Molecular Biology of the Cell
Vol. 21, 212–217, January 1, 2010

Hydrogen Sulfide Increases Hypoxia-inducible Factor-1 Activity Independently of von Hippel–Lindau Tumor Suppressor-1 in *C. elegans*

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Submitted March 11, 2009; Revised October 9, 2009; Accepted October 23, 2009
Monitoring Editor: William P. Tansey

Rapid alteration of gene expression in response to environmental changes is essential for normal development and behavior. The transcription factor hypoxia-inducible factor (HIF)-1 is well known to respond to alterations in oxygen availability. In nature, low oxygen environments are often found to contain high levels of hydrogen sulfide (H_2S). Here, we show that *Caenorhabditis elegans* can have mutually exclusive responses to H_2S and hypoxia, both involving HIF-1. Specifically, H_2S results in HIF-1 activity throughout the hypodermis, whereas hypoxia causes HIF-1 activity in the gut as judged by a reporter for HIF-1 activity. *C. elegans* require *hif-1* to survive in room air containing trace amounts of H_2S. Exposure to H_2S results in HIF-1 nuclear localization and transcription of HIF-1 targets. The effects of H_2S on HIF-1 reporter activity are independent of von Hippel–Lindau tumor suppressor (VHL)-1, whereas VHL-1 is required for hypoxic regulation of HIF-1 reporter activity. Because H_2S is naturally produced by animal cells, our results suggest that endogenous H_2S may influence HIF-1 activity.

INTRODUCTION

In nature, oxygen and hydrogen sulfide (H_2S) together create redox environments in which eukaryotes thrive (Fenchel and Finlay, 1995). In fiords, for example, ciliates and flagellates are most abundant at depths containing chemically reactive mixtures of oxygen and H_2S. In the terrestrial atmosphere, where oxygen is abundant, animal cells produce H_2S (Stipanuk and Beck, 1982). Although the role of endogenous H_2S is unclear, exposure to exogenous H_2S has profound physiological effects including improved outcome after myocardial infarction in mammals (Elrod *et al*., 2007; Simon *et al*., 2008) and increased life span in nematodes (Miller and Roth, 2007).

Mice exposed to trace amounts of H_2S consume 10-fold less oxygen and exhibit a corresponding reduction in basal metabolic rate (Blackstone *et al*., 2005). Treatment with H_2S also improves survival of mice in hypoxia (Blackstone and Roth, 2007). The response to hypoxia is coordinated by the evolutionarily conserved transcription factor hypoxia-inducible factor (HIF)-1 (Semenza, 2004). First identified as the protein responsible for the hypoxia-dependent transcription of erythropoietin (Wang and Semenza, 1993), HIF-1 activity increases as a function of decreasing oxygen levels. The activity of HIF-1 is contingent upon escaping degradation (Huang *et al*., 1998), nuclear localization (Wang and Semenza, 1993), and coactivator binding (Arany *et al*., 1996). Degradation of HIF-1 is mediated by an oxygen-dependent hydroxylation event and subsequent ubiquitin-dependent degradation (Epstein *et al*., 2001). In *C. elegans*, the enzymes regulating these events are the EGg Laying defective (EGL)-9 prolyl hydroxylase and the von Hippel–Lindau tumor suppressor (VHL)-1 ubiquitin ligase, respectively.

Here, we use *C. elegans* to study the influence of H_2S on HIF-1. We find that *hif-1* is required when nematodes are exposed to H_2S. In addition, an elevated level of HIF-1 activity dramatically increases the maximum tolerable concentration of H_2S. We show both H_2S and hypoxia cause an increase of both HIF-1 protein concentration and nuclear localization throughout the animal. However, H_2S and hypoxia treatments are distinct, with different patterns of HIF-1 transcriptional activity and H_2S can activate HIF-1 in the absence of *vhl-1*.

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09–03–0199) on November 4, 2009.

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MATERIALS AND METHODS

Strains

Wild-type *C. elegans* (N2 Bristol) and mutant strains were grown as described previously (Brenner, 1974) at room temperature on nematode growth medium (NGM) plates seeded with live *Escherichia coli* OP50 food. The following mutant strains were obtained from the *C. elegans* genetic stock center: CB5602, *vhl-1(ok161)*; JT307, *egl-9(sa307)*; ZG31 *hif01(ia04)*; CB6088, *egl-9(sa307)*, *hif-1(ia04)*; and CB6090, *vhl-1(ok161)*, *hif-1(ia04)*. The strain ZG120 *nhr-57::gfp(iaIs07)* was a gift from Jo Anne Powell-Coffman (Iowa State University) (Shen *et al*., 2006). Strains bearing the transgene were generated using standard genetic procedures.

Atmospheric Chambers

Atmospheric chambers were constructed as described previously (Nystul and Roth, 2004). In brief, atmospheres were constructed by mixing defined compressed H_2S stock mixtures with room air at a controlled rate to produce the desired final gas concentration. All compressed gas mixtures were obtained from Airgas (Radnor, PA) and were certified standard to within 2% of the indicated concentration. All experiments used continuous flow gas mixtures hydrated by using gas wash bottles. The 5 ppm H_2S atmosphere was constructed by mixing 500 ppm H_2S (balanced with nitrogen gas) with room air. All other H_2S containing atmospheres were constructed by mixing a 5000 ppm H_2S tank with room air. The hypoxic chamber was 5000 ppm O_2 balanced with nitrogen.

Viability Test

For age-dependent viability tests, nematodes were synchronized by hypochlorite treatment and arrested as starved L1 animals in M9 solution. The larvae were released onto NGM plates seeded with OP50 food and placed into the chamber at the time indicated. Survival was scored 24 h after introduction into the atmosphere.

For viability tests of mutant animals, L4 larvae were moved from a mixed stage NGM plate onto a fresh NGM plates seeded with live OP50 *E. coli*. These were moved into the H_2S containing chamber for 24 h, at which time worms were scored for survival to adulthood or death as L4 larvae. For adaptation experiments, worms were introduced into a chamber containing 50 ppm H_2S and grown for at least one generation in that atmosphere before challenge with a higher concentration of H_2S.

Antibody Production

A plasmid encoding the N terminus (amino acids 103–465) of *C. elegans* HIF-1 cloned into the pMal vector (New England Biolabs, Ipswich, MA) was a gift from Joanne Powell-Coffman. The plasmid was expressed in XL1 Blue cells and the recombinant protein was purified on an amylose column according to the manufacturer's instructions (New England Biolabs). The purified protein was used to immunize mice and generate the monoclonal antibody (mAb), by using established protocols (Wayner and Carter, 1987).

Immunostaining

Nematode strains were synchronized by hypochlorite treatment and grown to larval stage (L)4 on peptone-enriched NGM plates seeded with live *E. coli* NA22 food. Nematodes were rinsed in M9 solution until the supernatant looked clear of bacteria. The worms were placed onto unseeded NGM plates and exposed to gas. After 45-min exposure to H_2S or hypoxia, as indicated, the worms were washed off the plate, minced with a razor blade, collected onto glass slides, and frozen on dry ice as described previously (Moore *et al*., 1999). Fixation was in *N*,*N*-dimethylformamide at −20°C for 3.5 min and then rinsed two times with phosphate-buffered saline (PBS) with 1 mM $MgCl_2$ (PBSM). The specimens were ringed with a PAP pen (Thermo Fisher Scientific, Waltham, MA) and incubated in blocking solution (2% bovine serum albumin [BSA] in PBSM) for 30 min at room temperature. The slides were incubated with the conditioned media diluted 1:5 in blocking solution for 60 min at room temperature. The slides were then washed three times for 5 min in blocking solution and then incubated with Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin (Ig)G (Invitrogen, Carlsbad, CA) secondary antibody diluted 1:2000 with 4′,6-diamidino-3-phenylindole dihydrochloride (DAPI) for 60 min. The slides were then washed with blocking solution four times for 10 min each. Coverslips were mounted onto the slides with 50% glycerol in PBSM with DAPI. The slides were immediately examined on a 510 confocal microscope (Carl Zeiss, Thornwood, NY).

Confocal image stacks were acquired with a 40×/1.3 PlanNeofluar oil immersion objective, on a LSM 510 microscope (Carl Zeiss). Green fluorescent protein (GFP) and Alexa 488 fluorescence was excited with the 488 nm line of an Argon laser and imaged through a 500–550 nm bandpass filter. DAPI was excited in two-photon mode with a pulsed femtosecond Chameleon laser tuned to 780 nm (Coherent, Santa Clara, CA), and detected through a 435–485 nm bandpass filter.

Western Blot Analysis

Nematodes were synchronized by hypochlorite treatment and arrested as starved L1 animals in M9 solution. The worms were counted and 10,000 animals were dispensed onto each of three 15-cm NGM plates seeded with OP50 bacteria (1 plate per treatment). When the worms reach L4, they were exposed to 45 min of gas treatment and then pelleted, brought up in sample buffer (72.5 mM Tris, pH 6.8, 1.8% SDS, 9% glycerol, 9 mM EDTA, 10% 2-Mercaptoethanol, and a trace amount of bromphenol blue), frozen in liquid nitrogen, and stored at −80 C. The time between opening the atmospheric chamber and freezing did not exceed 90 s. This was repeated on three different days to generate replicate samples. Before loading, the samples were heated to 95°C for 15 min, vortexed vigorously, heated for another 15 min, and then centrifuged to remove insoluble matter. Polyacrylamide gel electrophoresis (PAGE) and transfer was performed in an XCell SureLock Mini-Cell and XCell II Blot Module system (Invitrogen). Electrophoresis was performed NuPAGE gel (4–12% Bis-Tris; 1.0 mm × 15 well) with 3-(*N*-morpholino)propanesulfonic acid buffer, by using 12.5 μl of sample. After electrophoresis, the protein was transferred to nitrocellulose, stained with Ponceau S, rinsed, and incubated with block (PBS/Tween 20 [PBST] with 2% normal goat serum and 5% heat-inactivated nonfat dried milk) for 1 h. The blot was then incubated with conditioned media diluted 1:5 in block for 1 h. The blot was rinsed three times with PBST and then incubated with goat anti-mouse IgG-horseradish peroxidase (HRP) (Southern Biotechnology Associates, Birmingham, AL) diluted 1:20,000 for 1 h. The blot was then washed eight times with PBST, incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical, Rockford, IL), and immediately exposed to film (F-BX810; Phenix Research Products, Candler, NC). The membrane was then stripped by placing it in a solution of 50 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol at 60°C for 30 min. The membrane was then rinsed three times and blocked for 30 min in PBST + 2% BSA. The membrane was then incubated with an anti-actin antibody (catalog no. sc-1616-R; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:3000 in PBST + 2%BSA for 1 h with rocking. The membrane was washed three times with PBST and then incubated with a secondary antibody-HRP (catalog no. 111-035-003; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:5000 in PBST + 2% BSA for 30 min. This was then washed eight times with PBST and incubated with ECL Western Blotting Substrate (catalog no. 32209; Pierce Chemical, Rockford, IL) and immediately exposed to film. The film was scanned into a computer, and the bands were quantified using the program Quantity One (Bio-Rad Laboratories, Hercules, CA).

mRNA Quantification

Synchronized L4 *C. elegans* were exposed to air, 60 ppm H_2S, or 0.5% O_2 for 45 min. Then, 1 ml of TRIzol (Invitrogen) was added to 0.1 ml of packed worms and the worms were frozen in liquid nitrogen. RNA was prepared as described in the TRIzol instruction manual. cDNA was synthesized using a Protoscript First Strand Synthesis kit (New England Biolabs) according to the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) was performed on an iCycler IQ system (Bio-Rad Laboratories) by using Platinum SYBR Green QPCR Supermix (Invitrogen) in 0.03-ml reactions. Standard curves were generated with cDNA, and data were analyzed using the Pfaffl analysis method (Pfaffl, 2001). cDNA levels were normalized to *sir-2.1* cDNA, which has been shown previously not to change expression in H_2S (Miller and Roth, 2007).

Live Animal Fluorescence Microscopy

Mixed stage NGM plates were sealed in the chamber for the time indicated, after which L4 *C. elegans* were picked into a drop of 1 mM levamisole in M9. After 5 min, the animals were pipetted onto a pad of 2% agarose in water. The nematodes were visualized on a 510 confocal microscope (Carl Zeiss).

RESULTS

Survival of *C. elegans* in H_2S

We exposed *C. elegans* to atmospheres containing H_2S in room air for 24 h. Animals either survived, seemed healthy, and were indistinguishable from untreated animals, or they died; sickly animals were not observed. Survival was dose dependent, with wild-type worms surviving 50 ppm H_2S but not 150 ppm H_2S.

Hif-1 Mediates Survival of Worms in Hydrogen Sulfide

C. elegans has a single conserved homologue of mammalian hypoxia-inducible factor α subunit, *hif-1* (Jiang *et al*., 2001). Animals with the *hif-1(ia04)* null mutation seem normal and are viable in normal culture conditions, but they display reduced embryonic and larval viability in hypoxia compared with wild-type animals (Jiang *et al*., 2001; Padilla *et al*., 2002; Nystul *et al*., 2003). We observed that *hif-1(ia04)* worms cannot survive exposure to 15 ppm H_2S (Table 1). In contrast, even at 50 ppm H_2S, wild-type worms survive with high viability and no obvious changes on growth rate or morphology (Miller and Roth, 2007). These results indicate that HIF-1 is necessary for responding appropriately to H_2S.

We wondered whether animals with elevated HIF-1 activity would be resistant to H_2S. HIF-1 protein is a transcription factor that is negatively regulated by EGL-9 and VHL-1 (Semenza, 2007); thus, mutations in either *egl-9* or *vhl-1* result in an overabundance of HIF-1. We found that animals with a null mutation either in *vhl-1(ok161)* or *egl-9(sa307)* were unaffected by 150 ppm H_2S, whereas wild-type worms are incapable of surviving (Table 1). Animals with the *egl-9(sa307)* tolerated up to 500 ppm, whereas *vhl-1(ok161)* null animals were only able to tolerate 150 ppm H_2S. To confirm that the H_2S resistance in these mutant animals is dependent on *hif-1*, we tested these mutant alleles in combination with *hif-1 (ia04)*. Both the *egl-9; hif-1* double mutant and the *vhl-l; hif-1* double mutant are sensitive to H_2S. Thus, an increase in HIF-1 creates resistance to H_2S.

Table 1. HIF-1 confers resistance to H_2S

Genotype	% animals surviving after 24 h ± SD (N)				
	Room air	15 ppm H_2S	50 ppm H_2S	150 ppm H_2S	500 ppm H_2S
Wild type	100* (106)	100* (95)	99 ± 2 (105)	0* (106)	0* (59)
hif-1(ia04)	100* (93)	1 ± 1 (91)	0* (110)	0* (109)	0* (63)
vhl-1(ok161)	100* (89)	100* (86)	100* (99)	99 ± 3 (96)	2 ± 4 (45)
egl-9(sa307)	100* (94)	100* (82)	100* (96)	100* (87)	100* (47)
hif-1(ia04); vhl-1(ok161)	100* (58)	0* (58)			
hif-1(ia04); egl-9(sa307)	100* (61)	2 ± 3 (62)			
Wild type (H_2S acclimated)	100* (49)		100* (68)	100* (47)	100* (38)

L4 worms were exposed to H2S for 24 h and scored for growth to adulthood. All surviving worms were identical to room air controls.
* No deviation was observed.

We hypothesized, given that *hif-1* is required for wild-type worms to survive in H_2S, that exposure to H_2S may stabilize and increase HIF-1 activity. In this case, HIF-1 activation may allow worms to survive subsequent exposure to higher levels of H_2S. To test this idea, we raised wild-type worms in low-level H_2S (50 ppm) and then transferred them to higher concentrations (150 and 500 ppm). Consistent with our hypothesis, we found that all naïve worms died in as little as 150 ppm H_2S, whereas acclimated worms survived exposure to 150 ppm and even 500 ppm H_2S (Table 1). We conclude from the above-mentioned information that H_2S may activate HIF-1.

Hydrogen Sulfide Exposure Results in an Increase of HIF-1 Protein Levels and HIF-1 Nuclear Localization

To further explore the possibility that H_2S can activate HIF-1 in vivo, we developed a mAb specific for *C. elegans* HIF-1. HIF-1 protein is detected at high levels in nuclei of *egl-9(sa307)* and *vhl-1(ok161)* mutant animals and is absent in *hif-1(ia04)* worms (Supplemental Figure 1). The large gut nuclei are most easily visible, but HIF-1 protein is accumulated in nuclei throughout the animal. This is in agreement with previous reports showing that the HIF-1 promoter region is ubiquitously active throughout the worm (Jiang *et al*., 2001). Western blot analysis of total protein detects two bands (molecular masses 106 and 113 kDa) present in extract prepared from *egl-9(sa307)* but absent in *hif-1(ia04)* worm extract, indicating that this antibody is specific for HIF-1 (Supplemental Figure 2).

HIF-1 protein levels increase during exposure to H_2S. As with hypoxia, H_2S exposure results in increased protein concentration in as little as 45 min (Figure 1). We also examined whether H_2S treatment alters the cytological location of HIF-1. Immunostaining reveals HIF-1 accumulation in the nuclei throughout the H_2S-treated worms, compared with untreated controls (Figure 2). We also exposed nematodes to hypoxia and determined that this causes a pattern of nuclear localization indistinguishable from H_2S exposure.

Hydrogen Sulfide and Hypoxia Treatments Result in Different Patterns of HIF-1 Activity

To determine whether nuclear localization of HIF-1 in H_2S is associated with transcription of *hif-1*–responsive genes, we examined expression of *nhr-57::gfp(iaIs07)*, which has HIF-1–dependent expression (Shen *et al*., 2006). After exposure to hypoxia, NHR-57::GFP was observed in the gut, as has been reported previously (Shen *et al*., 2006) (Figure 3). In contrast, H_2S treatment resulted in expression of NHR-57::GFP in the hypodermis but not in the gut. We tested various H_2S exposure lengths (1–3 h) to verify that this expression incongruity was unrelated to the amount of time that worms were exposed to H_2S. We found that reporter activity is transient, with maximal fluorescence experienced at 3 h of continuous exposure. We observed bright fluorescence in 100 of 100 worms exposed to H_2S, and we observed fluorescence in 0 of 100 untreated worms. We never observed gut fluorescence upon H_2S exposure, and we conclude that the pattern of HIF-1 activity in hypoxia and H_2S are mutually exclusive. In addition, *hif-1(ia04); nhr-57::gfp(iaIs07)* worms do not fluoresce when exposed to H_2S, showing that *hif-1* is required for H_2S-induced expression.

To confirm that HIF-1 activity increase is not an artifact of the transgene, we assayed the relative amounts of known HIF-1 target transcripts in wild-type worms. As with the transgene, H_2S exposure increases *nhr-57* mRNA levels (Figure 4A), suggesting that the transgene represents a transcriptional activation present in wild-type worms. To confirm that H_2S-dependent transcription is not limited to only one gene, we tested another known HIF-1 target, K10H10.2 (Figure 4B), and we found that it was also increased during H_2S exposure. Neither of these mRNAs are increased when assayed in a *hif-1(ia04)* mutant background, showing that *hif-1* is required for the induction. These results show that H_2S exposure increases transcription of HIF-1 targets in wild-type *C. elegans*.

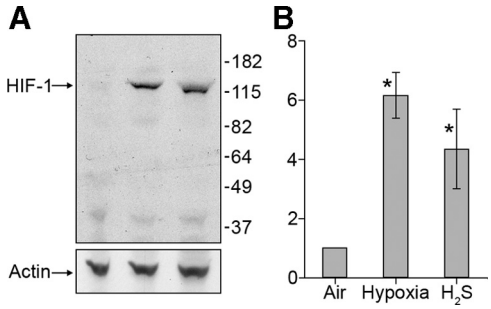

Figure 1. HIF-1 protein levels increase when worms are exposed to H_2S. (A) A representative Western blot analysis shows HIF-1 protein increases when worms are exposed to 50 ppm H_2S or 0.5% O_2. The blots were stripped and reprobed with an anti-actin antibody to confirm equivalent amounts of total protein. The size markers are shown in kilodaltons. (B) Average increase in signal relative to control ± SD of three independent experiments. *$p < 0.05$ (Student's *t* test) compared with air-treated animals.

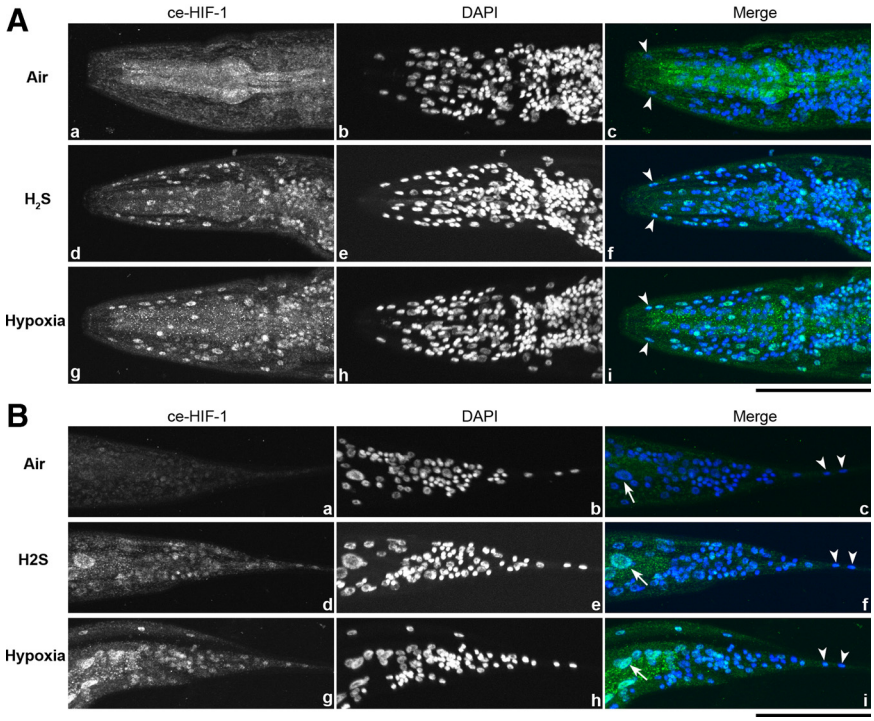

Figure 2. H_2S exposure causes HIF-1 nuclear localization. Both H_2S and hypoxia (0.5% O_2) treatment induce HIF-1 nuclear localization throughout the animal. Images are stacks of all nuclei visible with DAPI staining. Animals were stained with anti-Ce-Hif-1 antibody (a, d, and g) and DAPI (b, e, and h) to visualize nuclei. Hypodermal nuclei (tailless arrows) and intestinal nuclei (tailed arrows) are clearly observed in the merged images (c, f, and i). (A) Anterior of the worm. (B) Posterior of the worm. Bars, 50 μm.

Our previous results demonstrated that *egl-9* and *vhl-1* have distinct effects on survival, suggesting that they may have distinct effects on HIF-1 activation. To more critically define the relationship between *vhl-1* and *hif-1*, we examined the ability of these mutant worms to express NHR-57::GFP in the presence of H_2S (Figure 5). As expected, *egl-9(sa307); nhr-57::gfp(iaIs07)* mutant worms exhibit constitutive expression in both the gut and in the hypodermis. In contrast, H_2S exposure of *vhl-1(ok161); nhr-57::gfp(iaIs07)* worms results in hypodermal fluorescence that is absent in untreated worms. Thus, this H_2S-dependent regulation of HIF-1 is dependent on *egl-9* but independent of *vhl-l*. These results suggest that the activation of HIF-1 by H_2S is necessary and sufficient for survival and is distinct from hypoxia.

DISCUSSION

Increased H_2S or decreased oxygen both constitute a net change in the redox balance to which cells are exposed. We have shown that nematodes rapidly respond to changes in H_2S by HIF-1 activation. HIF-1 is also activated by decreased oxygen, which suggests that HIF-1 is important in coordinating the response to changes in environmental and organismal redox balance. Our results demonstrate that *vhl-1* is not required for H_2S-dependent increase in HIF-1 target gene expression. Previous studies in mammals show that the hypoxic HIF-1 response does require VHL (Iliopoulos *et al.*, 1996), suggesting that cells have multiple ways of activating the HIF responses.

Increased HIF-1 activity is positively correlated with survival of nematodes exposed to H_2S. *C. elegans* exposed to low levels of H_2S absolutely require *hif-1*, and increased levels of HIF-1 facilitate quantal survival in otherwise lethal concentrations of H_2S. *Hif-1* mutants are sensitive to H_2S concentrations that have no effect on wild type (15 ppm). Furthermore, *egl-9* and *vhl-1* mutants have increased HIF-1 levels and can survive higher concentrations of hydrogen sulfide than wild type. *Egl-9* mutant animals show elevated HIF-1 reporter activity compared with *vhl-1* mutants worms, consistent with the finding that *egl-9* mutant worms can tolerate higher H_2S concentrations than *vhl-1* mutant worms. The increased survival of *egl-9* mutant worms in H_2S is reminiscent of previous work which showed that a mutation in *egl-9* protects nematodes from cyanide toxicity (Darby *et al.*, 1999; Gallagher and Manoil, 2001). Both sulfide and cyanide disrupt cellular respiration as noncompetitive inhibitors of cytochrome oxidase (Cooper and Brown, 2008). Thus, it is plausible that HIF-1 helps overcome the toxicity by relieving the stress of impeded cytochrome oxidase activity. Indeed, HIF-1 has been shown to increase expression of genes encoding glycolytic enzymes (Chen *et al.*, 2001; Minchenko *et al.*, 2002; Obach *et al.*, 2004) as well as to down-regulate tricarboxylic acid cycle entry and oxygen consumption in mammals (Papandreou *et al.*, 2006).

The H_2S-induced expression of a reporter for HIF-1 activity does not require *vhl-1* but does require *egl-9*. This is in contrast to the increase of HIF-1 protein levels observed in both mutant backgrounds. This suggests that H_2S may be involved in two steps of HIF-1 activation: protein stabilization and transcriptional activation. A recent article demonstrated the existence of an alternative HIF-1 regulatory pathway in *C. elegans* (Shen *et al.*, 2006). This pathway does not require *vhl-1* but does require *egl-9* and a novel protein, *r*egulator of *hy*poxia-inducible factor (*rhy-1*). The exact function of *rhy-1* remains obscure, but perhaps it is important for responding to nonhypoxic impediments of the respiratory chain. In addition, VHL-independent, EGLN1-dependent HIF regulation has been noted in mammalian cells (Ozer *et al.*, 2005; To and Huang, 2005); thus, it will be interesting to investigate the potential for H_2S induction of HIF-1 activity in higher organisms.

We observed that hypoxia and sulfide treatment result in different expression patterns of expression of a reporter for HIF-1 activity. One possibility for the differences observed is

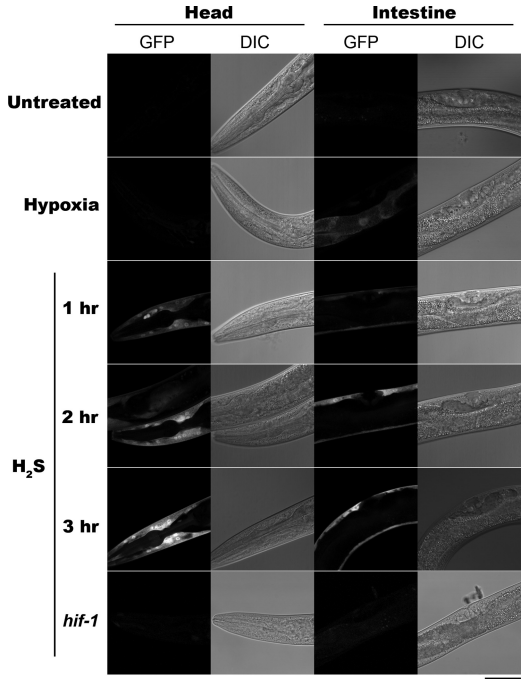

Figure 3. H_2S exposure increases expression of NHR-57::GFP. A midfocal plane of a fluorescent confocal photomicrograph and Nomarski micrograph. Fluorescence is not apparent in untreated L4 *nhr-57::gfp(iaIs07)* transgenic animals. An *nhr-57::gfp(iaIs07)* animal exposed to hypoxia exhibits HIF-1 activity in the in the gut but absent in the hypodermis. At 1 h into H_2S treatment, fluorescence is apparent in the hypodermis throughout the animal but undetectable in the gut. Fluorescence becomes more intense until 3 h into exposure. *nhr-57::gfp(iaIs07)*; *hif-1(ia04)* animals fail to show any fluorescence, regardless of H_2S treatment. Bar, 40 μm.

that the gut is more easily made hypoxic and the hypodermis is more easily exposed to H_2S. This model would predict that increasing the severity of hypoxia should increase the tissue with detectable HIF-1 activity. We were unable to find any concentrations of O_2, including anoxia, which would result in HIF-1 activity detected in more tissues. Likewise, increasing the concentration of H_2S also failed to increase HIF-1 activity in additional tissues. Also, the near perfect overlap of HIF-1 activity in hypoxia and *vhl-1* null animals argues that the observed differences reflect something intrinsic to the tissue, perhaps involving additional transcription factors.

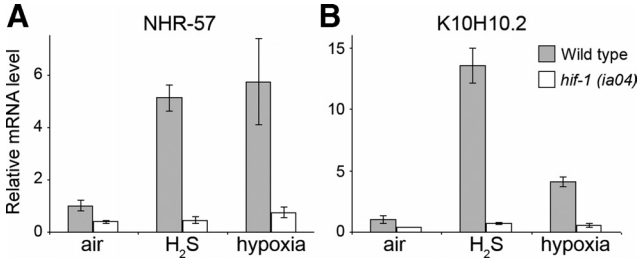

Figure 4. H_2S exposure increases mRNA levels of HIF-1 target genes. Real-time reverse transcription-PCR was used to quantitate mRNA levels of HIF-1 target genes in wild-type or *hif-1(ia04)* worms exposed to H_2S or hypoxia. The cDNA of *nhr-57* (A) or *K10H10.2* (B) is shown as the average increase ± SD relative to *sir-2.1* cDNA levels, which do not change during H_2S exposure.

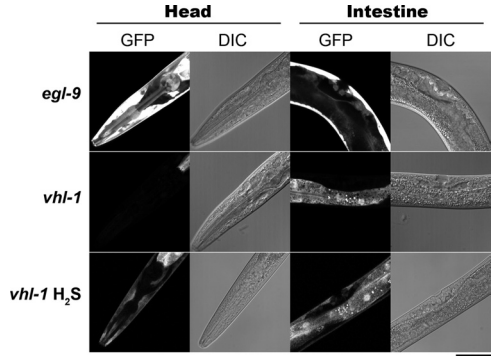

Figure 5. H_2S dependent HIF-1 activity is independent of VHL-1. *nhr-57::gfp(iaIs07)*; *egl-9(sa307)* exhibits intense fluorescence is apparent throughout the animal, including in the hypodermis and the gut. 100 Untreated *nhr-57::gfp(iaIs07)*; *vhl-1(ok161)* animals show basal GFP expression in the gut and H_2S treatment induces hypodermal expression. These results are representative of 100 of 100 worms examined for each panel. Bar, 40 μm.

Organisms survive transitions to new environments by changing their physiology to be better suited to the new environment, referred to here as acclimation. Acclimation to nonlethal environmental changes is known to improve the ability to survive otherwise lethal exposure to the same agent. For example, acclimation of nematodes to low concentrations of salt allows them survive subsequent exposure to high salt concentrations that are lethal for naïve animals (Lamitina *et al*., 2004). We suggest that HIF-1 may be involved in acclimation to H_2S containing environments. Wild-type nematodes acclimated to a sub-lethal concentration of H_2S (50 ppm), are able to withstand a 10-fold increase in H_2S concentration (500 ppm) that would kill all naïve animals. This is concordant with the increase in HIF-1 activity observed and suggests that HIF-1 activity may be essential for the acclimation to H_2S.

Here, we have shown that H_2S exposure increases HIF-1 activity and increases tolerance of further H_2S exposure. This is reminiscent of investigations into hypoxia preconditioning, in which brief exposure to hypoxia increases HIF-1 activity and protects against subsequent hypoxia. We now know that animals produce H_2S in a regulated manner and that the resultant sulfide is physiologically important (Kamoun, 2004). In fact, endogenous H_2S production has been suggested to be important during hypoxia preconditioning (Bian *et al*., 2006). In addition, several recent studies have described a protective role of H_2S with respect to hypoxia (Blackstone and Roth, 2007), hemorrhage (Morrison *et al*., 2008), and reperfusion injury (Elrod *et al*., 2007). It has been shown that overexpression of a sulfide-generating enzyme, cystathionine gamma lyase, results in cardiac protection, suggesting a protective effect of endogenous H_2S (Elrod *et al*., 2007). Although all of these studies implicate H_2S as a potentially useful therapeutic, the mechanism of action remains ill defined. Other studies have shown that increased HIF-1 levels also provide protection against reperfusion injury, and the data provided here provide a possible link between these phenomena.

ACKNOWLEDGMENTS

We thank members of the M.B.R. laboratory for helpful discussions; K. Chan, H. Frazier, D. Miller, and S. Lockett for critical reading; J. Priess for discussing worm anatomy; J. Powell-Coffman for strains and the recombinant ceHIF-1 construct; and the *C. elegans* Genetics Center for providing strains. This work

was supported by supported by the National Institutes of Health grant R01 GM-48435 (to M.B.R.).

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