAC (1 mmol/L). Moreover, evaluation of morphological
and nuclear condensation also demonstrated that both H$_2$S and NAC prevent BSO mediated sensitization of HCT116 cells to PEITC.

**DISCUSSION**

In the present investigation we sought to examine whether exogenous sources of the reductant H$_2$S could alter the response of human colon cancer cells to $\beta$-phenylethyl isothiocyanate induced apoptosis. PEITC is known to possess potent chemopreventive properties against carcinogen induced colon cancer in rodent models, perhaps mediated largely by the induction of carcinogen detoxification pathways, anti-inflammatory affects, and apoptosis$^{[8,11,18,19,45]}$. It is
PEITC, as well as other ITCs were not explored. However, whether these interactions could change the biological properties of PEITC remains to be explored. Cell viability, as determined by MTT or LDH leakage, in HCT116 cells following either co-treatment or pre-treatment (24 h) with H2S or NAC (125 µmol-1 mmol/L) as determined by MTT or LDH leakage. Data are expressed as mean±SD. (n = 8); D: Co-treatment with either H2S or NAC (125 µmol-1 mmol/L) inhibits PEITC induced caspase-3 activity as determined at 16 h and Data are representative of 3 or more separate experiments conducted on separate occasions. means±SD, P<0.01 vs PEITC treated groups.

Figure 3 A: Effects of low molecular weight sulfur compounds on PEITC mediated loss of cell viability. B and C: Inhibition of PEITC mediated loss of cell viability in HCT116 cells following either co-treatment or pre-treatment (24 h) with H2S or NAC (125 µmol-1 mmol/L) as determined by MTT or LDH leakage. Data are expressed as mean±SD, (n = 8); D: Co-treatment with either H2S or NAC (125 µmol-1 mmol/L) inhibits PEITC induced caspase-3 activity as determined at 16 h and Data are representative of 3 or more separate experiments conducted on separate occasions. means±SD, P<0.01 vs PEITC treated groups.

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interesting to note, that previous investigations have shown that colon cancer cells are more sensitive to ITC mediated cytotoxicity than other transformed counterparts. This property has been proposed to protect against the development of colorectal cancer by inhibiting the growth of transformed cell clones within the gastrointestinal mucosa. However, factors that may limit these beneficial effects have been largely unexplored.

The biological properties of ITCs, including PEITC, are predominantly mediated by the electrophilic central carbon atom of the isothiocyanate groups. The NCS residue readily reacts with nucleophilic centers of deprotonated thiols such as those present on glutathione (GSH) and N-acetylcysteine (NAC) leading to the formation of N-substituted mono-dithiocarbamate conjugates. Such interactions alter the intracellular redox state and activate phase II detoxification enzymes or apoptotic signaling pathways.

Considering that sulfur metabolizing bacteria generate appreciable levels of the thiol H2S in the GI tract, via metabolism of inorganic and organic sulfur compounds, we investigated whether H2S could act in a similar manner to GSH or NAC in the detoxification of PEITC. Early work conducted by Zuman and Zahradnik demonstrated that organic ITCs can reversibly react with the hydrosulfide ion of H2S forming N-mono-substituted dithiocarbamates, such findings supporting our hypothesis. However, whether these interactions could change the biological properties of PEITC, as well as other ITCs were not explored. H2S concentrations in the GI tract range between 0.2-1 mmol/L in mice and up to 3.4 mmol/L in human feces, therefore chemical interaction between H2S and PEITC are highly plausible. On the basis of the chemical nature of H2S and its similar biological characteristics to that of both GSH and NAC e.g. antioxidant properties and a deprotonated thiol group in aqueous solution, perhaps H2S at physiological concentrations may impair the biological properties of electrophilic chemopreventative agents against colon cancer cells.

In our study, PEITC reduced colon cancer cell viability in a concentration manner in all cell lines studied (Figure 1). In addition, we also confirmed that the loss of cell viability in HT29 and HCT116 cells was due to the induction of apoptosis as assessed by nuclear fragmentation and condensation, LDH leakage, caspase-3 like activation and an increase in the SubG1 population (Figure 2). We next addressed using the HCT116 cell line whether H2S could impair PEITC induced apoptosis. In each assay examined H2S significantly reduced the apoptotic response of HCT116 cells to PEITC at concentrations well within the physiological reported levels (up to 3.4 mmol/L). Indeed, H2S was almost as effective on a molar basis at inhibiting PEITC induced apoptosis as the antioxidant thiol N-acetylcysteine, a known inhibitor of PEITC intracellular uptake. We assume that the mechanism of inhibition by H2S is similar to NAC, although this requires further study.

Understanding the mechanisms by which PEITC mediates apoptosis and factors that may influence its in vivo efficacy is of considerable importance as PEITC mediated
Figure 4 Inhibition of PEITC mediated loss in cell viability in GSH depleted cells following pre-treatment with 0.5 mmol/L BSO for 24 h. Both H₂S and NAC inhibited PEITC induced loss of cell viability in concentration dependant manner as determined using the MTT assay (A) and (B) LDH leakage. (C) Respective photographs and DAPI stained cells showing cell morphological changes and nuclear condensation. Data are representative of 3 or more separate experiments conducted on separate occasions. mean±SD, *P<0.01 vs PEITC treated to control groups.
apoptosis is thought to contribute to its chemopreventative effects. In particular, extensive induction in apoptosis by PEITC has been observed in cell culture studies and rodent models of colon cancer. Similarly, an inverse correlation between ITC exposure and a reduction in colon cancer prevalence has been demonstrated in some epidemiological studies. Whether elevated levels of H_{2}S observed in colon cancer and ulcerative colitis impair chemopreventive or chemotherapeutic drug induced anti-inflammatory and apoptotic effects of has yet to be demonstrated.

In summary, our data reports for the first time that physiological concentrations of H_{2}S can inhibit the apoptotic effects of the chemopreventative agent PEITC. It is possible based on our data that changes in H_{2}S levels along the GI tract may influence the extent of PEITC mediated apoptosis in vivo. The potential interaction of H_{2}S with other dietary constituents as well as chemotherapeutic drugs requires further study.

REFERENCES

10. Zhang DD, Hennink 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


46 Musk SR, Johnson IT. Allyl isothiocyanate is selectively toxic to transformed cells of the human colorectal tumour line HT29. Carcinogenesis 1993; 14: 2079-2083

