Biochemical Pharmacology 83 (2012) 733–740

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Hydrogen sulfide-releasing aspirin modulates xenobiotic metabolizing enzymes *in vitro* and *in vivo*

Mitali Chattopadhyay [a], Ravinder Kodela [a], Niharika Nath [b], Cherease R. Street [a], Carlos A. Velázquez-Martínez [c], Daniel Boring [d], Khosrow Kashfi [a,*]

[a] Department of Physiology and Pharmacology, Sophie Davis School of Biomedical Education, City University of New York Medical School, New York, NY 10031, United States
[b] Department of Life Sciences, New York Institute of Technology, New York, NY 10023, United States
[c] Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada
[d] Division of Cancer Prevention, National Cancer Institute, Bethesda, MD 20892, United States

ARTICLE INFO

Article history:
Received 14 September 2011
Accepted 15 December 2011
Available online 24 December 2011

Keywords:
Xenobiotic metabolizing enzymes
GST
NOQ1
UGT
CYP 1A1
Hydrogen sulfide
NSAIDs

ABSTRACT

The balance between phase-I carcinogen-activating and phase-II detoxifying xenobiotic metabolizing enzymes is critical to determining an individual's risk for cancer. We evaluated the effect of Hydrogen sulfide-releasing aspirin (HS-ASA) on xenobiotic metabolizing enzymes in HT-29 human colon and Hepa 1c1c7 mouse liver adenocarcinoma cells and in Wistar rats. HS-ASA inhibited the growth of HT-29 and Hepa 1c1c7 cells, with an IC_{50} of 3.2 ± 0.3 μM and 4.2 ± 0.4 μM, respectively. The IC_{50} for ASA in both cell lines was greater than 5000 μM at 24 h. In these cell lines, HS-ASA caused a dose-dependent increase in activity and expression of the phase-II enzymes glutathione S-transferase (GST) and NAD(P)H:quinoneoxireductase (NQO1). It also caused an increase in UDP-glucuronosyltransferase (UGT) expression. The levels of CYP 1A1 a phase-I enzyme was increased by HS-ASA in both cell lines. Pretreatment of cells with NaF, an esterase inhibitor, abrogated the HS-ASA-mediated increases in NQO1 enzyme activity. HS-ASA increased the protein levels of the transcription factor Nrf2, which is a regulator of the phase-II enzymes. *In vivo*, HS-ASA at 100 mg/kg/day had no effect on rat's weights; it induced a 3.4-fold and 1.4-fold increase in hepatic GST and NQO1 enzyme activities, respectively. GST and NQO1 protein levels were also increased. In contrast to that in cultured cells, CYP 1A1 protein levels were not altered *in vivo*. Therefore, HS-ASA induces phase-II enzymes, at least in part, through the action of H_2S and by modulating Nrf2; these effects may be part of its mechanism of action against carcinogenesis.

1. Introduction

Environmental and endogenous chemical carcinogenic agents contribute to the process of carcinogenesis. Induction of enzymes that enhance the detoxification of such chemical carcinogens represents an important strategy for cancer chemoprevention. The xenobiotic-metabolizing machinery consists of phase-I cytochrome P-450 oxidative-metabolism enzymes and phase-II conjugating enzymes. In general, phase-II enzymes can reduce the cellular exposure to carcinogens whereas phase-I enzymes can increase it [1,2]. Therefore, the balance between the phase-I carcinogen-activating enzymes and the phase-II detoxifying enzymes is critical, and cancer susceptibility may, in part, be determined by altered functions of these enzymes [3].

The phase-I metabolic enzymes are the P450 super family, with the CYP1, 2, and 3 families being the major contributors to xenobiotic metabolism [1]. The phase-II enzymes include many enzyme super families such as NAD(P) H:quinoneoxireductase (NQO1), glutathione S-transferases (GSTs) and UDP-glucuronyltransferases (UGTs) [4]. NQO1 protects cells against the toxicity of quinones and their metabolic precursors by promoting the two-electron reduction of quinones to semiquinones [5]. GSTs detoxify a number of carcinogenic electrophiles by catalyzing their conjugation with reduced glutathione [6,7].

Pharmacological strategies that modulate the levels of phase-I and phase-II enzymes can enhance the elimination of the carcinogenic reactive species. In particular, identification of compounds that preferentially activate phase-II over phase-I enzymes can be beneficial as chemopreventive agents. Certain chemopreventive compounds, such as isothiocyanates and dithiolthiones have been shown to act as transcriptional activators of phase-II enzymes [8,9]. There is a consensus enhancer element, known as antioxidant response element (ARE) in the regulatory domains of many phase-II genes, and the ARE-binding transcription

* Corresponding author at: Department of Physiology and Pharmacology, City University of New York Medical School, 138th Street and Convent Avenue, New York, NY 10031, United States. Tel.: +1 212 650 6641; fax: +1 212 650 7692.
E-mail address: kashfi@med.cuny.edu (K. Kashfi).

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.12.020

factor Nrf2 have been implicated in the action of many chemopreventive agents [8]. Nrf2 is a regulator of the inducible expression of the enzymes GST and NQO1, which are important in catalyzing the detoxification of reactive electrophiles and oxidants (reviewed in [10]).

Non-steroidal anti-inflammatory drugs (NSAIDs) in general and aspirin in particular are prototypical anticancer agents [11]. Increasing evidence from human epidemiological studies, animal models, and *in vitro* experiments have demonstrated the potential of NSAIDs in the chemoprevention of colorectal cancer (CRC) [12,13]. However, the side effects from aspirin and other NSAIDs have reduced the interest for NSAIDs as cancer chemopreventive agents. A more recent approach to alleviate the gastrointestinal side effects of NSAIDs has been the development of compounds where the conventional NSAID is modified with active redox moieties such as endogenous gases, for example, nitric oxide releasing NSAIDs (NO-NSAIDs) and more recently hydrogen sulfide-releasing NSAIDs (HS-NSAIDs) (reviewed in [11]). Hydrogen sulfide (H_2S) at micro molar concentrations neutralize a variety of reactive species including oxy radicals [14], peroxynitrite [15], hypochlorous acid [16] and homocysteine [17]. H_2S has been recognized for its ability to affect key physiologic functions [18]. In the intestine, H_2S modulates epithelial secretion [19] and promotes resolution of colitis [20]. However, there are only a few reports describing actions of HS-NSAIDs. An H_2S-releasing derivative of diclofenac was reported to produce significantly less damage in the GI tract, while it was more effective than the parent drug in reducing inflammation [21,22].

In the previous accompanying manuscript, we demonstrated cell growth inhibition by various HS-NSAIDs in human cancer cell lines of different tissue types. In this study we explored further the effects of one such HS-NSAID, H_2S-releasing aspirin (HS-ASA) on colorectal cancer cells *in vitro* and *in vivo*, and evaluated its effects on xenobiotic metabolizing enzymes. We report its effect on phase-I and phase-II enzymes in liver and colon cell lines, and in the liver, kidney and colon of rats.

2. Materials and methods

2.1. Reagents

HS-ASA was synthesized and purified by us with ^{1}H NMR verification. Stock (100 mM) solutions of HS-ASA and ASA (Sigma Chemical, St Louis, MO) were prepared in dimethylsulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ). The final DMSO concentration was adjusted in all media to 1%.

2.2. Cell culture

HT-29 human colon adenocarcinoma and Hepa 1c1c7 mouse liver adenocarcinoma cell lines were obtained from American Type Tissue Collection (Rockville, MD). Cells were grown in 5% CO_2 at 37 °C in a MEM (Hepa 1c1c7) or McCoy 5A (HT-29) medium supplemented with 10% fetal calf serum, 10 000 IU/mL penicillin and 10 mg/mL streptomycin.

To assay GST activity and perform immunoblot analyses, cells were seeded in 10 cm cell culture dishes at a density of 5.0×10^5 cells/cm^2 in 10 mL of media and allowed to attach for 24 h. Cells were exposed to various concentrations of HS-ASA for 24 h, control cultures were supplemented with DMSO only. Cells were washed twice with ice-cold PBS and harvested using a rubber policeman. The cell pellet was stored dry at −70 °C until analyses. Cells were lysed by incubating them for 30 min on ice in lysis buffer (50 mM Tris–HCl, 0.8% digitonin and 2 mM EDTA, pH 6.8). After centrifugation, the lysate was assayed for GST enzyme activity and subjected to immunoblot analysis.

2.3. Animal treatments

Male Wistar rats were purchased from Charles River Laboratories (Wilmington, MA). After acclimation, the animals were housed and maintained according to the approved standards of Institutional Animal Care and Use Committee. HS-ASA was suspended (100 mg/mL, w/v) in a solution of 0.5% carboxy methyl cellulose (Sigma Chemical) immediately before treatment. Rats were divided into two groups of five animals each and treated *via* gavage either with vehicle or with HS-ASA, 100 mg/kg/day. After 21 days of treatment, animals were killed by CO_2 and cervical dislocation and their liver, kidneys and colon were removed rapidly for preparation of microsomes and cytosol. Liver and kidneys were immediately perfused with cold NaCl (0.9, w/v) and homogenized in 3 mM Tris buffer (pH 7.2) containing 0.25 M sucrose and 1 mM EGTA. For the liver and kidneys after removing the mitochondria as described previously [23], the post-mitochondrial supernatant was centrifuged at 20,000 × *g* for 20 min to remove light mitochondria and peroxisomes, and the resulting supernatant was centrifuged at 100,000 × *g* for 60 min to collect microsomes and cytosol. Microsomes were further purified and characterized as previously described [24]. The small intestine was homogenized in 50 mM Tris–HCl (pH 7.5) containing 1 mM DTT, 1 mM EDTA, 10 mM leupeptin, heparin 25 U/mL and 20% glycerol (v/v) [25]. Protein concentration was determined by the Bradford method.

2.4. Measurement of GST activity

GST enzyme activity was determined by measuring the enzyme's ability to catalyze the conjugation of reduced glutathione with 1-chloro-2,4-dinitrobenzene [26]. The conjugation was accompanied by an increase in absorbance at 340 nm. A GST activity assay kit was purchased from Cayman Chemical (Ann Arbor, MI) and followed in accordance with the manufacturer's instructions. The rate of increase was directly proportional to the increases in GST activity in the sample. The absorbance in the wells was kinetically measured at 340 nm at 1-min intervals for 5 min. The activity of GST was expressed as nmol/min/mg homogenate protein.

2.5. Measurement of NQO1 activity

In vitro NQO1 activity was assayed as described previously [27]. Briefly, Hepa 1c1c7 (10 000 cells/well) or HT-29 (15 000 cells/well) were plated into 96 well plates and incubated overnight. Cells were then treated with various concentrations of HS-ASA for 24 h. Cells were lysed in 50 mL 0.8% digitonin, after which we added to each well 200 μL of reaction mixture [25 mM Tris–HCl buffer (pH 7.4), 0.5% bovine serum albumin, 0.025% Tween-20, 5 mM FAD, 30 μM NADP, 1 mM glucose-6-phosphate, 2 U/mL glucose-6-phosphate dehydrogenase baker's yeast (Sigma), 0.3 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrozolium bromide (MTT)] containing 25 mM menadion (2-methyl-1,4 napthoquinone). The reaction was arrested after 5 min by adding 50 μL of solution containing 0.3 mM dicoumarol in 0.5% DMSO and 5 mM potassium phosphate (pH 7.4). Initial reaction rates were measured at 610 nm. *In vivo*, NQO1 activity was determined by adding the cytosolic fractions into the above reaction mixture (final volume 1 mL) in order to initiate the reaction. After 5 min, the absorbance at 610 nm was measured.

2.6. Measurement of glutathione levels

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were measured by a commercial kit by Cayman Chemical

(Ann Arbor, MI). The protocol measures both GSH and GSSG, therefore, the assay reflects total glutathione. The cell homogenate was prepared in ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 0.1 mM EDTA. Then the cell homogenates were centrifuged at 14,000 × *g* for 30 min at 4 °C and the supernatants were collected for glutathione assay. Protein concentrations were determined with a Bio-Rad protein concentration assay kit (Bio-Rad Laboratories). Briefly, 100 μL of cell supernatant was added to an equal volume of the metaphosphoric acid and then centrifuged at 2000 × *g* for 2 min to remove protein. Then 50 μL of 4 M triethanolamine per mL of the supernatant was added to increase the pH of the sample. For total GSH assay, 50 μL of sample was added to 150 μL of a reaction mixture containing 0.4 M 2-(N-morpholino) ethane sulfonic acid, 0.1 M phosphate (pH 6.0), 2 mM EDTA, 0.24 mM NADPH, 0.1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and 0.1 unit glutathione reductase (GR). The reaction was carried out at 37 °C for 25 min and then total glutathione was determined by absorbance at 412 nm using GSSG as standard. For the measurement of GSSG, GSH was removed from the reaction by adding 10 μL of 1 M 2-vinylpyridine solution per mL of supernatant. Then GSSG remained in the reaction was quantified as total GSH assay. The amount of reduced GSH was obtained by subtracting GSSG from total glutathione. Each assay was performed in duplicate and GSH and GSSG were expressed as μmol/g tissue.

2.7. Western blot analysis

After treatment with HS-ASA or vehicle, cells were harvested and lysed in buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% IGEPAL, and 10% glycerol in the presence of proteinase inhibitors. Proteins were fractionated by SDS-PAGE and transferred to supported nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Proteins were probed with primary antibody and visualized using an ECL kit according to the manufacturer's instructions (Amersham Pittsburg, PA). Goat polyclonal antibodies against NQO1 (1:500), rabbit polyclonal antibodies against Nrf2 (1:1000), P4501A1 (1:1000), P4502E1 (1:1000) and mouse monoclonal antibody against tubulin (1:1500) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies to GST (1:1000) were from Cayman Chemical (Ann Arbor, MI).

2.8. Statistics

Data are presented as means ± SEM for the number of observations indicated in the figure legends. Statistical evaluation of the data was performed by one-factor analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. HS-ASA inhibits the growth of HT-29 colon cancer cells and Hepa 1c1c7 murine hepatoma cells

The growth inhibitory effect of HS-ASA was evaluated by the MTT assay in human colon cancer cell line HT-29 and murine hepatoma Hepa1c1c7 cells (Fig. 1). HS-ASA had a significant growth inhibitory effect on HT-29 and Hepa1c1c7 cells in a concentration-dependent manner, with an IC_{50} of 3.2 ± 0.3 μM and 4.2 ± 0.4 μM, respectively (Table 1). The IC_{50} for ASA in both cell lines was greater than 5000 μM at 24 h. Therefore, the ratio ASA/HS-ASA is >1500 in the HT-29 cells and >1000 in Hepa1c1c7 cells, suggesting that HS-ASA is at least 1000–1500-fold more potent than traditional ASA. For further studies, we used the various fold amounts of the IC_{50} concentrations in the two cell lines. In HT-29 cells, the equivalent concentration used for 0.5×, 1×, and 2 × IC_{50} was 1.6, 3.2, and 6.4 μM, and in murine hepatoma Hepa 1c1c7 cells, the equivalent concentration used for 0.5×, 1×, and 2 × IC_{50} was 2.1, 4.2, and 8.4 μM.

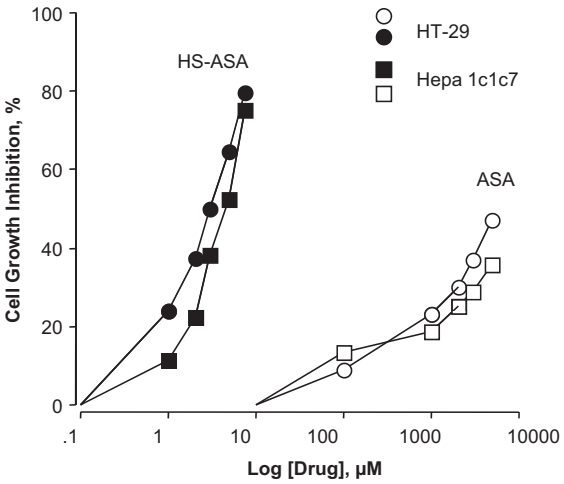

Fig. 1. Effects of HS-ASA on HT-29 human colon cancer and Hepa 1c1c7 cell growth. Cells were treated with various concentrations of HS-ASA or conventional aspirin for 24 h from which IC_{50}s for growth inhibition were determined (Table 1). Results are means ± SEM of 3 different experiments with triplicate plates. Error bars are within the symbols.

3.2. HS-ASA induces phase-II enzyme activity and protein levels

We examined the effect of HS-ASA on the activity of two major phase-II enzymes, NQO1 and GST, in mouse hepatoma Hepa 1c1c7 and human HT-29 colorectal cancer cells. Both cell lines are known to express NQO1 and GST enzymes [28,29]. Cells were treated with various concentrations of HS-ASA or equimolar concentrations of conventional ASA or 1 mM ASA for 24 h, followed by assay for change in GST and NQO1 activities. HS-ASA induced the activities of GST (Fig. 2A and B) and NQO1 (Fig. 3A and B) in these two cell lines. The induction of the enzyme activities were as follows: in Hepa 1c1c7 cells at 1 × IC_{50} HS-ASA induced GST activity by 8.8-fold, and in HT-29 cells at 1 × IC_{50} HS-ASA induced GST activity by 7.5-fold, compared to the basal levels. The parent compound ASA at equimolar concentrations had no effect on either NQO1 or GST enzyme activity in these cell lines (data not shown). However, at 1 mM, ASA caused approximately a 3-fold increase in GST activity in HT-29 cells, 1.83 ± 0.14 U/mg protein to 5.9 ± 0.3 U/mg protein for control and treated cell, respectively. NQO1 activity in Hepa 1c1c7 cells at 1 mM ASA was not determined.

We examined whether H_2S release from HS-ASA is required for induction of NQO1 activity. For this we used NaF, a carboxylesterase enzyme inhibitor, that we shown to inhibit the release of H_2S from HS-ASA in HT-29 cells (please see previous accompanying manuscript). Cells were incubated with 10 μM NaF for 1 h before

Table 1
IC_{50}s for cell growth inhibition.

	IC_{50} μM	
	HT-29	Hepa 1c1c7
ASA	>5000	>5000
HS-ASA	3.2 ± 0.3†	4.2 ± 0.4†
Enhanced potency	>1500	>1000

Colon HT-29 and liver Hepa 1c1c7 cells were treated for 24 h with various concentrations of ASA and HS-ASA as described in Section 2. From their growth curves, IC_{50} values for growth inhibition were determined. Results are means ± SEM of 4 different experiments performed in triplicates.

† $P < 0.001$ compared to ASA.

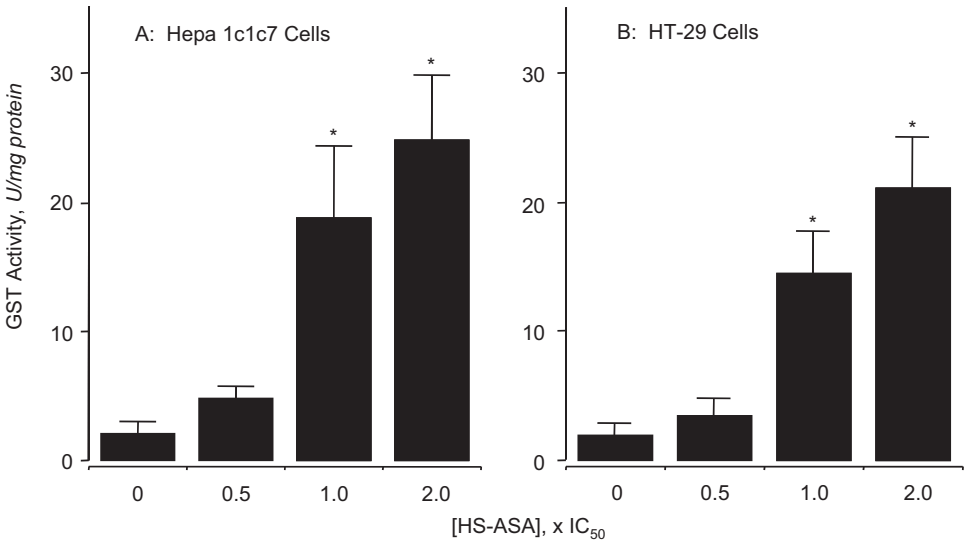

Fig. 2. Effect of HS-ASA on GST enzyme activity in cultured cells. Hepa 1c1c7 (panel A) or HT-29 (panel B) cells were treated with the indicated concentrations of HS-ASA for 24 h before enzyme activity was measured as described in Section 2. HS-ASA increased GST enzyme activity in a concentration dependent manner. Results are mean ± SEM of 3 different experiments performed in duplicates. $^{*}P < 0.05$ compared to untreated cells.

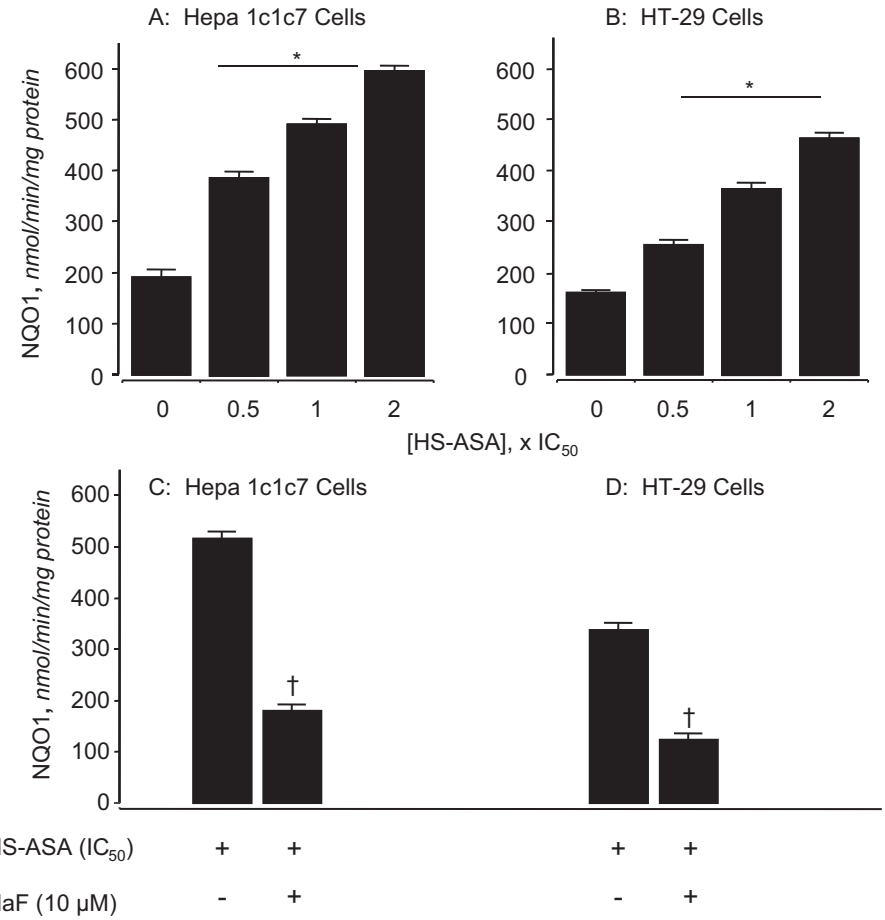

Fig. 3. The activity of the phase-II enzyme NQO1 is induced by HS-ASA. Hepa 1c1c7 (panel A) and HT-29 (panel B) cells were treated with the indicated concentrations of HS-ASA for 24 h. NQO1 enzyme activity was measured as described in Section 2. HS-ASA increased NQO1 enzyme activity is a concentration dependent manner. This increase in enzyme activity was dependent on H_2S since pre-incubation of the cells with NaF a carboxylesterase inhibitor abrogated the increase in enzyme activity back to control levels (panels C and D). The results are mean ± SEM of 3 different experiments performed in duplicate. $^{*}P < 0.02$ compared to untreated cells, $^{\dagger}P < 0.02$ compared to HS-ASA at its IC_{50} in absence of NaF.

treatment with various concentrations of HS-ASA for 6 h. Inhibition of esterase enzyme reversed HS-ASA-mediated induction of NQO1 activity to near basal levels (Fig. 3C and D) Therefore, H_2S release from HS-ASA is required for induction of NQO1 activity.

The effect of HS-ASA on protein levels of the phase-I enzyme CYP1A1 and phase-II enzymes NQO1, GST and UGT1 were examined in HT-29 and Hepa 1c1c7 cells. Cells were treated with HS-ASA for 24 h followed by immunoblotting and detection of protein levels. A dose-dependent increase in NQO1, GST and UGT1 protein levels was observed for 0.5×, 1×, and 2 × IC_{50} concentration of HS-ASA in both cell lines (Fig. 4 upper panel). The increase in protein expression was qualitatively in agreement with changes in enzyme activity. GST protein levels increased in both the colon and liver cell lines with a large basal expression in liver cells, as expected. NQO1 was induced many folds in both liver and colon cells, with a marginal excess in fold in mouse hepatoma cells than in colon cancer cells. This agrees with the enzymatic activity fold increase observed which was slightly more in the mouse hepatoma cells. While NQO1 (but not GST) is known to be more active in the liver cells compared with colon cells, here such a comparison could not be made due to different species tissue types. Therefore, HS-ASA-mediated induction of phase-II enzyme activity is associated with an increase in phase-II protein levels in both cell types.

Many phase-II enzyme inducers also co-induce phase-I enzymes [8]. Therefore, we evaluated the effects of HS-ASA on two P450 enzyme families. CYP1A1 is a very important phase-I metabolizing enzyme in humans. It is a polycyclic aromatic hydrocarbon hydroxylase which is mostly extra-hepatic, and is expressed in the small intestine, placenta, lung, and skin [30]. The enzyme CYP2E1, belonging to another P450 family, which is involved in alcohol metabolism. It is expressed in liver, kidney, intestine and the lung [30]. Interestingly, HS-ASA increased the protein levels of CYP1A1 and CYP2E1 in both HT-29 and Hepa 1c1c7 cells (Fig. 4).

3.3. Induction of phase-II enzymes is associated with Nrf2- increase

In the cytoplasm the Nrf2 transcription factor is bound to Keap1. Nrf2 translocates to the nucleus when certain inducers disrupt the complex formation with Keap1, where it leads to the transcription of phase-II genes. We examined whether HS-ASA-mediated induction of phase-II enzymes occurs by modulating Nrf2. For a preliminary study we determined nuclear level of Nrf2 in response to HS-ASA in Hepa 1c1c7 and HT-29 cells. In both cell types, HS-ASA increased the expression of Nrf2 in a concentration-dependent manner (Fig. 4 lower panel).

3.4. HS-ASA induces the enzymatic activities of NQO1 and GST while it also increases protein levels of NQO1, GSH and GST in vivo

To determine whether HS-ASA induces hepatic phase-II enzymes *in vivo*, we measured its effect on the enzymatic activities of GST and NQO1; the total GSH levels, and also on the protein levels of NQO1, GSH and GST in male Wistar rats. As

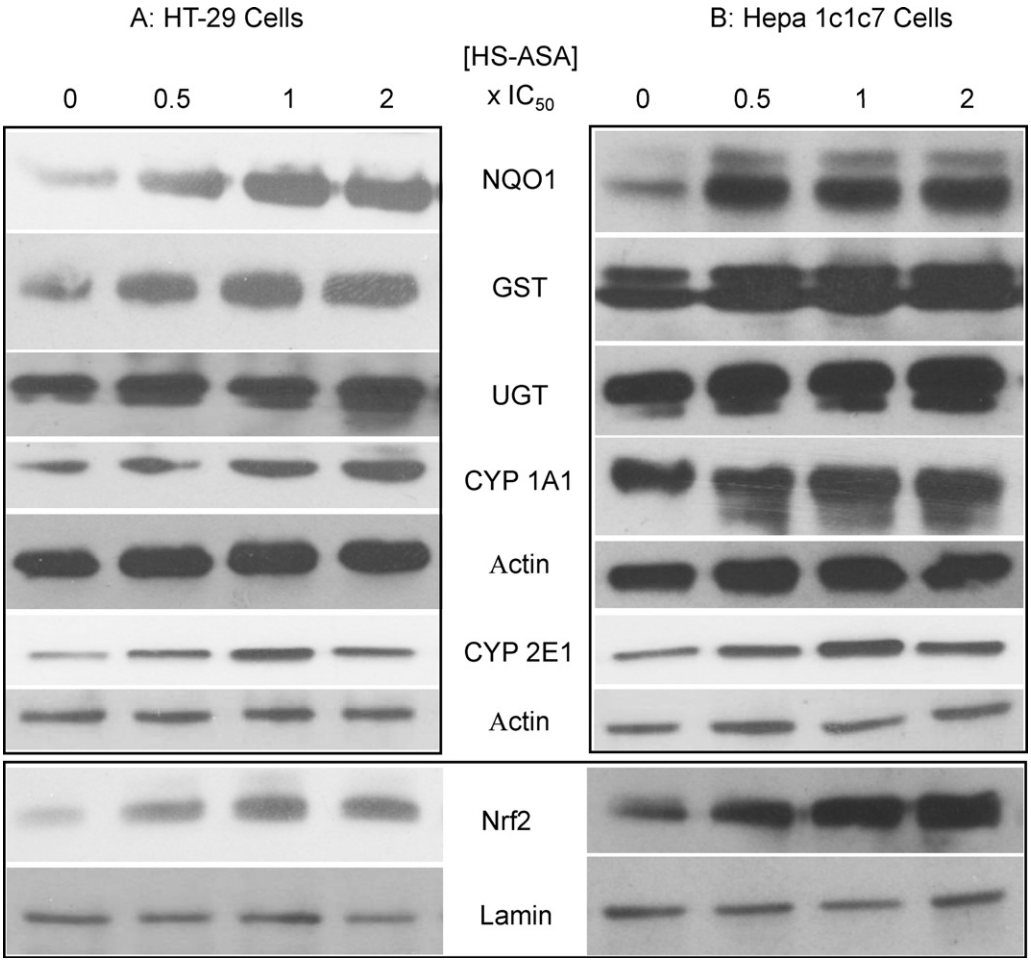

Fig. 4. Effect of HS-ASA on phase-I and phase-II metabolizing enzymes. Hepa 1c1c7 (panel A) or HT-29 (panel B) cells were incubated with the indicated concentrations of HS-ASA for 24 h, after which cell extracts were prepared for immunoblotting as described in Section 2. Representative blots (one of two determinations) are shown here. HS-ASA increased NQO1, GST, UGT, CYP1A1 and CYP2E1 protein levels. Nuclear proteins were also immunoblotted for Nrf2 and Lamin (bottom panel).

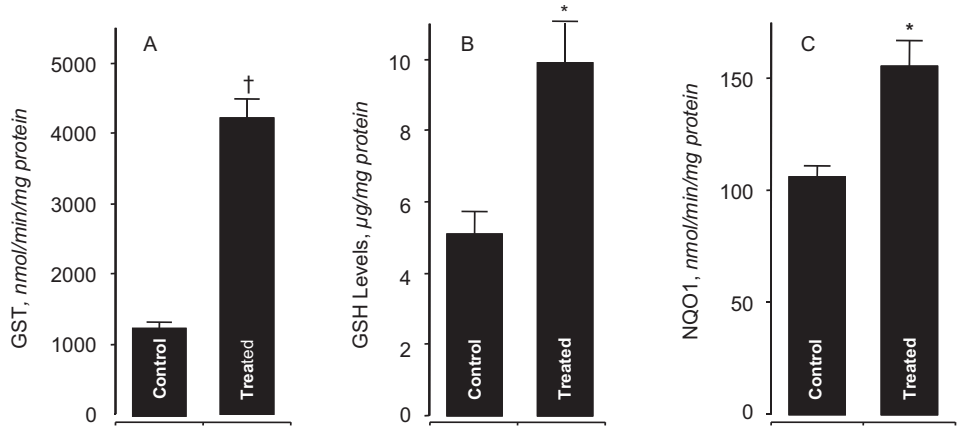

Fig. 5. HS-ASA induces phase-II enzyme activity and GSH levels *in vivo*. Male Wistar rats ($N = 4$ per group) were treated with HS-ASA (100 mg/kg body weight) for 3 weeks after which liver microsomes and cytosol were prepared as described in Section 2. HS-ASA treatment induced GST and NQO1 enzyme activities panels A and C, respectively. GSH levels were also increased in the livers of the HS-ASA-treated rats (panel B). Results are mean ± SEM for 4 rats in each group. $^{*}P < 0.05$; $^{\dagger}P < 0.01$ compared to vehicle treated rats.

Table 2
GST and NQO1 activity in rat extra hepatic tissues.

Tissue	GST (nmol/min/mg)		NQO1 (nmol/min/mg)	
	Untreated	Treated	Untreated	Treated
Kidney	796 ± 53	918 ± 51	197 ± 8	320 ± 10*
Colon	487 ± 22	755 ± 35*	48 ± 3	73 ± 4*

GST and NQO1 enzymatic activity were measured in kidney and colon of untreated and HS-ASA treated rats as described in Section 2. Results are means ± SEM of 4 different animals in each group.
* $P < 0.05$ compared to untreated rats.

shown in Fig. 5A, compared with controls, after 4 weeks of treatment, HS-ASA significantly induced the activity of GST by 3.4-fold, from 1234 ± 106 in control to 4218 ± 261 nmol/min/mg protein in the treated rats, respectively ($P < 0.01$). GSH levels were increased 2-fold, from 5.2 ± 0.5 to 9.8 ± 0.8 μg/mg protein. NQO1 enzyme activity was increased 1.5-fold from 101 ± 5.2 to 155 ± 9.7 nmol/min/mg protein in control and treated animals, respectively ($P < 0.05$, Fig. 5C). We also evaluated the effects of HS-ASA on extra hepatic tissues of rats, namely the kidneys and the colon. Similar to the liver, HS-ASA significantly increased ($P < 0.05$), the activity of GST (1.5-fold) and NQO1 (1.6-fold) in the colon (Table 2). In the kidneys, NQO1 enzyme activity was also increased (1.6-fold), however, GST enzyme activity although it was increased in the treated animals, this increase was not statistically significant (Table 2). Immunoblot analysis (Fig. 6) demonstrated that HS-ASA moderately increased the liver cytosolic GST, NQO1, and substantially increased UGT1A1 protein levels. The levels of the phase-I enzymes, CYP1A1 and CYP2E1, did not differ between the untreated and treated groups. This finding strongly suggests that *in vivo* HS-ASA is a monofunctional inducer of phase-II metabolizing enzymes.

4. Discussion

The present study identifies an H_2S-donating derivative of aspirin (HS-ASA) as an inducer of phase-II metabolizing enzyme in HT-29 human colon cancer cells and in Hepa 1c1c7 mouse hepatoma cells. The importance of phase-II enzyme induction in cancer prevention has been well established [31]. HS-ASA increased NQO1 and GST protein levels and enzymatic activities. In these cell types, it also increased the protein levels of P450 1A1 a phase-I metabolizing enzyme. HS-ASA was also effective at inducing the phase-II enzyme response *in vivo*. Rats treated by gavage with 100 mg/kg body weight for 7 days had an increase in NQO1, GSH and GST levels in the kidney, colon, and liver. Although, the kidney and colon also participate in the detoxification process, the liver is the major site for xenobiotic metabolism and transformation. HS-ASA also demonstrated remarkable improvement in cell growth inhibitory activity and potency as compared with the parent compound ASA, implying an active pharmacological role for H_2S in this molecule. Based on these properties of HS-ASA in HT-29 cells and Hepa 1c1c7 mouse hepatoma cells, HS-ASA has a good potential as a colon cancer preventive agent. Current understanding of the biology of the gasotransmitter H_2S is still in the developmental stages compared to that of nitric oxide. It is well established that the H_2S rapidly travels through cell membranes without using specific transporters, exerts a host of biological effects on various biological targets ranging from cytotoxic to cytoprotective effects [15,32–36]. Among the cellular processes, H_2S can inhibit the adhesion and activation of neutrophil granulocytes [35] and platelet aggregation [37–41]. At the mechanistic level, upregulation of anti-inflammatory genes such as *HO1*, and downregulation of inflammatory genes (*COX2*, *FOS*, *IL1*β) are known to occur [33,42].

The results reported in this study demonstrate that HS-ASA increases GST enzyme activity and protein levels *in vitro.* It also increases GST and GSH levels *in vivo.* GSTs catalyze the conjugation of electrophilic compound with reduced glutathione [43]. The enhancement of GST enzyme activity and its protein levels is the working mechanism of several anticarcinogens [44,45]. Substances that specifically increase conjugation systems are considered to be more potent inhibitors of cancer, thereby explaining in part, the anticarcinogenic properties of NSAIDs in the upper gastrointestinal tract [46,47]. Allyl sulfides which are constituents of garlic and generate H_2S have also been shown to induce GSTs in animal models [48,49]. In addition to GST induction and anti-cancer activity, compounds that release H_2S have been used in multiple models of inflammation [38,42,50–55].

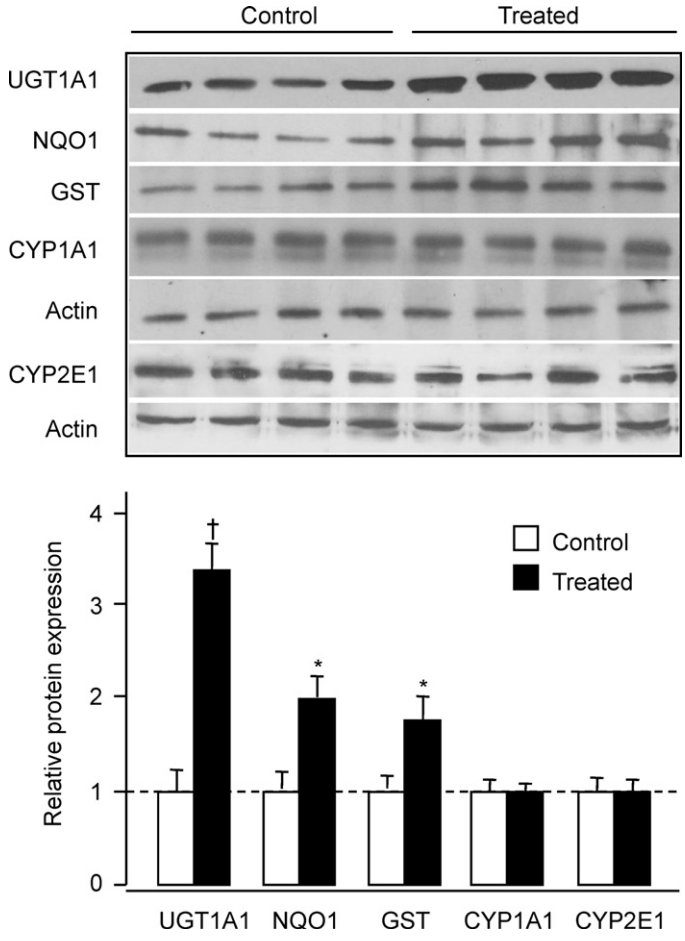

Fig. 6. HS-ASA is a mono-functional inducer of hepatic phase-II enzymes. Upper panel: Liver microsomes and cytosol from HS-ASA-treated Wistar rats were subjected to immunoblotting for phase-I (CYP1A1 and CYP2E1) and phase-II (GST, UGT1A1, NQO1) enzymes. Actin was used as a housekeeping gene. The four lanes represent individual rats. Lower panel: The expression of each protein (fold over control) for each group of animals (mean ± SEM, $N = 4$). $^{*}P < 0.05$, $^{\dagger}P < 0.01$ compared with the corresponding control.

Two important considerations regarding HS-ASA and the role of H_2S emerge. First, it is clear that H_2S release from HS-ASA is required for the induction of NQO1 activity, since NaF reversed this induction. Second, the pharmacological role of H_2S conjugation with ASA is highlighted by the inability of the parent compound ASA to induce GST and NQO1 activities at equimolar doses.

HS-ASA increased UGT1A1 protein expression in both the HT-29 and Hepa 1c1c7 cells. UGTs are phase-II enzymes that are membrane-bound, localized in the endoplasmic reticulum of liver and extrahepatic tissues [56]. The primary role of UGT1A1 activity is the glucuronidation of various exogenous drugs and endogenous substrates such as bilirubin and estrogen. NQO1 is primarily cytosolic and catalyzes the reduction of a variety of quinones. Down-regulation of *UGT1A* gene expression also was found in an early stage of hepatocarcinogenesis [57]. Generally, the ability to induce phase-II enzymes without inducing or decreasing phase-I enzyme activities appears to be an ideal quality of a chemopreventive agent. However, phase-II enzyme inducers may also induce phase I enzymes [8]. In this respect, a promising observation in our *in vivo* study was the ability of HS-ASA to induce phase-II enzymes without affecting the phase-I enzyme P4501A1 and P4502E1, although there was some increases in cultured cells. This observation underscores the dangers of translating *in vitro* data to whole animals.

Several detoxifying and cytoprotective enzymes are under the regulatory control of Nrf2 [58], such as expression of certain GSTs and NQO1. It is possible that HS-ASA-mediated increases in GST and NQO1 are probably brought about, in part, through increase in Nrf2 protein levels. This is evidenced by the concentration-dependent increase of Nrf2 expression by HS-ASA in cultured cells. It may be envisaged that the translocation of overexpressed Nrf2 occurs to the nucleus, followed by induction of ARE-containing genes, as has been reported for sulforaphane treated cancer cells [59–61]. Although our data do not demonstrate translocation to the nucleus, the ability of HS-ASA to upregulate Nrf2, NQO1, UGT, and GST levels, suggests that HS-ASA can potentially prevent carcinogenesis. In conclusion, HS-ASA co-induces multiple phase-II metabolizing enzymes and thus may be regarded as a monofunctional inducer. This represents one mechanism by which HS-ASA exhibits chemopreventive properties. The mechanism(s) of action including glutathione-dependent and independent pathways, and H_2S enzymatic release and its implications warrant further study.

Acknowledgment

Supported in part by the National Cancer Institute through a subcontract from ThermoFisher, contract #FBS-43312-26.

References

[1] Gonzalez FJ, Gelboin HV. Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. Drug Metab Rev 1994;26:165–83.
[2] Raunio H, Husgafvel-Pursiainen K, Anttila S, Hietanen E, Hirvonen A, Pelkonen O. Diagnosis of polymorphisms in carcinogen-activating and inactivating enzymes and cancer susceptibility—a review. Gene 1995;159:113–21.
[3] Wilkinson Jt, Clapper ML. Detoxication enzymes and chemoprevention. Proc Soc Exp Biol Med 1997;216:192–200.
[4] Jana S, Mandlekar S. Role of phase II drug metabolizing enzymes in cancer chemoprevention. Curr Drug Metab 2009;10:595–616.
[5] Ross D, Siegel D. NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase), functions and pharmacogenetics. Methods Enzymol 2004;382:115–44.
[6] Chasseaud LF. The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents. Adv Cancer Res 1979;29:175–274.
[7] Yang G, Wu L, Wang R. Pro-apoptotic effect of endogenous H2S on human aorta smooth muscle cells. FASEB J 2006;20:553–5.
[8] Lee JS, Surh YJ. Nrf2 as a novel molecular target for chemoprevention. Cancer Lett 2005;224:171–84.
[9] Morimitsu Y, Nakagawa Y, Hayashi K, Fujii H, Kumagai T, Nakamura Y, et al. A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway. J Biol Chem 2002;277:3456–63.
[10] Slocum SL, Kensler TW. Nrf2: control of sensitivity to carcinogens. Arch Toxicol 2011;85:273–84.
[11] Kashfi K. Anti-inflammatory agents as cancer therapeutics. Adv Pharmacol 2009;57:31–89.
[12] Cooper K, Squires H, Carroll C, Papaioannou D, Booth A, Logan RF, et al. Chemoprevention of colorectal cancer: systematic review and economic evaluation. Health Technol Assess 2010;14:1–206.
[13] Half E, Arber N. Colon cancer: preventive agents and the present status of chemoprevention. Expert Opin Pharmacother 2009;10:211–9.
[14] Geng B, Chang L, Pan C, Qi Y, Zhao J, Pang Y, et al. Endogenous hydrogen sulfide regulation of myocardial injury induced by isoproterenol. Biochem Biophys Res Commun 2004;318:756–63.
[15] Whiteman M, Armstrong JS, Chu SH, Jia-Ling S, Wong BS, Cheung NS, et al. The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite 'scavenger'. J Neurochem 2004;90:765–8.
[16] Whiteman M, Cheung NS, Zhu YZ, Chu SH, Siau JL, Wong BS, et al. Hydrogen sulphide: a novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain. Biochem Biophys Res Commun 2005;326:794–8.
[17] Yan SK, Chang T, Wang H, Wu L, Wang R, Meng QH. Effects of hydrogen sulfide on homocysteine-induced oxidative stress in vascular smooth muscle cells. Biochem Biophys Res Commun 2006;351:485–91.
[18] Wang R. Two's company, three's a crowd: can H2S be the third endogenous gaseous transmitter. FASEB J 2002;16:1792–8.
[19] Schicho R, Krueger D, Zeller F, Von Weyhern CW, Frieling T, Kimura H, et al. Hydrogen sulfide is a novel prosecretory neuromodulator in the Guinea-pig and human colon. Gastroenterology 2006;131:1542–52.
[20] Wallace JL, Vong L, McKnight W, Dicay M, Martin GR. Endogenous and exogenous hydrogen sulfide promotes resolution of colitis in rats. Gastroenterology 2009;137:569–78.

[21] Li L, Rossoni G, Sparatore A, Lee LC, Del Soldato P, Moore PK. Anti-inflammatory and gastrointestinal effects of a novel diclofenac derivative. Free Radic Biol Med 2007;42:706–19.
[22] Wallace JL, Caliendo G, Santagada V, Cirino G, Fiorucci S. Gastrointestinal safety and anti-inflammatory effects of a hydrogen sulfide-releasing diclofenac derivative in the rat. Gastroenterology 2007;132:261–71.
[23] Gamble MS, Cook GA. Alteration of the apparent Ki of carnitine palmitoyltransferase for malonyl-CoA by the diabetic state and reversal by insulin. J Biol Chem 1985;260:9516–9.
[24] Park EA, Mynatt RL, Cook GA, Kashfi K. Insulin regulates enzyme activity, malonyl-CoA sensitivity and mRNA abundance of hepatic carnitine palmitoyltransferase-I. Biochem J 1995;310(Pt 3):853–8.
[25] Kashfi K, McDougall CJ, Dannenberg AJ. Comparative effects of omeprazole on xenobiotic metabolizing enzymes in the rat and human. Clin Pharmacol Ther 1995;58:625–30.
[26] Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 1974;249:7130–9.
[27] Prochaska HJ, Santamaria AB. Direct measurement of NAD(P)H:quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. Anal Biochem 1988;169:328–36.
[28] Beaumont PO, Moore MJ, Ahmad K, Payne MM, Lee C, Riddick DS. Role of glutathione S-transferases in the resistance of human colon cancer cell lines to doxorubicin. Cancer Res 1998;58:947–55.
[29] O'Dwyer PJ, Yao KS, Ford P, Godwin AK, Clayton M. Effects of hypoxia on detoxicating enzyme activity and expression in HT29 colon adenocarcinoma cells. Cancer Res 1994;54:3082–7.
[30] McKinnon RA, McManus ME. Localization of cytochromes P450 in human tissues: implications for chemical toxicity. Pathology 1996;28:148–55.
[31] Talalay P, Fahey JW. Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. J Nutr 2001;131:3027S–33S.
[32] Nagai Y, Tsugane M, Oka J, Kimura H. Hydrogen sulfide induces calcium waves in astrocytes. FASEB J 2004;18:557–9.
[33] Oh GS, Pae HO, Lee BS, Kim BN, Kim JM, Kim HR, et al. Hydrogen sulfide inhibits nitric oxide production and nuclear factor-kappaB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. Free Radic Biol Med 2006;41:106–19.
[34] Kimura Y, Dargusch R, Schubert D, Kimura H. Hydrogen sulfide protects HT22 neuronal cells from oxidative stress. Antioxid Redox Signal 2006;8:661–70.
[35] Rinaldi L, Gobbi G, Pambianco M, Micheloni C, Mirandola P, Vitale M. Hydrogen sulfide prevents apoptosis of human PMN via inhibition of p38 and caspase 3. Lab Invest 2006;86:391–7.
[36] Rose P, Moore PK, Ming SH, Nam OC, Armstrong JS, Whiteman M. Hydrogen sulfide protects colon cancer cells from chemopreventative agent beta-phenylethyl isothiocyanate induced apoptosis. World J Gastroenterol 2005; 11:3990–7.
[37] Zagli G, Patacchini R, Trevisani M, Abbate R, Cinotti S, Gensini GF, et al. Hydrogen sulfide inhibits human platelet aggregation. Eur J Pharmacol 2007;559:65–8.
[38] Zhu YZ, Wang ZJ, Ho P, Loke YY, Zhu YC, Huang SH, et al. Hydrogen sulfide and its possible roles in myocardial ischemia in experimental rats. J Appl Physiol 2007;102:261–8.
[39] Sodha NR, Clements RT, Feng J, Liu Y, Bianchi C, Horvath EM, et al. The effects of therapeutic sulfide on myocardial apoptosis in response to ischemia-reperfusion injury. Eur J Cardiothorac Surg 2008;33:906–13.
[40] Elrod JW, Calvert JW, Morrison J, Doeller JE, Kraus DW, Tao L, et al. Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. Proc Natl Acad Sci USA 2007;104:15560–5.
[41] Sivarajah A, McDonald MC, Thiemermann C. The production of hydrogen sulfide limits myocardial ischemia and reperfusion injury and contributes to the cardioprotective effects of preconditioning with endotoxin, but not ischemia in the rat. Shock 2006;26:154–61.
[42] Zhang H, Zhi L, Moore PK, Bhatia M. Role of hydrogen sulfide in cecal ligation and puncture-induced sepsis in the mouse. Am J Physiol Lung Cell Mol Physiol 2006;290:L1193–201.
[43] Kashfi K, Rimarachin JA, Weksler BB, Dannenberg AJ. Differential induction of glutathione S-transferase in rat aorta versus liver. Biochem Pharmacol 1994;47:1903–7.
[44] Beckett GJ, Hayes JD. Glutathione S-transferases: biomedical applications. Adv Clin Chem 1993;30:281–380.
[45] Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol 1995;30:445–600.
[46] van Lieshout EM, Tiemessen DM, Peters WH, Jansen JB. Effects of nonsteroidal anti-inflammatory drugs on glutathione S-transferases of the rat digestive tract. Carcinogenesis 1997;18:485–90.
[47] Van der Logt EM, Roelofs HM, van Lieshout EM, Nagengast FM, Peters WH. Effects of dietary anticarcinogens and nonsteroidal anti-inflammatory drugs on rat gastrointestinal UDP-glucuronosyltransferases. Anticancer Res 2004; 24:843–9.
[48] Srivastava SK, Hu X, Xia H, Zaren HA, Chatterjee ML, Agarwal R, et al. Mechanism of differential efficacy of garlic organosulfides in preventing benzo(a)-pyrene-induced cancer in mice. Cancer Lett 1997;118:61–7.
[49] Gudi VA, Singh SV. Effect of diallyl sulfide, a naturally occurring anti-carcinogen, on glutathione-dependent detoxification enzymes of female CD-1 mouse tissues. Biochem Pharmacol 1991;42:1261–5.
[50] Szabo C, Kiss L, Pankotai E. Cytoprotective and antiinflammatory effects of hydrogen sulfide in macrophages and in mice. Crit Care 2007;11:P2.
[51] Zanardo RC, Brancaleone V, Distrutti E, Fiorucci S, Cirino G, Wallace JL. Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB J 2006;20:2118–20.
[52] Johansen D, Ytrehus K, Baxter GF. Exogenous hydrogen sulfide (H2S) protects against regional myocardial ischemia-reperfusion injury—evidence for a role of K ATP channels. Basic Res Cardiol 2006;101:53–60.
[53] Distrutti E, Sediari L, Mencarelli A, Renga B, Orlandi S, Russo G, et al. 5-Amino-2-hydroxybenzoic acid 4-(5-thioxo-5H-[1,2]dithiol-3yl)-phenyl ester (ATB-429), a hydrogen sulfide-releasing derivative of mesalamine, exerts antinociceptive effects in a model of postinflammatory hypersensitivity. J Pharmacol Exp Ther 2006;319:447–58.
[54] Blackstone E, Roth MB. Suspended animation-like state protects mice from lethal hypoxia. Shock 2007;27:370–2.
[55] Fiorucci S, Antonelli E, Distrutti E, Rizzo G, Mencarelli A, Orlandi S, et al. Inhibition of hydrogen sulfide generation contributes to gastric injury caused by anti-inflammatory nonsteroidal drugs. Gastroenterology 2005; 129:1210–24.
[56] King CD, Rios GR, Green MD, Tephly TR. UDP-glucuronosyltransferases. Curr Drug Metab 2000;1:143–61.
[57] Strassburg CP, Manns MP, Tukey RH. Differential down-regulation of the UDP-glucuronosyltransferase 1A locus is an early event in human liver and biliary cancer. Cancer Res 1997;57:2979–85.
[58] Wai CY, Miller DS. Urinary bladder cancer. Clin Obstet Gynecol 2002;45: 844–54.
[59] Shapiro TA, Fahey JW, Wade KL, Stephenson KK, Talalay P. Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans. Cancer Epidemiol Biomarkers Prev 2001;10:501–8.
[60] Kelloff GJ. Perspectives on cancer chemoprevention research and drug development. Adv Cancer Res 2000;78:199–334.
[61] Sporn MB, Suh N. Chemoprevention of cancer. Carcinogenesis 2000;21: 525–30.