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Revsbech, I. G., Shen, X., Chakravarti, R., Jensen, F. B., Thiel, B., Evans, A., . . . Fago, A. (2014). Hydrogen sulfide and nitric oxide metabolites in the blood of free-ranging brown bears and their potential roles in hibernation. *Free Radical Biology & Medicine, 73*, 349-357.

doi: 10.1016/j.freeradbiomed.2014.05.025

Hydrogen sulfide and nitric oxide metabolites in the blood of free-ranging brown bears and their potential roles in hibernation

Inge G. Revsbech[a1], Xinggui Shen[b1], Ritu Chakravarti[c], Frank B. Jensen[d], Bonnie Thiel[e], Alina L. Evans[f], Jonas Kindberg[g], Ole Fröbert[h], Dennis J. Stuehr[c], Christopher G. Kevil[b], and Angela Fago[a2]

[a]Department of Bioscience, Aarhus University, 8000 Aarhus C, Denmark; [b]Department of Pathology, LSU Health Sciences Center, Shreveport LA 71130, USA; [c]Department of Pathobiology, Cleveland Clinic Lerner Research Institute, Cleveland OH 44195, USA; [d]Department of Biology, University of Southern Denmark, 5230 Odense M, Denmark; [e]Department of Medicine, Case Western Reserve University, Cleveland OH 44106, USA; [f]Department of Forestry and Wildlife Management, Faculty of Applied Ecology and Agricultural Sciences, Hedmark University College, Campus Evenstad, 2418 Elverum, Norway; [g]Department of Wildlife, Fish, and Environmental Studies, Swedish University of Agricultural Sciences, 90183 Umeå, Sweden; [h]Department of Cardiology, Örebro University Hospital, 70362 Örebro, Sweden

[1]I.G.R. and X.S. contributed equally to this work

[2]Corresponding author: Angela Fago, Department of Bioscience, Aarhus University, C. F. Møllers Allé 3, 8000 Aarhus C, Denmark; phone +45 871 55992; e-mail: angela.fago@biology.au.dk

Abstract

During winter hibernation, brown bears (*Ursus arctos*) lie in dens for half a year without eating while their basal metabolism is largely suppressed. To understand underlying mechanisms of metabolic depression in hibernation, we measured type and content of blood metabolites of two ubiquitous inhibitors of mitochondrial respiration, hydrogen sulfide (H_2S) and nitric oxide (NO), in winter hibernating and summer active free-ranging Scandinavian brown bears. We found that levels of sulfide metabolites were overall similar in summer active and hibernating bears but their composition in the plasma differed significantly, with a decrease of bound sulfane sulfur in hibernation. High levels of unbound free sulfide correlated with high levels of cysteine (Cys) and with low levels of bound sulfane sulfur, indicating that during hibernation H_2S, besides being formed enzymatically from the substrate Cys, may also be regenerated from its oxidation products, including thiosulfate and polysulfides. In the absence of any dietary intake, this shift in the mode of H_2S synthesis would help preserve free Cys for synthesis of glutathione (GSH), a major antioxidant found at high levels in the RBCs of hibernating bears. In contrast, circulating nitrite and erythrocytic S-nitrosation of the glyceraldehyde 3-phosphate dehydrogenase, taken as markers of NO metabolism, did not change appreciably. Our findings reveal that remodeling of H_2S-metabolism and enhanced intracellular GSH levels are hallmarks of the aerobic metabolic suppression of hibernating bears.

Keywords: metabolic depression, adaptation, hypothermia, thiosulfate, polysulfide

Introduction

Hibernating bears lie in dens for almost half a year without eating or drinking while relying on body fat reserves before they emerge relatively unharmed in the spring [1-3]. During winter hibernation, bears become essentially self-containing units with little or no exchange with the environment, and, to prolong body energy reserves, they reach a profound hypometabolic state, with lowered body temperatures and minimum metabolic rates down to ~25% of the basal levels [3,4]. During hibernation, lowered heart and ventilation rates [4,5] and increased blood O_2 affinity, due in part to the reduced body temperature and in part to reduced levels of red cell 2,3-diphosphoglycerate [6] reduce O_2 supply, thus matching the reduced tissue O_2 consumption. Because of these adjustments, hibernators most likely remain essentially aerobic and experience little or no hypoxia [2]. While in small hibernators body temperature drops to only a few degrees above zero [3], bears hibernate at much less reduced body temperatures (i.e. with regular oscillations between ~30-36 °C) [4,7], despite having the same weight specific low metabolic rate as small hibernators [3]. This suggests a significant temperature-independent component in the metabolic depression of hibernating bears [4].

The remarkable ability of bears and other mammalian species to hibernate has remained poorly understood in terms of the underlying mechanisms. After some early attempts to identify a circulating “trigger” molecule in the blood from hibernators [8,9], a first clue to understand the key to metabolic depression in hibernation came from experiments [10] showing that mice inhaling ~80 ppm hydrogen sulfide (sulfane, H_2S)[1] underwent drastic but fully reversible reductions in metabolic rate, body temperature, lung ventilation, O_2 consumption and CO_2 production. The dramatic changes observed, albeit artificial, were strikingly similar to those of natural hibernators. This hypometabolic effect has been ascribed to the known ability of H_2S to reversibly inhibit mitochondrial cytochrome c oxidase when present at low levels [10-12]. However, it was not known whether levels of H_2S and of its physiological in vivo metabolites in fact change in natural hibernators like bears.

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[1] Abbreviations used are: H_2S, hydrogen sulfide (sulfane); NO, nitric oxide (nitrogen monoxide); CSE, cystathionine γ-lyase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RBC, red blood cell; DTPA, diethylenetriaminepentaacetic acid; NEM, N-ethylmaleimide; SNO, S-nitrosothiol; ROS, reactive oxygen species; GSH, reduced glutathione; GSSG, oxidized glutathione;BSS, bound sulfane sulfur; SBD-F, 4-fluoro-7-sulfobenzofurazan; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; RP-HPLC, reverse-phase high performance liquid chromatography.

Suppression of O_2 consumption in hibernation necessarily originates from the mitochondria, where ~90% of whole-animal O_2 consumption takes place [13]. In principle, other signaling molecules capable of reversible inhibition of cytochrome c oxidase could also be involved in the metabolic suppression of hibernators. One such molecule is nitric oxide (nitrogen monoxide, NO). H_2S and NO are ubiquitous signaling molecules synthetized by naturally occurring enzymes (including cystathionine γ-lyase for H_2S and nitric oxide synthases for NO) with profound physiological effects on mitochondrial respiration, blood pressure regulation and cytoprotection [14,15]. Because of their reactivity, both these signaling molecules generate in vivo a broad range of oxidative products, each with distinctive biological activities. The complex in vivo effects of NO and its products, in particular nitrite and S-nitrosothiols (SNO, formed when Cys thiols are modified by NO), are known in good detail [16,17] since highly sensitive (e.g. chemiluminescence and biotin switch) methods have been available for some time for the detection of their low nanomolar in vivo levels [18,19]. These methods have revealed important roles of circulating nitrite as a storage pool of NO, from where NO can be regenerated during hypoxia and contribute to vasodilation and cytoprotection [16,20,21], and of S-nitrosation as a site-specific redox-dependent protein modification in mammals [17] and in ectotherm vertebrates [22-24]. In contrast, the biological roles of H_2S and its metabolites in vivo have remained more elusive due to technical limitations for their detection [25,26] and new methods are being currently developed to obtain reliable measures of physiological levels of H_2S and related compounds [14,27].

As fluctuations in respiratory rates are associated with oxidative stress, physiological metabolic suppression is tightly linked with antioxidant capacity. Hibernating bears most likely possess enhanced tolerance against oxidative stress and regenerative capacity as known for other animals capable of prolonged metabolic suppression [2,28]. Enhanced oxidative stress typically occurs whenever mitochondrial activity varies independently from available O_2 and potentially damaging reactive oxygen species (ROS) are generated as a product [29]. The ubiquitous tripeptide glutathione (GSH) is a key element in the thiol-dependent cellular defense against ROS and redox imbalance. For instance, ectotherms experiencing seasonal periods of prolonged hypoxia and severe oxidative stress at arousal are known to possess much higher levels of GSH compared to their hypoxia-intolerant counterparts [28].

In this study, we report measurements of a large number (23 in total) of different blood parameters taken in the same individuals of winter hibernating and summer active free-ranging brown bears, with the intent to identify which ones could be involved in hibernation. Specifically, we have examined circulating levels of major H_2S and NO metabolites, activity of the enzyme cystathione γ-lyase (CSE), an important enzyme catalyzing the production of H_2S from L-Cys in the circulation, levels of free L-Cys and GSH and other thiols. We have also investigated levels, activity and S-nitrosation of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme known to undergo S-nitrosation [30] as a marker of targeted S-nitrosation-dependent control of energy metabolism and potentially involved in the reduction of downstream 2,3-diphosphoglycerate in RBCs during hibernation [6]. All of the investigated parameters have been subject to a stringent statistical analysis to test for significant differences and mutual correlations between hibernating and summer active individual bears. The findings of this exploratory study unveil distinct potential roles of H_2S and NO-dependent signaling in physiological metabolic suppression.

Materials and Methods

Animals and Blood Samples

Animal handling and sampling were approved by the Swedish Ethical Committee on Animal Research (C212/9) and the Swedish Environmental Protection Agency. Blood samples were collected from the same seven 3-5 years old (2 males and 5 females) free-ranging anesthetized Eurasian brown bears (*Ursus arctos*) in Dalarna and Gävleborgs Counties, Sweden. Bears were previously equipped with global positioning system (GPS) collars as well as radio transmitters for tracking. Bears were immobilized by darting in the den during winter (February 2013) and again by darting from a helicopter during summer (June 2013). Anesthetics used were in winter a mixture of tiletamine-zolazepam (1.1 mg/kg), medetomidine (0.03 mg/kg) and ketamine (1.3 mg/kg), and in summer a mixture of tiletamine-zolazepam (4.7 mg/kg) and medetomidine (0.09 mg/kg) [7]. The medetomidine was antagonized with a 5 mg antisedan for each mg medetomdine after the procedures were finished, and after placing the bears back into the dens in winter [6,7]. Blood was taken from the jugular vein using heparinized vacuum tubes and immediately centrifuged in the field (4 min, 9,000 g) to separate plasma from RBCs. For each individual, RBC aliquots were immediately frozen in dry ice for later measurements of GAPDH activity or treated before freezing as described below under *H_2S products* and *NO products*. All processing and freezing of blood samples was done in the field within 10 min from blood sample collection. Samples were protected from light during processing. All chemicals were from Sigma-Aldrich unless otherwise stated.

H_2S products

Biochemical forms of H_2S were measured using the HPLC monobromobimane (MBB) assay as previously reported [27,31]. Aliquots of RBCs and plasma from individual bears were immediately diluted 1:5 in rubber cap-sealed anaerobic eppendorf vials containing previously degassed 100 mM Tris-HCl buffer pH 9.5, 0.1 mM diethylenetriamine pentaacetic acid (DTPA) and frozen in dry ice for later measurements of H_2S products, total GSH, cysteine and homocysteine concentrations [27,31,32]. Additional RBC and plasma samples were frozen without further treatment immediately after centrifugation for measurement of CSE activity. All samples were stored in liquid N_2 and analyzed within two weeks of collection [33].

Measurements of total GSH, Cys and homocysteine

Total GSH, Cys and homocysteine were measured after thiol reduction and derivatization with 4-fluoro-7-sulfobenzofurazan (SBD-F) as described [32,34]. Briefly, samples were reduced by incubation with 30 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at room temperature for 30 min. 100 mg/ml trichloroacetic acid was added to precipitate proteins. After centrifugation for 10 min, the supernants were derivatized with 90 mM SBD-F and the fluorescent thiol derivatives were separated on a C_{18} column by reverse-phase high performance liquid chromatography (RP-HPLC) and detected by fluorescence (extinction 385 nm, emission 515 nm).

CSE activity

CSE activity was measured as previously reported [35]. Briefly, tissue lysates were mixed with 2 mM cystathionine, 0.25 mM pyridoxal 5′-phosphate in 100 mM Tris–HCl buffer and incubated for 60 min at 37 °C. Trichloroacetic acid (10%) was added to the reaction mixture and spun down. The supernatant was then mixed with 1% ninhydrin reagent and incubated for 5 min in a boiling-water bath. The solution was then cooled on ice for 2 min and the absorbance measured at 455 nm using a Smart Spect Plus spectrophotometer (Bio-Rad). CSE activity was expressed as nanomoles of cystathionine consumed per milligram of total protein per hour of incubation.

NO Products

Aliquots of RBCs from individual bears were diluted 1:5 with a SNO/nitrite-stabilizing solution containing 4 mM ferricyanide ($K_3FeIII(CN)_6$), 10 mM N-ethylmaleimide (NEM), 0.1 mM DTPA [18] and frozen in dry ice. Thawed hemolysate and plasma was vortexed, and centrifuged (2 min, 16,000 *g*, 4°C), and supernatants were immediately measured. NO metabolites were measured by reductive chemiluminescence using a Sievers (Boulder, CO) Nitric Oxide Analyzer (model 280i) and previously described procedures [18,36]. Levels of SNO, iron-nitrosyl and N-nitrosyl compounds were found to be below detection limit (approx. 10 nM with the used volume and dilution). Nitrite was subsequently determined on samples deproteinized with ice-cold ethanol (1:1). Nitrite peaks were integrated with the software Origin (OriginLab Corporation, Northampton, MA, USA).

Biotin Switch

RBC samples were stored at -80 °C in stabilization solution containing 10 mM N-ethylmaleimide. The biotin switch assay was performed as described [19,37] and pull-downs from Neutravidin^{TM} resin (Pierce, USA) were probed for presence of GAPDH by western blotting. Total GAPDH was tested in RBC samples before processing samples for biotin-switch.

GAPDH Activity

GAPDH activity was assessed at 25 °C in the absence and presence of 0.3 mM dithiothreitol (DTT) by monitoring the time-dependent decrease in NADH absorbance at 340 nm (extintion coefficient 6.22 mM^{-1} cm^{-1}) following the Sigma enzymatic assay protocol (EC 1.2.1.12, Sigma Aldrich) [37]. The assay proceeds in two subsequent steps, catalyzed by 3-phosphoglyceric phosphokinase (step 1) and GAPDH (step 2)

1) 3-phosphoglyceric acid + ATP → 1,3-DPG + ADP
2) 1,3-DPG + NADH → Glyceraldehyde-3P + NAD^+ + Pi

Reactions were completed after 150 sec. For each sample, the GAPDH activity reported is expressed as units per mg total hemoglobin, measured at 540 nm and 576 nm using known extinction coefficients [38].

Statistical Analysis

Statistical differences between plasma and RBC parameters of the same seven individual winter hibernating and summer active bears were assessed by parametric paired t-test with a significance level set at $P<0.002$ to account for multiple tests. Because of the high number of parameters measured on the same samples, a low $P<0.002$ (~0.05/23) is required to validate that differences between parameters measured in hibernating and summer active bears are significant. Non-parametric paired Wilcoxon signed rank tests produced similar results (significance level $P<0.016$). To search for relationships between pairs of individual parameters measured in winter hibernating and summer active bears, pairwise Pearson correlations were calculated. The most interesting pairs were determined based on the strength ($r>0.7$ or $r<-0.7$) and significance ($P<0.05$) of the correlation. Statistical analyses were performed with SAS software, version 9.3 (SAS Institute, Cary, NC).

Results

We used the recently developed monobromobimane (MBB) method for the detection of physiological levels of different H_2S metabolites [31] in plasma and RBCs isolated from the same individual hibernating and summer active brown bears. H_2S metabolites can be divided into 1) acid-labile sulfur, which mainly contains Fe-S clusters and persulfides and is converted into H_2S under acidic conditions, 2) bound sulfane sulfur (BSS), which contains thiosulfate and polysulfides and is converted into H_2S under reducing conditions, and 3) free sulfide, containing mainly freely dissolved H_2S and HS^- [14]. We found that in winter hibernating bears, plasma contained significantly ($P<0.002$) lower BSS and free sulfide and significantly ($P<0.002$) higher acid-labile sulfane than in summer active bears (Fig. 1A; Supplementary Material Table S1, S2). Marked changes were also found when measuring thiols. Levels of plasma Cys (the most abundant plasma thiol) and total GSH decreased significantly ($P<0.002$) during hibernation (Fig. 1B, Table S1, S2). Less pronounced changes were found in other plasma parameters, such as an increase in CSE activity ($P=0.033$) (Fig. 1C) and a decrease in total sulfane sulfur ($P=0.0035$) and nitrite ($P=0.043$) (Table S1, S2), all approaching significance. Conversely, in RBCs, all parameters examined remained relatively constant with the exception of total GSH that increased significantly ($P<0.002$) during hibernation (Fig. 2A,B; Table S3, S4). CSE activity was higher in RBCs than in plasma, and possibly increased in hibernating bears (Fig. 2C) ($P=0.05$). RBC nitrite also increased during hibernation, albeit not significantly ($P=0.05$) (Table S3, S4). Despite the level of total SNO compounds was below the detection limit, S-nitrosated GAPDH was detected in RBCs by the biotin switch method (Fig. 3A), but its levels did not change significantly during hibernation and showed a large individual variation (Fig. 3B, Table S3, S4). Consistently, GAPDH activity was similar in hibernating and summer active bears (Table S3, S4). Changes approaching statistical significance were also found in RBC bound and total sulfane sulfur (P=0.0072 and 0.0087, respectively), total homocysteine (P=0.03) and GAPDH content (P=0.0032) (Table S3, S4).

We then analyzed all pairwise Pearson correlations for plasma and RBC variables (Fig. S1, S2) within individual hibernating and summer active bears to search for significant patterns (r higher than 0.7 for direct correlations and less than -0.7 for inverse correlations). Selected correlations are shown in Figure 4. In hibernating bears plasma, BSS and free sulfide were inversely correlated (r=-0.77) (Fig. 4A), whereas Cys was positively correlated with GSH (r=0.89) (Fig. 4B) and free sulfide (r=0.87) (Fig. 4C). Within RBCs, GSH was positively correlated with Cys (r=0.82) in hibernating bears (Fig. 4D). None of these parameters were significantly correlated in summer (Fig. 4A-D). These correlations suggest that during hibernation plasma Cys availability is important for generation of free sulfide and GSH (as also in RBCs) and that plasma BSS is used as a source of free sulfide. Furthermore, nitrite and SNO-GAPDH levels were not correlated (Fig. S2), suggesting a nitrite-independent mechanism for S-nitrosation in this enzyme. Other correlations albeit significant, are not clearly interpretable in terms of seasonal patterns of their variations. We note however that a strong correlation between two parameters indicates that they are equivalent in what they are measuring. For example, in both hibernating and summer active bears, total and bound sulfane sulfur were tightly correlated (r=0.91 in plasma and 0.94 and 0.96 in RBCs; Fig. S1, S2), meaning that the amount of variation in one variable is largely due to the other variable (as given by r^2). In other words, in these samples measuring total sulfane sulfur is largely equivalent to measuring bound sulfane sulfur.

Discussion

How brown bears and other mammalian hibernators are capable of drastically reducing their metabolic rate for long periods of time while still preserving organ integrity is largely unknown. A major finding of this study is that hibernation in free-ranging brown bears is associated with highly significant changes in plasma H_2S metabolites and enhanced intracellular GSH levels.

The possible origin of H_2S in hibernation

Overall, total sulfide did not change significantly in either plasma (Fig. 1A) or RBCs (Fig. 2A) upon hibernation, indicating that the balance between H_2S generation and consumption is largely the same. Plasma values of ~5 µM total sulfide in bears are about the same as those found in mice (~4.5-4.8 µM) [31]. These results indicate that it is not a general increase in H_2S levels that is associated with hibernation, but rather a shift in the way it is produced and consumed. Consistent with this interpretation, the relative composition of H_2S metabolites changed markedly in plasma (Fig. 1A) but not in RBCs (Fig. 2A), with more sulfide present in the plasma as acid labile fraction and less as free sulfide or BSS (Fig. 1A). The significant decrease in the plasma BSS pool (Fig. 1A) and the negative correlation between BSS and free sulfide found in hibernating bears (Fig. 4A) are interesting as these results suggest that H_2S is generated at the expenses of the BSS pool, while in summer bears, there is no obvious correlation between these two parameters. Polysulfides and thiosulfate ($S_2O_3^-$), are major products of H_2S oxidation contained in the BSS fraction [14,25,39] that can be recycled back to H_2S under reducing conditions [14,25], and enzymes catalyzing the conversion of thiosulfate to H_2S, including a ubiquitous GSH-dependent thiosulfate reductase [25] and mitochondrial rhodanase and 3-mercaptopyruvate sulfur transferase [40], have been identified. A recent study [40] has reported H_2S formation from thiosulfate and various reducing agents in tissue homogenates, indicating a biological role for thiosulfate in its reduction to H_2S. Although future studies will be needed to identify the BSS source for H_2S in hibernating bears, the regeneration of H_2S from one or more of its oxidative products would be of particular physiological importance for the hibernating bear, as it would help preserve levels of Cys, for protein and GSH synthesis during hibernation. This strategy would also contribute to preservation of body nitrogen stores and sustain protein synthesis in spite of absence of dietary intake of amino acids [9,41].

Figure 5 shows a plausible model for H_2S origin and fate consistent with our findings. In the blood of summer active bears, H_2S generated in RBCs from the CSE-catalyzed conversion of Cys freely diffuses out into plasma and is rapidly metabolized to generate thiosulfate and other oxidized products [14,39]. Due to its propensity to become oxidized [42,43], at normal O_2 levels most H_2S generated would be inactivated before reaching cytochrome c oxidase in the mitochondria of perfused tissues. During hibernation, part of the plasma BSS pool is transferred to the RBC, where it is converted to H_2S, a reaction that is favored by reduced GSH [25]. The low arterial O_2 tension [7] and high hemoglobin O_2 affinity [6] in hibernating brown bears indicate conditions of low O_2, where H_2S would be able to diffuse into nearby cells and contribute to suppress mitochondrial respiration. It can be envisaged that similar O_2-linked processes could also take place in cells and tissues other than blood.

Generation of H_2S from Cys

Although the BSS may function as an alternative source of H_2S, the positive correlation between plasma free sulfide and Cys (Fig. 4C) indicates that H_2S is still produced by erythrocytic CSE, possibly even functioning at higher rates (Fig. 2C), whereas the lower CSE activity in plasma (Fig. 1C) would reflect release from hepatocytes and endothelium [44]. Other enzymes, including cystathionine β-synthase (CBS), 3-mercaptopyruvate sulfurtransferase (3MST), and cysteine aminotransferase (CAT), may also synthesize H_2S from Cys in various tissues and cellular compartments [45], and CSE may even translocate to mitochondria and improve ATP production in vascular muscle cells [46], a process that may well occur in vivo in hibernating animals. Taken together, these results indicate that plasma may contain circulating available pools of BSS and Cys for uptake into RBCs and tissues where they can be used in H_2S synthesis and of mitochondrial function during hibernation.

Effects of H_2S on mitochondrial respiration

Several studies have consistently reported lower mitochondrial respiration rates in several hibernating ground squirrel species, especially in the liver [47,48] and in the skeletal muscle in 13-lined ground squirrels [49]. In this latter species, the suppressed O_2 consumption was not due to phosphorylation of respiratory complexes [50], thus supporting that a soluble factor, such as H_2S, could be involved. Interestingly, mitochondrial respiration in cardiac muscle and brain was not depressed in hibernating ground squirrels [51], suggesting that during winter hibernation energy resources are preferentially allocated to these two vital organs.

H_2S is a weak reversible inhibitor of ferrous heme of cytochrome c oxidase in mitochondria, with estimated affinity constants in the physiological µ-molar range (0.2-12.5 µM) [11,12]. In binding to cytochrome c oxidase with low affinity, it is readily displaced by stronger ligands, such as O_2. Inhalation of ~80 ppm gaseous H_2S that induced suspended animation in mice [10] correspond to ~1 µM H_2S [39], levels which are compatible with those found in the present study (1.08 and 2.00 µM free sulfide for winter hibernating and summer active bears, respectively; Table

S1). The results of this study extend the original conclusion that H_2S is involved in hibernation [10] by showing that in hibernating brown bears H_2S may in part originate from plasma BSS.

Effects of H_2S on the circulation

Besides inhibiting mitochondrial respiration, H_2S has also marked effects on the circulation, by acting as a hypoxic vasoconstrictor or vasodilator in the pulmonary and systemic circulation, respectively [42]. Although H_2S is a potent vasodilator of isolated systemic vessels [52], its effect in living hibernating animals would be likely overwhelmed by a strong adrenergic tone that may constrict peripheral systemic blood vessels. In the systemic circulation, an increase in the peripheral resistance would then maintain adequate blood pressure in spite of the reduced cardiac output and prolong body energy stores by redistributing blood flow to most demanding organs, representing a conserved adaptive trait in diving and hibernating mammals [1,2]. Conversely, in the lung circulation, H_2S may contribute to hypoxic vasoconstriction [42] thereby helping maintaining a high arterial O_2 saturation at the low ventilation rates occurring during hibernation [2,4]. Understanding the physiological processes occurring in hibernating bears, including the effects of H_2S on metabolism and circulation, will help improving therapeutic applications of hypothermia and hypometabolism in human diseases and preventing organ damage during cardiac arrest [53], immune suppression [54,55] and major surgery [56].

GSH and hibernation

Another important finding of this study is the large increase in erythrocytic total GSH found in hibernating bears (Fig. 2B). Total GSH was highly dependent on the availability of Cys in plasma and RBCs (Fig. 4B, 4D) and consistent with these results, the rate of synthesis of GSH is rate limited by the levels of Cys present in human plasma and RBCs, where Cys enters through a Na^+-dependent transporter [57]. Thiol-containing GSH is an essential component of the defense against oxidative stress in that it reacts with ROS to generate oxidized GSSG, which is then reduced back to GSH via NADPH-dependent GSH reductase or actively exported from the erythrocyte [57]. While relying on aerobic metabolism, hibernating animals undergo periodic oscillations in their metabolic rates, with inevitable mismatches between local O_2 supply and consumption, and resulting generation of potentially damaging ROS [4,28]. Although we could not measure oxidized vs. reduced GSH because of time delay in the collection and analysis of samples from the free ranging bears, a large pool of total GSH available in RBCs (Fig. 2B) and likely in other tissues during the hibernation period would help limit periodic oxidative damage. We note that reduction of any oxidized GSSG back to GSH predicts that sufficient NADPH is available as reducing agent, whereby glucose reserves (not the primary energy fuel during hibernation) might be diverted away from glycolysis to fuel the NADPH-generating pentose-phosphate pathway. In this process, reversible inactivation of phosphofructokinase, a key enzyme of glycolysis, mediated by low temperature and pH may well play a role in hibernating bears as found in a small hibernating rodent [58]. This would also explain our earlier finding of a substantial reduction in RBC 2,3-diphoshoglycerate, a side product of glycolysis, during hibernation in brown bears [6].

H_2S and NO: A comparison between two signaling molecules

Perhaps surprisingly, we did not find significant changes in the major NO metabolite nitrite in plasma or RBCs nor in S-nitrosation levels or activity of erythrocytic GAPDH, although we cannot rule out that some of the changes (for example plasma nitrite with P=0.043; Table S2) may become statistically significant in separate studies with a lower number of parameters investigated (where $P<0.05$ instead of $P<0.002$ is sufficient). Other parameters that were not investigated here may also reveal a role of NO in the control of hibernation, such as changes in NOS activity, nitrate or targeted S-nitrosation of key proteins or enzymes, in blood or other tissues. Previous studies have shown that H_2S and NO share some important characteristics: they both originate enzymatically from amino acids (L-Cys and L-Arg, respectively) [59,60], can be regenerated from their respective oxidative products [40,61] and interact in the control of vasodilation [62] and in cytoprotection [63]. However, results from this and previous investigations suggest that these two signaling molecules may operate in different physiological and pathological contexts [64]. Under hypoxia, enzymatic rate of NO synthesis from L-Arg decreases (as O_2 is a co-substrate) while conversion of nitrite to NO increases, a process also favored by acidic conditions. Such conditions are present during acute exercise and heart ischemia in mammals, or even during prolonged acclimation to extreme hypoxic and anoxic conditions as achieved by some fish and turtles [28]. Accordingly, in these hypoxia-tolerant ectotherms, levels of plasma nitrite are constitutively higher than in mammals and hypoxia-intolerant species, and plasma nitrite is shifted to tissues and used for NO synthesis during hypoxia and anoxia [22,36,65]. Conversely, mammalian hibernators use stored fat as the major energy fuel to sustain a hypometabolic state where little O_2 is consumed and supplied, without becoming hypoxic. As a result, blood pH and lactate remain relatively stable under hibernation in brown bears [6,7]. Although the role of NO in mammalian hibernation appears less clear than that of H_2S, we speculate that H_2S-dependent inhibition may prevail in aerobic metabolic suppression, as it occurs in hibernating bears, whereas NO-dependent inhibition may be dominant in hypoxic or even anoxic metabolic suppression, as it occurs in a few ectotherm species, such as crucian carp [22] and turtle [23,65] that overwinter in total lack of O_2. These complementary abilities of H_2S and NO to induce controlled and reversible hypometabolic states and to protect cells and organs against O_2 deprivations would be of far reaching consequences in biology and medicine.

Conclusions

In summary, our study is the first to show that in a hibernating species in its natural environment, hibernation is associated with 1) a significant remodeling of H_2S metabolism consistent with generation of H_2S from both BSS and Cys, and 2) a large increase in the intracellular GSH pool available. While the role of NO in hibernation remains to be conclusively established, these findings underscore the emerging importance of sulfane metabolism in metabolic depression and antioxidant defense and provide a rare snapshot into the physiological processes underlying the fascinating phenomenon of mammalian hibernation.

Acknowledgments

We thank Dr. Rakesh P. Patel for helpful input on research design. We thank Mr. Sven Brunberg and the personnel of the Scandinavian Brown Bear Project for organizing captures of the bears and logistics during the fieldwork, and Mr. John Glawe for sample processing. This study was supported by research grants from the Danish Council for Independent Research, Natural Sciences (10-084565 to A.F.), National Institute of Health (GM097041 to D.J.S and HL113303 to C.G.K.), Nordforsk (44042 to O.F.) and the Lundbeck Foundation (R126-2012-12408 to O.F.). The Scandinavian Brown Bear Research Project is funded by the Swedish Environmental Protection Agency, the Norwegian Directorate for Nature Management, the Swedish Association for Hunting and Wildlife Management, WWF Sweden and the Research Council of Norway.

Figure Legends

Figure 1. Plasma concentrations of H_2S metabolites and thiols and CSE activity in summer active and winter hibernating free-ranging bears. Concentrations (means ± SEM) of (A) sulfide (free sulfide, acid labile sulfide, bound sulfane sulfur and total sulfide), (B) total thiols (Cys, homocysteine and total GSH) and (C) CSE activity in plasma of the same summer active and winter hibernating bears (n=7). Significant differences between summer and winter values are indicated (*P<0.002). The composition of H_2S metabolites but not of total sulfide (A) and levels of Cys and total GSH changed significantly upon hibernation (B), whereas CSE activity increased (C), albeit not significantly (P=0.033).

Figure 2. RBC concentrations of H_2S metabolites and thiols and CSE activity in summer active and winter hibernating free-ranging brown bears. RBC concentrations (means ± SEM) of (A) sulfide (free sulfide, acid labile sulfide, bound sulfane sulfur and total sulfide) and (B) total thiols (Cys, homocysteine and GSH) and (C) CSE activity in the same summer active and winter hibernating bears (n=7). Significant differences between summer and winter values are indicated (*P<0.002). Whereas H_2S metabolites did not change (A), total GSH increased significantly in RBCs upon hibernation (B). CSE activity increased, but not significantly (P=0.05).

Figure 3. (A) RBC S-nitrosated GAPDH was pulled down by Neutravidin resin after lysates were treated with biotin-HDPD in the presence and absence of ascorbate. Eluates and cell lysates were probed for GAPDH. GAPDH was observed in the eluates from ascorbate-treated lysates but not in the absence of ascorbate. Cell lysates showed similar GAPDH content in summer active and winter hibernating bears. (B) Densitometric analysis of the western blots showed not significantly different S-nitrosated GAPDH normalized to total GAPDH between summer active and winter hibernating bears.

Figure 4. Correlations between selected pairs of parameters in individual winter hibernating (*closed symbols*) and summer active (*open symbols*) free-ranging brown bears. Pairwise Pearson correlations were selected based on the strength (r>0.7 or r<-0.7) and significance (P<0.05). (A) Plasma bound sulfane sulfur and free sulfide (r=-0.77); (B) plasma Cys and GSH (r=0.89); (C) plasma Cys and free sulfide (r=0.87); (D) RBC Cys and GSH (r=0.82). The shown pairs of variables were significantly correlated in hibernating bears (shown by continuous lines) but not in

summer active ones. All other pairwise correlations are reported in Supplementary Material Fig. S1 and S2.

Figure 5. Proposed role of blood H_2S in the control of metabolic rate in summer active and hibernating brown bears. Cys enters the RBC through a Na^+-dependent membrane transporter and is converted to GSH and to H_2S by the enzyme cystathionine γ-lyase (CSE). In summer active bears (*left panel*), H_2S freely diffuses through membranes and is largely inactivated (*discontinuous arrow*) before reaching cytochrome c oxidase in the inner membrane of perfused tissues mitochondria, whereby O_2 consumption is not inhibited. H_2S is also oxidized to bound sulfane sulfur (BSS) and exported to plasma. In winter hibernating bears (*right panel*), RBC, H_2S originates in part from the reduction of plasma BSS, a reaction that is favored by reduced GSH, and in part from the CSE-mediated conversion of Cys. Plasma BSS may then function as an available pool of H_2S bioactivity. The low substrate Cys concentration in the RBCs of winter hibernating bears available for the CSE-catalyzed reaction (*discontinuous arrow*) suggests that Cys is preferentially used to generate high levels of GSH. At low tissue O_2 levels during hibernation, H_2S generated (from either BSS or Cys) may inhibit mitochondrial O_2 consumption and contribute to metabolic depression during hibernation.

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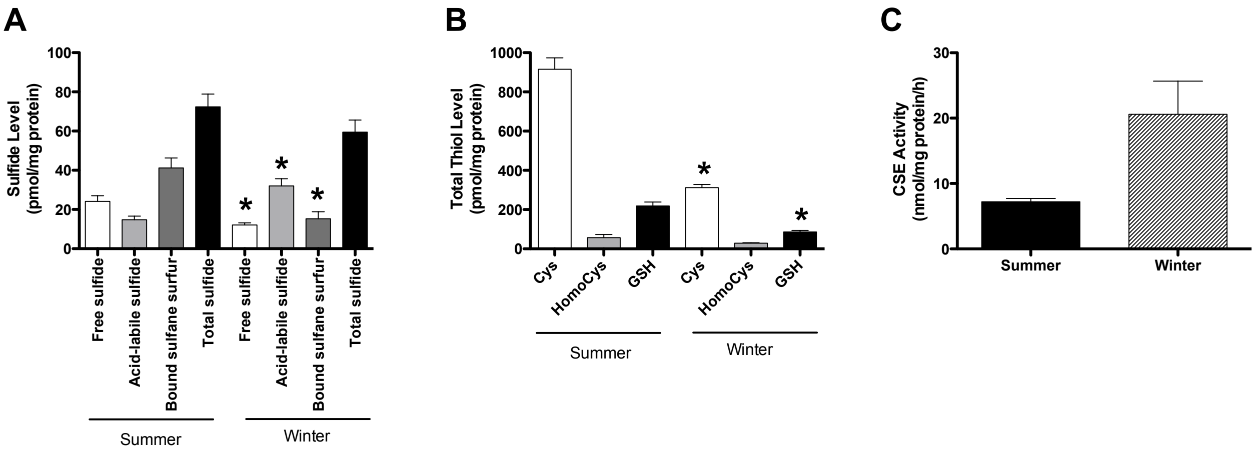
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Total Thiol Level (pmol/mg protein)
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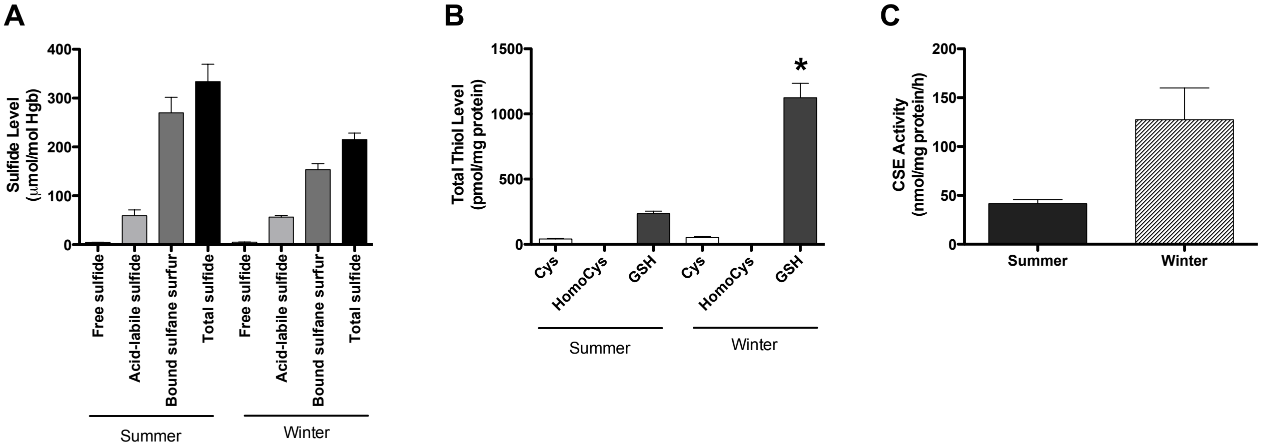
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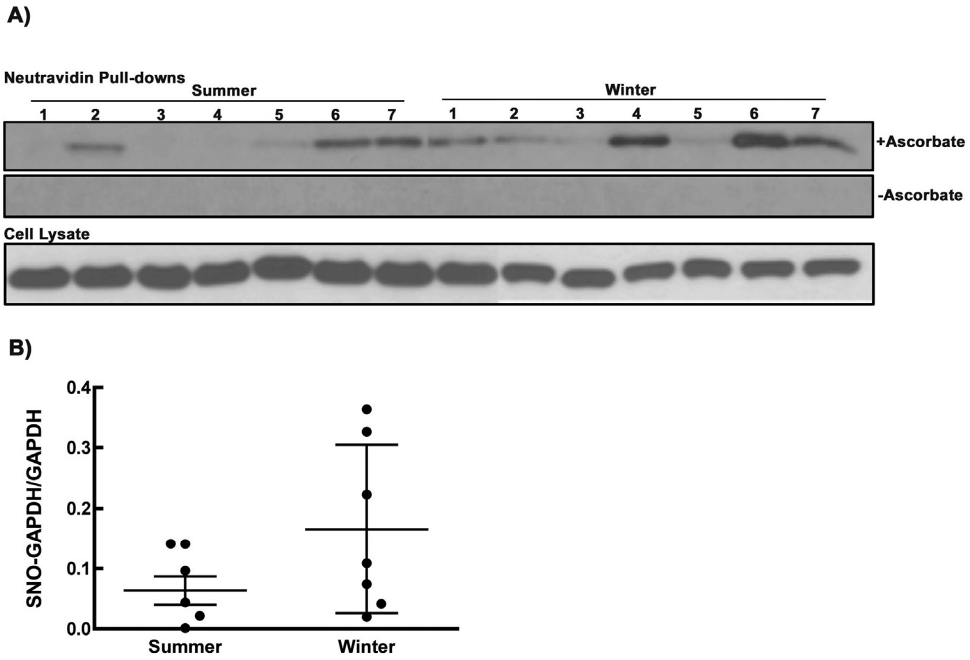
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+Ascorbate
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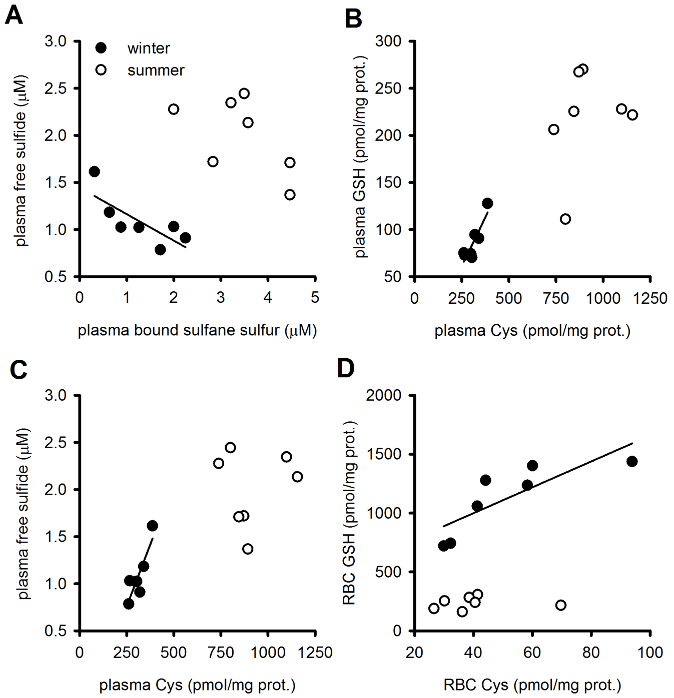
Summer
Winter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plasma GSH (pmol/mg prot.)
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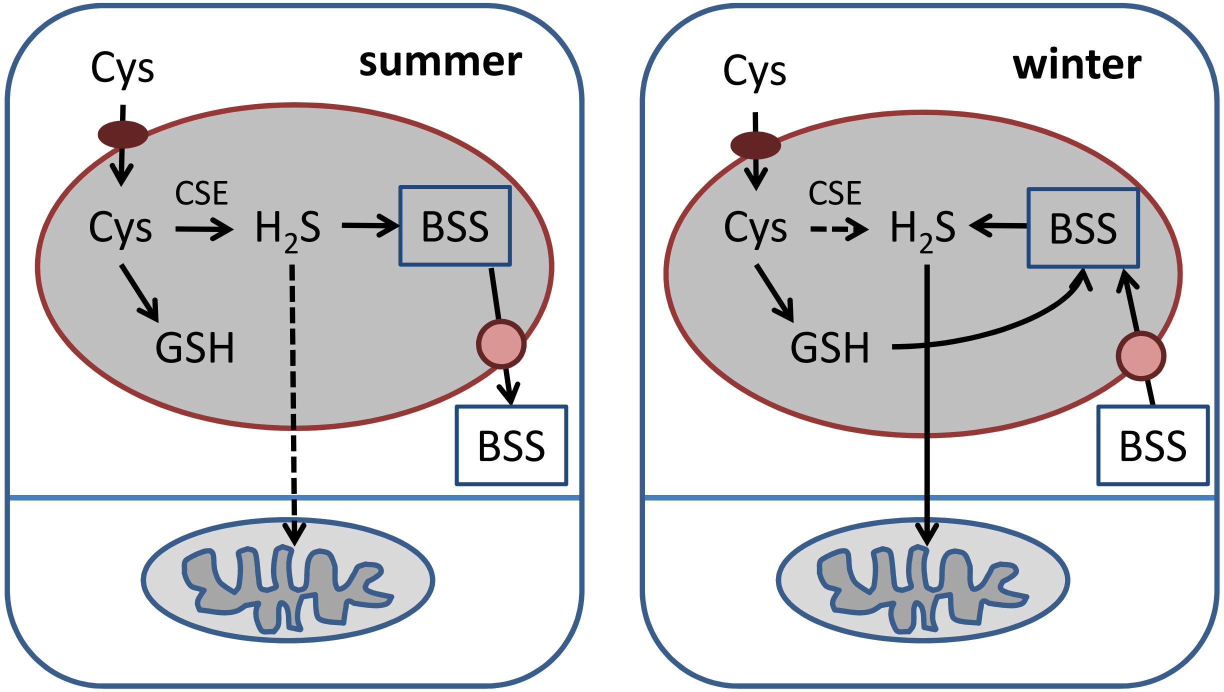
Cys
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