Hydrogen sulfide induces serum-independent cell cycle entry in nontransformed rat intestinal epithelial cells[1]

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SPECIFIC AIM

Hydrogen sulfide (H_2S), produced by commensal sulfate-reducing bacteria, is an environmental insult that potentially contributes to chronic intestinal epithelial disorders. The objectives of this study were to characterize molecular responses of nontransformed intestinal epithelial cells (IEC-18) to physiological concentrations of the bacterial catabolite and reducing agent H_2S.

PRINCIPAL FINDINGS

1. Hydrogen sulfide reduces the intracellular redox environment and triggers cell cycle entry

Exposure of IEC-18 cells to H_2S (1 mM NaHS) rapidly increased ($P<0.05$) the NAD(P)H/NAD(P) ratio, reduced ($P<0.05$) the intracellular redox environment, and inhibited ($P<0.05$) mitochondrial respiratory activity. The addition of 0.2–5 mM NaHS for 4 h increased the IEC-18 proliferative cell fraction ($P<0.05$), as evidenced by flow cytometric analysis of the cell cycle and PCNA expression (**Fig. 1*A, B***), whereas apoptosis occurred only at the highest concentration of H_2S (5 mM NaHS).

2. Steady-state c-*Jun* mRNA concentrations are increased in a dose-dependent manner by H_2S

The presence of NaHS increased ($P<0.05$) steady-state c-*Jun* mRNA concentrations two- to threefold in a dose-responsive manner after 30 min exposure compared with controls (**Fig. 2*A, B***).

3. Inhibition of mitogen-activated protein kinase (MAPK) decreases H_2S-induced PCNA expression

To determine the involvement of MAP kinases in mitogenic signaling by H_2S, cells were pretreated with MAP kinase-specific inhibitors for 90 min, then incubated with 1 mM NaHS for 4 h. Inhibition of either extracellular signal-regulated kinase (ERK) or p38 kinase in the presence of NaHS decreased ($P<0.05$) the percentage of PCNA-positive cells to levels similar to cells treated with NaHS alone. Inhibition of Jun-N-terminal kinase (JNK) did not significantly alter NaHS-induced PCNA expression (Fig. 1*C*).

4. Microarray analysis confirmed an increase in MAPK-mediated proliferative activity

To further resolve molecular responses of IEC-18 cells to H_2S, microarray analysis was performed using the rat genome U34A Array™. Both control and NaHS-treated IEC-18 cells expressed 49% (4243 of 8600) of the Affymetrix gene set. Of these 4243 genes, 19% (818) were sensitive to NaHS. Seventeen transcripts (0.40%) displayed 2- to 3.8-fold greater expression in NaHS-treated than control cells whereas 20 transcripts (0.47%) displayed 2- to 3.4-fold lower expression in NaHS-treated vs. control cells. Because replicate microarray experiments were performed, fold expression changes lower than 2 that were statistically significant were also considered to avoid the exclusion of potentially important molecular responses. Using a cutoff probability value of 0.05, an additional 8.4% (356 of 4243) and 10% (424 of 4243) of the genes were respectively up- or down-regulated in H_2S-treated vs. control cells. Up- and down-regulated genes were classified according to Gene Ontology (http://www.geneontology.org/) and categorized by function. Microarray analysis confirmed an increase ($P<0.05$) in MAPK-mediated proliferative activity likely reflecting the reduced redox environment of NaHS-treated cells, as the expression of numerous other genes involved in growth and proliferation, intracellular signaling pathways (e.g., Ras-MAPK pathway, TGF-β pathway), electron transport and ATP generation, and redox homeostasis was differentially affected by the presence of this bacterial-derived reductant.

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[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.02-0883fje; doi: 10.1096/fj.02-0883fje

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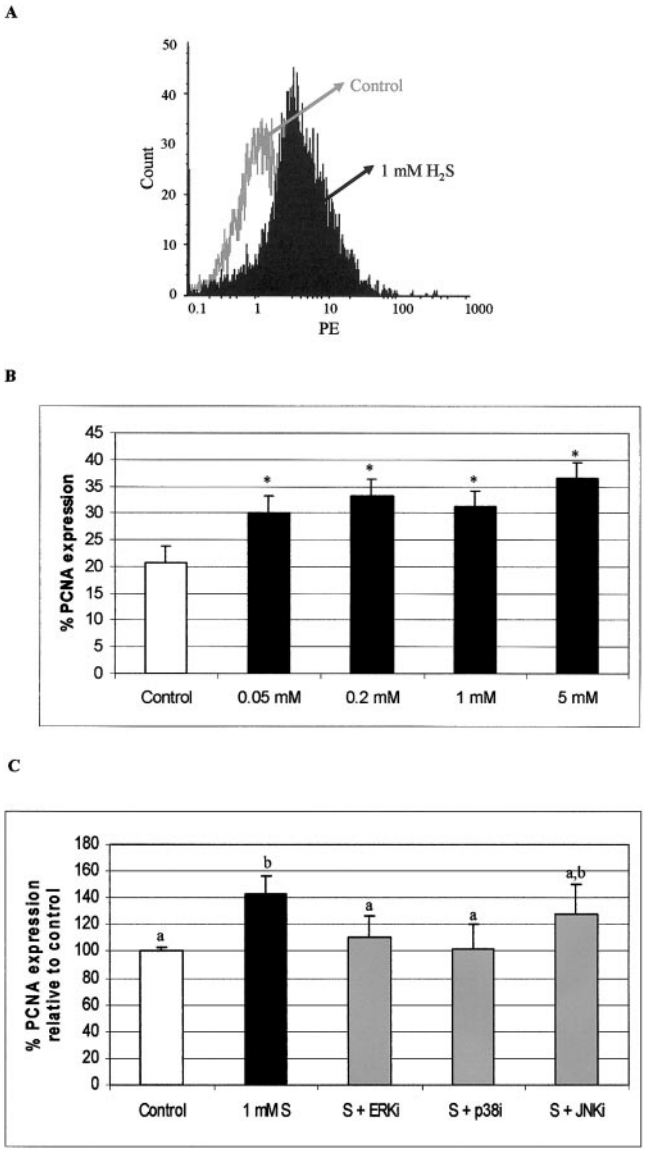

Figure 1. Flow cytometric analysis of PCNA expression in control and H_2S-treated IEC-18 cells with or without MAPK inhibitors: *A)* Representative flow cytometric analysis of PCNA expression in control and H_2S (1 mM NaHS) -treated cells. *B)* Quantitative comparison of PCNA expression in control and H_2S-treated cells. Hydrogen sulfide induced early cell cycle entry in nontransformed intestinal epithelial cells. Data shown are mean values ± SE of 2 independent experiments (n=6). *Significantly different compared with respective controls (P<0.05). *C)* PD98059 (10 μM), a specific blocker of ERK (ERKi), SP600125, inhibitor of Jun N-terminal kinase (JNKi), and SB202190 (100 nM), an antagonist of p38 MAP kinase (p38 K_i). ERK and p38 MAPK inhibitors reduce the H_2S-induced increase in PCNA expression. Data shown are mean values ± SE of 2 independent experiments (n=6). Columns not sharing a common letter are significantly different (P<0.05).

CONCLUSIONS AND SIGNIFICANCE

Ulcerative colitis (UC) and sporadic colorectal cancer are thought to require genetic and environmental determinants, and both are characterized by a hyperproliferative epithelium. Despite the clear evidence for involvement of the luminal environment, specific agents that contribute to the development of UC or sporadic colorectal cancer in susceptible individuals remain undefined. This study provides molecular support that H_2S may be one such agent.

In agreement with previous data, H_2S in concentrations commonly found in the intestine inhibited mitochondrial respiratory activity of IEC-18 cells, leading to a more reduced intracellular redox environment. When IEC-18 cells were treated with varying concentrations of NaHS, a rapid but transient increase of steady-state c-*Jun* mRNA concentrations was observed compared with control cells. These findings are consistent with studies demonstrating similar kinetics of c-*Jun* expression in cells subjected to hypoxia or treated with metabolic inhibitors such as rotenone and cyanide. The resulting alteration in redox homeostasis may have promoted early cell cycle entry mediated by MAPK, which is consistent with the observed up-regulation of genes encoding proteins in the MAPK signaling pathway and others related to proliferative activity. These results are also consistent with the previously reported involvement of ERK and p38 MAP kinase in hypoxia-mediated proliferation in a variety of cell types.

Combined, the data indicate that H_2S-induced hyp-

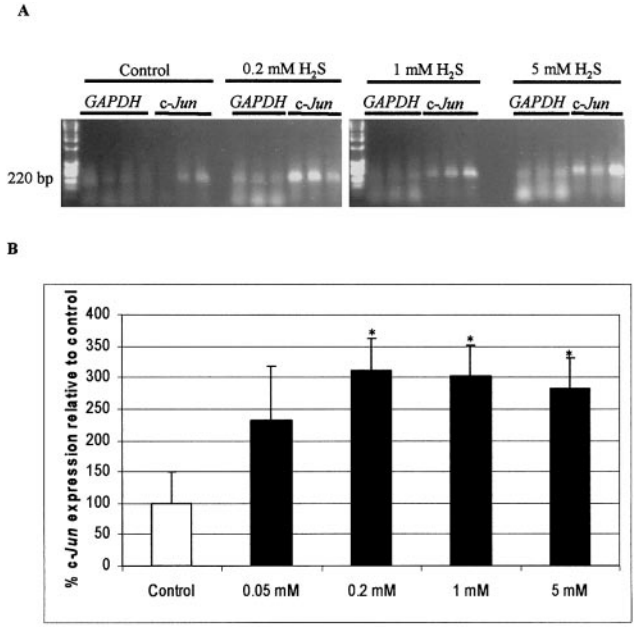

Figure 2. c-*Jun* mRNA expression in control and H_2S-treated IEC-18 cells. Cells were exposed to 0, 0.05, 0.2, 1, and 5 mM NaHS for 30 min, after which RNA was extracted and analyzed by quantitative RT-PCR. *A)* Representative agarose gels of quantitative RT-PCR amplicons (220 bp) of *GAPDH* and c-*Jun* for control and 0.2–5 mM NaHS-treated samples. *B)* Histogram of relative amounts of c-*Jun* mRNA detected by quantitative RT-PCR amplification after normalization for *GAPDH* concentrations. Hydrogen sulfide induces the immediate-early accumulation of c-*Jun* mRNA. Data shown are mean values ± SE for 2 independent experiments performed in triplicate (n=6). *Significantly different from control (P<0.05).

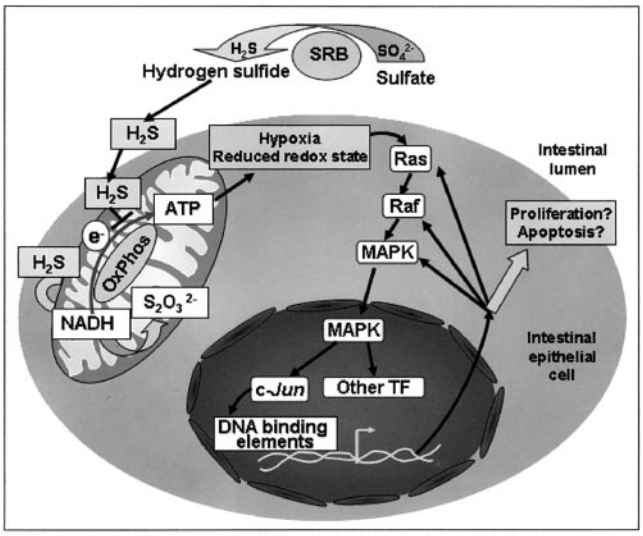

Figure 3. Overview of the main findings and testable hypotheses formulated from the experimental work. Sulfate derived from endogenous secretions (sulfomucins, taurocholic acid, inorganic sulfate) or the diet is transformed into H_2S through anaerobic respiration by sulfate-reducing bacteria. Hydrogen sulfide diffuses into intestinal epithelial cells, where it can inhibit the metabolic respiratory activity of mitochondria, which attempt to detoxify H_2S via its active oxidation to less harmful thiosulfate. The resulting reduced redox environment then triggers the Ras/MAPK pathway, resulting in activation of c-*Jun* and other undefined transcription factors (TF) that up-regulate genes involved in both proliferation and apoptosis. Dependent on genetic background, this event may result in hyperproliferation, a phenotype common to UC and colorectal cancer.

oxia may trigger nontransformed intestinal cells to proliferate via a MAP kinase-dependent mechanism, which activates c-*Jun* expression, culminating in increased PCNA expression and transition to the proliferative phases of the cell cycle. However, a similar up-regulation of c-*Jun* and PCNA expression has been described in cells "en route to apoptosis," indicating that early apoptotic cells, which lack obvious physiological signs of apoptosis, also undergo events typical of early cell cycle entry. Although late apoptotic events were not observed in NaHS-treated cells except in response to the 5 mM NaHS treatment, the possibility that the observed molecular responses may instead reflect an early apoptotic response to H_2S exposure cannot be excluded.

An interesting finding was the up-regulation of several genes associated with the transforming growth factor β (TGF-β) signaling pathway. Although the latter observation is in apparent conflict with the up-regulation of genes involved in cell cycle progression given the anti-proliferative properties of TGF-β signaling, this response may reflect the molecular expression of efforts of NaHS-treated cells to prevent uncontrolled proliferation. The observed down-regulation of c-*Myc* is consistent with this hypothesis, as decreased c-*Myc* expression is commonly observed in most cells exhibiting an antiproliferative response to TGF-β. Inhibition of proliferation in response to TGF-β is only effective during the early G_1 phase, and cells committed to DNA replication in the late G_1 phase will therefore proceed undisturbed by TGF-β until cells reenter the G_1 phase. It has been suggested that TGF-β serves as a rescue mechanism to eliminate preneoplastic cells, as may have been the case in NaHS-treated IEC-18 cells. Loss of cell responsiveness to the antiproliferative properties of TGF-β because of mutations in TGF-β pathway components may then predispose to cancer. Mutations in genes coding for TGF-β pathway components are found in the majority of colorectal cancers.

Together, results from this study identify functional pathways by which H_2S may initiate epithelial dysregulation and thus provide a working model to further define molecular mechanisms by which H_2S may contribute to the development of UC or potentially malignant transformation of intestinal epithelial cells. It appears crucial to understand how genetic background may affect intestinal epithelial cell responses to the bacterial-derived environmental insult H_2S. FJ