Endogenous Hydrogen Sulfide Production Is Essential for Dietary Restriction Benefits

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Summary

Dietary restriction (DR) without malnutrition encompasses numerous regimens with overlapping benefits including longevity and stress resistance, but unifying nutritional and molecular mechanisms remain elusive. In a mouse model of DR-mediated stress resistance, we found that sulfur amino acid (SAA) restriction increased expression of the transsulfuration pathway (TSP) enzyme cystathionine γ-lyase (CGL), resulting in increased hydrogen sulfide (H_2S) production and protection from hepatic ischemia reperfusion injury. SAA supplementation, mTORC1 activation, or chemical/genetic CGL inhibition reduced H_2S production and blocked DR-mediated stress resistance. *In vitro*, the mitochondrial protein SQR was required for H_2S-mediated protection during nutrient/oxygen deprivation. Finally, TSP-dependent H_2S production was observed in yeast, worm, fruit fly and rodent models of DR-mediated longevity. Together, these data are consistent with evolutionary conservation of TSP-mediated H_2S as a novel mediator of DR benefits with broad implications for clinical translation.

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Introduction

Dietary restriction (DR) encompasses a variety of nutritional interventions with overlapping functional benefits including increased stress resistance and extended longevity in a number of organisms across evolutionary boundaries (Fontana et al., 2010). In mammals, dietary regimens associated with these benefits are diverse, including reduced daily food intake, intermittent fasting and reduced protein or essential amino acid intake. While common features exist amongst DR regimens, including reduced adiposity and improved sensitivity to growth factors, differences exist as well. Thus, whether overlapping functional benefits of DR regimens share a common underlying nutritional and/or molecular basis remains unclear. Despite strong evidence of DR benefits in humans (Levine et al., 2014), difficulties with compliance prevent widespread clinical applications. Uncovering common mechanisms is thus of great significance for targeted dietary and/or pharmacological interventions.

Mitohormesis represents a potential unifying molecular hypothesis of DR action (Sinclair, 2005). Based on the concept of hormesis (Calabrese and Mattson, 2011), in which low level stressors promote adaptive changes resulting in stress resistance, the mitohormesis hypothesis of DR posits that increased reactive oxygen and nitrogen species (RONS) derived from increased mitochondrial fatty acid oxidation induce mild oxidative stress, thus driving adaptive mechanisms of antioxidant protection (Tapia, 2006). SKN1/NRF2 has emerged as a candidate mediator of adaptive protection based on its activation by RONS and function as a key transcription factor in the cytoprotective Phase II antioxidant and detoxification response (Hine and Mitchell, 2012). Gene targets of NRF2 include heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase quinone 1 (NQO-1), glutathione transferases (GSTs) and additional genes that utilize glutathione (GSH) for resolving oxidative stress. SKN1/NRF2 in worms is required for stress resistance and longevity benefits of DR (Bishop and Guarente, 2007). In mammals, NRF2 is required for DR-mediated protection from chemically induced carcinogenesis but not for DR-mediated longevity (Pearson et al., 2008). Functionally, mitohormesis can be blocked by Vitamin C, Vitamin E, and/or N-acetyl cysteine (NAC), presumably due to their antioxidant capacity (Ristow and Schmeisser, 2011).

Restriction of sulfur amino acids (SAA) methionine (Met) and cysteine (Cys) is common to numerous DR regimens across evolutionary boundaries and is thus a potential shared nutritional trigger of DR benefits. In yeast, Met restriction extends longevity (Ruckenstuhl et al., 2014). In flies, restriction of essential amino acids (EAA), and in particular Met, controls DR longevity benefits (Grandison et al., 2009). Dietary Met further interacts with overall protein levels in longevity control (Lee et al., 2014). In rodents, diets lacking the non-essential amino acid (NEAA) Cys and restricted for Met (Met restriction, or MetR) extend longevity and increase hepatic stress resistance (Miller et al., 2005; Orentreich et al., 1993).

The TSP controls the conversion of Met into Cys and is required for DR-mediated lifespan extension in flies (Kabil et al., 2011a). Two key enzymes of this evolutionarily conserved pathway are cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL). Under normal physiological conditions, CBS converts serine and homocysteine, a product of Met methyl transfer, into cystathionine. Cystathionine is then converted into α-ketobutyrate and Cys by

CGL (Kabil et al., 2011b). Although Cys is required for *de novo* synthesis of GSH, thus potentially linking TSP to NRF2-mediated antioxidant responses, the molecular mechanism underlying the genetic requirement for a functional TSP in DR-mediated benefits is unknown.

A product of the TSP with potential to mediate physiological benefits including stress resistance and extended longevity is the water and fat-soluble gas H_2S (Cuevasanta et al., 2012; Zhang et al., 2013). While toxic at high levels, H_2S produced at low concentrations by degradation of Cys or homocysteine by CGL or CBS acts on the vasculature and the brain as a signaling molecule to reduce blood pressure (Yang et al., 2008) and prevent neurodegeneration (Paul and Snyder, 2012). Exogenous H_2S can also extend lifespan of worms (Miller and Roth, 2007) and induce suspended animation in mammals (Blackstone et al., 2005). Although diet can impact H_2S production (Predmore et al., 2010), neither the dietary requirements for increased endogenous H_2S production, nor the potential role of H_2S in the benefits of DR, are currently known.

Ischemia reperfusion injury (IRI) is initiated by lack of nutrients and oxygen due to occlusion of blood flow (ischemia) followed by activation of pro-oxidation pathways and inflammatory mediators in damaged tissues upon return of blood flow (reperfusion). IRI represents a major clinical concern in controlled (tissue resection, organ transplantation) and uncontrolled settings (stroke, heart attack). Various short-term (3–14 days) DR regimens improve outcome in models of kidney, liver and brain IRI (Harputlugil et al., 2014; Mitchell et al., 2010; Peng et al., 2012; Varendi et al., 2014). Here we used dietary preconditioning against hepatic IRI as a model system to probe dietary and molecular mechanisms underlying protection.

Results

NAC, but not NRF2 deficiency, abrogates benefits of DR against IRI

50% DR for 7 days significantly decreased body weight, % fat mass, serum triglycerides (TG) (Figure 1A–C) and blood glucose (BG) (Supplemental Figure 1A) while increasing hepatic expression of FAO-related genes (Figure 1D) and the rate of peroxisomal FAO (Figure 1E) relative to the *ad libitum* (AL) fed group. Consistent with the mitohormesis hypothesis, hepatic RONS and NRF2 target gene expression were increased (Figure 1F,G), and the latter blocked by NAC administration during the DR period. Total GSH was also decreased upon DR (Figure 1H).

The functional relevance of increased RONS and NRF2 activation in DR-mediated stress resistance was tested in a model of hepatic IRI. Wildtype (WT) mice were preconditioned on AL or DR regimens +/− NAC for 1wk prior to IRI. NAC treatment was halted 24hrs prior to IRI to avoid any direct antioxidant effects of this short-lived compound on outcome. Neither DR nor NAC had any significant effect on liver damage markers in serum prior to IRI (data not shown). After reperfusion, liver damage markers remained significantly lower in DR serum indicative of protection from injury (Figure 1I). NAC had no effect on outcome in the AL group, but significantly reduced protection in the DR group. Macroscopic and histological analysis of hemorrhagic necrosis in livers excised 24hrs after reperfusion

(Figure 1J, Supplemental Figure 1B) were consistent with serum damage markers (Figure 1I).

To test the requirement for the NRF2-dependent Phase II antioxidant response, we compared NRF2 knockout (KO) mice to wildtype (WT) littermate controls. NRF2KO mice subject to DR had decreased hepatic GSH similar to WT (Supplemental Figure 1C), but failed to upregulate Phase II antioxidant response genes as expected (Supplemental Figure 1D). Surprisingly, benefits of DR against hepatic IRI did not require NRF2, with similar reductions in liver damage markers in serum (Figure 1K) and macroscopic evidence of hemorrhage 24hrs post reperfusion upon DR in both WT and NRF2KO mice (Supplemental Figure 1E). To confirm and extend this result, we tested the requirement for NRF2 in DR-mediated protection from renal IRI. While AL-fed NRF2KOs had slightly elevated damage and decreased renal function upon IRI relative to WT mice as reported previously (Liu et al., 2009), both gained similar benefits upon DR (Supplemental Figure 1F).

Sulfur amino acids control the benefits of DR and PR

NRF2 independence of DR benefits despite abrogation by NAC is consistent either with an alternate RONS-dependent mechanism of DR-mediated protection, or an antioxidant-independent activity of NAC. NAC is also a source of Cys, and NAC supplementation to the DR group above serendipitously returned dietary Cys content close to AL levels. To separate the functional consequences of antioxidant capacity from SAA content, we supplemented restricted diets separately with either antioxidants (Vitamin C and E, VitC&E) or a 2x concentration of Met and Cys (2xMet&Cys) for 1wk prior to hepatic IRI. Following reperfusion, protection afforded by DR was maintained despite VitC&E antioxidant treatment, but abrogated by 2xMet&Cys (Figure 2A, B) without affecting food intake, weight loss or expression of FOXO target genes (Supplemental Figure 2A, B, C), consistent with the relative importance of the SAA aspect of NAC in abrogation of DR benefits.

Protein and carbohydrates are isocaloric and partially interconvertible as dietary energy sources, rendering distinctions between calorie restriction and protein/amino acid restriction upon DR challenging. To test the relative contribution of energy restriction, protein restriction (PR), and specifically SAA restriction to DR-mediated protection against hepatic IRI, we preconditioned mice on a complete or isocaloric protein-free diet fed either AL or 35% restricted +/− 2xMet&Cys. Following hepatic IRI, PR mice displayed significant protection from liver damage (Figure 2C, D) independent of total calorie intake or weight loss (Supplemental Figure 2D, E). Importantly, 2xMet&Cys abrogated significant benefits of combined protein/energy restriction (Figure 2C, D) without affecting weight loss (Supplemental Figure 2E). Because MetR regimens offering overlapping functional benefits with DR are also restricted or deficient in Cys, we also tested Cys reconstitution alone. Following reperfusion, protection observed in the DR group was abrogated upon 2xCys supplementation (Figure 2E, F), consistent with PR and Cys restriction as critical aspects of DR-mediated resistance to hepatic IRI.

DR stimulates endogenous H_2S production via repression of mTORC1 and activation of the TSP

Expression and activity of TSP genes CBS and CGL increase when Cys is low (Stipanuk and Ueki, 2011) to allow *de novo* synthesis from Met (Figure 3A). Hepatic CGL and CBS to a lesser degree were increased on the mRNA and protein levels upon 1wk DR (Figure 3B, C), and this was abrogated by 2xMet&Cys or NAC supplementation (Figure 3B).

A metabolomics approach was used to identify candidate TSP metabolites involved in DR-mediated protection. To facilitate identification of potentially causative vs. correlative changes, we included a genetic model that fails to gain the benefits of DR against hepatic IRI due to liver-specific genetic ablation of the mTORC1 repressor, tuberous sclerosis complex 1 (TSC1) (Harputlugil et al., 2014) (Figure 3D, Supplemental Figure 3A). GSH and taurine are two downstream metabolites with potential to protect against IRI, however neither was increased by DR specifically in WT mice (Figure 3D). A third protective TSP metabolite is hydrogen sulfide (H_2S) (Kang et al., 2009). Although not detected in our metabolomic screen, associated by-products homoserine and serine (Kabil et al., 2011b) were significantly increased upon DR in WT but not TSC1KO livers (Figure 3D).

For direct measurement of H_2S, we leveraged the specific reaction between H_2S and lead acetate to form a black precipitate (lead sulfide) that can be trapped and visualized on filter paper containing lead acetate (Supplemental Figure 3B). In the presence of excess CGL/CBS substrate Cys and co-factor pyridoxal-5′-phosphate (PLP), H_2S gas produced by liver extracts was greater from DR than AL fed mice (Figure 3E). This phenomenon was independent of NRF2 status (Supplemental Figure 3C). Met&Cys add-back during DR blocked the DR-mediated increase in H_2S production capacity (Figure 3F), correlating with a loss of protection against hepatic IRI (Figure 2A) and normalization of hepatic GSH levels (Supplemental Figure 3D).

Using a less specific but more easily quantifiable sulfide probe-based method calibrated against known H_2S concentrations (Supplemental Figure 3E, F), liver extracts of DR mice displayed a 4-fold increase in H_2S production capacity compared to AL mice (Figure 3G). Endogenous hepatic H_2S levels measured by insertion of the sulfide probe into the right and median lobes of anesthetized mice were in the low micromolar range relative to the appropriate standard curve (Supplemental Figure 3G, H), and nearly 3-fold higher in the livers of DR relative to AL fed mice (Figure 3H).

Consistent with a link between DR, TSP, H_2S and protection from IRI, DR failed to increase hepatic CGL protein and H_2S production capacity in livers of TSC1KO mice (Figure 3I, J), suggesting that constitutive mTORC1 activation blocks DR-mediated TSP upregulation.

H_2S is necessary and sufficient to confer DR-mediated stress resistance *in vivo*

We next determined if exogenous H_2S is sufficient to confer protection in our model of hepatic IRI. AL-fed mice injected with the H_2S precursor NaHS or vehicle 30min prior to ischemia had decreased liver damage relative to vehicle-treated controls (Figure 4A, B). Similarly, non-invasive H_2S delivery in the drinking water for 1wk prior to hepatic IRI using the slow releasing/long-lived donor GYY4137 (Lee et al., 2011) (but not the fast

releasing/short-lived donor NaHS) decreased liver damage without affecting animal weight, food intake or water consumption (Supplemental Figure 4A, B, C, D). Thus, exogenous H_2S is sufficient to protect against hepatic IRI.

A specific inhibitor of CGL, DL-propargylglycine (PAG), was employed to determine if endogenous H_2S is necessary for DR-mediated stress resistance. PAG treatment blocked hepatic H_2S production capacity (Supplemental Figure 4E) and decreased endogenous hepatic H_2S levels upon DR (Supplemental Figure 4F), consistent with the dominant role of CGL in hepatic H_2S production. PAG treatment had no effect on IRI outcome in AL mice, but abrogated benefits of DR (Figure 4C, D). Re-addition of H_2S via IP injection of 30min prior to induction of ischemia in DR+PAG-treated mice restored protection (Figure 4C, D).

Two genetic models were used to test the specificity of the TSP and H_2S in protection against hepatic IRI (Figure 4E–L). The necessity of CGL in DR-mediated benefits was tested with whole-body CGLKO mice (Yang et al., 2008). CGLKO mice responded to DR like WT mice with respect to changes in body weight, food intake, body composition and blood glucose (Supplemental Figure 4G–J) despite the lack of CGL protein in liver or capacity of liver extracts to produce H_2S (Figure 4E, F). However, while the absence of CGL had no apparent impact on the outcome of hepatic IRI in AL fed mice, CGLKO mice failed to gain protection upon DR (Figure 4G, H). To test the sufficiency of increased TSP activity and endogenous H_2S production, CGL was overexpressed via adenoviral-mediated gene delivery (Ad-CGL). Hepatic CGL protein levels and H_2S production capacity were both significantly increased (Figure 4I, J) independent of body mass or food intake relative to control adenovirus (Ad-Null) infected mice (Supplemental Figure 4K, L). Ad-CGL infection resulted in improved outcome after hepatic IRI relative to Ad-Null infected mice (Figure 4K, L). Together, these results suggest that increased TSP activity and endogenous H_2S production via CGL are sufficient, and in the case of DR, necessary, for protection against the acute surgical stress of hepatic IRI.

H_2S is necessary and sufficient to confer DR-mediated stress resistance *in vitro* via sulfhydration and antioxidant properties

To determine if TSP gene expression and H_2S production can be induced in a cell autonomous manner by SAA restriction, we cultured mouse hepatocyte-derived Hepa1-6 cells overnight in complete media or media lacking SAA (–Met&Cys). Similar to DR *in vivo*, SAA deprivation increased CBS and CGL expression and H_2S production capacity *in vitro* (Figure 5A, B).

To determine if exogenously added H_2S can protect cultured Hepa1-6 cells against simulated IRI, warm ischemia was simulated by replacing growth media with saline and incubating in a 37°C hypoxic chamber for up to 4hrs, and reperfusion by replacing the growth media under normoxic conditions. Addition of H_2S decreased Hepa1-6 cell damage under both normoxic and hypoxic conditions during the ischemic phase (Figure 5C) and restored MTT activity during the reperfusion phase (Figure 5D), indicating that exogenous H_2S is sufficient to induce significant protection against both nutrient/energy deprivation and hypoxia *in vitro*.

To assess if endogenous H_2S induced by SAA restriction afforded similar protection, primary hepatocytes were cultured +/− Met&Cys overnight prior to simulated IRI. LDH release during ischemia was decreased (Figure 5E) and MTT activity during reperfusion increased in hepatocytes preconditioned in Met&Cys deficient media (Figure 5F). Similar protection afforded by overnight preconditioning of Hepa1-6 cells in -Met&Cys media was blocked by addition of PAG during the preconditioning period and was partially rescued by exogenous addition of H_2S during ischemia (Supplemental Figure 5A).

H_2S has been proposed to prevent cell damage/death under stress in part by sulfhydration of target proteins (Paul and Snyder, 2012) including the mitochondrial inner membrane component Sulfide Quinone Oxidoreductase (SQR), which allows transfer of electrons from H_2S to Coenzyme Q and the electron transport chain (ETC) (Módis et al., 2013; Olson et al., 2013) (Figure 5G). To test the potential role of the SQR in H_2S-mediated protection, we partially knocked down the SQR in Hepa1-6 cells (Supplementary Figure 5B) prior to simulated IRI +/− exogenous H_2S. H_2S addition reduced LDH release during the ischemic phase and upon reperfusion (Figure 5H, I); the latter was significantly altered upon SQR KD (Figure 5I). Additionally, the increase in MTT activity during the reperfusion phase in the presence of H_2S was significantly reduced by SQR knockdown (KD) (Figure 5J). SQR KD did not significantly alter the effects of H_2S addition on nutrient/growth factor withdrawal in the absence of hypoxia (Figure 5H–J).

Because oxidation of H_2S by SQR results in production of thiosulfate ($S_2O_3^{-2}$) (Hildebrandt and Grieshaber, 2008), which itself can be utilized in mammalian tissues under hypoxic conditions for further H_2S production (Olson et al., 2013), we next tested if this downstream metabolite could afford similar protection against simulated IRI in Hepa1-6 cells. While thiosulfate had a negative impact on LDH release upon nutrient/growth factor withdrawal under normoxia, increasing thiosulfate concentrations decreased cell damage during the ischemic phase under hypoxia (Supplemental Figure 5C) and improved MTT activity during the reperfusion phase (Supplemental Figure 5D). SQR KD partially blocked thiosulfate-mediated protection against simulated IRI (Figure 5K and Supplemental Figure 5E), suggesting that the protective effect of thiosulfate during hypoxia can be partially attributed to its conversion to H_2S.

H_2S also has antioxidant properties (Calvert et al., 2010) that could contribute to cytoprotection independent of target protein sulfhydration. At 100μM but not 10μM final concentration, H_2S protected Hepa1-6 cells against acute oxidative stress delivered by H_2O_2 exposure (Supplemental Figure 5F, G). Similar protection was found with 100μM H_2S against H_2O_2 exposure in primary smooth muscle cells (Supplemental Figure 5H, I). Thus, H_2S protects from acute ischemic insult and oxidative stress via multiple mechanisms.

Increased endogenous H_2S production in diet-induced longevity extension models across evolutionary boundaries

While exogenous H_2S can extend longevity in worms, the potential role of endogenous H_2S in DR-mediated lifespan extension has not been reported. To test the hypothesis that increased endogenous H_2S is a common feature of DR regimens that lead to extended

longevity across evolutionary boundaries, we measured H_2S production capacity in four different organisms subject to species-appropriate DR longevity regimens.

Adult mice subject to long-term MetR (Figure 6A), EOD fasting or 20–30% DR (Figure 6B) had increased H_2S production capacity in liver and kidney extracts compared to AL-fed complete diet controls. In mice subject to 1wk 50% DR, kidney, spleen and carotid artery additionally demonstrated increased H_2S production capacity (Supplemental Figure 6A), while brain, skeletal muscle, heart and aorta tested under the same conditions did not (Supplemental Figure 6B). Nonetheless, primary smooth muscle cells prepared from aorta displayed increased H_2S production capacity upon Met&Cys depletion (Supplemental Figure 6C), suggesting tissue/cell type specificity in the regulation of the TSP in response to diet. Although we failed to observe an increase in H_2S production capacity in mouse brain upon 1wk 50% DR, 3 days of water-only fasting increased H_2S production capacity (Supplemental Figure 6D).

In the fruit fly *D. melanogaster*, maximal H_2S production capacity of whole-body extracts of flies subject to varying levels of dietary protein and Met restriction (Figure 6C) correlated with maximal lifespan extension (Lee et al., 2014).

In the nematode worm *C. elegans*, the *eat-2* mutant serves as a genetic model of lifespan extension by DR due to defects in pharyngeal function resulting in decreased food intake (Lakowski and Hekimi, 1998). Extracts of *eat-2* mutants produced more H_2S than extracts of age-matched WT N2 worms (Figure 6D). To assess the genetic requirements for TSP factors in longevity extension, we performed individual RNAi-mediated KD of the two worm CGL homologs CTH-1 and CTH-2, the CBS homolog CBS-1, and a fourth worm TSP gene, CBL-1. Individual RNAi KD of CBL-1 and CBS-1 had no effect on longevity (Supplemental Figure 6E), but RNAi KD specifically of CBS-1 significantly decreased *eat-2* mediated lifespan extension, while overexpression of CBS-1 significantly extended median lifespan of WT N2 controls (Figure 6F).

Finally, DR-mediated extension of chronological lifespan in the yeast *S. cerevisiae* can be accomplished by reducing glucose in the growth media from 2% to 0.5% (Figure 6G) (Wu et al., 2013). Under these conditions, H_2S production from the glucose-restricted cultures was detected within 24–48hrs and continued to increase for up to 96hrs in three different yeast strains (Figure 6H and Supplemental Figure 6F, G). As the majority of H_2S production in yeast occurs via an assimilatory pathway not present in metazoans in which extracellular sulfate is converted to sulfide by the combined enzymatic activities of MET14, MET16, and MET5/10 (Figure 6I), we next asked if increased H_2S upon DR occurred through this assimilatory pathway or the conserved TSP pathway in yeast. Deletion of MET5, 14, or 16 resulted in inhibition of H_2S production under 2.0% glucose, but did not block H_2S induction (Figure 6J) or chronological longevity extension (Supplemental Figure 6H) upon glucose restriction. To further confirm Met and/or Cys, rather than inorganic sulfur, as the primary source of increased H_2S under conditions upon glucose restriction, we examined H_2S production in a met14 line lacking a functional assimilatory pathway, with differing concentrations of Met and Cys in the media. Complete deprivation of either Met or Cys resulted in reduced H_2S production (Figure 6K). Thus, under conditions of glucose

restriction, H_2S is produced from SAAs via the TSP. Because TSP-deficient CGL/STR1 and CBS/STR4 deletion strains grow abnormally in culture, it was not possible to test their requirement for increased H_2S production or extended chronological longevity under low glucose conditions. However, exogenous H_2S donors NaHS and GYY4137 added early after inoculation were sufficient to significantly increase chronological lifespan (Figure 6L).

Discussion

H_2S as unifying mechanism of DR regimens and benefits

As presented in the model in Figure 7, multiple DR regimens, including total food restriction, PR and SAA restriction/MetR, confer overlapping functional benefits in a number of diverse organisms from yeast (Johnson and Johnson, 2014) to worms (Cabreiro et al., 2013) to flies (Grandison et al., 2009; Troen et al., 2007) to rodents (Miller et al., 2005; Orentreich et al., 1993). In each of these experimental organisms, we found an increase in TSP-mediated H_2S production upon application of species-appropriate longevity regimens, ranging from EOD and MetR in mice to glucose restriction in yeast. Furthermore, in rodents and worms we demonstrated that genetic repression of TSP components blocked DR benefits, while overexpression could mimic benefits in the absence of any dietary intervention. In yeast we found the TSP, rather than the assimilatory pathway, to be a major source of H_2S production upon glucose restriction. Although future studies are required to directly demonstrate the necessity of H_2S production for various DR benefits, our data demonstrate increased H_2S production via the TSP is an evolutionarily conserved response to DR from yeast to mammals with the potential to mediate multiple DR benefits including stress resistance and longevity. We note that the ability of SAA restriction to confer benefits in no way rules out other dietary triggers or downstream mechanisms of DR benefits, and that future studies will be required to determine if benefits attributed to different DR regimens, including Trp restriction, impact the TSP or H_2S production.

Regulation of TSP gene expression and H_2S production

Cys deprivation triggers increased TSP gene expression possibly via the integrated stress response through GCN2 activation, eIF2α phosphorylation and ATF4 stabilization (Lee et al., 2008). We confirmed the ability of DR to increase hepatic CGL expression *in vivo*, and Cys or NAC addback to block this induction. Whether or not GCN2 is required for this remains to be determined; however, our data suggest a novel role for mTORC1 in CGL regulation, as constitutive mTORC1 expression in the TSC1KO livers blocked diet-induced CGL expression.

Because the logic underlying TSP activation upon Cys deprivation is presumably to increase Cys biosynthesis, the notion that Cys degradation into H_2S increases when Cys concentrations are limiting is potentially counterintuitive. However, the *in vivo* source and identity of substrates for CGL or CBS-derived H_2S upon DR are not known. Free Cys released upon autophagy, rather than that produced *de novo* by TSP, could be a major fuel source for H_2S production. Interestingly, the benefits of MetR on longevity in yeast require autophagy (Ruckenstuhl et al., 2014), but whether autophagy plays a role in H_2S production remains to be determined.

Potential mechanisms of H_2S action in DR

By what mechanism(s) could endogenous H_2S give rise to pleiotropic DR benefits? We found that resistance to simulated IRI *in vitro* required the mitochondrial ETC component SQR. The SQR is thought to be of eubacterial origin and conserved from the early evolution of eukaryotes in an anoxic and sulfidic world (Theissen et al., 2003). H_2S could interact with SQR to lend protection against ischemia by donating electrons to the ETC via coenzyme Q, thus potentially serving as a source of electrons during ischemia. Interestingly, thiosulfate generated as a product of H_2S oxidation via SQR can undergo further chemical modification in a thioredoxin reductase dependent reaction using glutathione to produce sulfite and/or sulfate (Olson et al., 2013), which are used in some unicellular organisms as terminal electron acceptors for ATP production instead of oxygen, resulting in H_2S generation (Muyzer and Stams, 2008). Whether this can occur in mammalian cells remains to be explored; however, the addition of thiosulfate to hypoxic mammalian tissues results in the production of H_2S by unknown mechanisms (Olson et al., 2013).

Vasodilatory effects of H_2S could contribute to healthspan and lifespan extension in mammals by counteracting atherosclerosis, hypertension, and trauma. Exogenous H_2S protects the vasculature from oxidative damage (Yan et al., 2006), while a lack of CGL and/or endogenous H_2S production leads to hypertension (Yang et al., 2008). Fasting and PR protect the vasculature against intimal hyperplasia as a result of carotid focal stenosis (Mauro et al., 2014), correlating with increased H_2S production capacity upon DR in the carotid artery *in vivo* and vascular smooth muscle cells *in vitro*. Fasting protects the brain against IRI in rat models of transient middle cerebral artery occlusion (Varendi et al., 2014) and increased H_2S production capacity in mouse brain (Supplemental Figure 6D). Future studies are required to address the specific role of increased TSP activity and H_2S production in DR-mediated effects on the vasculature and other organ systems.

Clinical Significance

The use of exogenous H_2S as a DR mimetic is appealing for applications in which patient compliance with restricted food intake is an issue, but nonetheless remains challenging due to the short half-life of the gas *in vivo* and risks of toxicity at high levels. Modulation of endogenous H_2S production by diet offers advantages in terms of safety and continuous delivery in planned applications with brief duration, for example in the context of preconditioning prior to elective surgery. In this context, H_2S may have pleiotropic advantages on surgical outcome, including vasodilatory and anti-inflammatory effects on the systemic level, as well as benefits against ischemic injury that may arise as part of the procedure itself or an unintended complication including heart attack or stroke.

Conclusion

H_2S was in vogue for centuries past as a cure-all before being viewed as a poisonous toxin with little or no acceptable level of exposure. Recently, H_2S has re-emerged as a potential therapeutic agent addressing numerous health issues (Zhang et al., 2013). Here, we identified DR as a trigger of increased endogenous H_2S production, and H_2S as a molecular mediator of pleiotropic DR benefits including longevity and stress resistance. These findings

have broad implications for our basic understanding of DR and its potential clinical applications.

Material and Methods

Mice

All experiments were performed with IACUC approval. 8–10 week old male B6D2F1 hybrids (Jackson Laboratories) were used for all experiments unless otherwise indicated. Male and female NRF2 KO and littermate controls on a mixed 129/C57BL/6 background (Martin et al., 1998), LTsc1KO and littermate controls on a C57BL/6 background (Harputlugil et al., 2014) and CGLKO and control mice on a mixed 129/C57BL/6 background (Yang et al., 2008) were described previously.

Preconditioning Regimens

Dietary—Mice were given AL access to food and water unless otherwise indicated. Experimental diets were based on Research Diets D12450B with approximately 18% of calories from protein (hydrolyzed casein or individual crystalline amino acids (Ajinomoto) in the proportions present in casein), 10% from fat and 72% from carbohydrate. AL food intake was monitored for several days and used to calculate 50% DR. Mice were fed daily with fresh food between 6 and 7pm.

Pharmacological—NAC (600mg/kg/day total in food and drinking water) and VitC&E (1000 and 250mg/kg/day, respectively, in food and drinking water) supplementation was halted 16–24hrs prior to organ harvest and/or induction of IRI. NaHS (1mM) or GYY4137 (260μM final) were delivered in the drinking water; PAG (10mg/kg) and NaHS (3–5mg/kg) were administered by IP injection.

Adenoviral-mediated gene delivery—CGL was overexpressed by IV injection of 10^{10} PFUs of Ad-CMV-CGL (ADV-256305) or control Ad-CMV-Null virus (Vector Biolabs) 7 days prior to hepatic IRI.

Cells—In vitro DR was performed in mouse Hepa1-6 cells, primary hepatocytes or primary aortic smooth muscle cells by incubating in DMEM lacking Met and Cys (Invitrogen) with 10% dialyzed FBS for up to 20hrs.

Ischemia reperfusion models

In vivo—Warm hepatic or renal IRI was induced and damage assessed as previously described (Peng et al., 2012). ALT, AST, and/or LDH values were normalized to the experimental control, usually the WT group fed a complete diet AL, for each time point (3, 24hrs post reperfusion) and combined into a single value per animal.

In vitro—For simulated ischemia, growth or preconditioning media was replaced with saline to mimic nutrient/energy deprivation and placed in an airtight chamber flushed with nitrogen gas at 37°C for 4hrs. Saline supernatant was removed after simulated ischemia for LDH measurement. Reperfusion was simulated by adding back fresh complete

DMEM-12320 (Invitrogen) containing 0.5 mg/mL MTT under normoxia at 37°C for an additional 3–4hrs.

Metabolic Parameters

Body composition was determined with an Echo MRI. Blood glucose was measured with an Easy Step Glucometer (Home Aide Diagnostics), serum triglycerides with a Serum Triglyceride Determination Kit (Sigma), liver RONS and GSH from left hepatic lobes normalized by wet weight using OxiSelect *In Vitro* Green Fluorescence ROS/RNS Assay Kit (Cell Biolabs) and the fluorimetric Glutathione Assay Kit (Sigma), and liver metabolites via mass spectrometry and normalized to total protein content. Peroxisome β-oxidation of lipids was performed on freshly harvested liver (Hu et al., 2005; Lazarow, 1981).

mRNA and Protein Analysis

Total RNA was isolated from tissues and cells using miRNeasy Mini Kit (Qiagen) and cDNA synthesized by random hexamer priming with the Verso cDNA kit (Thermo). qRT-PCR was performed with SYBR green dye (Lonza) and TaqPro DNA polymerase (Denville). Fold changes were calculated by the C_t method using Hprt and/or Rpl13 as standards and normalized to the experimental WT AL control. Protein expression was analyzed in tissues homogenized in passive lysis buffer (Promega), separated by SDS-PAGE, transferred to PVDF membrane (Whatman) and blotted for CGL (ab151769 Abcam), CBS (ab135626 Abcam) or Actin (13E5 Cell Signaling) followed by HPRT conjugated secondary anti-rabbit antibody (Dako).

H$_2$S measurements

Lead sulfide method—H$_2$S production capacity was measured in 100mg fresh tissue, or 100–300μg freeze-thaw homogenate in passive lysis buffer (Promega) of tissues, flies or worms, in PBS supplemented with 10mM Cys and 10μM-6mM PLP. A lead acetate H$_2$S detection paper (Sigma; or made by soaking blotting paper (VWR) in 20mM lead acetate (Sigma) and then vacuum drying) was placed above the liquid phase in a closed container (mirocentrifuge tube or covered 96 well plate) and incubated 2–5hrs at 37°C until lead sulfide darkening of the paper occurred. In live Hepa1-6 cells in 96-well plates, growth media was supplemented with 10mM Cys and 10μM PLP, and a lead acetate paper placed over the plate for 2–24hrs of further incubation in a CO$_2$ incubator at 37°C. In live yeast culture, lead acetate papers were placed above liquid cultures at the time of inoculation.

Sulfide probe method—A micro-sulfide ion electrode probe (Lazar Research Laboratories) and volt meter (Jenco) were used to measure H$_2$S production capacity in extracts *in vitro* and endogenous H$_2$S concentrations in liver *in vivo* upon direct insertion into median and left lobes of anesthetized mice prior to sacrifice.

Fly Conditions

D. melanogaster were maintained as described previously (Lee et al., 2014). Flies used in the H$_2$S production capacity assay were held on the designated diet and transferred to fresh vials without anesthesia every 3 days until 18 days of age.

Worm Conditions

N2 and DA1116 (*eat-2(ad1116)* II.) strains were grown following standard procedures, and all lifespan experiments carried out as previously described (Mair et al., 2011). RNAi clones were from the Ahringer RNAi library. CBS transgenic strains overexpressing the *cbs-1* isoform a (ZC373.1a) from the ubiquitous *sur-5* promoter and *unc-54* 3′UTR were generated by microinjection into gonads of young adult hermaphrodites.

Yeast Conditions

Experiments were carried out in WT strains BY4742 (MATα his3 1 leu2 0 lys2 0 ura3 0), W303 (MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15), and DBY746 (MATα leu2-3, 112 his3 1 trp1-289 ura 3-52 GAL+). Met deletion strains in BY4742 (met5, met14 and met16) were purchased from EUROSCARF. For chronological aging experiments, cells were inoculated to an OD600 of 0.05, and grown at 28°C in SCD media and cell survival determined by clonogenicity at the given time points in at least 3 independent cultures and values normalized to survival on day one. Longevity effects of exogenous H_2S were tested by adding 100μM GYY4137 (in DMSO) to BY4742 cultures at the time of inoculation and 5μM NaHS at 6, 24 and 48hrs after inoculation, with DMSO-treated BY4742 as a control.

Statistical Analyses

Data are displayed as means +/− SEM and statistical significance assessed in GraphPad Prism using Student's t tests to compare values and one-sample t test to compare means to a hypothetical value of 1 or 100 when data was normalized to the average value of the control group. A P-value of 0.05 or less was deemed statistically significant.

A more detailed Materials and Methods section is included in Supplemental Materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Zhang Y, Tang ZH, Ren Z, Qu SL, Liu MH, Liu LS, Jiang ZS. Hydrogen sulfide, the next potent preventive and therapeutic agent in aging and age-associated diseases. Mol Cell Biol. 2013; 33:1104–1113. [PubMed: 23297346]

Highlights

- Sulfur amino acid restriction is a key nutritional determinant of dietary restriction

- Cysteine restriction enhances endogenous CGL activity and H_2S production

- Increased H_2S is necessary and sufficient for DR-mediated stress resistance

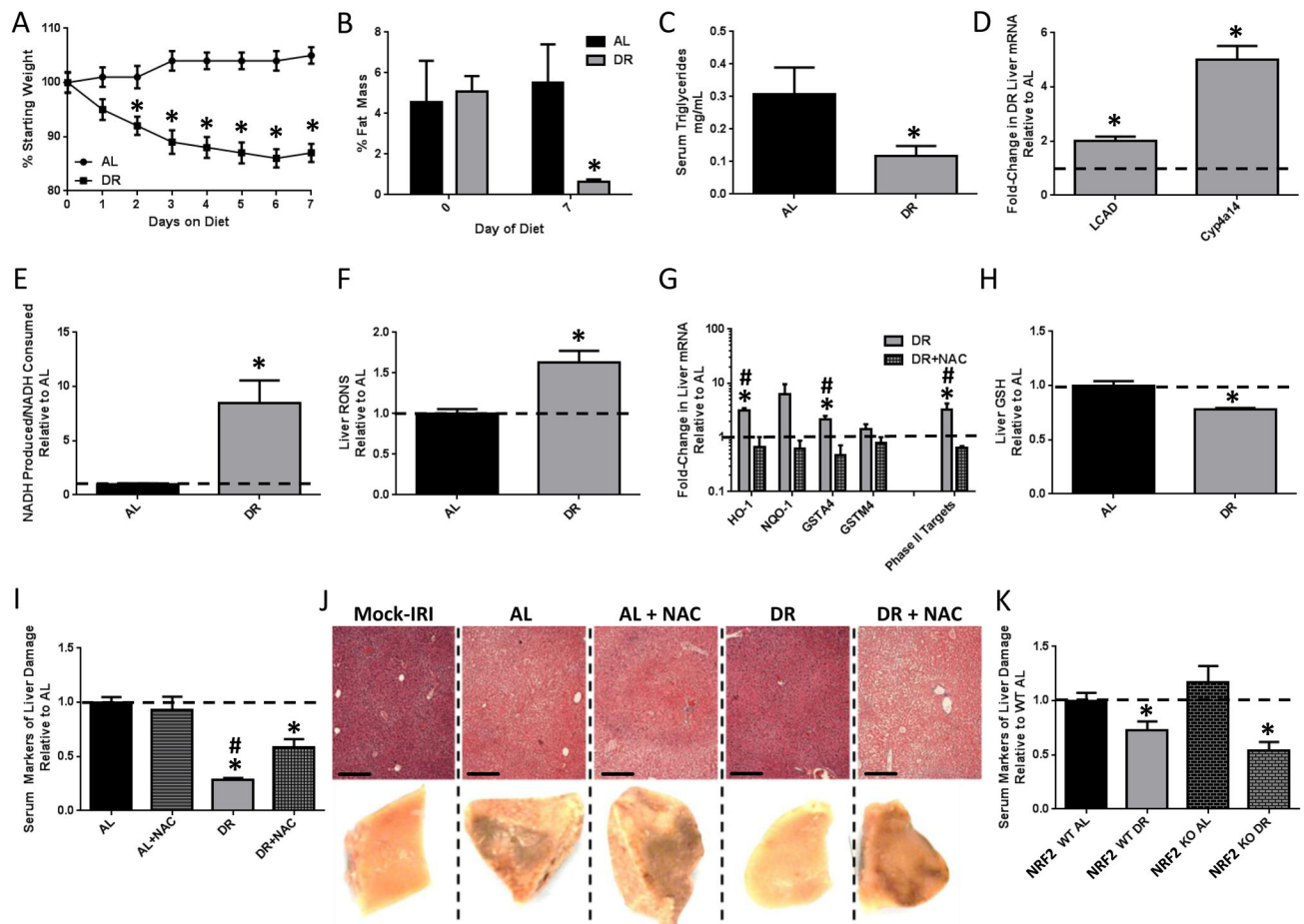- Increased H_2S is shared by DR regimens in yeast, worms, flies and rodents

Figure 1. NAC abrogates benefits of DR against acute stress independent of NRF2
(A–H) Effects of 1wk of 50% DR vs. AL feeding +/− NAC as indicated on body weight
(n=15–17/group, **A)**; %fat mass (n=5/group, **B)**; serum triglycerides (n=4–7/group, **C)**;
hepatic FAO-associated gene expression (n=3/group, **D)**; peroxisomal FAO capacity in liver
extracts (n=3/group, **E)**; hepatic RONS (n=6–8/group, **F)**; hepatic NRF2 target gene
expression (n=4/group, **G);** and hepatic GSH levels (n=7/group, **H). (I, J)** Combined serum
markers of liver damage normalized to the average of the AL control group **(I)** and liver
pathology from injured left liver lobes on the microscopic level stained with hematoxylin
and eosin (250μm scale bar, above) and the macroscopic level (1x magnification, below)
showing fixed tissue 24hrs after reperfusion **(J)** in mice (n=5–6/group) preconditioned as
indicated before hepatic IRI or mock injury. Asterisk indicates the significance of the
difference between AL and DR, and pound sign between DR and DR+NAC; */#p<0.05. **(K)**
Serum markers of liver damage in WT (n=5–6/group) or NRF2KO (n=7–12/group)
preconditioned as indicated. Asterisk indicates the significance of the difference between
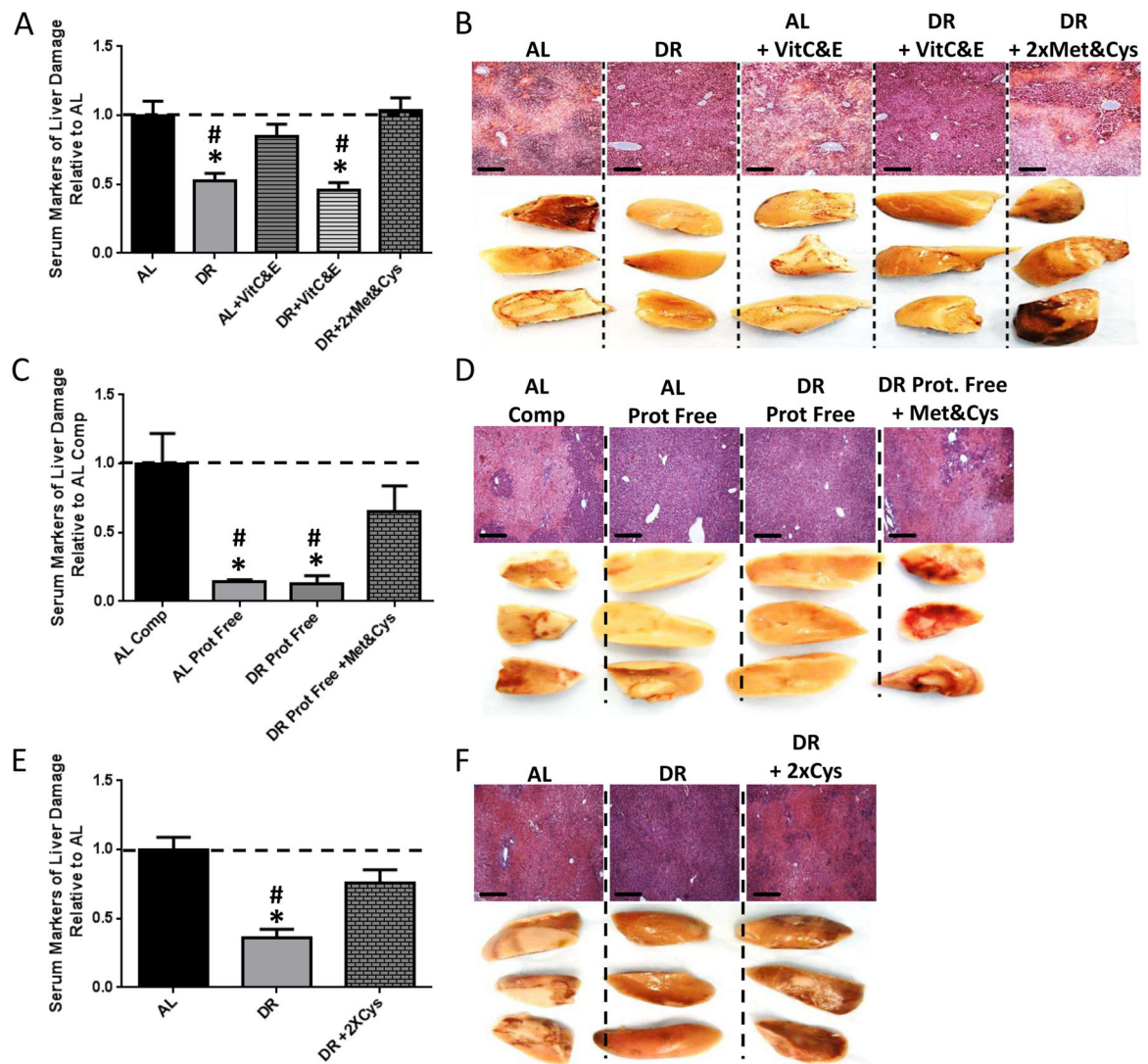AL and DR within genotype; *p<0.05. See also Suppl. Fig. 1.

Figure 2. Sulfur amino acids control the benefits of DR and PR

(A, B) Serum markers of liver damage (A) and liver pathology after reperfusion (B) in mice (n=5/group) preconditioned on complete diets fed AL or 50% DR +/− supplementation with vitamins C&E or Met&Cys as indicated. Asterisk indicates the significance of the difference vs. AL and pound sign vs. DR+2xMet&Cys; */#p<0.05. (C, D) Serum markers of liver damage (C) and liver pathology after reperfusion (D) in mice (n=5/group) preconditioned on complete (Comp) or protein free (Prot. Free) diets fed AL or 35% DR with Met&Cys addition as indicated prior to hepatic IRI. Asterisk indicates the significance of the difference vs. AL complete and pound sign vs. DR Prot. Free+Met&Cys; */# p<0.05. (E, F) Serum markers of liver damage (E) and liver pathology after reperfusion (F) in mice (n=5/group) preconditioned on complete diets fed AL or 50% DR with 2xCys added as indicated. Asterisk indicates the significance of the difference vs. AL and pound sign vs. DR+2xCys; */#p<0.05. See also Suppl. Fig. 2.
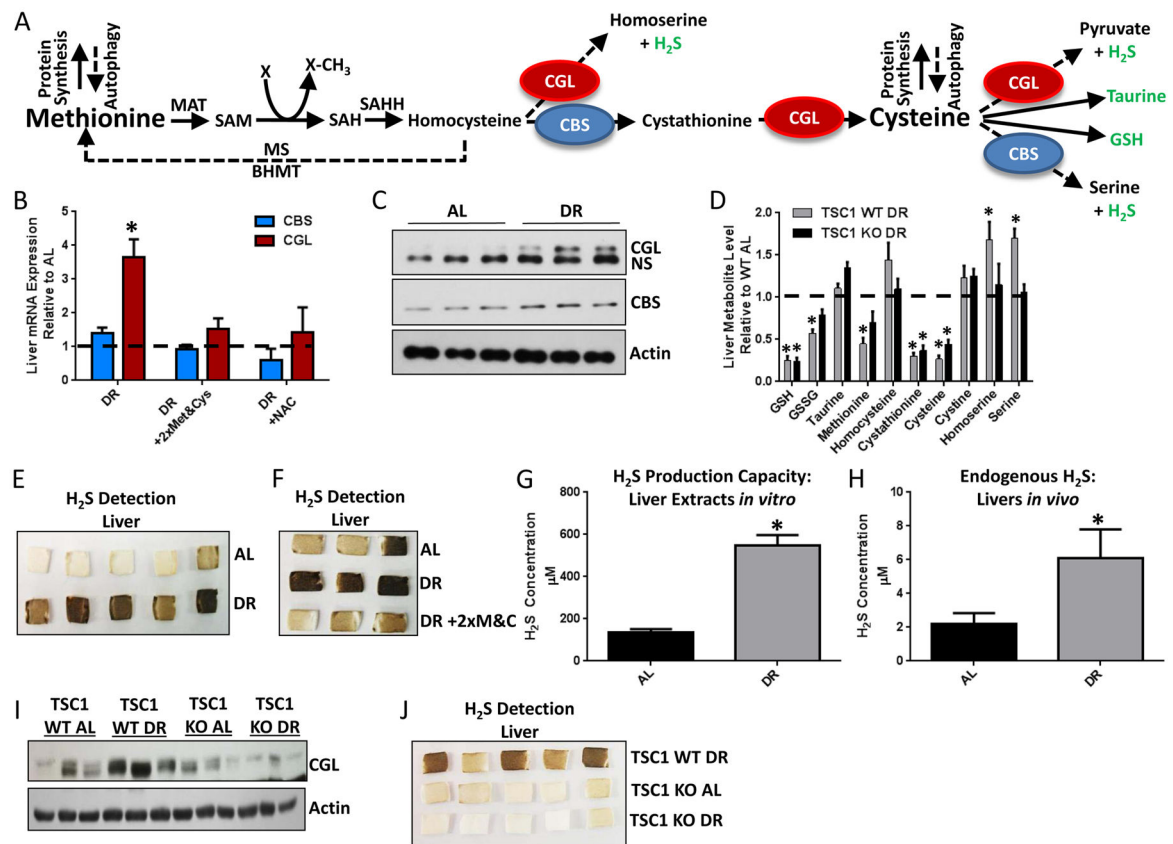
Figure 3. DR stimulates endogenous H₂S production via the TSP

(**A**) Model of the transmethylation and TSP. Arrows trace sulfur from Met to Cys and downstream cellular processes via Cystathionine Beta-Synthase (CBS) and Cystathionine Gamma-Lyase (CGL). Metabolites in green (taurine, GSH and H₂S) have demonstrated potential to protect against IRI. MAT: methionine adenosyl transferase, SAM: S-Adenosylmethionine, SAH: S-Adenosylhomocysteine, SAHH: S-adenosylhomocysteine hydrolase, MS: Methionine synthase, BHMT: Betaine homocysteine methyltransferase. (**B**) Hepatic CBS and CGL gene expression upon DR +/− 2xMet&Cys or NAC as indicated; n=3/group. (**C**) Immunoblots of CGL, CBS and actin in liver extracts from AL or DR mice as indicated; NS, non-specific protein band. (**D**) Liver metabolites in WT or LTsc1KO mice fed 35% DR on a protein free diet relative to the AL fed complete diet group; n=5/group. (**E, F**) H₂S production capacity from liver extracts of AL or DR mice (**E**) +/− 2xMet&Cys (**F**) as detected by the black precipitate, lead sulfide. (**G**) H₂S production capacity as in (**E**), but using a micro-sulfide probe to detect H₂S. (**H**) Endogenous hepatic H₂S in mice on the indicated diet using a micro-sulfide probe inserted into liver lobes; n=3 mice/group, 2 lobes/mouse. Asterisk indicates the significance of the difference vs. AL; *p<0.05. (**I, J**) Immunoblots of liver CGL (**I**) and H₂S production (**J**) in WT and LTsc1KO mice fed AL or 35% DR on a protein free diet. See also Suppl. Fig. 3.
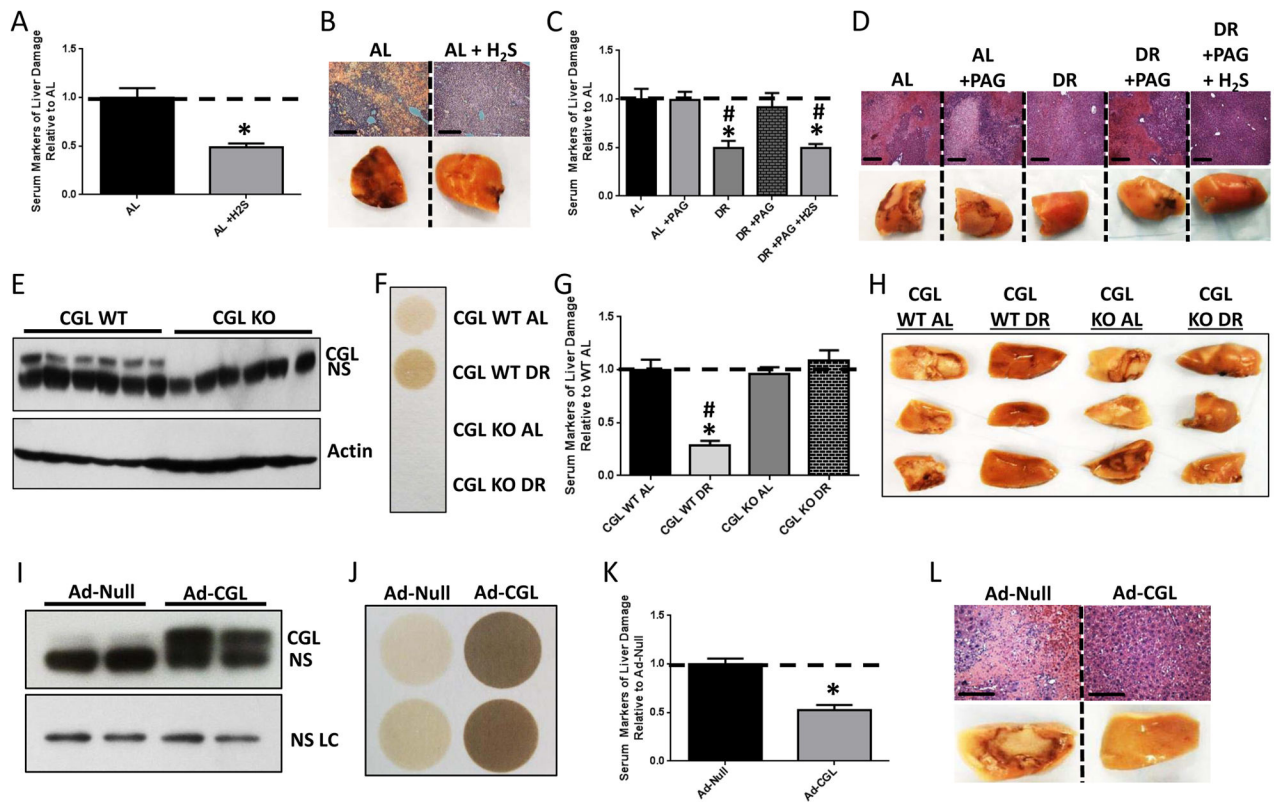
Figure 4. H₂S is necessary and sufficient to confer DR benefits against hepatic IRI

(**A, B**) Serum markers of liver damage (**A**) and liver pathology after reperfusion (**B**) in mice (n=4–5/group) treated with vehicle or H₂S 30 min prior to surgery. Asterisk indicates the significance of the difference vs. AL; *p<0.05. (**C, D**) Serum markers of liver damage (**C**) and liver pathology after reperfusion (**D**) in mice (n=3–4/group) treated with PAG during AL or DR preconditioning with a single H₂S injection prior to surgery as indicated. Asterisk indicates the significance of the difference vs. AL and pound sign vs. DR+PAG; */#p<0.05. (**E**) Immunoblot of CGL in liver extracts of WT or CGLKO mice as indicated. NS, non-specific protein. (**F**) H₂S production capacity of liver extracts from WT or CGLKO mice preconditioned as indicated. (**G, H**) Serum markers of liver injury (**G**) and liver pathology after reperfusion (**H**) in WT or CGLKO mice (n=5–8/group) preconditioned as indicated. Asterisk indicates the significance of the difference vs. WT AL and pound sign vs. CGLKO DR; */#p<0.05. (**I**) Immunoblot of CGL in liver extracts from WT mice infected with control (Ad-Null) or CGL-overexpressing (Ad-CGL) adenovirus as indicated; NS LC, non-specific loading control protein. (**J**) H₂S production capacity of liver extracts of Ad-Null or Ad-CGL infected mice. (**K, L**) Serum markers of liver damage (**K**) and liver pathology after reperfusion (**L**) in Ad-Null or Ad-CGL infected mice (n=6/group). Asterisk indicates the significance of the difference vs. Ad-Null; *p<0.05. See also Suppl. Fig. 4.
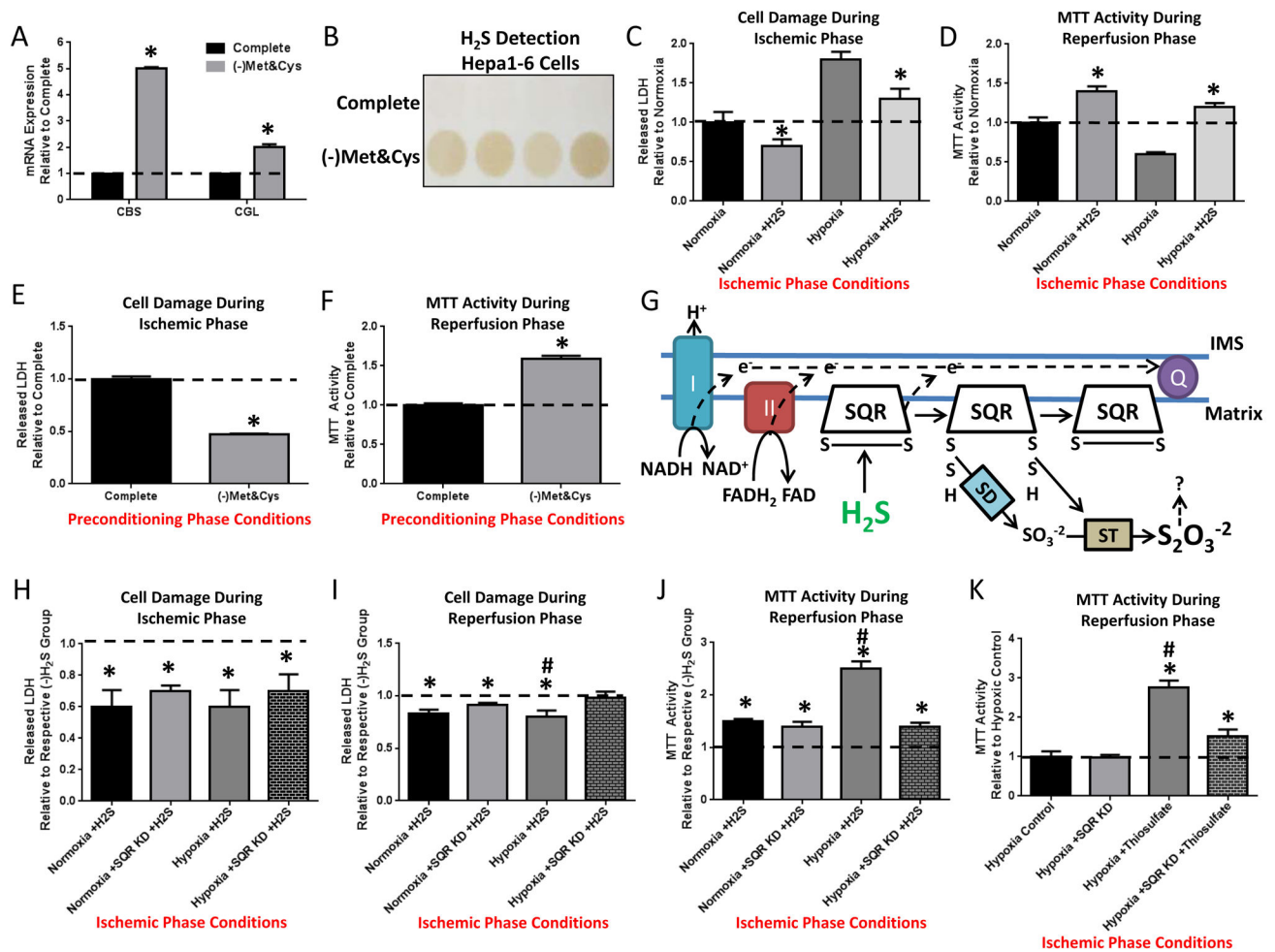
Figure 5. Mitochondrial SQR is required for cytoprotective effects of H$_2$S during ischemia
(**A, B**) Cell autonomous increase in TSP enzymes and H$_2$S production. (A) CBS and CGL gene expression in Hepa1-6 cells cultured overnight in complete or -Met&Cys media. Asterisk indicates the significance of the difference vs. complete; *$p< 0.05$. (**B**) H$_2$S production in live Hepa1-6 cells preconditioned in complete or -Met&Cys media in quadruplicate for 16–24hrs as indicated before readdition of Cys and PLP for H$_2$S detection. (**C, D**) Cell autonomous effects of exogenous H$_2$S on simulated IRI in Hepa1-6 cells *in vitro;* H$_2$S was added during the ischemic phase and removed upon simulated reperfusion. (**C**) LDH release during the ischemic phase consisting of 3hr incubation in saline (simulated nutrient/energy deprivation) under normoxic or hypoxic (simulated ischemia) conditions +/− exogenous H$_2$S as indicated. (**D**) MTT activity during the reperfusion phase consisting of readdition of complete media under normoxic conditions (simulated reperfusion). (**E, F**) Cell autonomous effects of overnight Met&Cys withdrawal (preconditioning phase conditions) on simulated IRI in primary hepatocyes *in vitro*. LDH release (**E**) during simulated ischemia and MTT activity (**F**) upon simulated reperfusion. (**G**) Schematic of H$_2$S oxidation to thiosulfate by SQR in mitochondria. Q, Coenzyme Q; IMS, inner membrane space; I and II, Complex I and II of the mitochondrial ETC; SD, Sulfur Dioxygenase; ST, Sulfur Transferase; S$_2$O$_3^{-2}$, thiosulfate. (**H–J**) Effects of exogenous H$_2$S on simulated IRI

upon SQR KD in Hepa1-6 cells. LDH release during the ischemic phase **(H)** in saline under the indicated normoxic or hypoxic conditions $+/-$ SQR KD, and during the reperfusion phase **(I)** in complete media expressed relative to respective group not receiving H_2S during the ischemic phase; and MTT activity **(J)** during the reperfusion phase. Asterisk indicates the significance of the difference in the indicated group $+/-$ H_2S treatment; hash mark indicates the significance of the difference between control and SQR KD within a given treatment group; */#$p<0.05$. **(K)** Cell autonomous effects of exogenous thiosulfate on MTT activity during the reperfusion phase following simulated IRI in Hepa1-6 cells $+/-$ SQR KD. Asterisk indicates the significance of the difference in the indicated group $+/-$ thiosulfate treatment; hash mark indicates the significance of the difference between control and SQR KD within a given treatment group; */#$p<0.05$. See also Suppl. Fig. 5.
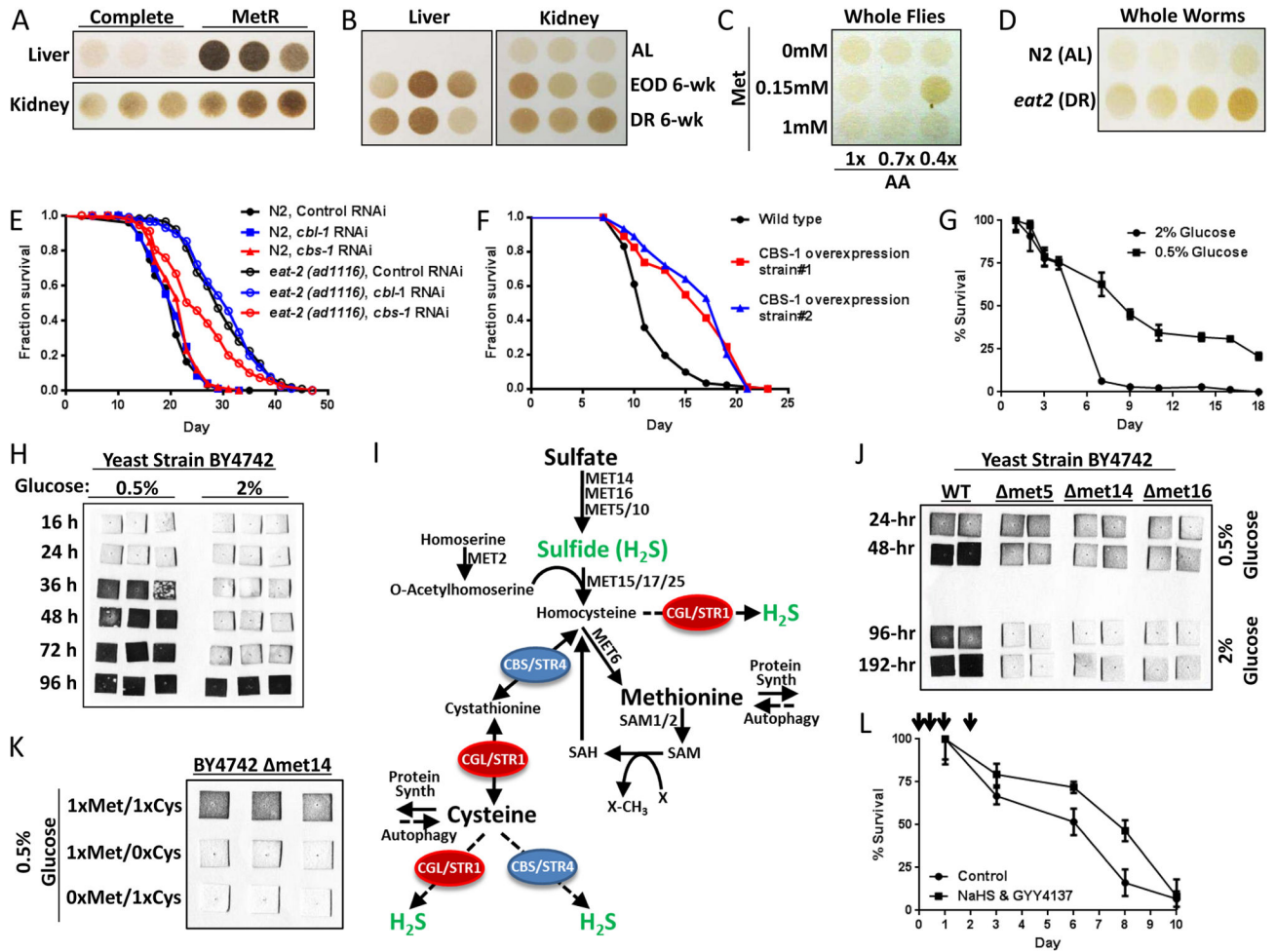
Figure 6. Increased endogenous H2S production in diet-induced longevity extension models crosses evolutionary boundaries

(A–B) H_2S production capacity of liver and kidney extracts from mice with AL access to complete or MetR diets as indicated for 4mos (A) and in mice with AL, every-other-day (EOD) fasting or 20–30% restricted diets for 6wks (B). Each circle represents H_2S production from an individual animal, normalized for extract protein content. (C) H_2S production capacity of whole fly lysates normalized for protein content from populations subject to the indicated dietary amino acid (AA) and Met concentrations. Maximal H_2S production capacity correlated with the optimal diet for longevity extension (0.4x AA with 0.15mM Met) (Lee et al., 2014). (D) H_2S production capacity of N2 and *eat-2* whole worm lysates in quadruplicate. (E) Lifespan of N2 and *eat-2* worms subject to RNAi-mediated KD of the TSP genes *cbs-1* or *cbl-1* as indicated. (F) Lifespan analysis of WT or transgenic worms overexpressing CBS-1. (G) Chronological lifespan of budding yeast grown in 2% or 0.5% glucose as indicated. (H) H_2S production in yeast cultures grown in 2% or 0.5% glucose as detected by black lead sulfide accumulation on lead acetate papers inserted into the caps of the growth flasks and removed at the indicated time point. (I) Schematic of transmethylation, TSP and H_2S production in budding yeast. (J) H_2S production in WT and sulfur assimilatory pathway mutant strains grown in 2% or 0.5% glucose for the indicated

time. **(K)** H_2S production in the met14 sulfur assimilatory mutant grown in 0.5% glucose and the indicated relative amount of Met or Cys. **(L)** Chronological lifespan in 2% glucose +/− H_2S donors NaHS and GYY4137 added during early culture growth at indicated time points (arrows). See also Suppl. Fig. 6.
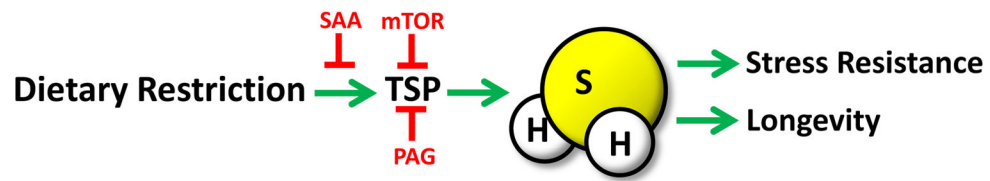
Figure 7. Model of DR-mediated benefits through increased H$_2$S production

DR regimens leading to extended longevity across evolutionary boundaries include PR, SAA restriction and Cys restriction, leading to increased TSP activity via CGL and/or CBS family members, and increased endogenous H$_2$S production. Specific SAA addition, increased activity of the nutrient/energy sensing kinase, mechanistic target of rapamycin (mTOR), or the pharmacological agent, PAG, can block TSP upregulation and H$_2$S production. H$_2$S can readily diffuse through tissues and has pleiotropic, overlapping beneficial effects on the cellular, tissue and organismal levels with the potential to contribute to stress resistance and longevity benefits of DR.