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(RESEARCH ARTICLE)

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Putative pathways fueling anaerobic mitochondrial respiration

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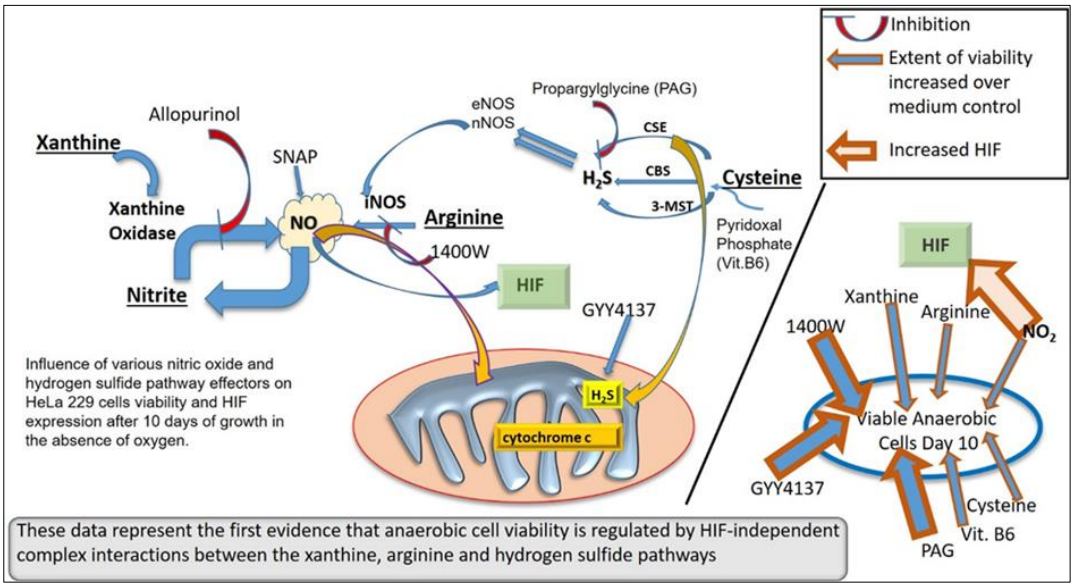
Abstract

Tumor interiors undergo prolonged anoxia; however, the pathways involved have not been identified. Since NO and H_2S function in prokaryotic anaerobic respiration, the effect their pathway elements have on HeLa 229 cell viability was measured after 10 days anaerobic incubation. Arginine or xanthine (NO pathway precursors) increased cell viability (13.1- and 4.4-fold, respectively). The H_2S pathway precursor, cysteine, also enhanced viability (9.8-fold), as did H_2S donor GYY4137, or inhibitor of glutathione synthesis, propargylglycine, (40- and 85-fold, respectively). These results demonstrate that cell viability after extended anaerobic incubation (10 days) can be modulated by affecting NO or H_2S pathways.

Graphical abstract



Keywords: Cysteine; Hydrogen Sulfide; Arginine; Xanthine; Nitric Oxide; Hypoxia Inducible Factor (HIF); Anoxia

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1. Introduction

Prior to the oxygenation of the oceans, eucaryotic cellular function evolved for millions of years under anaerobic conditions [1-3]. This continued preference for oxygen levels significantly below that of atmospheric oxygen levels is evidenced by *in situ* oxygen levels ranging from completely lacking in oxygen (anoxic/anaerobic) to hypoxic levels (less than 13%) [4, 5]. For example, intestinal epithelium experiences oxygen fluctuations that range from severely hypoxic to anoxic [6, 7]. This broad *in vivo* oxygen range (0 to ~13%) is generally referred to as physioxic oxygen levels. Experimentally, however, most studies examining the effects of oxygen stress generally ignore the metabolic rewiring that occurs in response to even short-term anaerobic conditions (3 hrs) when nitrite and fumarate are shown to function in mitochondrial ATP production [8-13].

While there has been a push towards characterizing cell metabolism under physioxia, cell metabolism under strict anoxic microenvironmental conditions has not been studied. Metabolic rewiring under anaerobic growth conditions has principally focused on the Warburg effect that is regulated by HIF and involves anaerobic glycolysis, which is analogous to microbial fermentation [14-17]. This pathway is functionally present for short periods of time when cells are deprived of oxygen. However, based on the shared evolutionary environment and common ancestor, it is likely that eucaryotic cells evolved anaerobic respiratory pathways comparable to those expressed by microbes which use alternative electron acceptors [8, 10-13, 18, 19]. To date, assays performed in an anaerobic environment are typically initiated using media containing atmospheric oxygen levels that deplete over time; thus, yielding incubation conditions that cover an oxygen concentration spectrum, the nadir of which is not measured [20]. Until recently, the ability to study long-term anoxic cell growth has been elusive due to an inability to culture cells *ex vivo* for extended periods of time in the absence of oxygen. We pioneered a system for the study of anaerobic cell physiology and the resultant oxygen-triggered metabolic rewiring. In this model system, both cancerous and noncancerous cell lines retained viability for over 2 weeks (maximum time tested), replicated, and autophagy was suppressed [21, 22]. In addition, HIF expression cycled on and off in response to cellular release of ROS. *In vitro* support of the role anaerobic rewiring may play in tumorigenesis is the finding that HeLa cells grown anaerobically shift their cytokine secretome expression to one that is pro-angiogenic [22]. Thus, characterization of anaerobic cell physiology is essential, particularly since in addition to mucosal surfaces, stem cell niches and solid tumors contain anaerobic areas [23-25]. Therefore, it is crucial to characterize this aspect of cancer cell metabolism to understand metastasis and drugs resistance fully.

A potential link between anaerobic respiration and tumorigenesis is the detection of gaseous intercellular transmitters, i.e., nitric oxide (NO) and hydrogen sulfide (H_2S), which are reported to serve critical roles in tumor microenvironments and acquired chemotherapy resistance [26-29]. Since bacteria, which undergo anaerobic respiration, use NO and H_2S as terminal electron acceptors of their respiratory electron transport chain, we focused on determining whether NO and H_2S generating pathways play a role in sustaining anaerobic cancer cell viability using our established HeLa 229 cell system [13, 30-32].

2. Material and methods

2.1. Reagents

Effectors for nitric oxide synthesis used in experiments were S-nitroso-N-acetylpenicillamine (SNAP; 25, 50, 100 μM; Cayman Chemical), arginine (10 mM; Sigma), N omega-nitro-L-arginine methyl ester hydrochloride (L-NAME; 0.15 mM; Sigma), N-([3-(aminomethyl)phenyl] methyl) ethanimidamide dihydrochloride (1400W; 100 μM; Sigma), xanthine ($4x10^{-5}$ M; Sigma), nitrite (50, 100, 500 mM; Sigma), and allopurinol (200 μM; Sigma). Effectors for hydrogen sulfide synthesis used in experiments were cysteine (0.4 mM; Sigma), JK-2 (300 μM; Sigma), P-(4-Methoxyphenyl)-P-4-morpholinyl-phosphinodithioic acid (GYY4137; 400 μM; Cayman Chemical), pyridoxal phosphate (PLP; 2 μM; Sigma), and DL-propargylglycine (PAG; 0.5 mM; Sigma).

2.2. Anoxic Cell Culture

Previous studies show that regardless of cell line screened, all cells can replicate for extended periods of time (14-17 days minimum) in the absence of oxygen [21]. For this initial study to assess the roles various pathways and their mediators play in supporting anaerobic cell viability, HeLa 229 cells were used, based on their replication rate and consistency as the model cell line, for initial determinations of anaerobic metabolic changes. HeLa 229 cells were grown to 80% confluence (2.24×10^5 cells/well) in 24 well plates (normoxic conditions, 5% CO_2 in air; 10% FBS, 0.05 mg/ml gentamicin (Hyclone); high glucose (4.5 g/l) DMEM medium with glutamine and pyruvate (584 and 110 mg/l, respectively; Cellgro) for 24 hrs. These cells were then transferred to an anaerobic chamber (Whitley A35; anaerobic gas mixture: H_2, CO_2, N_2; 37°C), and medium replaced with degassed low glucose (1 g/l) homologous medium without

gentamicin, as previously described [21]. The lack of oxygen (0%) in the degassed medium and culture plate wells were confirmed by oxygen electrode measurements (Microelectrodes, Inc. oxygen probe; Pod-Vu software).

2.3. Nitric Oxide and Hydrogen Sulfide Pathway Constituents and Selected Inhibitors

Nitric oxide and hydrogen sulfide producing pathways could participate in supporting anaerobic mitochondrial respiration. To test this hypothesis, these pathways' precursors, inhibitors, and agonists were tested for their effect on cell viability and hypoxia inducible factor (HIF) expression. Test chemicals were dissolved in the degassed medium under anaerobic conditions. Upon transferring the cells to the anaerobic chamber (Day 0), aerobic DMEM was removed by aspiration and replaced with anaerobic degassed DMEM alone, or containing chemical supplement indicated. At day 10, the total number of viable cells per condition were determined by standard trypan blue dye exclusion (Countess™ II Automated Cell Counter) and HIF expression measured (see below). The trypan blue assay was the most appropriate assay for anaerobic viability measurement since luciferase-based tests require oxygen and the tetrazolium-based MTT assay is adversely affected by ROS, which are intermittently released from cells during anaerobic incubation [21, 33]. Controls for all tests consisted of cells grown in anaerobic medium alone. Medium with DMSO (0.05%) was used as the control for medium containing supplements requiring DMSO for solution preparation (allopurinol, 1400W, and GYY4137).

2.4. HIF-1α Analysis

HIF expression for each growth condition was determined. Immediately, post-viability measurements were taken, plate well contents (on ice) from each condition were pooled for six wells (4°C), then centrifuged (2000 RPM, 5 min, 4°C). Supernatants were removed. HIF-1α protein was extracted from cell pellets and confirmed using Human Simple Step ELISA kit (Abcam) according to manufacturer's instructions.

2.5. Data Analysis

Data were analyzed (GraphPad Prism) by two-way ANOVA ($p < 0.05$). Where appropriate, Tukey post-hoc tests were performed. Significant points ($p < 0.05$) are designated with an asterisk and standard error of the mean (SEM) are included on all graphs.

3. Results and discussion

Within solid tumors and stem cell niches, there are microenvironments that are anaerobic [23-25]. As previously reported, replicating HeLa cells at 10 days anaerobic incubation produce reactive oxygen species (ROS), as detected by DCFDA and CellRox Green fluorescence, and lack expression of the autophagy marker LC3B [21].

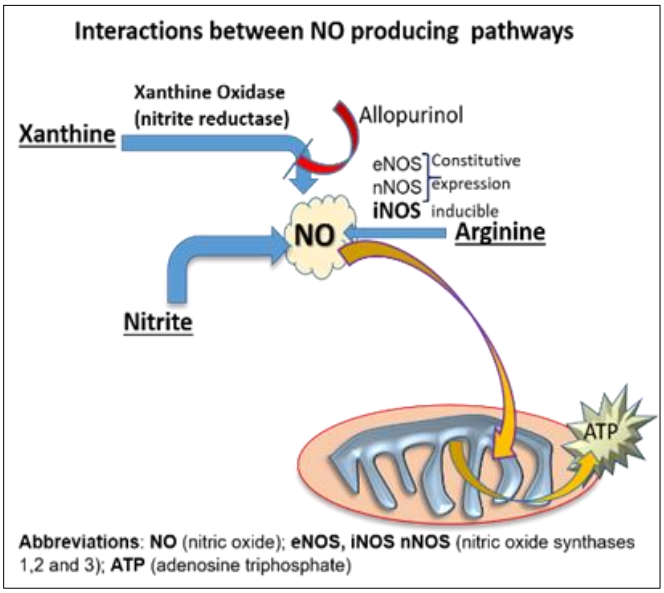

Figure 1 Schematic overview of nitric oxide (NO) generating pathways [34, 35]

In addition, they accumulate MitoTracker Red FM, an indicator of mitochondrial function (data not shown). Two important pathways that result in NO cellular release are the oxidation of L-arginine by nitric oxide synthase (NOS), and the nitrite reductase pathway (xanthine oxidase, XO) that can utilize either xanthine, or nitrite as a precursor (Figure 1) [34, 35]. In bacteria and plants, nitrite can fuel ATP production via the mitochondrial electron transport chain with NO as the terminal electron acceptor [13, 36, 37]

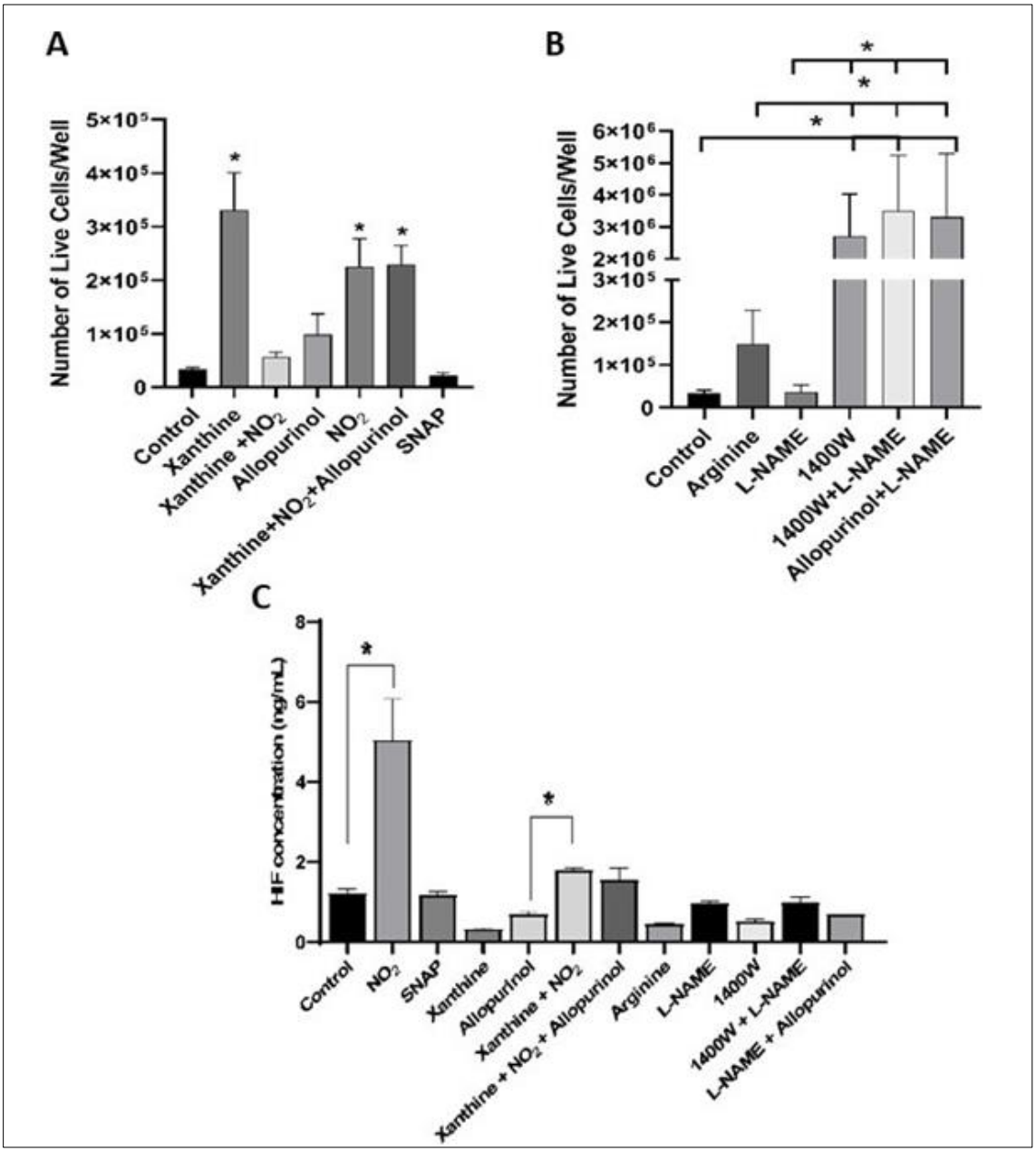

Figure 2 Anaerobic HeLa cell viability modulation by mediators of the nitric oxide generating pathways. (A, B) Number of viable cells/well present after 10 days of anaerobic incubation with various mediators of the pathways responsible for nitric oxide generation. Treatments (Mean ± SEM; n=6, repeated once to twice); * = $p < 0.05$ considered significant; (C) Hypoxia inducible factor (HIF) expression by HeLa cells grown anaerobically in the presence of various mediators which promote, or inhibit, the generation of nitric oxide. Each HIF is a pooled sample from viability measurements.

The ability of nitric oxide generating pathway constituents to support long-term (10 days) anaerobic cell viability was determined (Figure 2A; 2B). The addition of each of the three pathway precursors (nitrite, arginine, and xanthine) resulted in a reproducible pattern of increased cell viability. Xanthine, nitrite, and arginine increased cell viability by 13, 6.6 and 4.4-fold, respectively, as compared to supplement-free medium control. Interestingly, addition to medium of xanthine and nitrite, which compete for XO activity, results in a viability level similar to that of the medium control. The

increased viability due to the presence of NO precursors can be restored when allopurinol, an inhibitor of xanthine oxidase, is added to the xanthine and nitrite mixture. This effect of allopurinol indicates that XO is not essential for nitrite enhancement of anaerobic cell viability. In addition, SNAP, a NO donor, caused a slight reduction in viability, possibly due to a chemical interaction of NO with hydrogen sulfide (H_2S) [38, 39].

In contrast to the effects of nitrite and xanthine pathways on viability, targeting the essential enzyme of the arginine-NO pathway, i.e., nitric oxide synthase (NOS), caused a significant increase in viability that was NOS isoform dependent (Figure 2B). The addition of the iNOS inhibitor 1400W alone, and when combined with L-NAME, an inhibitor of the constitutive NOS (cNOS), enhanced cell viability 80-fold and 103-fold, respectively, while L-NAME alone had no effect on cell viability, as compared to control. Interestingly, when both xanthine oxidase and cNOS were inhibited, via the combination of allopurinol and L-NAME, cell viability increased by 97-fold. In contrast, addition of SNAP, a nitric oxide donor (50 and 100 μM) depressed viability levels to 0.6-fold of control (data for 50 μM shown in Figure 2A), while viability levels in response to 25 μM SNAP were similar to that of supplement-free control.

As previously reported, HIF expression in long-term anaerobically cultured cells cycles on and off over time [21]. At day 10 of anaerobic culture, HIF protein levels were depressed, as compared to medium control, with the exception of medium supplemented with nitrite, alone or in combination with other supplements (Figure 2C). Based on reports that nitrite stabilizes HIF, this finding of nitrite-mediated enhanced HIF expression is expected [40]. Thus, with respect to NO pathways, inhibition of NO production via 1400W, L-NAME and allopurinol had the most robust effect on increasing cell viability. Another interesting finding relative to anaerobic metabolism, that differentiates it from what is reported for cell metabolism under hypoxic conditions, is that the change in viability appears disconnected from HIF expression.

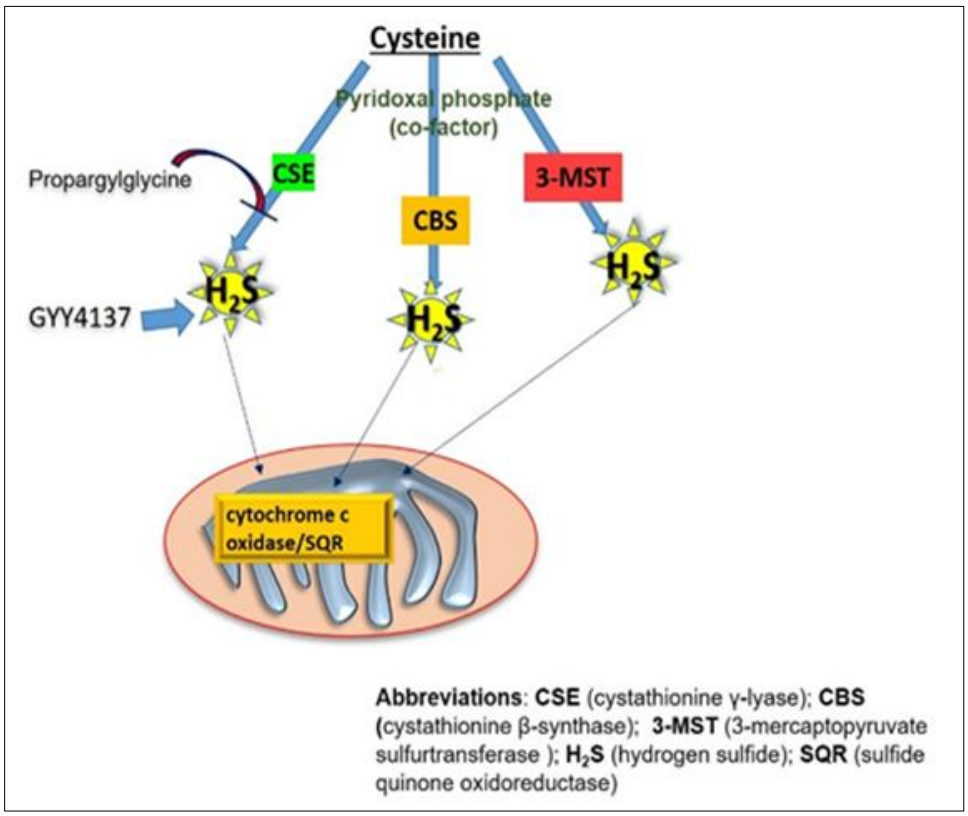

Figure 3 Schematic overview of hydrogen sulfide (H_2S) generating pathways [41]

Similar to cellular NO generation, multiple parallel pathways exist for cellular H_2S synthesis from the precursor cysteine (Figure 3) [41]. These pathways include the cystathionine-β-synthase (CBS) branch, located in the central nervous system; the cystathionine γ-lyase (CSE) branch, located in the vasculature, liver, and kidney; and the 3-mercaptopyruvate sulfur transferase (3-MST) branch, which is found in kidney, liver, lung, heart, muscle, spleen, and brain [42]. H_2S oxidation, which occurs widely throughout eukaryotes, is coupled to ATP synthesis by a mitochondrial electron transport chain through a sulfide quinone oxidoreductase [43, 44]. H_2S also regulates expression of cytochrome

c oxidase [45-47]. The cysteine/CSE/H_2S pathway has been shown to promote cell cycle progression in squamous cell (oral) carcinoma cells *in vitro* and melanoma progression [48-51].

Depending on the organ system, and type of NO synthase present, H_2S exhibits contradictory effects [38, 39, 52-54]. These effects include inhibition of NO produced by the neuronal form of nitric oxide synthase (nNOS), or increased NO production by iNOS and eNOS [55, 56]. In addition, H_2S appears to function as a feedback inhibitor of iNOS and nNOS, presumably due to its function as a free radical scavenger [39, 57]. To determine if constituents of H_2S producing pathways support HeLa cell anaerobic viability, cysteine, H_2S donors JK-2 (short term) and GYY4137 (slow release), pathway cofactor pyridoxal phosphate (vitamin B6), or propargylglycine (PAG) which is the irreversible inhibitor of cystathionine γ-lyase and subsequent glutathione synthesis, were tested (Figure 4A).

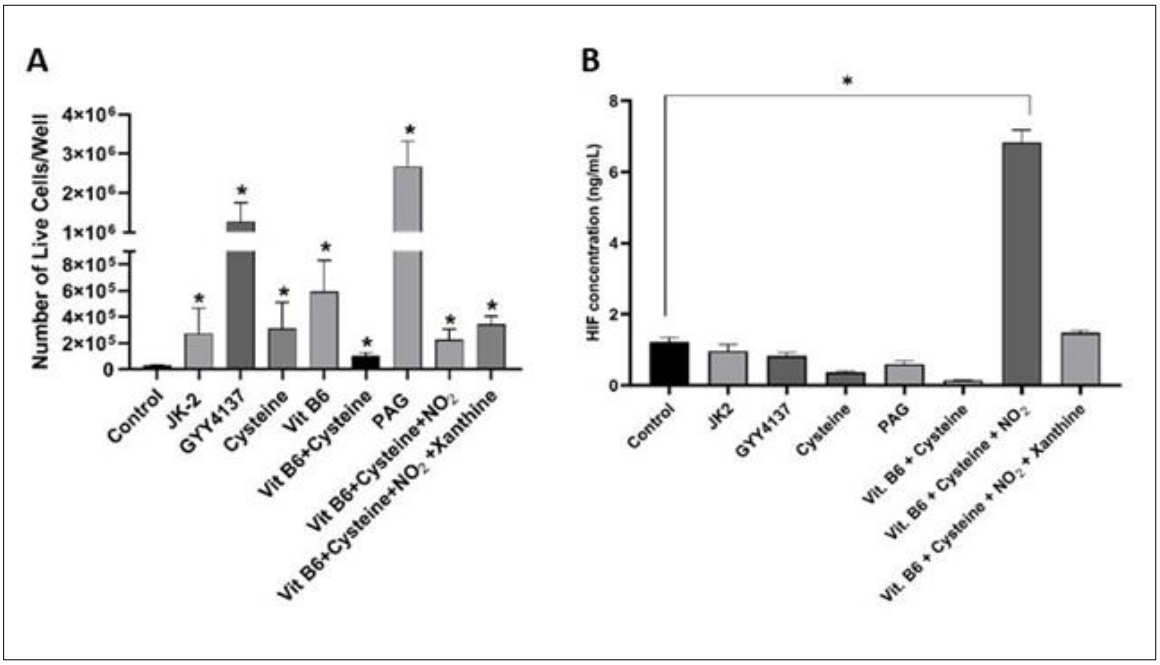

Figure 4 Anaerobic HeLa cell viability modulation by mediators of the hydrogen sulfide generating pathways. (A) Number of viable cells/well present after 10 days of incubation with various mediators of the hydrogen sulfide pathways under anaerobic conditions; (B) Expression of hypoxia inducible factor (HIF) response to the various mediators after growth under anaerobic conditions. Each HIF measurement is a pooled sample from viability measurements. Mediators and HIF (Mean ± SEM; n=6, repeated once to twice); * = $p < 0.05$ considered significantly different from control.

In the *in vitro* anaerobic model system, all treatments that increased cellular H_2S levels caused significantly ($p < 0.05$) enhanced HeLa cell viability, as compared to medium alone (Figure 4A). The most potent enhancers of cell viability were the slow H_2S donor GYY4137 (40.4-fold viability increase), and PAG, which inhibits CSE; thus, blocking glutathione, a consumer of H_2S, synthesis (83.8-fold viability increase) [40, 41, 58]. The enhancement of viability by PAG is in contrast to the negative effect inhibition of CSE expression has on viability, via either siRNA, or after exposure to an oxidizing agent (hydrogen peroxide) [59]. This is a further indication of the oxygen-mediated metabolic rewiring. In addition, cysteine, JK-2 (rapid H_2S donor), and vitamin B6 increased cell viability by 9.8-, 8.8-, and 18.7-fold, respectively. Since under anaerobic culture there is minimal need for the antioxidative properties of H_2S to combat cellular reactive oxygen species generated during aerobic respiration, the higher available H_2S levels may be utilized to drive mitochondrial ATP production [21, 29, 41]. However, H_2S does not appear to work in isolation, and it is likely that interactions with both ROS and NO are likely. This may occur at a variety of levels, from influencing the generation of ROS and/or NO, interacting directly with them, or competing for control of downstream signaling events. In addition, H_2S, like arginine and xanthine, had no significant effect on HIF expression (Figure 4B). Interestingly, the level of HIF in cells grown with cysteine alone, and with its cofactor vitamin B6, was 30% and 11% that of cells grown in control medium, respectively. This depressed HIF level indicates that factors which lead to H_2S production may further destabilize HIF protein in cells incubated for extended periods (10 days) in the absence of oxygen. As was measured for nitric oxide pathways, nitrite was the sole supplement responsible for increasing HIF expression compared to control (Figure 4B).

4. Conclusion

This is the first report of the effects various gaseous chemical signaling molecules and their inhibitors have on the viability of anaerobically grown cells, and the major role H_2S plays in sustaining viability in the absence of oxygen. Metabolic rewiring of cells grown long term in the absence of oxygen appears to be supported by two compensatory systems, i.e., those pathways required for NO and H_2S production. However, since free H_2S and not NO enhances viability, it is likely that the NO generating pathways serve a more regulatory/rescue function, depending on the nutrients available in the anaerobic microenvironments of the host. Under anaerobic growth conditions, neither arginine nor xanthine pathways for NO production nor H_2S production pathways affected HIF expression. This finding supports our previous observation that HIF expression is only present intermittently in cells growing long-term anaerobically appearing to serve in a bridge function, in contrast to its importance in hypoxically-grown cells [21, 60]. Further studies on the role various pathway enzymes play in anaerobic metabolism and how these various pathways affect anaerobic mitochondrial function are ongoing.

Compliance with ethical standards

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Author's contribution

Conceptualization, B.P. and I.S.; methodology, B.P. and I.S.; validation, B.P, I.S., and A.K.; formal analysis, B.P, I.S., and A.K.; investigation, B.P, I.S., and A.K.; resources, B.P.; data curation, B.P. and A.K.; writing—original draft preparation, B.P.; writing—review and editing, I.S., A.K. and M.K.; visualization, B.P. and A.K; supervision, B.P. and I.S.; project administration, B.P.; funding acquisition, B.P. All authors have read and agreed to the published version of the manuscript.

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Disclosure of conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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