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Original Contribution

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

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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 the 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 in 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, in addition to 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 red blood cells of hibernating bears. In contrast, circulating nitrite and erythrocytic S-nitrosation of 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.

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]. Whereas 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 and 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].

Abbreviations: CSE, cystathionine γ-lyase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RBC, red blood cell; DTPA, diethylenetriaminepentaacetic acid; SNO, *S*-nitrosothiol; ROS, reactive oxygen species; GSH, reduced glutathione; GSSG, oxidized glutathione; BSS, bound sulfane sulfur; SBD-F, 4-fluoro-7-sulfobenzofurazan; HPLC, high-performance liquid chromatography

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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 understanding the key to metabolic depression in hibernation came from experiments [10] showing that mice inhaling ~80 ppm hydrogen sulfide (sulfane, H_2S) 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 such as bears.

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 synthesized 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 ($SNOs^2$; formed when Cys thiols are modified by NO), are known in good detail [16,17] because 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 for 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 for 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 owing 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 independent of 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 blood parameters taken from winter-hibernating and summer-active free-ranging brown bears (the same individuals in winter and summer), with the intent to identify which parameters could be involved in hibernation. Specifically, we examined the circulating levels of major H_2S and NO metabolites; the activity of the enzyme cystathione γ-lyase (CSE), an important enzyme catalyzing the production of H_2S from L-Cys in the circulation; and the 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 were subjected 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 Ethics Committee on Animal Research (C212/9) and the Swedish Environmental Protection Agency. Blood samples were collected from seven 3- to 5-year-old (two males and five females) free-ranging anesthetized Eurasian brown bears (*Ursus arctos*) in Dalarna and Gävleborgs Counties, Sweden. The bears were previously equipped with global-positioning system collars as well as radio transmitters for tracking. Bears were immobilized by darting in the den during winter (February 2013) and the same bears again by darting from a helicopter during summer (June 2013). Anesthetics used in winter were 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 5 mg antisedan for each milligram of medetomidine 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, 9000*g*) to separate plasma from RBCs. For each individual, RBC aliquots were immediately frozen in dry ice for later measurement of GAPDH activity or treated before freezing as described below under *H_2S products* and *NO products*. All processing and freezing of blood samples were done in the field within 10 min of 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 measurement 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 2 weeks of collection [33].

Measurement 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

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