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

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Abstract

AIM: Hydrogen sulfide (H_2S) is a prominent gaseous constituent of the gastrointestinal (GI) tract with known cytotoxic properties. Endogenous concentrations of H_2S 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, H_2S may interact with chemical agents and alter the response of colonic epithelium 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 chemopreventative properties of the phytochemical agent β-phenyethyl 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 H_2S 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 H_2S (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 H_2S, apoptosis was abolished. These data suggest that H_2S may play a significant role in the response of colonic epithelial cells to beneficial as well as toxic agents present within the GI tract.

Key words: Apoptosis; Colon cancer; Hydrogen sulfide; β-phenylethyl isothiocyanate

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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[3,4]. 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[5,6]. 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[7] and induce phase II detoxification enzymes[8-12]. 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[13-19].

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[20-24]. 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_2S) 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[25]. The toxic effect of H_2S exposure are associated with its ability to bind reversibly to the heme site of cytochrome c aa3 site of cytochrome c oxidase thus inhibiting oxidative phosphorylation, although other mechanism(s) may also be involved. In addition, endogenous sites of H_2S production also exist in mammals[26]. Catabolism of the amino acids L-cysteine and homocysteine by cystathionine-β-synthetase (CBS) generates appreciable levels of H_2S in the brain. CBS is highly expressed in the hippocampus and cerebellum and CBS knockout mice have no detectable amounts of H_2S in brain tissues. In contrast, H_2S 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_2S through the actions of enteric sulfate reducing bacteria in the GI tract[27]. Recent, studies have shown that H_2S concentrations in the large intestine of mice ranges between 0.2-1 mmol/L and are comparable to the concentration of H_2S in human feces (0.3-3.4 mmol/L)[28,29]. Considering the high *in vivo* levels of H_2S it is plausible that such concentrations may directly affect the GI environment. Indeed, several clinical trials have shown a correlation between H_2S levels and the development of colorectal cancer and ulcerative colitis, such studies highlighting a potential deleterious effect of H_2S exposure[30]. However, it is not currently known whether H_2S is the causative factor or a consequence of such diseases. In a more recent study Deplancke and Gaskins[31] demonstrated that H_2S promotes cell cycle entry, MAPkinase activation, and cell proliferation in non-transformed intestinal IEC-18 cell *in vitro*. This data suggests a link between H_2S exposure and cell cycle dysregulation which perhaps contributes to colon cancer and ulcerative colitis development. However, considering the potential toxic effects of H_2S the GI tract has developed mechanism(s) to reduce the adverse effects of H_2S such as increased rhodanese expression and activity, detoxification via oxidation to thiosulfate and bicarbonate secretion[32-34]. Therefore, H_2S production in health subjects may not lead to disease formation due to adequate protective mechanisms. Recent studies have shown that H_2S prevents glutamate induced oxidative stress in human neurons by stimulating glutathione biosynthesis[35]. H_2S 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 *Saccharomyces cerevisiae* and deep sea hydrothermal vent *Archea* use H_2S 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_2S in the GI tract we have systematically evaluated whether H_2S 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_2S, at non-toxic concentrations, may act indirectly on colonic 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 ℃ in 50 mL/L CO_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_2S as it dissociates to Na+and HS- in solution, then HS-associates with H+ to produce H_2S. We herein use the term H_2S to reflect the sum of the species H_2S, HS-or S2-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 propodium iodide staining followed by flow cytometry analysis as described[41]. 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 (Scripps 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[42,43]. Cell homogenates were incubated with synthetic substrate 0.2 mmol/L at 37 ℃/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[14,16]. 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 24h 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).

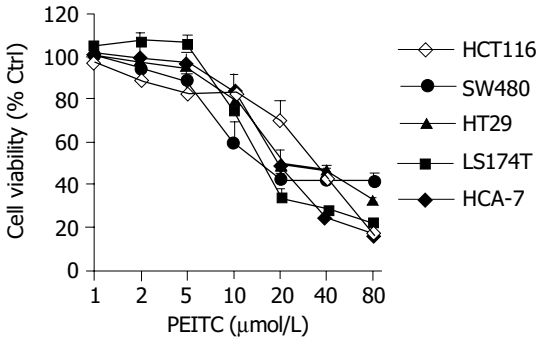

Figure 1 (A) Concentration dependant loss of cell viability induced by PEITC in colon cancer cells as determined at 24 h using the MTT viability assay. Data are representative of three or more separate experiments (mean±SD).

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[14,44]. 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 endogeneous 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-DEVD-CHO (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 propodium 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 (H_2S) and its metabolic by-products sulfate (SO_4), sulfite (SO_3) and thiosulfate ($S_2O_3^{2-}$) 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 (SO_3, SO_4, $S_2O_3^{2-}$) at 1 mmol/L concentrations failed to prevent the apoptotic effects of PEITC (40 μmol/L). In contrast, H_2S 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 H_2S and NAC was a significant reduction in caspase activity (Figure 3D).

H_2S 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 H_2S 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 H_2S or NAC (1 mmol/L). Moreover, evaluation of morphological

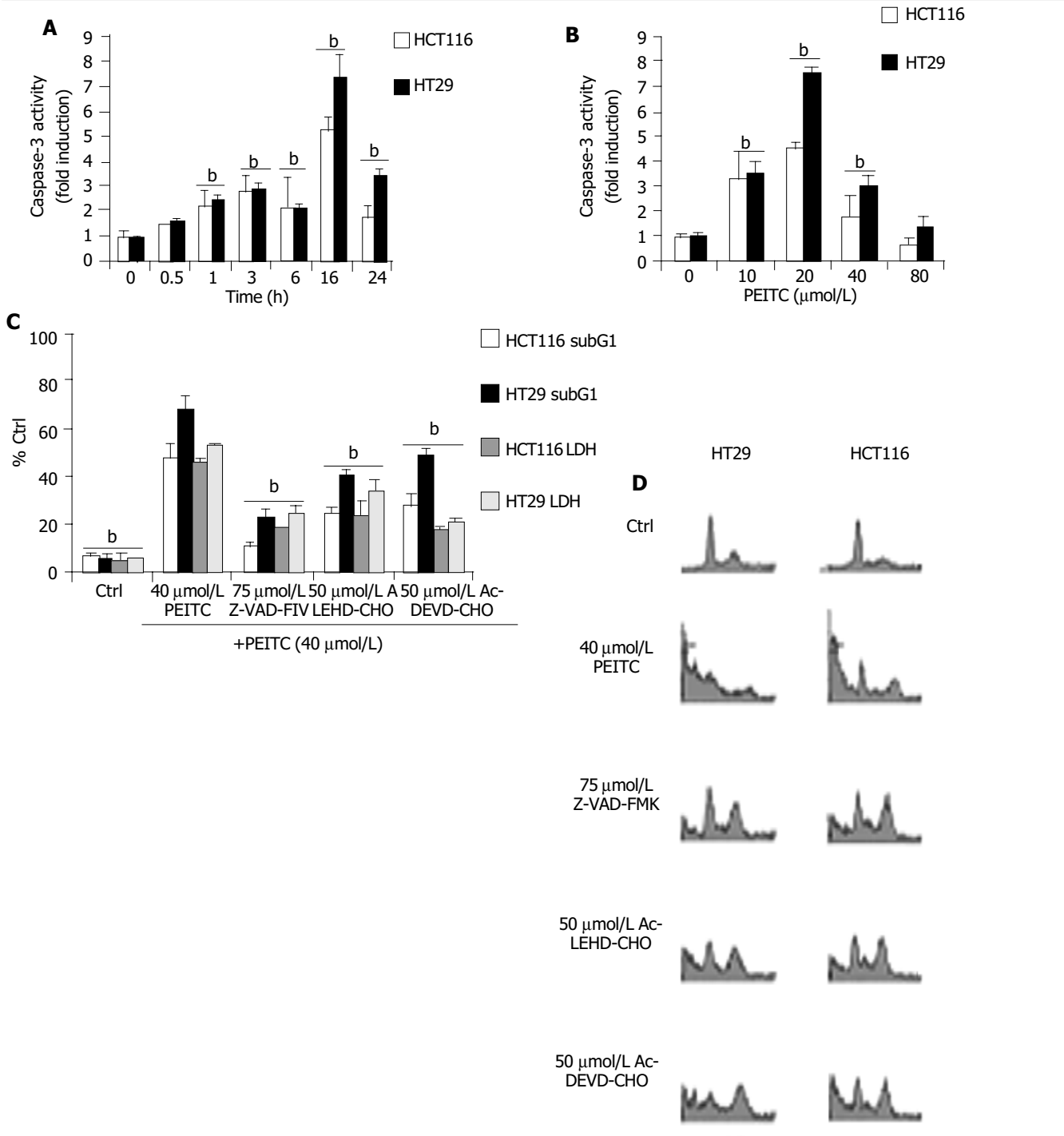

Figure 2 **A**: Time dependant Induction of caspase -3 activities in HCT116 and HT29 cells treated with 40 μmol/L PEITC as determined at 0.5, 1, 3, 6, 16 and 24 h. **B**: Concentration dependant activation of caspase-3 as determined at 24 h after incubation with PEITC (10-80 μmol/L). **C**: Z-VAD-FMK, Ac-LEHD-CHO and Ac-DEVD-CHO inhibition on PEITC mediated apoptosis as determined at 24 h. $^bP<0.01$, significant difference compared to control groups. Cells were treated with individual caspase inhibitors for 1 h prior to the addition of 40 μmol/L PEITC. SubG1 populations were determined by propodium iodide staining followed by flow cytometry analysis as described in the materials and methods. $^bP<0.01$, comparing PEITC (alone) with control groups. **D**: Representative flow cytometry analysis of HCT116 and HT29 cells was used to determine the extent of DNA fragmentation in cells treated with PEITC (40 μmol/L) in the presence of caspase inhibitors. Data were determined by *PI staining* at 24 h. Figure shows representative example of at least 3 experiments. In each analysis, 10 000 events were recorded. $^bP<0.01$, PEITC (alone) *vs* control and caspase inhibitor treated groups.

and nuclear condensation also demonstrated that both H_2S 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_2S could alter the response of human colon cancer cells to β-phenylethyl isothiocyanate induced apoptosis. PEITC is known to possess potent chemopreventative 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

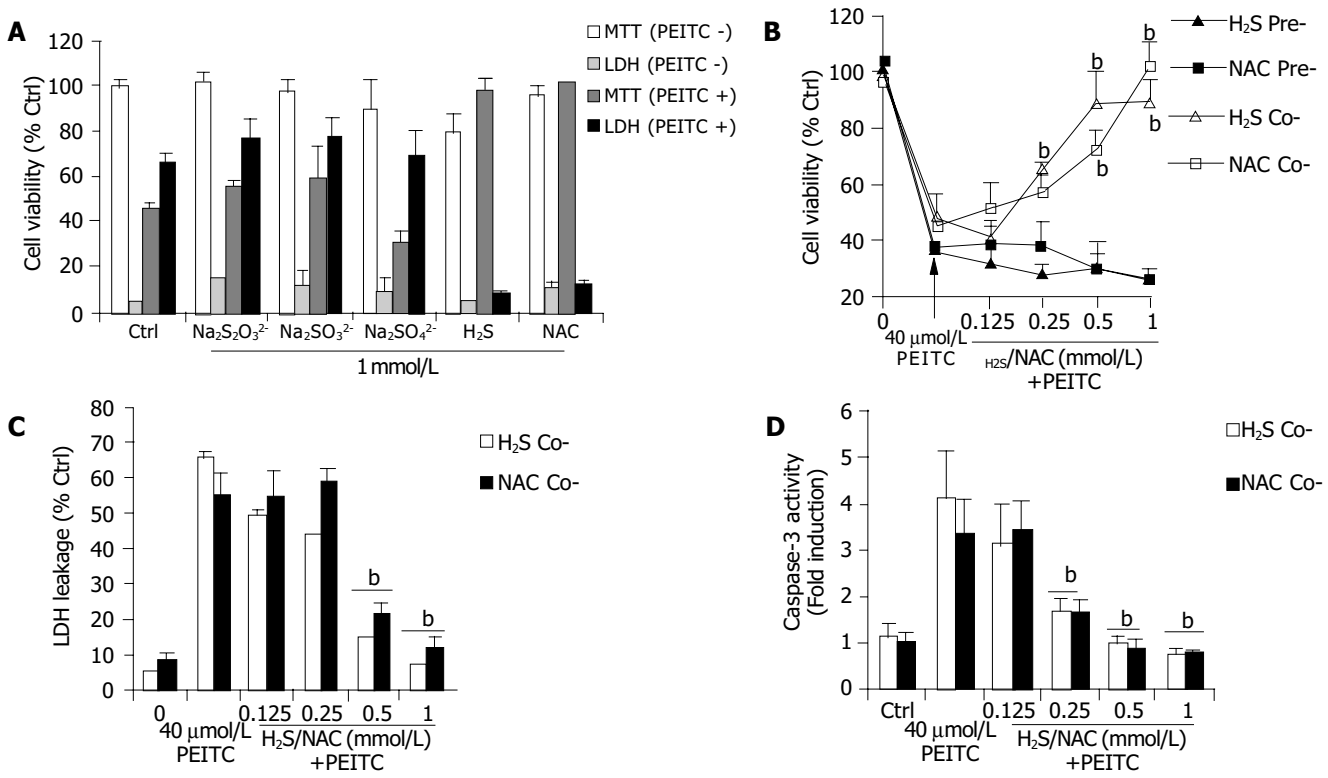

Figure 3 **A**: Effects of low molecular weight sulfur compounds on PEITC mediate loss of cell viability; **B** and **C**: Inhibition of PEITC mediated loss of cell viability in HCT116 cells following either co-treatment or pre-treatment (24 h) with H_2S or NAC (125 μmol-1 mmol/L) as determined by MTT or LDH leakage. Data are expressed as mean±SD, (n = 8); **D**: Co-treatment with either H_2S 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. mean±SD, $^bP<0.01$ *vs* PEITC treated groups.

interesting to note, that previous investigations have shown that colon cancer cells are more sensitive to ITC mediated cytotoxicity than there detransformed 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[46,47]. However, factors that may limit these beneficial effects have been largely unexplored.

The biological properties of ITCs, including PEITC, are predominantly mediated by the electophilic central carbon atom of the isothiocyanate groups (R-N = C = S)[48]. 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 H_2S in the GI tract, via metabolism of inorganic and organic sulfur compounds, we investigated whether H_2S could act in a similar manner to GSH or NAC in the detoxification of PEITC. Early work conducted by Zuman and Zahradnik[49] demonstrated that organic ITCs can reversibly react with the hydrosulfide ion of H_2S forming *N*-mono-substituted dithiocarbomates, such findings supporting our hypothesis. However, whether these interactions could change the biological properties of PEITC, as well as other ITCs were not explored. H_2S 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 H_2S and PEITC are highly plausible. On the basis of the chemical nature of H_2S 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 H_2S at physiological concentrations may impair the biological properties of electrohilic 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 H_2S could impair PEITC induced apoptosis. In each assay examined H_2S 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, H_2S 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 up-take[12]. We assume that the mechanism of inhibition by H_2S 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

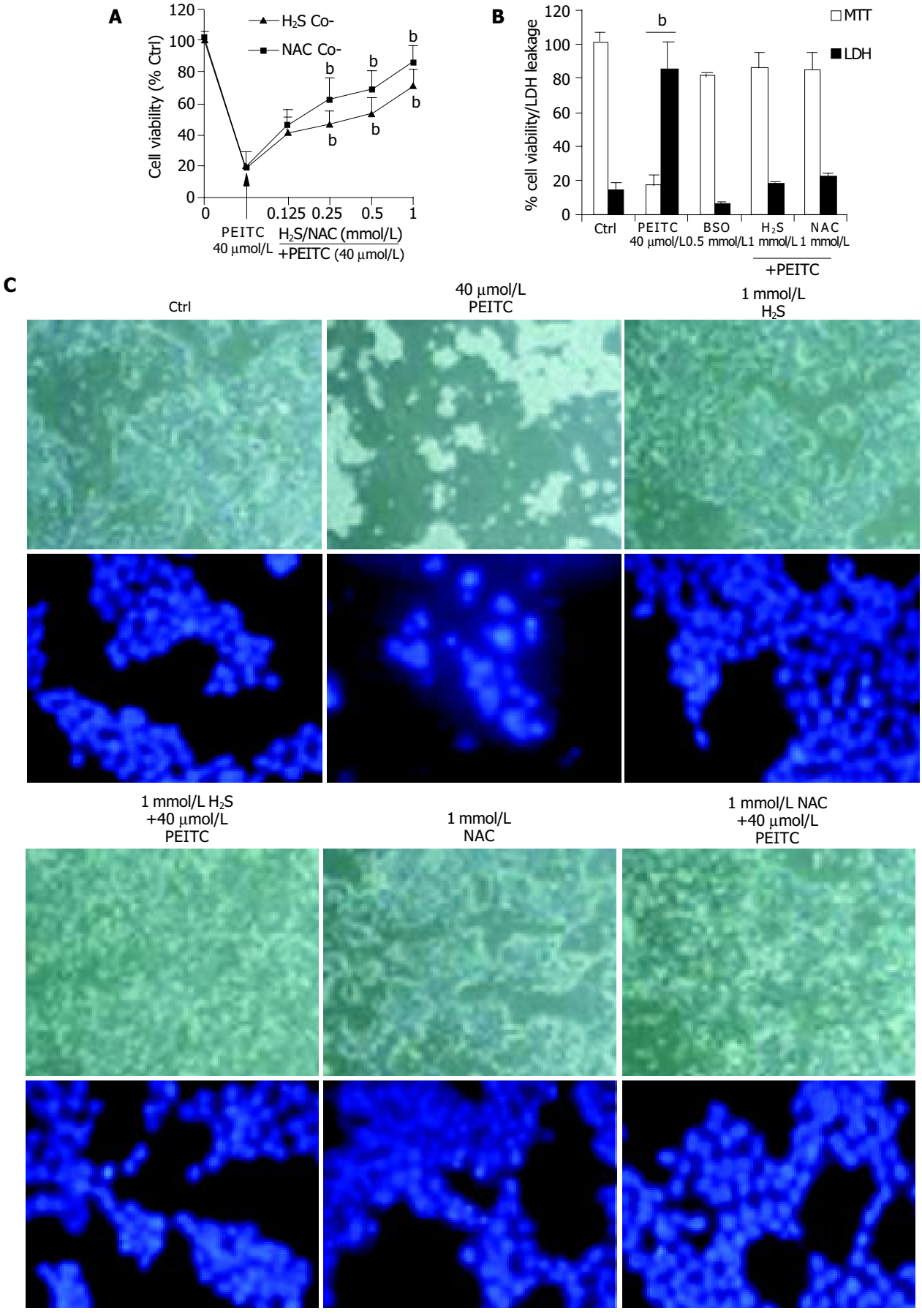

Figure 4 Inhibition of PEITC mediated loss in cell viability in GSH depleted cells following pre-treatment with 0.5 mmol/L BSO for 24 h. Both H_2S and NAC inhibited PEITC induced loss of cell viability in concentration dependant manner as determined using the MTT assay (**A**) and (**B**) LDH leakage. (**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, $^bP<0.01$ *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_2S observed in colon cancer and ulcerative colitis impair chemopreventative 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_2S can inhibit the apoptotic effects of the chemopreventative agent PEITC. It is possible based on our data that changes in H_2S levels along the GI tract may influence the extent of PEITC mediated apoptosis *in vivo*. The potential interaction of H_2S with other dietary constituents as well as chemotherapeutic drugs requires further study.

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