The hydrogen sulfide signaling system: changes during aging and the benefits of caloric restriction

Benjamin L. Predmore · Maikel J. Alendy ·
Khadija I. Ahmed · Christiaan Leeuwenburgh ·
David Julian

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Abstract Hydrogen sulfide gas (H_2S) is a putative signaling molecule that causes diverse effects in mammalian tissues including relaxation of blood vessels and regulation of perfusion in the liver, but the effects of aging on H_2S signaling are unknown. Aging has negative impacts on the cardiovascular system. However, the liver is more resilient with age. Caloric restriction (CR) attenuates affects of age in many tissues. We hypothesized that the H_2S signaling system is negatively affected by age in the vasculature but not in the liver, which is typically more resilient to age, and that a CR diet minimizes the age affect in the vasculature. To investigate this, we determined protein and mRNA expression of the H_2S -producing enzymes cystathionine γ -lyase (CSE) and cystathionine β -synthase (CBS), H_2S production rates in the aorta and liver, and the contractile response of aortic rings to exogenous H_2S . Tissue was collected from Fisher 344×Brown Norway rats from 8–38 months of age, which had been maintained on an ad libitum (AL) or CR diet. The results demonstrate that age and diet have differential effects on the H_2S signaling

system in aorta and liver. The aorta showed a sizeable effect of both age and diet, whereas the liver only showed a sizeable effect of diet. Aortic rings showed increased contractile sensitivity to H_2S and increased protein expression of CSE and CBS with age, consistent with a decrease in H_2S concentration with age. CR appears to benefit CSE and CBS protein in both aorta and liver, potentially by reducing oxidative stress and ameliorating the negative effect of age on H_2S concentration. Therefore, CR may help maintain the H_2S signaling system during aging.

Keywords H_2S · Gasotransmitter · Aging · Caloric restriction · Aorta · Liver

Introduction

Hydrogen sulfide (H_2S) is proposed to function as a gasotransmitter (Wang 2002) in the nervous, cardiovascular, and gastrointestinal systems. The two other recognized gasotransmitters are nitric oxide (NO) and carbon monoxide (CO). All three gasotransmitters share several similarities: they are endogenously produced, small gas molecules that are capable of physiological action; they can easily diffuse across cell membranes to exert their function; and they do not require a mechanism of degradation or reuptake because they are all very reactive (Wang 2002). In terms of the dose response, H_2S is the least potent of the known gasotransmitters, having physiological

B. L. Predmore · M. J. Alendy · K. I. Ahmed ·
D. Julian (✉)
Department of Biology, University of Florida,
Gainesville, FL 32611, USA
e-mail: djulian@ufl.edu

C. Leeuwenburgh
Department of Aging and Geriatrics, University of Florida,
Gainesville, FL 32611, USA

effects at micromole per liter concentrations, whereas the physiological effects of CO and NO occur at nanomole per liter concentrations (Pryor et al. 2006).

Abe and Kimura (Abe and Kimura 1996) first demonstrated that H₂S is produced in the brain and that it increases *N*-methyl-D-aspartic acid receptor-mediated responses and facilitates hippocampal long-term potentiation. Since then a variety of physiological roles have been proposed for H₂S (Lowicka and Beltowski 2007; Szabo 2007), and at least some capacity for H₂S production has been demonstrated in several vertebrate tissues (Dombkowski et al. 2006; Olson 2005; Olson et al. 2006; Zhao et al. 2003), including human mammary artery (Webb et al. 2008). When exogenously applied to isolated blood vessels of vertebrates, H₂S elicits responses that range from a monophasic relaxation or contraction, to multiphasic contraction-relaxation-contraction responses, depending on the phylogenetic class and type of vessel examined (Dombkowski et al. 2005). Vascular response to H₂S also depends on other experimental factors, such as the presence of an intact endothelium, the method of pre-constriction used, and the oxygen tension of the tissue baths (Koenitzer et al. 2007; Lim et al. 2008; Olson et al. 2008). In the liver, H₂S has been proposed to regulate perfusion and maintain portal venous pressure, with deficiencies in H₂S production pathways resulting in increases in intra-hepatic resistance (Fiorucci et al. 2005b). H₂S may also have a role in regulating biliary bicarbonate secretion (Fujii et al. 2005).

H₂S is endogenously produced from L-cysteine predominantly by the enzymes cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) in a variety of animals, from invertebrates to mammals (Fiorucci et al. 2005b; Gainey and Greenberg 2005; Geng et al. 2004b; Julian et al. 2005; Julian et al. 2002; Kimura 2000; Kimura et al. 2005; Wang 2002; Webb et al. 2008). Recently, 3-mercaptopyruvate sulfurtransferase (3-MST), in conjunction with cysteine aminotransferase, has also been found to produce H₂S from cysteine (Shibuya et al. 2009; Shibuya et al. 2008). In mammals, CSE and CBS are differentially expressed (Zhao et al. 2003; Zhao et al. 2001): of the two enzymes, CSE expression is higher in the cardiovascular system, whereas CBS expression is higher in the nervous system (Ebrahimkhani et al. 2005; Geng et al. 2004b; Hosoki et al. 1997; Kimura et al. 2005). In liver and kidney, CSE and CBS are

both expressed in high amounts compared with other tissues (Fujii et al. 2005; Lowicka and Beltowski 2007). 3-MST is expressed in vascular endothelial cells and is highly expressed in the brain (Shibuya et al. 2009; Shibuya et al. 2008).

Aging influences the action and/or efficacy of many signaling molecules, including norepinephrine and the more well-studied gasotransmitter NO (Lakatta 1993; Tanaka et al. 2006; van der Loo et al. 2000). A recent study of plasma H₂S concentration in humans over 50–80 years of age reported that H₂S levels may decline with age (Chen et al. 2005), but whether aging affects the H₂S signaling system is unknown. Furthermore, any affect of age on the H₂S signaling system may differ among tissues. For example, substantial physiological changes occur with age in the cardiovascular system, leading to endothelial dysfunction (Castello et al. 2005) and dysregulation of blood vessel tone (Anantharaju et al. 2002; Kitani 1991; Kitani 1994). In comparison, the liver is less susceptible to functional changes with age. While liver size and the number of proliferating hepatocytes decline with age, the liver enzymatic activity does not appear to change substantially until very old age (Anantharaju et al. 2002; Kitani 1991; Kitani 1994; Schmucker 1998; Zeeh and Platt 2002).

Caloric restriction (CR) is an intervention that attenuates many effects of aging (Anderson and Weindruch 2010; Labinskyy et al. 2006; Leeuwenburgh and Prolla 2006), including cardiovascular morbidity (van der Loo et al. 2000), cross-linking of cardiac and skeletal muscle proteins (Leeuwenburgh et al. 1997), mitochondrial dysfunction (Aspnes et al. 1997; Payne et al. 2003), loss of skeletal muscle mass (Payne et al. 2003; van der Loo et al. 2000), and endothelial dysfunction (Barja 2002; Castello et al. 2005; Gredilla et al. 2004; Gredilla et al. 2001; Taddei et al. 2006). Furthermore, CR attenuates the age-related impairment of the NO signaling system (Minamiyama et al. 2007; Sharifi et al. 2008; Yang et al. 2004). However, it is unknown whether CR affects the H₂S signaling system and whether CR specifically attenuates any affects of age on the H₂S signaling system.

In this study, we investigated the effects of aging and CR on the H₂S signaling system in liver and aorta in the Fisher 344×Brown Norway hybrid rat. We hypothesized that the H₂S signaling system is negatively affected by age in the vasculature but not in the liver, which is typically more resilient to age,

and that a CR diet minimizes the age affect in the vasculature. Liver and aorta tissue obtained from rats of various ages were assayed for CSE and CBS protein and mRNA expression and H₂S production rates. Contraction of aorta smooth muscle was used as a functional indicator to investigate the effects of age and CR on functional responses to H₂S.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except where noted otherwise.

Animals

Two groups of male Fisher 344×Brown Norway hybrid rats of 8, 18, 29, 38 months of age and 14, 27, 34 months of age were obtained from the National Institute on Aging (NIA) rodent colony. These animals were part of larger studies on the biological markers of skeletal muscle fitness and physical function and the relationship between cognition and brain fitness. Additional data resulting from these animals has also been published (Carter et al. 2009; Hofer et al. 2009; Marzetti et al. 2008). At NIA, all rats were fed the NIH31 diet ad libitum (AL) until 14 weeks of age. For CR animals, the calorie restricted regimen was incrementally established by 10% CR per week for 4 weeks, reaching 40% CR by 17 weeks. The NIH31 diet provided 60% of the calories and 100% of the vitamins compared with the diet consumed by AL animals. After arriving at our facilities (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care), animals were housed under standard conditions (12 h light, 12 h dark) and provided with food and water using either the NIH31 diet or the NIH31-NIA fortified diet at 40% CR, for AL and CR animals, respectively. Myography studies were performed on tissues from animals of 14, 27, and 34 months of age. All other studies were performed on tissues from animals of 8, 18, 29, and 38 months of age. The method of anesthesia affects the H₂S response in vascular tissues from a variety of vertebrates (Dombkowski et al. 2005). To reduce this potential artifact, euthanasia was by guillotine under IACUC-approved methods for all animals used in this study.

Western blotting

Western blotting was used to evaluate CSE and CBS protein expression in liver and aorta samples. Liver and aorta were removed within 5 min of euthanasia, frozen in liquid nitrogen and stored at -80°C. Samples were suspended in a lysis buffer consisting of 50 mmol/L Tris base, 2% (w/v) sodium dodecyl sulfate, 10 mmol/L ethylenediaminetetraacetic acid, 10 mmol/L dithiothreitol, 0.5 mmol/L sodium tetraborate, and 1% (v/v) Halt protease inhibitor cocktail (Thermo Fisher Scientific Rockford, IL USA). Western blotting was performed using commercially available monoclonal antibodies for CSE and CBS (Abnova, Neihu District, Taipei City, Taiwan), a monoclonal antibody for β-actin (Cell Signaling Technology, Danvers, MA, USA), and a secondary antibody conjugated to alkaline phosphatase (Sigma-Aldrich St. Louis, MO, USA). Protein was separated using SDS-PAGE and transferred onto PVDF membrane (Immobilon P, Millipore, Billerica, MA USA) using a semidry blotter (BioRad, Hercules, CA USA). Using the Snap-ID system (Millipore, Billerica, MA USA) at room temperature, membranes were blocked in 0.05% non-fat milk in phosphate-buffered saline with 0.05% Tween-20 (PBST), washed in PBST and incubated 10 min with appropriate primary antibodies (diluted 1:333 in PBST). Membranes were then washed in PBST four times and subsequently incubated 10 min with secondary antibody diluted 1:3,333 in blocking buffer. Membranes were washed two times in PBST, once in phosphate-buffered saline, and once in 100 mmol/L Tris-HCl, pH9.5. Chemiluminescent substrate (DuoLux, Vector Laboratories, Burlingame, CA USA) was used to generate a signal that was captured with a digital imager (GeneSnap, Syngene, Frederick, MD USA). Images were analyzed using commercial software (Quantity One, BioRad, Hercules, CA USA), with which band intensity was determined using local background subtraction.

RNA extraction

RNA was extracted from liver and aorta samples to evaluate mRNA expression for CSE and CBS using real-time polymerase chain reaction (PCR), for comparison to the protein expression data. Liver and aorta tissues were disrupted into powder under liquid nitrogen. All samples for each tissue were processed on the

same day. For liver, total RNA was extracted using an Arum total RNA mini kit (BioRad, Hercules, CA USA). For aorta, total RNA was extracted using Tri reagent and a glass homogenizer (Kontes Glass, Vineland, NJ USA).

Real-time PCR

Relative quantitative real time PCR was run in 96-well PCR plates in an Applied Biosystems 7300 Real-time PCR system (Life Technologies, Carlsbad, CA USA). Primers were designed using commercial software (Premier Biosoft International, Palo Alto, CA USA) and synthesized by Integrated DNA Technologies (Coralville, IA USA). The primers used were as follows: CSE sense 5'-TGGGCTTAGTGTCTGTTAATTCC-3', CSE antisense 5'-GGCAGCAGAGGTAACAATCG-3', CBS sense 5'-TGCCTGAGAAGATGAGTATG-3', CBS antisense 5'-GGTCCAGAATGTGAGAATTG-3', Actin sense 5'-CTCCCAGCACACTTAAGTCTAG C-3', Actin antisense 5'-AAAGCCACAAGAAACA CTCAGG-3', 18S rRNA sense 5'-CGAGGAATTCC CAGTAAGTGC-3', 18S rRNA antisense 5'-CCATC CAATCGCTAGTAGCG-3'. For both liver and aorta plates, a standard curve for the housekeeping genes β -actin (run with CSE samples) or 18S rRNA (run with CBS samples) was run with a standard curve for the gene of interest (GOI), with all samples in triplicate. The remainder of the plate was filled with eight random samples plus one calibrator sample for plate-to-plate corrections, loaded in triplicate for both the housekeeping gene and the GOI. Additional plates were run containing the calibrator sample on each plate until all samples had been processed. All plates for a given tissue-GOI combination were made and run on the same day, using the same real-time PCR machine.

Hydrogen sulfide production

H₂S production was measured essentially as previously described (Dombkowski et al. 2006; Julian et al. 2002; Stipanuk and Beck 1982). Frozen tissues were ground into a fine powder under liquid nitrogen with mortar and pestle. For each sample, two small scoops (approximately 50 mg wet weight) were added to 1.5 mL 100 mmol/L potassium phosphate buffer (pH 7.4) and homogenized with a Polytron homogenizer (Kinematica, Bohemia, NY USA). A 1 mL aliquot of this homogenate solution was placed in the outer well of a 25 mL glass flask containing an additional plastic

center well (Kontes Glass, Vineland, NJ USA), which contained 0.5 mL 1% (w/v) zinc acetate and a piece of chromatography paper (Whatman grade 3MM Chr, Maldstone England) cut to 2.5 by 4.5 cm and folded into a fan shape. The CSE and CBS substrate L-cysteine (10 mmol/L) and cofactor pyridoxal-5'-phosphate (PLP; 2 mmol/L) were added to the outer well, after which the flask was sealed with a septum stopper and flushed with N₂ gas for 30 s. Each flask was incubated on a shaker at 37°C for 1.5 h for liver and 8 h for aorta. After incubation, 1 mL 50% (w/v) trichloroacetic acid was added to the outer well to stop enzyme activity and to convert all S²⁻ and HS⁻ to H₂S. The zinc acetate solution on the chromatography paper reacts with volatilized H₂S to form zinc sulfide, which is relatively stable. The flasks were then incubated for an additional hour to allow remaining H₂S to volatilize and form zinc sulfide. The filter paper was then removed from the center well and placed in a test tube containing 3.5 mL of de-ionized water, 0.4 mL 20 mmol/L *N,N*-dimethyl-*p*-phenylenediamine oxalate in 7.2 mol/L HCl and 0.4 mL of 30 mmol/L FeCl₃ in 1.2 mol/L HCl. The test tubes were gently vortexed and incubated for 20 min at room temperature. Absorbance of the solution in the test tube was read at 670 nm with a plate reader (BioTek Instruments, Inc., Winooski, VT USA). Absorbance measurements were calibrated against a standard curve generated from NaHS in deoxygenated, de-ionized water that was added to the outside wells, incubated at 37°C for 1.5 h, and assayed as above. To ensure that all measured H₂S production is by tissue enzymes, several controls were run in addition to sample homogenates: a negative control of homogenate but no cofactors or substrate; and a blank of all cofactors and substrate in phosphate buffer, but without homogenate. Inhibitors of CSE (20 mmol/L propargylglycine, Pgly) and CBS (1 mmol/L aminooxyacetic acid, AOAA) were added to confirm the H₂S production was through these, and not alternate pathways (Julian et al. 2002). Protein concentration was quantified with a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA USA), to calculate nanomole H₂S production per hour per milligram (nmol H₂S/h/mg) protein.

Myography

The effect of H₂S on vascular tone was measured in 5 mm width aorta rings from 41 animals, with five to

eight rings tested for each age $\times$ diet treatment combination. Immediately after euthanasia, the aorta was removed, cleaned of fat and connective tissue, and sectioned into rings. Rings were attached with stainless steel wire to force transducers and mounted in a 37°C tissue bath system (Radnoti Glass Technology, Monrovia, Ca USA) containing Krebs bicarbonate buffer. Rings were allowed to equilibrate after mounting for a minimum of 1 h, and a baseline tension of 1.5 g was maintained throughout each experiment. At the start of an experiment, each ring was maximally contracted with two sequential additions of 80 mmol/L KCl, with a wash step in between each addition. Acetylcholine (1 μ mol/L) was used to check for a functional endothelium. If the aortic rings did not constrict to KCl or relax to acetylcholine, they were assumed to be damaged and were discarded. After washing with Krebs buffer and returning to baseline tension, the rings were incubated with 10 μ mol/L propranolol (to block β -adrenergic receptor relaxation and maximize α -adrenergic receptor contraction), and precontracted with 1 μ mol/L norepinephrine (NE). After the NE precontraction stabilized, rings were exposed to 100 μ mol/L H₂S (diluted from a 100 mmol/L NaHS stock solution). After 30–45 min, the tissue bath was drained and the rings were washed twice with Krebs buffer and allowed to return to baseline tension. This sequence of propranolol, NE and H₂S addition was repeated for 300, 600, and 900 μ mol/L H₂S. Data for H₂S-induced contractions for each aortic ring were standardized to the weight of the ring.

Actual total H₂S concentrations, as measured by a methylene blue assay (Gilboa-Garber 1971), were approximately 75% of the predicted value (presumably due mostly to oxidation). In physiological saline at pH 7.4, the dissociation of H₂S results in approximately 1/3 of total H₂S as H₂S and 2/3 as HS[−] (Beauchamp et al. 1984), and it is unknown whether the physiological effects of hydrogen sulfide are mediated only through H₂S gas, or if the HS[−] anion is involved as well (Olson and Donald 2009).

Statistics

All statistical analyses were performed using JMP statistical software (JMP 7.0, SAS Institute, Cary, NC, USA), with $\alpha \leq 0.05$ considered significant. Two-way analyses of variance (ANOVAs) were performed with a Tukey' post-hoc test, when possible. However,

the functional response data were not always normally distributed, and therefore these data violated the assumptions of the ANOVA. In those cases, significant effects of age and diet were tested using the Kruskal–Wallis nonparametric ANOVA, and post-hoc testing between these groups was done with a Wilcoxon's nonparametric *t* test. If the two-way ANOVA showed no significant effects or interactions, data were pooled and run using a one-way ANOVA (pooled by age) or *t* test (pooled by diet). For real-time PCR, statistical analyses were performed on the average cycle threshold (Ct) of each sample, since the Ct's are normally distributed (Wood et al. 2005).

Results

CSE and CBS protein expression

As expected, the relative expression of CSE and CBS protein differed between aorta and liver. In aorta, CSE protein expression (relative to β -actin) was 1.43 fold higher than CBS protein expression on average, whereas in liver, CBS protein expression (relative to β -actin) was 1.18 fold higher than CSE protein expression, on average.

CSE In both aorta and liver, CSE protein expression was significantly affected by age, diet, and an interaction between age and diet (Fig. 1a, b). In aorta, CSE protein expression increased with age in AL animals, but it was unchanged with age in CR animals (Fig. 1a, bars; 2-way ANOVA with Tukey's post-hoc, $F=13.9$, $p<0.0001$). Specifically, at ages 18, 29, and 38 months, CSE protein expression was significantly lower in CR animals than in AL animals, whereas CSE expression at 8 months was not affected by diet (Fig. 1a, asterisks). In liver, CSE protein expression was unchanged with age in AL animals but increased with age in CR animals (Fig. 1b, bars; 2-way ANOVA with Tukey's post-hoc, $F=4.90$, $p=0.0041$). At 38 months, AL CSE protein expression was significantly lower than CR (Fig. 1b, asterisks).

CBS In aorta but not liver, CBS expression was significantly affected by age, diet, and an interaction between age and diet (Fig. 1c, d). In aorta, CBS protein expression at 38 months was significantly higher in AL animals than in CR animals (Fig. 1c,

Fig. 1 Effect of age and diet on CSE and CBS protein expression. CSE expression is shown in aorta (**a**, $n=3-5$ per group) and liver (**b**, $n=3$ per group). CBS expression is shown in aorta (**c**, $n=3-4$ per group) and liver (**d**, $n=3$ per group). The y-axis shows CSE (**a** and **b**) or CBS (**c** and **d**) protein expression relative to β -actin. The x-axis shows the age in

months (mo). AL data are in *white columns* and CR data are in *grey columns*. Representative blots are aligned along the top, above each column. Data are represented as mean $\pm$ SE and were analyzed by 2-way ANOVA with Tukey's post-hoc tests. Means that do not share a common letter are significantly different

asterisks; 2-way ANOVA with Tukey post-hoc, $F=4.34$, $p=0.0056$). In liver, there was no significant effect of age, diet, or an interaction between age and diet on CBS expression (Fig. 1d, 2-way ANOVA, $F=1.44$, $p=0.257$).

CSE and CBS mRNA expression

There were no interactions between age and diet on mRNA expression of CSE or CBS in aorta or liver using a two-way ANOVA (Fig. 2). Therefore, data were pooled by age and pooled by diet and tested with one-way ANOVA and t tests, respectively. CSE

and CBS mRNA expression was affected by diet, but not age. In aorta, the CR diet significantly increased expression of both CSE mRNA (Fig. 2a, one-tailed t test, $p=0.0013$) and CBS mRNA (Fig. 2c, one-tailed t test, $p=0.0258$) compared with the AL diet. In liver, diet did not affect the expression of CSE mRNA (Fig. 2b, pooled t test, $p=0.142$) or CBS mRNA (Fig. 2d, pooled t test, $p=0.261$).

Hydrogen sulfide production

There was no effect of age, diet, or an interaction between age and diet on the capacity for H_2S production in either aorta or liver using a two-way ANOVA

Fig. 2 Effect of diet on CSE and CBS mRNA expression. CSE expression is shown in aorta (**a**, $n=13$ AL, $n=12$ CR) and liver (**b**, $n=12$ per group). CBS expression is shown in aorta (**c**, $n=12$ AL, $n=11$ CR) and liver (**d**, $n=12$ per group). The y-axis shows CSE (**a** and **b**) protein expression relative to β -actin and

CBS (**c** and **d**) protein expression relative to 18S rRNA. The x-axis shows the diet. AL data are in white columns and CR data are in grey columns. Data are represented as mean $\pm$ SE and were analyzed by one-tailed or pooled *t* tests. Means that are significantly different are indicated by asterisks

(Fig. 3). Therefore, data were pooled by age and pooled by diet and tested with one-way ANOVA and *t* tests, respectively. The capacity for H_2S production was affected by diet, but not age. In both aorta and liver, H_2S production was higher in tissues of CR animals than AL animals (Fig. 3a, one-tailed *t* test, $p=0.0407$ and Fig. 3b, one-tailed *t* test, $p=0.0411$, respectively). Inhibitors of the pyridoxal-5'-phosphate (PLP)-dependent enzymes CSE and CBS were added to confirm that H_2S production was occurring via the actions of these enzymes ($N=4$). Addition of the CSE inhibitor Pgly (20 mmol/L) decreased H_2S production by 57% in aorta (Ctrl= 0.143 ± 0.006 nmol H_2S /hr/mg protein vs. Pgly= 0.061 ± 0.002 nmol H_2S /h/mg protein) and by 94% in liver (Ctrl= 0.439 ± 0.026 nmol H_2S /h/mg protein vs. Pgly= 0.024 ± 0.005 nmol H_2S /h/mg protein). Addition of the CBS inhibitor AOAA (1 mmol/L) decreased H_2S production by 50% in aorta (Ctrl= 0.143 ± 0.006 nmol H_2S /h/mg protein vs. AOAA= 0.071 ± 0.008 nmol H_2S /h/mg protein) and by 81% in

liver (Ctrl= 0.331 ± 0.041 nmol H_2S /h/mg protein vs. AOAA= 0.064 ± 0.011 nmol H_2S /h/mg protein).

Contractile response to hydrogen sulfide in aorta

Application of KCl and NE initiated contractions that were used to ascertain ring viability and responsiveness. Age did not significantly affect the contractions in response to KCl or norepinephrine (data not shown, one-way ANOVA, $p>0.05$ for both). However, the contractions in response to KCl and NE were significantly stronger in rings from CR animals than from AL animals (Fig. 4a, b, one-tailed *t* test, $p=0.0282$ and $p<0.0001$, respectively).

Application of H_2S to isolated aorta rings typically elicited a triphasic, contraction-relaxation-contraction response (Fig. 5). The contraction in the third phase was larger in magnitude and longer lasting than the contraction in the first phase, although at the lowest H_2S concentration used (100 μ mol/L) the third phase

Fig. 3 Effect of diet on H₂S production. H₂S production rates are shown in aorta (**a**, $n=17$ AL, $n=21$ CR) and liver (**b**, $n=12$ per group). The y-axis shows production as nmol H₂S/h/mg protein. The x-axis shows the diet. AL data are in *white columns* and CR data are in *grey columns*. Data are represented as mean $\pm$ SE and were analyzed by one-tailed *t* tests. Means that are significantly different are indicated by *asterisks*

contraction was typically very weak or absent. Contractile response data that were not normally distributed were analyzed using non-parametric tests (see “[Materials and methods](#)”).

Age significantly affected the first phase of the triphasic contract-relax-contraction response to H₂S. After application of 100 μ mol/L H₂S, the first-phase contrac-

Fig. 5 Effect of age the first-phase contraction and second-phase relaxation to 100 μ M H₂S. The first-phase contraction (**a**, $n=12$, 15, and 14, from 14–34 month, respectively) and second phase relaxation (**b**, $n=12$, 15, and 14, from 14–34 month, respectively) to H₂S significantly increase in magnitude with age at 100 μ mol/L H₂S addition. The y-axis represents the H₂S-induced tension of aortic rings (grams per milligram) pooled from both diets. The x-axis shows the age (month). Data are presented as means $\pm$ SE (**a**) or as *box plots* showing the median as a horizontal line, the *box* covering the 25th and 50th percentiles, and *error bars* defining the 10th and 90th percentiles (**b**). Data were analyzed by one-way ANOVA with Tukey's post-hoc test (**a**) or a Kruskal–Wallis ANOVA with a Wilcoxon's *t* test for post-hoc comparison (**b**). Means that do not share a common letter are significantly different

Fig. 4 Representative tension tracing of a rat aorta ring pre-contracted with norepinephrine (NE) and exposed to H₂S (H₂S, 300 μ mol/L). Scale bar shows time in minutes and tension in grams

Fig. 6 Effect of diet on KCl- and norepinephrine (NE)-induced contractions. CR causes an increase in contraction magnitude in response to KCl (**a**, $n=41$) and NE (**b**, $n=41$). The y-axis represents the KCl- or NE-induced tension of aortic rings (grams per milligram) pooled from all ages. The x-axis represents age diet: ad libitum (AL) or caloric restricted (CR). AL data are in white columns and CR data are in grey columns. Data are represented as mean $\pm$ SE and were analyzed by one-tailed t tests. Means that are significantly different are indicated by asterisks

tion was stronger in rings from 34 month rats than from 14 month rats (Fig. 6a, one-way ANOVA with Tukey's post-hoc, $p=0.0062$), even though the first-phase contraction to 300–900 $\mu\text{mol/L}$ H_2S did not significantly increase with age (data not shown; one-way ANOVA, $p\geq 0.182$ for 300–600 $\mu\text{mol/L}$; Kruskal–Wallis ANOVA, $p=0.216$ for 900 $\mu\text{mol/L}$). As with the first-phase contraction, age significantly affected the second phase relaxation after application of 100 $\mu\text{mol/L}$ H_2S (Fig. 6b, Kruskal–Wallis ANOVA, $p=0.038$) and post-hoc testing with a Wilcoxon's non-parametric t test showed significant differences between 14 and 34 months ($p=0.0109$), but there was no effect of age at 300–900 $\mu\text{mol/L}$ H_2S (data

not shown; one-way ANOVA, $p\geq 0.255$ for 300–600 $\mu\text{mol/L}$; Kruskal–Wallis ANOVA, $p=0.0792$ for 900 $\mu\text{mol/L}$). The third-phase contraction was not significantly affected by age at any H_2S concentration tested (data not shown; Kruskal–Wallis ANOVA, $p=0.147$ for 100 $\mu\text{mol/L}$; one-way ANOVA, $p\geq 0.517$ for 300–900 $\mu\text{mol/L}$).

Diet significantly affected all phases of the triphasic response to H_2S . Compared with rings from AL animals, CR increased the magnitude of the first-phase contraction after application of 300–900 $\mu\text{mol/L}$ H_2S (Fig. 7a, pooled t test, $p=0.0006$, $p=0.0079$, and 0.0192 , respectively), the magnitude of the second-phase relaxation after application of 300 and 600 $\mu\text{mol/L}$ H_2S (Fig. 7b, one-tailed t test, $p=0.0492$ and Wilcoxon's test, $p=0.0027$, respectively), and the magnitude of the third-phase contraction after application of 300–900 $\mu\text{mol/L}$ H_2S (Fig. 7c, pooled t test, $p<0.0001$, $p=0.0025$, and $p=0.0008$, respectively).

Discussion

These data support our hypothesis that the H_2S signaling system is negatively affected by age in the vasculature but not in the liver, which is typically more resilient to age, and that a CR diet minimizes the age affect in the vasculature. We also observed a difference in the effect of age between CSE and CBS protein, and finally, we observed a positive effect of the CR diet that led to increased H_2S production in aorta and liver homogenates and increased vascular reactivity in aorta rings. Increased age was associated with increased expression of CSE protein in both aorta and liver, whereas CBS protein remained unchanged. Increased age was not associated with changes in mRNA expression of CSE and CBS or changes in H_2S production in aorta and liver. Compared with an AL diet, the CR diet resulted in decreased expression of CSE and CBS protein in aorta, whereas liver from CR rats showed increased expression of CSE protein at 38 months of age. CR also substantially increased mRNA expression of CSE and CBS in aorta but not liver and increased H_2S production in both aorta and liver. Age and diet both affected the triphasic response of aorta to H_2S : the first-phase contraction and second-phase relaxation each increased in magnitude with age, and all three phases of the aortic response increased in magnitude with CR.

Fig. 7 Effect of diet on H₂S-induced contractions. CR causes an increase in contraction magnitude in response to H₂S in the first-phase contraction (**a**, $n=18$ AL, $n=23$ CR), second-phase relaxation (**b**, $n=18$ AL, $n=23$ CR) and third-phase contraction (**c**, $n=18$ AL, $n=23$ CR). The y-axis represents the H₂S-induced tension of aortic rings (grams per milligram) pooled from all ages. The x-axis represents H₂S concentration. Columns show diet for ad libitum (AL, white) or caloric restricted (CR, grey) animals at each concentration tested. Data are presented as box plots showing the median as a horizontal line, the box covering the 25th and 50th percentiles, and error bars defining the 10th and 90th percentiles. Data were analyzed by one-tailed *t* test, pooled *t* test, or Wilcoxon's test. Medians that are significantly different are indicated by asterisks

CSE and CBS protein expression

In aorta, CSE protein expression increased with age in rats maintained on an AL diet, but remained constant in rats maintained on a CR diet. CBS protein expression in aorta showed a similar trend, but with lower expression levels. It is perhaps not surprising that changes were more evident for CSE than for CBS, since CSE is expressed at higher levels in the endothelium and smooth muscle cells of aorta and is believed to make the largest functional contribution to regulation of vascular tone (Hosoki et al. 1997; Wang et al. 2008; Zhao et al. 2003; Zhao et al. 2001).

The increased CSE protein expression with age in aorta is similar to changes in eNOS protein expression with age in peripheral arteries, although the increase in eNOS expression is typically larger (Cernadas et al. 1998; Goettsch et al. 2001; van der Loo et al. 2005; van der Loo et al. 2000). It has been proposed that the change in eNOS is a homeostatic correction for decreased NO bioavailability or NO concentration, and that this is caused by increased superoxide reacting with NO to form a peroxynitrite (van der Loo et al. 2005; van der Loo et al. 2000). Interestingly, NO and H₂S can interact, and therefore changes in one system could impact the other (Hosoki et al. 1997; Wang et al. 2008). Additionally, H₂S may also react with, and be quenched by, increased superoxide (Geng et al. 2004a) as well as increased peroxynitrite produced from the reaction of NO with superoxide (Whiteman et al. 2004). CR has been proposed to reduce age-related endothelial dysfunction by reducing the increase in superoxide production associated with aging, thereby maintaining a more youthful state (Barja 2002; Castello et al. 2005; Gredilla et al. 2004; Gredilla et al. 2001; Taddei et al. 2006). Consistent with the beneficial effects of CR, the expression of CSE and to a lesser extent CBS did not significantly increase with age in CR animals.

In liver, CSE protein expression increased with age in CR animals whereas CBS protein expression did not change. In addition to producing H₂S, CSE, and CBS are involved in cysteine metabolism (Zhao et al. 2001) and are present in relative abundance in the liver compared with other tissues. However, since CBS is the primary enzyme responsible for H₂S production in the liver (Zhao et al. 2003), any changes in CSE expression may have only a small effect on

H₂S signaling. These data are also not surprising given that liver enzymatic activity generally does not change substantially until very old age (Anantharaju et al. 2002; Kitani 1991; Kitani 1994).

CSE and CBS mRNA expression

While there were complex changes with age and diet in CSE protein expression in aorta and liver, there were no significant changes in mRNA message for CSE or CBS with age, but CR did increase CSE and CBS mRNA levels in aorta. This seems to contradict the protein expression data, in which CR reduced the expression of those proteins. Conversely, liver CSE and CBS mRNA message did not significantly change with age or diet, which was more consistent with the protein data. We cannot account for the discrepancy between mRNA and protein data, except to note that mRNA message does not always translate directly into protein (Gygi et al. 1999). We conclude that while CR causes increases in mRNA for CSE and CBS in the vasculature, this does not translate into the synthesis of more new protein.

Hydrogen sulfide production

Extracellular concentrations of total H₂S (representing the sum of H₂S, HS⁻, and S²⁻) have been reported in the range of 50–160 μmol/L in the brain (Abe and Kimura 1996; Goodwin et al. 1989) and ~50 μmol/L in the plasma (Zhao et al. 2001). However, more recent measurements in plasma indicate that true H₂S concentrations are much lower (<100 nmol/L) (Whitfield et al. 2008). This discrepancy is likely due to methodological differences in how free H₂S is differentiated from bound or “acid-labile” H₂S (Olson 2009).

While age did not affect H₂S production in homogenates of aorta or liver, diet did, with CR increasing H₂S production in both aorta and liver. For aorta, this is despite CR causing decreased CSE and CBS protein in aorta compared with AL. The fact that CR animals with low expression of CSE and CBS can produce as much or more H₂S than AL animals with higher expression of CSE and CBS implies that CR maintains CSE and CBS function with age. The mechanism for this is unknown, but the reduction of oxidative stress may reduce the amount of oxidative damage to these enzymes that would otherwise occur

with an AL diet, allowing maintenance of enzyme function.

Addition of the CBS inhibitor AOAA and the CSE inhibitor Pgly reduced but did not completely block H₂S production in both aorta and liver. Therefore, CSE and CBS both contribute to H₂S production in these tissues, but a small amount of the measured H₂S production may have come from PLP-independent sources, such as “acid labile” H₂S. The recovery rate of H₂S using the assay is probably 33–59%, depending on the H₂S production rate of the tissue (Julian et al. 2002). Therefore, these rates should only be used for comparisons between the treatment groups and not as an absolute rate of H₂S production. Furthermore, note that the H₂S production rates were calculated as nmol H₂S/h/mg protein, and that when H₂S production is instead calculated as nanomole H₂S production per hour per gram tissue or nanomole H₂S production per hour per milliliter of homogenate, liver has a higher production rate than aorta.

Contractile response to hydrogen sulfide in aorta

While aorta was used in this study and is commonly used to examine the vascular actions of hydrogen sulfide (Dombkowski et al. 2005; Hosoki et al. 1997; Koenitzer et al. 2007; Olson et al. 2006; Yang et al. 2008; Zhao et al. 2001), it should be noted that this is a conduit vessel, not a resistance vessel. Changing the tone of the aorta will effectively alter blood pressure and flow to the entire vascular system, not specific vascular beds, and therefore would not be as effective in regulating blood flow as it would in a resistance vessel. However, the triphasic response we observed in aorta has been observed before in pulmonary arteries (Dombkowski et al. 2005). Therefore, we propose that it is reasonable to use the aorta as an experimental proxy for the responses to H₂S in smaller resistance vessels.

Although triphasic responses to H₂S application have been reported in rat pulmonary artery (Olson et al. 2006), previous studies of rat aorta have shown only a monophasic relaxation or contraction with H₂S (Dombkowski et al. 2005; Kubo et al. 2007; Zhao and Wang 2002). We found that the development of all three phases typically required 15–30 min to develop after a bolus of H₂S. Consequently, the triphasic response may not have been observed in previous studies of aorta because in those studies vessel tone

was measured soon after H₂S addition. Additional methodological variations, such as tissue preparation (ring vs. strip), method of euthanasia, and lag time between tissue dissection and H₂S application, may have also contributed to differences in aorta response.

High O₂ concentrations in the tissue bath may lead to formation of vasoactive H₂S oxidation products, leading to contraction of the vessel instead of relaxation (Koenitzer et al. 2007). Since our experiments were run at ambient O₂ concentrations, oxidation products may have contributed to the triphasic response. However, H₂S-induced contractions in the dorsal aorta of cyclostomes are enhanced at low O₂ concentrations, suggesting that H₂S oxidation products are not the only cause of H₂S-induced contractions (Olson et al. 2008). Additionally, in rat aorta rings we observed the same triphasic response during hypoxia (Predmore et al. unpublished data), which is presumably mediated in part by reduced oxidation of H₂S under the extremely low oxygen conditions (Olson et al. 2006).

We found that the response of rat aorta rings to H₂S increases in magnitude with age, particularly in the first-phase contraction but also in the second phase relaxation. The mechanism of the first-phase contraction is unknown, but a likely candidate is the reaction of H₂S with NO to form a nitrosothiol (Whiteman et al. 2006), which would occur rapidly, reducing free NO and thereby lead to contraction (Ali et al. 2006). At higher H₂S concentrations, sufficient H₂S may remain to activate K_{ATP} channels, causing the second phase relaxation (Zhao et al. 2001). The concentration of NO is reduced with age (Cernadas et al. 1998; Goettsch et al. 2001; van der Loo et al. 2005; van der Loo et al. 2000), and therefore it follows that less H₂S would be required to significantly reduce free NO in tissue of older animals. This would have the effect of increasing the apparent sensitivity of the first-phase contraction to H₂S, consistent with our observations.

CR dramatically increased the response to H₂S in all three phases, as well as the response to KCl. Therefore, rather than representing a specific action on H₂S-signaling, the effect of CR may represent an overall enhancement of the smooth muscle function, as has been reported in skeletal muscle (Payne et al. 2003). CR is proposed to prevent endothelial dysfunction, by reducing oxidative stress (Raitakari et al. 2004; Sasaki et al. 2002) at least in part by the nuclear

transcription factor nuclear factor-E2-related factor 2 (Nrf-2), and by maintaining the tonic release of NO in blood vessels (Ungvari et al. 2008). This would protect the vasculature by inhibiting platelet aggregation and inflammatory cell adhesion to endothelial cells, as well as by inhibiting pro-inflammatory cytokine-induced signaling pathways (Ungvari et al. 2008). While we are not implying that CR works via H₂S, it would not be surprising if reduced oxidative stress, enhanced endothelial function and NO release were to result in enhanced function of the H₂S-signaling system, given the synergy between NO and H₂S (Hosoki et al. 1997; Zhao et al. 2001). In fact, H₂S has anti-oxidant and anti-inflammatory effects (Fiorucci et al. 2005a; Wallace 2007; Zanardo et al. 2006). For example, by acting through Nrf-2 and other cytoprotective signaling pathways, H₂S can upregulate phase II detoxifying genes and a variety of antioxidant enzymes (Calvert et al. 2009). Therefore, the possibility exists that H₂S may also play a role in the enhanced function observed with CR.

Limitations

While this study is the first to investigate the effects of both aging and caloric restriction on the hydrogen sulfide-signaling system, it is not without its limitations. Hydrogen sulfide is produced in the human cardiovascular system, but the evidence is largely from animal models that do not undergo the same cardiovascular pathology as humans; i.e., rats do not develop atherosclerotic plaques with progression of cardiovascular disease or age. This necessarily limits inferences to a human model. Additionally, since conduit vessels were used rather than resistance vessels, these data may not represent the changes in reactivity that are occurring in the vascular beds responsible for fine blood pressure regulation. Finally, the CR regimen imposed on the experimental animals was quite strict (40%) and is difficult to attain in humans as a therapeutic intervention. Therefore investigation at a lower level of caloric restriction (5–10%) may be warranted.

Conclusions

These results demonstrate that age and diet have differential effects on the H₂S signaling system, with age and CR causing greater changes in aorta than

liver. Aging leads to increased free radical production, and oxidatively damaged CSE and CBS protein may lead to decreased H₂S production and concentration, potentially reducing the chemical interactions between H₂S and other compounds, such as NO. CR seems to benefit CSE and CBS protein in both aorta and liver, potentially ameliorating the negative effect of age on H₂S concentration. Therefore, CR may help maintain the H₂S signaling system during aging. The sensitization of the aorta's triphasic contractile response to H₂S with CR may result from indirect effects on the NO signaling system. Therefore, elucidating the interactions between the known gasotransmitters (NO, CO, and H₂S) will be essential to understating the ramifications of aging and diet on gas signaling.

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