Hydrogen sulfide modulates eukaryotic translation initiation factor 2α (eIF2α) phosphorylation status in the integrated stress-response pathway

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Hydrogen sulfide (H2S) regulates various physiological processes, including neuronal activity, vascular tone, inflammation, and energy metabolism. Moreover, H2S elicits cytoprotective effects against stressors in various cellular models of injury. However, the mechanisms of the signaling pathways mediating effects against stressors in various cellular models of injury. H2S for treatment of cardiovascular diseases and inflammation was excreted in urine (7). H2S is a weak acid and ionizes to HS⁻ and H⁺ with a pKa of 6.9 (8) resulting in an estimated 80% of it being in the ionized form at physiological pH. Although HS⁻ is likely confined to cells in which it is produced, H2S gas can diffuse across membranes (9) to initiate paracrine signaling, in other sites remote from its production site. H2S-based signaling is mediated by formation of persulfides at reactive cysteine residues on target proteins to change activity (10). Persulfides can form via reaction of HS⁻ with oxidized cysteine on proteins such as cysteine sulfenic acid or by the reaction of cysteine thiylates on oxidized sulfide species such as HSSH and polysulfides (11).

Besides regulating vascular tone (12–18) and neuronal activity (3, 19), H2S provides profound cytoprotective effects. H2S treatment reduces oxidative injury during ischemia–reperfusion in various organ systems (20–22) and protects from heart failure in disease models (23–26). It protects neuronal cells from oxidative stress (27–31) and from the cytotoxic effect of β-amyloid peptides (32, 33). H2S treatment also increases resistance to ER stress in neuronal cells (34, 35), cardiomyocytes (36), and endothelial cells (37). It suppresses ER-induced endothelial to mesenchymal transdifferentiation (37), a pathological factor for progression of cardiac fibrosis (38), and reprograms cellular energy production in pancreatic beta cells in response to chronic ER stress (39). Although the mechanism of H2S-induced cytoprotection is not well understood, a growing body of evidence suggests that the effect of H2S is mediated by induction of anti-oxidative (27, 29, 40) and anti-apoptotic pathways (40). However, the mechanism by which H2S orchestrates a cellular defense response is not well studied.

2 The abbreviations used are: CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; ISR, integrated stress response; ER, endoplasmic reticulum; MEF, mouse embryonic fibroblast; eIF2α-P, eIF2α phosphorylation; AMPK, AMP-activated protein kinase; Tg, thapsigargin; NEM, N-ethylmaleimide; mTOR, mammalian target of rapamycin.

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Cells respond to endogenously produced and external stressors that perturb cellular homeostasis by activating stress-response pathways to adapt to stressful conditions and to minimize damage to cellular components. Recent findings have uncovered a significant regulatory role for H2S signaling on this front and fueled a growing pharmacological interest in H2S for treatment of cardiovascular diseases and inflammation where stress-induced cell injury contributes significantly to disease progression (1, 2). H2S is a signaling molecule produced endogenously from sulfur-containing amino acids, cysteine and homocysteine, by the actions of two enzymes in the trans-sulfuration pathway, cystathionine β-synthase (CBS) (2) and cystathionine γ-lyase (CSE) (3), and from 3-mercaptopyruvate catalyzed by mercaptopyruvate sulfurtransferase (4, 5). Metabolic removal of H2S involves its oxidation in mitochondria to thiosulfate and sulfate (6), which are excreted in urine (7). H2S is a weak acid and ionizes to HS⁻ and H⁺ with a pKa of 6.9 (8) resulting in an estimated 80% of it being in the ionized form at physiological pH. Although HS⁻ is likely confined to cells in which it is produced, H2S gas can diffuse across membranes (9) to initiate paracrine signaling, i.e. at sites remote from its production site. H2S-based signaling is mediated by formation of persulfides at reactive cysteine residues on target proteins to change activity (10). Persulfides can form via reaction of HS⁻ with oxidized cysteine on proteins such as cysteine sulfenic acid or by the reaction of cysteine thiylates on oxidized sulfide species such as HSSH and polysulfides (11).

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Regulation of integrated stress-response pathway by H2S

The key biochemical step for ISR is the induction of eIF2α phosphorylation, an evolutionarily conserved cytoprotective response, which has broad cellular consequences including translational and transcriptional reprogramming (41, 42). Phosphorylation of eIF2α at Ser-51 is catalyzed by four kinases, GCN2, PERK, HRI, and PKR, each responding to different stresses (42). Phosphorylation of eIF2α blocks global mRNA translation, whereas translation of select cytoprotective proteins, including ATF4, continues via regulatory uORFs in their 5′-UTRs (42). ATF4 activates expression of stress-response genes and promotes proteostasis via a feedback loop that involves induction of GADD34, a regulatory subunit of protein phosphatase-1 (PP1c), which dephosphorylates eIF2α-P. Low basal level of eIF2α-P in unstressed cells is maintained by the action of PP1c in complex with the constitutively expressed regulatory subunit CrEP (43–45).

In this study, we tested the hypothesis that H2S regulates the ISR signaling pathway. Herein, we describe the cellular response to H2S and show that exogenous H2S or induction of its endogenous synthesis leads to increased eIF2α phosphorylation. H2S leads to increased eIF2α-P levels by inhibiting PP1c phosphatase via persulfidation, which in turn leads to transient suppression of global translation and activation of ATF4 expression.

Results

H2S induces phosphorylation of eIF2α

To test whether H2S modulates eIF2α phosphorylation, we treated mouse embryonic fibroblast (MEF) cells and HeLa cells with 100 μM NaHS for 2 h. NaHS treatment resulted in a ~2.5-fold increase in eIF2α phosphorylation in both cell types, whereas the total eIF2α levels did not change (Fig. 1, a and b). The increase in eIF2α phosphorylation was evident as early as 1 h after H2S exposure, and it decayed to baseline levels after 8–12 h (Fig. 1, c and d). A 25 μM concentration of NaHS was sufficient to increase eIF2α-P levels, and no further increase was seen at concentrations up to 200 μM (Fig. 1, e and f). In comparison, the ER stress-inducing agent, tunicamycin (Tn), resulted in a more robust increase in eIF2α phosphorylation (Fig. 1a). However, repeated exposure to H2S (100 μM NaHS added every 4 h) resulted in a gradual increase in eIF2α phosphorylation with no change in eIF2α level (Fig. 1g).

Next, we tested whether induction of endogenous H2S production elicits similar effects on eIF2α phosphorylation levels. We have recently described a regulatory switch whereby inhibition of CBS by CO increases H2S production by CSE (46). We exploited this regulatory strategy by overexpressing heme oxygenase-2 (HO-2), a CO-producing enzyme, in HEK293 cells. Transient overexpression of HO-2 increased endogenous H2S levels, as detected in live cells using the fluorescent H2S probe, 7-azido-4-methylcoumarin that was sensitive to propargylglycine, an inhibitor of CSE (Fig. 2a), and resulted in a 4-fold increase in basal eIF2α-P level compared with cells transfected with an empty plasmid (p = 0.02) (Fig. 2b). We obtained similar results by overexpressing nitric-oxide synthase (NOS), a source of NO, which also inhibits CBS activity (Fig. 2d) (47). Although the increase in eIF2α-P levels in response to a single dose of exogenous H2S was transient, induction of endogenous H2S production by HO-2 overexpression resulted in a persistent increase in eIF2α-P (compare Fig. 2, b and c, with Fig. 1, a and b). This result reveals that both induction of endogenous H2S synthesis and exogenous H2S addition are associated with increased eIF2α-P levels. To further test whether the increase in eIF2α-P level by overexpression of CO- or NO-producing enzymes is mediated by CBS inhibition, we analyzed eIF2α-P levels in liver tissue homogenates prepared from CBS−/− mice, as described previously (48). An ~2-fold higher level of eIF2α-P levels was consistently seen in CBS−/−/liver compared with wild-type control (Fig. 2, e and f).
Inhibition of global protein synthesis by H\(_2\)S

We tested whether H\(_2\)S-induced eIF2\(\alpha\)-P phosphorylation leads to inhibition of protein translation. For this, we monitored incorporation of [\(\text{\textsuperscript{35}S}\)]methionine into the protein pool in MEF cells (\(\pm 100 \ \mu\text{M} \ \text{NaHS} \) treatment) and in HEK293 cells transiently overexpressing HO-2 in the absence of exogenous H\(_2\)S. We observed a significant decrease in translation in H\(_2\)S-treated cells (\(p = 0.007\)) and in HO-2-overexpressing HEK293 cells (\(p = 0.005\)) compared with controls (Fig. 3, a and b). Because exogenous H\(_2\)S increased eIF2\(\alpha\)-P levels transiently (Fig. 1d), we determined whether the kinetics of translational suppression was correlated with this behavior. For this, we monitored the time-dependent changes in protein translation in MEF cells exposed to a single dose of H\(_2\)S (100 \(\mu\text{M} \ \text{NaHS}\)). H\(_2\)S-induced inhibition of protein synthesis was exerted over 4 h and returned to baseline levels over 8–12 h mirroring the pattern of H\(_2\)S-induced increase in eIF2\(\alpha\)-P levels (Figs. 3c and 1c). This result is consistent with the involvement of H\(_2\)S-induced eIF2\(\alpha\) phosphorylation in translational suppression.

Next, we tested whether H\(_2\)S induced ATF4 expression, which is associated with increased eIF2\(\alpha\)-P levels and inhibition of global translation. ATF4 was increased in MEF cells after a single dose of H\(_2\)S treatment (Fig. 4a), and in cells stably overexpressing HO-2, compared with control cells (Fig. 4b) confirming induction of ATF4 expression by H\(_2\)S.

Inactivation of protein phosphatase-1 by persulfidation

The transient increase in eIF2\(\alpha\) phosphorylation levels in response to H\(_2\)S treatment can result from activation of one of the four upstream kinases and/or by inhibition of the phosphatase, PP1c. We hypothesized that the increase in eIF2\(\alpha\)-P levels by H\(_2\)S results from inhibition of the basal activity of PP1c for the following reason. H\(_2\)S-induced increase in eIF2\(\alpha\)-P levels was lower compared with the effect of ER stress-inducing
agents (Fig. 1a) and was independent of \( H_2S \) concentration between 25 and 200 \( \mu M \) NaHS (Fig. 1e). Additional increase in \( eIF2\alpha\)-P levels required either repeated exposure to \( H_2S \) (Fig. 1g) or sustained \( H_2S \) overproduction (Fig. 2, b and c). These results suggested to us that the increase in \( eIF2\alpha\)-P levels upon \( H_2S \) exposure is limited by its rate of basal phosphorylation.

To test our hypothesis, we expressed and purified recombinant human PP1c to \( 95\% \) purity and analyzed the effect of \( H_2S \) on dephosphorylation of \( eIF2\alpha\)-P in extracts prepared from cells exposed to ER stress. Addition of PP1c to extracts reduced \( eIF2\alpha\)-P (\( p = 0.007 \)), whereas NaHS-treated PP1c had no effect (Fig. 5, a and b). PP1c contains 13 cysteines, including several reactive ones, Cys-127, Cys-273, and Cys-291 (49). We hypothesized that the observed decrease in PP1c activity in the presence of \( H_2S \) was due to persulfidation, which was characterized by mass spectroscopic analysis. A single cysteine, corresponding to Cys-127, was identified as being persulfidated (Fig. 6). To test whether Cys-127 mediates the effect of \( H_2S \) on PP1c activity, we substituted Cys-127 with serine. Whereas PP1c-C127S efficiently dephosphorylated \( eIF2\alpha\)-P in cell extracts (\( p = 0.01 \)), it was unresponsive to \( H_2S \) treatment (Fig. 5, c and d) consistent with the importance of Cys-127 in mediating the \( H_2S \) effect. To further validate these results, we overexpressed wild-type and C127S-PP1c in HEK293 cells. Overexpression of wild-type and mutant PP1c significantly reduced \( eIF2\alpha\)-P levels (Fig. 5, e and f) as expected (43, 44). Treatment of these cells with 100 \( \mu M \) \( H_2S \) increased the \( eIF2\alpha\)-P level in cells overexpressing wild-type PP1c but not in cells overexpressing C127S-PP1c (Fig. 5e) confirming that Cys-127 is required to mediate \( H_2S \) inhibition of PP1c activity. Interestingly, \( H_2S \) had no effect on dephosphorylation when \( p \)-nitrophenyl phosphate or a phosphopeptide corresponding to residues 45–56 of \( eIF2\alpha\), \( I(LLEL(pS)RRRIR) \), was used as substrates (data not shown). This difference in \( H_2S \) effects on PP1c presumably results from differences in its interaction between the phosphopeptide and full-length protein substrates as also demonstrated for \( eIF2\alpha \) phosphorylation, which requires at least 80 amino acids from the N terminus (50).
were quantified. The ratio of eIF2\alpha-P to eIF2\alpha was contributed by increased phosphorylation of PERK due to inhibition of PTP1B by H2S. For this, we analyzed levels of eIF2\alpha-P and eIF2\alpha in the presence or absence of NaHS. EP denotes empty plasmid.

Western blot analysis. Involvement of PERK kinase in H2S-induced phosphorylation of eIF2\alpha. H2S reportedly inhibits PTP1B phosphatase during ER stress (51). PTP1B dephosphorylates the PERK kinase, an ER stress sensor that autophosphorylates and induces the PERK signaling pathways to transiently induce eIF2\alpha-P levels independent of PERK activation.

Discussion

In this study, we have characterized an H2S signaling pathway using a cellular model system where endogenous H2S production was induced by overexpressing HO-2. Our results indicate that H2S is a physiological modulator of eIF2\alpha phosphorylation status and that it exerts its effect via a mechanism involving persulfidation and concomitant inhibition of PP1c. Phosphorylation of eIF2\alpha is typically induced under stress conditions by activation of upstream kinases to guard against dysregulation of cellular homeostasis. However, the existence of signaling pathways to transiently induce eIF2\alpha phosphorylation in the absence of overt stress is largely unexplored. Herein, we show that H2S-induced inhibition of PP1c provides an alternative route to modulate eIF2\alpha-P levels independent of upstream kinases. We propose that although a transient increase in H2S production induces an acute response, which is consistent with its cytoprotective effects, continuous exposure results in a persistent increase in eIF2\alpha-P levels but is tolerated in cells due to the presence of an efficient H2S oxidation pathway present in mitochondria. Consistent with this model, disruption of the first sulfide oxidation pathway enzyme in Caenorhabditis elegans leads to death upon H2S exposure resulting from both ER and mitochondrial stress (54).

A cytoprotective effect for H2S-induced inhibition of PP1c leading to a transient increase in basal eIF2\alpha-P levels is consistent with other reports. For instance, inhibition of PP1c activity by knockdown of CREP activates the ISR and is cytoprotective against stressors, including oxidative and ER stress (43). Similarly, inhibition of PP1c interaction with the regulatory subunits by salubrinal (55) or by mutagenesis (44) and by GADD34 knock-out (56) increases eIF2\alpha-P levels, inhibits translation,
and in some systems has a protective role against stress (43, 55, 56). Our results might be relevant for understanding the protective effect of H₂S on protein metabolism in response to hypoxia-induced stress as discussed below (57).

PP1c is rich in cysteine residues and contains several reactive ones. Cys-273 is critical for activity, and microcystin, an inhibitor of PP1c, functions by binding covalently to the sulfur in Cys-273 (49, 58). In the crystal structure of PP1c, Cys-127 is oxidized to sulfinic or sulfonic acid, and Cys-291 forms a mixed disulfide with mercaptoethanol (49). Our results validate that Cys-127 in PP1c is a target of persulfidation, which was initially picked up in a persulfide proteomic analysis under ER stress conditions that is accompanied by increased H₂S synthesis (39).

We predict that PP1c activity is sensitive to cysteine modifications, and its inhibition by persulfidation results in increased eIF2α-P levels and to modulation of global translation.

PP1c associates with a variety of regulatory subunits that dictate target specificity (59). Although the dependence of the H₂S effect on the identity of the regulatory domains remains to be demonstrated, our model is consistent with the reported effect of H₂S on increasing phosphorylation levels of AMP-activated protein kinase (60), which is dephosphorylated by PP1c in complex with the regulatory subunit R6 (61). AMP-activated protein kinase functions as an energy sensor, and its phosphorylation under hypoxia activates the AMPK/TSC2/Rheb/mTOR signaling pathway, which inhibits mTOR activity. These changes lead to suppression of the initiation and elongation phases of translation (62, 63). Although it is not understood how H₂S treatment modulates this signaling network during hypoxia, we speculate that H₂S-induced inhibition of PP1c activity might increase AMPK phosphorylation for inhibition of eIF2B activity.

In summary, we have shown that transient exposure of cells to H₂S leads to increased eIF2α phosphorylation by PP1c persulfidation at Cys-127, which leads to its inhibition. This study reveals a previously unknown mode of regulation for the eIF2α-P level that may underlie the cytoprotective effects of H₂S. The ISR/ATF4 program mediates metabolic reprogramming of cells exposed to ER stress via H₂S-mediated protein persulfidation (39). The current findings suggest that H₂S might also contribute to the outcome of ISR in part by modulating translational recovery required for transcriptional reprogramming and adaptation (64, 65). Translational recovery depends on the phosphorylation status of eIF2α and is critical in most chronic stress conditions as uncontrolled translational recovery decreases survival of stressed cells (39, 66, 67). Our data suggest that inhibition of PP1c by H₂S can potentially dampen translational recovery and be important in delaying the onset of diseases involving chronic stress.

**Experimental procedures**

**Materials**

Rabbit polyclonal anti-eIF2α (catalog no. 9722), rabbit monoclonal anti-PERK (catalog no. 3192), and rabbit monoclonal anti-PERK-P (catalog no. 3179) were purchased from Cell Signaling Technology. Rabbit monoclonal anti-eIF2α-P (catalog no. ab32157) was purchased from Abcam. Rabbit polyclonal anti-HO-2 (catalog no. LS-C48375) was purchased from LSBIO. Rabbit polyclonal anti-ATF4 (catalog no. cs-200) was purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-PP1c (catalog no. 55150-1-AP) was purchased from Proteintech. All other chemicals were purchased from Sigma unless otherwise noted.

**Cell culture**

ATF4+/− and ATF4−/− MEFs were obtained from Dr. Ronald Wek (Indiana University School of Medicine) and were described previously (41, 68). HEK293, RWPE (prostate cells), and HeLa cells were grown in DMEM. LNCaP cells (prostate cancer cells) were grown in RPMI 1640 media. Perk+/− MEF
**Regulation of integrated stress-response pathway by H$_2$S**

Cells were treated with thapsigargin for >24 h to induce eIF2$\alpha$ phosphorylation. Dephosphorylation of eIF2$\alpha$ was performed in a 50-μl reaction mixture containing 50 mM Tris, pH 7.4, 100 mM KCl, 4 mM MgCl$_2$, and 50–100 μg of protein from cell extracts prepared as described above from Tg-treated cells but without protease inhibitors. Reactions were started by adding 5–10 μg of purified wild-type or mutant PP1c with or without H$_2$S pretreatment (250 μM final) and incubated at room temperature for 10 min. At the end of the incubation time, SDS denaturing buffer was added to the reaction mixtures and boiled for 5 min at 95°C. Proteins were separated on 10–12% SDS-polyacrylamide gel and then transferred to a PVDF membrane. Membranes were blocked with 5% non-fat dry milk in TTBS (Tris-buffered saline containing 0.1% Tween 20) for 1 h at room temperature with shaking and washed four times with TTBS before overnight incubation with the primary antibody at 4°C. Membranes were washed for 4–5 times, 20 min each, with TTBS and then incubated with the secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Signal intensities for specific proteins were estimated using ImageJ (National Institutes of Health) software. Antibody dilutions were as follows: anti-eIF2α, 1:1000; anti-eIF2α-P, 1:1000; anti-HO-2, 1:1000; anti-PP1c, 1:1000; and anti-ATF4, 1:500.

**Western blot analysis**

Cells were washed three times with PBS on ice and scraped in 50 mM Tris, pH 7.4, containing 0.1% Triton X-100, complete protease inhibitor mixture (Sigma) or in RIPA buffer supplemented with protease inhibitor mixture. Extracts were incubated on ice for 30 min before centrifuging at 13,000 × g for 10 min at 4°C. Aliquots of supernatants were added to SDS denaturing buffer and boiled for 5–10 min. Equal amounts of proteins were separated on 10–12% SDS-polyacrylamide gel and then transferred to a PVDF membrane. Membranes were blocked with 5% non-fat dry milk in TTBS (Tris-buffered saline containing 0.1% Tween 20) for 1 h at room temperature with shaking and washed four times with TTBS before overnight incubation with the primary antibody at 4°C. Membranes were washed for 4–5 times, 20 min each, with TTBS and then incubated with the secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Signal intensities for specific proteins were estimated using ImageJ (National Institutes of Health) software. Antibody dilutions were as follows: anti-eIF2α, 1:1000; anti-eIF2α-P, 1:1000; anti-HO-2, 1:1000; anti-PP1c, 1:1000; and anti-ATF4, 1:500.

**Western-based eIF2α dephosphorylation assay**

Cells were grown in either 6-cm plates or in 12-well plates to a confluency of 80%. Then they were labeled with 15 or 30 μCi/ml $^{35}$S-methionine (PerkinElmer Life Sciences) and continued to grow for 1–2 h depending on the experiment. Fresh medium was added to plates 1 h before radiolabel addition. At the end of the labeling time, cells were washed twice with PBS and scraped off the plates in RIPA buffer. After two freeze/thaw cycles, 10% (v/v) of trichloroacetic acid (TCA) was added to precipitate proteins. Aliquots from extracts were used to measure protein concentration. The protein precipitate was washed twice with 5% TCA and dissolved in 200 μl of 1 M NaOH, and the radioactivity was counted in a liquid scintillation counter. Radioactivity was normalized to protein concentration measured using Bradford reagent (Bio-Rad) with bovine serum albumin as the standard. For radioactive gels, extracts were denatured in SDS dye loading buffer and boiled for 5 min before electrophoresis. Equal amounts of protein were loaded in each well. Radioactive gels were dried in a gel dryer attached to a vacuum pump, placed on a phosphor storage screen cassette for 24 h, and imaged on a STORM 860 phosphorimager. Autoradiograms were quantified using the software ImageJ.

**Figure 7. Perk kinase activation is not involved in H$_2$S-induced increase in eIF2α phosphorylation.** a, Western blot analysis for eIF2α-P level in extracts from Perk$^{-/-}$ MEF cells along with wild-type control cells with and without NaHS treatment, 100 μM for 1 h, b, quantification of signal intensities for eIF2α-P and eIF2α from three independent experiments. c, rate of protein synthesis in Perk$^{-/-}$ MEF cells along with wild-type controls were determined by radiolabel incorporation from $^{35}$S Met in the presence or absence of 100 μM NaHS. Error bars represent S.D. from three independent experiments.

**Metabolic labeling and determination of protein translation rate**

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Fluorescence microscopy

H₂S was visualized in HEK293 cells transfected with mammalian expression construct for HO-2 or with an empty plasmid using 7-azido-4-methylcoumarin as described previously (69). Briefly, 30–36 h post-transfection, 7-azido-4-methylcoumarin was added to the culture medium to a final concentration of 50 μM, and incubation was continued for 30 min. Then, the cells were washed three times with PBS and visualized using an IX70 inverted microscope, connected with Photometrics CoolSNAP HQ2 camera, using 720-nm laser excitation. Metamorph software was used to acquire and analyze images. Propargylglycine, an inhibitor of the H₂S-producing enzyme, CSE, was added 6 h prior to imaging at a 2 μM final concentration.

Cloning, expression, and purification of PP1c

The mammalian expression vector containing PP1c, pEZ-M01, was purchased from GeneCopoeia (Rockville, MD). The PP1c-coding region was PCR-amplified using the forward (5’-GGAGGAGTTCCGACATGCGGGATTGATAAACTCAACATCG) and reverse (5’-GGCGCGCCGACTCCGAGCTATTTCCTTGGTCTTTGTGATCACAC) primers containing the XhoI and NdeI restriction sites, which were used for subcloning into the pET28b bacterial expression vector to generate the expression construct pET28b-PP1c. E. coli BL21 (DE3) cells transformed with the pET28b-PP1c construct were grown overnight at 37 °C in 100 ml of lysogeny buffer containing 20 mM sodium phosphate, pH 7.4, 150 μg of lysozyme, 10 mM MgCl₂, 500 mM NaCl, 20 mg of DNase, and 10% glycerol. The cell suspension was stirred at 4 °C for 30 min and then sonicated at a power setting of 7 for 10 min in 30-s intervals separated by 1 min of cooling. The sonicate was centrifuged at 17,000 g for 30 min to obtain the soluble fraction.

The N-terminal His-tagged PP1c was affinity-purified using a nickel-nitrilotriacetic acid column in 20 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, 10% glycerol. Further purification was achieved by gel filtration chromatography using a pre-packed Hiload 16/60 Superdex 200 column (GE Healthcare) using the same buffer.

Mass spectrometry

Recombinant PP1γ (20 μg) was treated with NaHS (1 mM) at room temperature for 30 min and then incubated with the thiol-blocking buffer containing 20 mM NEM for 30 min. NEM-alkylated PP1γ was subjected to a non-reducing SDS gel and stained with Coomassie Blue. The PP1γ protein band was excised and digested with trypsin and chymotrypsin. The peptide samples were analyzed by capillary column LC-tandem MS and the CID spectra searched against the human reference sequence database using the program Mascot and more specifically against the protein sequences with the program Sequest. All analyses utilized the standard LC gradient from 2 to 70% acetonitrile in 110 min.
Cell viability analysis

Because H$_2$S can impact metabolic rate and energy production, we used the trypan blue assay for staining and counting live and dead cells under the microscope using a hemocytometer. Flow cytometry was used for MEF to determine the effect of H$_2$S on cell viability using propidium iodide (Calbiochem) to stain dead cells. Cells were harvested by trypsinization and washed twice with PBS. To the cell suspensions, 1% BSA (w/v) was added to prevent cell aggregation and to stain dead cells. Cells were harvested by trypsinization and alternately, mixed with trypan blue for counting under the microscope. The data are presented as percent dead or live cells.

Statistical analysis

The statistical significance of observed differences was evaluated using paired $t$ test.

Author contributions—V. Y. performed the majority of the experiments and analyzed the data. X.-H. G. contributed his expertise in preparing samples for detection of PP1c persulfidation. B. W. performed the mass spectroscopy analysis with LC-MS/MS. M. H. contributed to designing the study related to ISR, data analysis, and manuscript preparation. R. B. contributed to data analysis and helped in manuscript preparation. O. K. conceived and designed the study, analyzed and interpreted the data, performed experiments, and prepared the manuscript. All authors approved the final version of the manuscript.

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References

1. Lavu, M., Bhushan, S., and Lefer, D. J. (2011) Hydrogen sulfide-mediated cardioprotection: mechanisms and therapeutic potential. Clin. Sci. 120, 219–229
2. Gong, Q. H., Shi, X. R., Hong, Z. Y., Pan, L. L., Liu, X. H., and Zhu, Y. Z. (2011) A new hope for neurodegeneration: possible role of hydrogen sulfide. J. Alzheimers Dis. 24, 173–182
3. Abe, K., and Kimura, H. (1996) The possible role of hydrogen sulfide as an endogenous neuromodulator. J. Neurosci. 16, 1066–1071
4. Shibuya, N., Tanaka, M., Yoshida, M., Ogasawara, Y., Togawa, T., Ishii, K., and Kimura, H. (2009) 3-Mercaptopropionate sulfurate transferase produces hydrogen sulfide and bound sulfane sulfur in the brain. Antioxid. Redox Signal. 11, 703–714
5. Yadav, P. K., Yamada, K., Chiku, T., Koutmos, M., and Banerjee, R. (2013) Structure and kinetic analysis of H2S production by human mercaptopropyvurate sulfurate transferase. J. Biol. Chem. 288, 20002–20013
6. Hildebrandt, T. M., and Grieshaber, M. K. (2008) Three enzymatic activities catalyze the oxidation of sulfide to thiosulfate in mammalian and invertebrate mitochondria. FEBS J. 275, 3352–3361
7. Beauchamp, R. O., Jr., Bus, J. S., Popp, J. A., Boreiko, C. J., and Andjelkovich, D. A. (1984) A critical review of the literature on hydrogen-sulfide toxicity. Crit. Rev. Toxicol. 13, 25–97
8. Vorobets, V. S., Kovach, S. K., and Kolbasov, G. Y. (2002) Distribution of ion species and formation of ion pairs in concentrated polysulfide solutions in photoelectrochemical transducers. Russ. J. Appl. Chem. 75, 229–234
9. Mathai, J. C., Missner, A., Kügler, P., Sarapov, S. M., Zeidel, M. L., Lee, J. K., and Pohl, P. (2009) No facilitator required for membrane transport of hydrogen sulfide. Proc. Natl. Acad. Sci. U.S.A. 106, 16633–16638
10. Mustafa, A. K., Gadalla, M. M., Sen, N., Kim, S., Mu, W., Gazi, S. K., Barrow, R. K., Yang, G., Wang, R., and Snyder, S. H. (2009) H2S signals through protein S-sulfhydration. Sci. Signal. 2, ra72
11. Kabli, O., and Banerjee, R. (2014) Enzymology of H2S biogenesis, decay and antagonizing. Antioxid. Redox Signal. 20, 770–782
12. Hosoki, R., Matsuki, N., and Kimura, H. (1997) The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. Biochem. Biophys. Res. Commun. 237, 527–531
13. Zhao, W., Zhang, J. L., and Wang, R. (2001) The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO J. 20, 6008–6016
14. Yang, G., Wu, L., Jiang, B., Yang, W., Qi, J., Cao, K., Meng, Q., Mustafa, A. K., Mu, W., Zhang, S., Snyder, S. H., and Wang, R. (2008) H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine γ-lyase. Science 322, 587–590
15. Ali, M. Y., Ping, C. Y., Mok, Y. Y., Ling, L., Whiteman, M., Bhatai, M., and Pohl, P. (2006) Regulation of vascular nitric oxide in vitro and in vivo; a new role for endogenous hydrogen sulphide? Br. J. Pharmacol. 149, 625–634
16. Mustafa, A. K., Sikka, G., Gazi, S. K., Steppan, J., Jung, S. M., Bhunia, A. K., Barodka, V. M., Gazi, F. K., Barrow, R. K., Wang, R., and Amzel, L. M., Berkowitz, D. E., and Snyder, S. H. (2011) Hydrogen sulfide as endothelium-derivered hyperpolarizing factor sulfhydrates potassium channels. Circ. Res. 109, 1259–1268
17. Geng, B., Yang, J., Qi, Y., Zhao, J., Pang, Y., Du, J., and Tang, C. (2004) H2S generated by heart in rat and its effects on cardiac function. Biochem. Biophys. Res. Commun. 313, 362–368
18. Cheng, Y., Ndisang, J. F., Tang, G., Cao, K., and Wang, R. (2004) Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats. Am. J. Physiol. Heart Circ. Physiol. 287, H2316–H2323
19. Delo Russo, C., Tringali, G., Ragazzoni, E., Maggiano, N., Menini, E., Vaiano, M., Preziosi, P., and Navarra, P. (2000) Evidence that hydrogen sulfide can modulate hypothalamo-pituitary-adrenal axis function: in vitro and in vivo studies in the rat. J. Neuroendocrinol. 12, 225–233
20. Elrod, J. W., Calvert, J. W., Morrison, J., Doeller, J. E., Kraus, D. W., Tao, L., Jiao, X., Scalia, R., Kiss, L., Szabo, C., Kimura, H., Chow, C. W., and Lefer, D. J. (2007) Hydrogen sulfide attenuates myocardial ischemia–reperfusion injury by preservation of mitochondrial function. Proc. Natl. Acad. Sci. U.S.A. 104, 15560–15565
21. Nicholson, C. S., and Calvert, J. W. (2010) Hydrogen sulfide and ischemia–reperfusion injury. Pharmacol. Res. 62, 289–297
22. Ansari, S. B., and Kurian, G. A. (2016) Hydrogen sulfide modulates subcellular susceptibility to oxidative stress induced by myocardial ischemic reperfusion injury. Chem. Biol. Interact. 252, 28–35
23. Calvert, J. W., Elston, M., Nicholson, C. K., Gundewar, S., Jha, S., Elrod, J. W., Ramachandran, A., and Lefer, D. J. (2010) Genetic and pharmacologic hydrogen sulfide therapy attenuates ischemia-induced heart failure in mice. Circulation 122, 11–19
24. Barr, L. A., Shimizu, Y., Lambert, J. P., Nicholson, C. K., and Calvert, J. W. (2015) Hydrogen sulfide attenuates high fat diet-induced cardiac dysfunction via the suppression of endoplasmic reticulum stress. Nitric Oxide 46, 145–156
25. Polhemus, D. J., Kondo, K., Bhushan, S., Bir, S. C., Kevil, C. G., Murohara, T., Lefer, D. J., and Calvert, J. W. (2013) Hydrogen sulfide attenuates cardiac dysfunction after heart failure via induction of angiogenesis. Circ. Heart Fail. 6, 1077–1086
26. Kondo, K., Bhushan, S., King, A. L., Prabhu, S. D., Hamid, T., Koenig, S., Murohara, T., Predmore, B. L., Gojon, G., Sr., Gojon, G., Jr., Wang, R., Karusula, N., Nicholson, C. K., Calvert, J. W., and Lefer, D. J. (2013) H2S protects against pressure overload-induced heart failure via upregulation of endothelial nitric oxide synthase. Circulation 127, 1116–1127
27. Kimura, Y., and Kimura, H. (2004) Hydrogen sulfide protects neurons from oxidative stress. FASEB J. 18, 1165–1167
28. Kimura, Y., Dargusch, R., Schubert, D., and Kimura, H. (2006) Hydrogen sulfide protects HT22 neuronal cells from oxidative stress. Antioxid. Redox Signal. 8, 661–670

29. Kimura, Y.,goto, Y. and Kimura, H. (2010) Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. Antioxid. Redox Signal. 12, 1–13

30. Whiteman, M., Armbrust, J. S., Chu, S. H., Jia-Ling, S., Wong, B. S., Cheung, N. S., Halliwell, B., and Moore, P. K. (2004) The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite ‘scavenger’. J. Neurochem. 90, 765–768

31. Whiteman, M., Armstrong, J. S., Chu, S. H., Jia-Ling, S., Wong, B. S., Armstrong, J. S., and Moore, P. K. (2005) Hydrogen sulfide: a novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain? Biochem. Biophys. Res. Commun. 326, 794–798

32. Tang, X. Q., Yang, C. T., Chen, J., Yin, W. L., Siau, J. L., Wong, B. S., Armstrong, J. S., and Moore, P. K. (2006) Hydrogen sulfide: a novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain? Biochem. Biophys. Res. Commun. 326, 794–798

33. Li, X. H., Deng, Y. Y., Li, F., Shi, J. S., and Gong, Q. H. (2016) Neuroprotective effects of sodium hydrosulfide against β-amyloid-induced neurotoxicity. Int. J. Mol. Med. 38, 1152–1160

34. Xie, L., Tiong, C. X., and Bian, J. S. (2012) Hydrogen sulfide protects SH-SY5Y cells against 6-hydroxydopamine-induced endoplasmic reticulum stress. Am. J. Physiol. Cell Physiol. 303, C81–C91

35. Li, X., Zhang, K. Y., Zhang, P., Chen, L. X., Wang, L., Xie, M., Wang, C. Y. and Tang, X. Q. (2014) Hydrogen sulfide inhibits formaldehyde-induced endoplasmic reticulum stress in PC12 cells by upregulation of SIRT-1. PLoS ONE 9, e89856

36. Wei, H., Zhang, R., Jin, H., Liu, D., Tang, X., Tang, C., and Du, J. (2010) Hydrogen sulfide attenuates hyperhomocysteinemia-induced cardiomyocytic endoplasmic reticulum stress in rats. Antioxid. Redox Signal. 12, 1079–1091

37. Ying, R., Wang, X. Q., Yang, Y., Gu, Z. J., Mai, J. T., Qiu, Q., Chen, Y. X., and Wang, J. F. (2016) Hydrogen sulfide suppresses endoplasmic reticulum stress-induced endothelial-to-mesenchymal transition through Src pathway. Life Sci. 144, 208–217

38. Zeisberg, E. M., Tarnavski, O., Zeisberg, M., Dorfman, A. L., McMullen, J. R., Gustafsson, E., Chandraker, A., Yuan, X., Wu, T. T., Roberts, A. B., Neeson, E. G., Sayegh, M. H., Izumo, S., and Kalluri, R. (2007) Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat. Med. 13, 952–961

39. Gao, X. H., Krokowski, D., Gao, B. J., Bederman, I., Majumder, M., Paules, R., Stojdl, D. F., Bell, J. C., Hettmann, T., Liu, M., Arvan, P., and Hatzoglou, M. (2015) Quantitative H2S-mediated protein sulfhydrylation reveals metabolic reprogramming during the integrated stress response. eLife 4, e10067

40. Jha, S., Calvert, J. W., Duranski, M. R., Ramachandran, A., and Lefer, D. J. (2008) Hydrogen sulfide attenuates hepatic homocysteine–reperfusion injury: role of antioxidant and antiapoptotic signaling. Am. J. Physiol. Heart Circ. Physiol. 295, H801–H806

41. Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., Sadri, N., Yun, C. R., Fox, P. L., Gerson, S. L., Hoppel, C. L., Liu, M., Arvan, P., and Hatzoglou, M. (2015) Quantitative H2S-mediated protein sulfhydrylation reveals metabolic reprogramming during the integrated stress response. eLife 4, e10067

42. Baird, T. D., and Wek, R. C. (2012) Eukaryotic initiation factor 2 phosphorylation and translational control in metabolism. Adv. Nutr. 3, 307–321

43. Jousse, C., Oyadomari, S., Novoa, I., Lu, P., Zhang, Y., Harding, H. P., and Ron, D. (2003) Inhibition of a constitutive translation initiation factor 2α phosphatase, CREP, promotes survival of stressed cells. J. Cell Biol. 163, 767–775

44. Novoa, I., Zeng, H., Harding, H. P., and Ron, D. (2001) Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2α. J. Cell Biol. 153, 1011–1022

45. Bollen, M., Peti, W., Ragusa, M. J., and Beullens, M. (2010) The extended PPII1 toolkit: designed to create specificity. Trends Biochem. Sci. 35, 450–458

46. Kabil, O., Yadav, V., and Banerjee, R. (2016) Heme-dependent metabolite switching regulates H2S synthesis in response to endoplasmic reticulum (ER) stress. J. Biol. Chem. 291, 16418–16423

47. Taoka, S., and Banerjee, R. (2001) Characterization of NO binding to human cytosolateine β-synthase: possible implications of the effects of CO and NO binding to the human enzyme. J. Inorg. Biochem. 87, 245–251

48. Gupta, S., Kühnisch, J., Mustafa, A., Lhotak, S., Schlachterman, A., Silifier, M. I., Klein-Santo, A., High, K. A., Austin, R. C., and Kruger, W. D. (2009) Mouse models of cytosolateine β-synthase deficiency reveal significant threshold effects of hyperhomocysteimia. FASEB J. 23, 883–893

49. Goldberg, J., Huang, H. B., Kwon, Y. G., Greengard, P., Nairn, A. C., and Kuriyan, J. (1995) Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature 376, 745–753

50. Majumder, M., Mitchell, D., Merkulov, S., Wu, J., Guan, B. J., Nairn, A. C., and Lefer, D. J. (2008) Effect of hydrogen sulphide on NADPH oxidase-dependent inactivation of eNOS. Biochem. Biophys. Res. Commun. 360, 233–238

51. Cohen, P. T. (2002) Protein phosphatase 1–targeted in many directions. J. Cell Sci. 115, 241–256

52. Lee, H. J., Mariapann, M. M., Feliers, D., Cavaglieri, R. C., Sataranatarajan, K., Abboud, H. E., Choudhury, G. G., and Kasinath, B. S. (2012) Hydrogen sulfide inhibits formaldehyde-induced matrix protein synthesis by activating AMP-activated protein kinase in renal epithelial cells. J. Physiol. 590, 233–244

53. Lee, H. J., Mariapann, M. M., Feliers, D., Cavaglieri, R. C., Sataranatarajan, K., Abboud, H. E., Choudhury, G. G., and Kasinath, B. S. (2012) Hydrogen sulfide inhibits formaldehyde-induced matrix protein synthesis by activating AMP-activated protein kinase in renal epithelial cells. J. Physiol. 590, 233–244

54. Arsham, A. M., Howell, J. J., and Simon, M. C. (2003) A novel hypoxia-inducible factor α subunit target of retinoic acid receptor (RAR) family members. J. Biol. Chem. 278, 4451–4461

55. Garcia-Haro, L., Garcia-Gimeno, M. A., Neumann, D., Beullens, M., Bollen, M., and Sanz, P. (2010) The PPI1-R6 protein phosphatase holoenzyme is involved in the glucose-induced dephosphorylation and inactivation of AMP-activated protein kinase, a key regulator of insulin secretion, in MIN6 β cells. FASEB J. 24, 5080–5091

56. Arsham, A. M., Howell, J. J., and Simon, M. C. (2003) A novel hypoxia-inducible factor–dependent hypoxic response regulating mammalian target of rapamycin and its targets. J. Biol. Chem. 278, 29655–29660

57. Liu, L., Cash, T. P., Jones, R. G., Keith, B., Thompson, C. B., and Simon, M. C. (2006) Hypoxia-induced energy stress regulates mRNA translation and cell growth. Mol. Cell 21, 521–531

58. Novoa, I., Zhang, Y., Zeng, H., Jungreis, R., Harding, H. P., and Ron, D. (2003) Stress-induced gene expression requires programmed recovery from translational repression. EMBO J. 22, 1180–1187

59. Novoa, I., Zhang, Y., Zeng, H., Jungreis, R., Harding, H. P., and Ron, D. (2003) Stress-induced gene expression requires programmed recovery from translational repression. EMBO J. 22, 1180–1187

60. Novoa, I., Zhang, Y., Zeng, H., Jungreis, R., Harding, H. P., and Ron, D. (2003) Stress-induced gene expression requires programmed recovery from translational repression. EMBO J. 22, 1180–1187
Regulation of integrated stress-response pathway by H₂S

65. Kojima, E., Takeuchi, A., Haneda, M., Yagi, A., Hasegawa, T., Yamaki, K., Takeda, K., Akira, S., Shimokata, K., and Isobe, K. (2003) The function of GADD34 is a recovery from a shutoff of protein synthesis induced by ER stress: elucidation by GADD34-deficient mice. *FASEB J.* 17, 1573–1575

66. Han, J., Back, S. H., Hur, J., Lin, Y. H., Gildersleeve, R., Shan, J., Yuan, C. L., Krokowski, D., Wang, S., Hatzoglou, M., Kilberg, M. S., Sartor, M. A., and Kaufman, R. J. (2013) ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nat. Cell Biol.* 15, 481–490

67. Krokowski, D., Jobava, R., Guan, B. J., Farabaugh, K., Wu, J., Majumder, M., Bianchi, M. G., Snider, M. D., Bussolati, O., and Hatzoglou, M. (2015) Coordinated regulation of the neutral amino acid transporter SNAT2 and the protein phosphatase subunit GADD34 promotes adaptation to increased extracellular osmolarity. *J. Biol. Chem.* 290, 17822–17837

68. Jiang, H. Y., Wek, S. A., McGrath, B. C., Lu, D., Hai, T., Harding, H. P., Wang, X., Ron, D., Cavener, D. R., and Wek, R. C. (2004) Activating transcription factor 3 is integral to the eukaryotic initiation factor 2 kinase stress response. *Mol. Cell. Biol.* 24, 1365–1377

69. Chen, B., Li, W., Lv, C., Zhao, M., Jin, H., Jin, H., Du, J., Zhang, L., and Tang, X. (2013) Fluorescent probe for highly selective and sensitive detection of hydrogen sulfide in living cells and cardiac tissues. *Analyst* 138, 946–951