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Comparative Biochemistry and Physiology Part A 133 (2002) 885–892

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Comparison between the antioxidant status of terrestrial and diving mammals☆

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Received 2 February 2002; received in revised form 11 June 2002; accepted 27 August 2002

Abstract

Many diving mammals are known for their ability to deal with nitrogen supersaturation and to tolerate apnea for extended periods. They are all characterized by high oxygen-carrying capacity in blood together with high oxygen storage in their muscle mass due to large myoglobin concentrations. The above properties theoretically also imply a high tissue antioxidant defenses (AD) to counteract reactive oxygen species (ROS) generation associated with the rapid transition from apnea to reoxygenation. Different enzymatic (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, and glutathione *S*-transferase), and non-enzymatic (levels of glutathione) AD as well as cellular damage (thiobarbituric acid-reactive substances contents, as a measure of lipoperoxidation) were measured in blood samples obtained from anesthetized animals, and also in blood obtained from recently dead diving mammals, and compared to some terrestrial mammals ($n=5$ in both groups). The results confirmed that diving mammals have, in general, higher antioxidant status compared to non-diving mammals. Apparently, to avoid exposure of tissues to changing high oxygen levels, and therefore to avoid an oxidative stress condition related to antioxidant consumption and increased ROS generation, diving mammals possess constitutive high levels of antioxidants in tissues. These data are in agreement with short-term AD adaptations related to torpor and to animals that experience large daily changes in oxygen consumption. These data are similar to the long-term adaptations of animals that undergo hibernation, estivation, freezing–thawing and dehydration–rehydration processes. In summary, animals that routinely face high changes in oxygen availability and/or consumption seem to show a general strategy to prevent oxidative damage by having either appropiate high constitutive AD and/or the ability to undergo arrested states, where depressed metabolic rates minimize the oxidative challenge.

Keywords: Antioxidants; Oxidative stress; Reactive oxygen species; Diving mammals; Diving response; Ischemia–reperfusion; Adaptation

☆ This paper was originally presented at 'Chobe 2001'; The Second International Conference of Comparative Physiology and Biochemistry in Africa, Chobe National Park, Botswana – August 18–24, 2001. Hosted by the Chobe Safari Lodge and the Mowana Safari Lodge, Kasane; and organised by Natural Events Congress Organizing (information@natural-events.com).

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1095-6433/02/$ - see front matter
PII: S1095-6433(02)00253-2

1. Introduction

Oxygen metabolism in aerobic organisms implies the formation of reactive oxygen species (ROS). These ROS can oxidize biologically important molecules leading to alterations in normal cell and tissue functions (Halliwell and Gutteridge, 1999; Mc Cord, 2000). This oxidation is functionally minimized by a fairly complex panoply of antioxidant defenses (AD). Therefore, a steady-state rate of ROS production, molecule oxidation, and antioxidant consumption is continuously taking place in aerobic cells and tissues. These three aspects need to be understood if comparisons dealing with ROS metabolism in different vertebrate species are to be made (Wilhelm Filho et al., 2000).

Enzymatic and non-enzymatic AD are essential for all aerobic organisms to prevent or attenuate the deleterious effects promoted by ROS. Beside the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase, the most important non-enzymatic antioxidants include, among others, the ubiquitous endogenous glutathione, nutritional antioxidants such as tocopherols, carotenoids, flavonoids, and ascorbate, which can be synthesized by most vertebrates, as well as bile pigments, some hormones and amino acids, and urate (Halliwell and Gutteridge, 1999). Most of these antioxidants accompany the adaptive responses to different respiratory conditions related to normal activities in nature such as feeding and reproduction, but they also play an important role involved in pathological events and environmental contaminants (Halliwell and Gutteridge, 1999; Mc Cord, 2000).

Many diving mammals can tolerate relatively long periods of apnea (Schreer and Kovacs, 1997), due to specific morphological, physiological (especially peripheral vasoconstriction and bradycardia) and biochemical adaptations (Berta and Sumich, 1999; Hochachka et al., 1999). The so-called diving response is a hypoxic-tolerance response also displayed by human and other mammalian neonates (Singer, 1999). Consequently, diving animals are able to continuously switch from hypoxia to normoxia. Upon diving some organs and tissues that rely more upon aerobic metabolism such as skeletal muscle, kidney and liver routinely experience dramatic flow restrictions (Castellini, 1991) and, therefore, they theoretically must cope with frequent high rates of ROS generation due to the alternation of apnea/reoxygenation and ischemia–reperfusion processes (Halliwell and Gutteridge, 1999), making diving mammals ideal models to study antioxidant status.

On the other hand, data on the antioxidant status of diving mammals are very scarce in the literature, and most studies deal with non-enzymatic antioxidants present in the serum of captive animals (Cook et al., 1990; Schweigert et al., 1991; Schweigert and Stobo, 1994; Crissey and Wells, 1999), excepting two studies that compared the antioxidant status of organs from seal and pig after experimental ischemia (Elsner et al., 1995, 1998). On the other hand, beside the studies performed on laboratory animals, the literature contains numerous reports dealing with wild mammals, despite the fact that most of the data available are related to animals kept in confinement (Wilhelm Filho et al., 2000). The main objective of the present study was to compare the blood antioxidant status of terrestrial and diving mammals.

2. Material and methods

Blood samples from elephant seals *Mirounga leonina* (Order Carnivora, family Phocidae) were obtained in heparinized syringes through supravertebral sinus puncture of animals captured at Ponta Delgada (Peninsula Valdes, Chubut, Argentina; 42°30′S; 64°30′W). Samples were centrifuged ($5000 \times g$ for 5 min) to separate red cells and plasma, and 7 aliquots were stored in liquid nitrogen until they were sent (on dry ice) to Buenos Aires and Florianópolis, where they were analysed. Samples from the marine manatee *Trichechus manatus manatus* were obtained through caudal vein puncture of a female specimen born in confinement (Projeto Peixe-boi, Centro de Mamíferos Aquáticos, Itamaracá, PE, Brazil). Animals were imobilized with Zoletil 50, a mixture of chlorhydrates of tiletamine and chlorhydrate of zolazepan (3.5–7.0 mg kg^{-1}, according to the species). The same procedure to obtain blood samples was performed with specific terrestrial mammalian species ($n=5$) held in CETRAS (Centro de Triagem e Recuperação de Animais Silvestres, Polícia Ambiental) located at Florianópolis, Santa Catarina state, southern Brazil (27°34′S; 48°33′W). The following non-diving mammalian species (one specimen of each species) were studied: a monkey species *Cebus apella* (Order Primates, family Cebidae); a

ferret *Grison vittatus* (Order Carnivora, family Mustelidae); a racoon *Procyon cancrivorous* (Order Carnivora, Family Procyonidae); an anteater *Tamandua tetradactyla* (Order Edentata, family Myrmecophagidae), and a deer *Ozotocerus besoarticus* (Order Artiodactyla, family Cervidae).

Blood samples were obtained through cardiac puncture, brachial vein, or dorsal intervertebral sinus, according to anatomical convenience, using minimal amounts of heparin. Samples destined for glutathione (GSH) analysis were rapidly precipitated with trichloroacetic acid 12% (1:3 v:v; blood: acid) and kept on ice. After centrifugation ($3000\times g$, for 5 min), the supernatants were used for evaluation of glutathione concentrations. Plasma samples were used for thiobarbituric acid-reactive substances (TBARS) content determinations. Red cells were washed twice with cold saline solution, centrifuged, and hemolysates were obtained by freezing/thawing; these were separated in aliquots and then stored in liquid nitrogen, until separate examination for each enzyme (Wilhelm Filho et al., 1993).

Some samples were also obtained from blood of recently dead (approx. 1 h after death) marine diving mammals from the following species belonging to Order Cetacea: the dwarf minke whale *Balaenoptera acutorostrata* (young, female); the striped dolphin *Stenella clymene* (adult, female); and the Franciscana dolphin *Pontoporia blainvillei* (young, female).

Enzymatic antioxidant assays: superoxide dismutase activity was measured by cytochrome *c* reduction (Flohé and Ötting, 1984). Catalase activity was evaluated by measuring the decrease in hydrogen peroxide concentration at 240 nm (Aebi, 1984). Glutathione peroxidase activity was measured according to Flohé and Gunzler (1984) using *tert*-butyl hydroperoxide as substrate, and glutathione reductase activity according to Carlberg and Mannervick (1985), measuring the rate of NADPH oxidation. Glutathione *S*-transferase was determined using CDNB as substrate (Habig et al., 1976).

2.1. Glutathione assay

Reduced glutathione (GSH) was measured using Ellmann's reagent (DTNB: 2-dithionitrobenzoic acid) (Beutler et al., 1963). Acid extracts were obtained by the addition of 12% TCA followed by centrifugation. Supernatants from the acid extracts were added to 0.25 mM DTNB in 0.1 M $NaPO_4$, pH 8.0, and the formation of thiolate anion determined at 412 nm. Total glutathione was measured according to the enzymatic method of Tietze (1969).

2.2. Lipid oxidation

Determination of thiobarbituric TBARS was used to assess endogenous lipid oxidation in plasma according to Bird and Draper (1984). Tissue acid extracts were obtained by the addition of the plasma to 12% trichloroacetic acid (1:4 v/v), followed by centrifugation. Supernatants were centrifuged at $5000\times g$ for 3 min, added to 0.67% (w/v) 2-thiobarbituric acid, maintained in boiling water for 60 min, cooled at 5 °C for 30 min, and then measured spectrophotometrically at 535 nm. Absorbances were expressed as equivalent to nmol TBARS g tissue^{-1} ($E_{535}=153$ mM^{-1} cm^{-1}).

Statistical analysis for the comparison between terrestrial and diving mammals was carried by the Student's *t* test, using a confidence interval of 5% ($P<0.05$).

3. Results and discussion

When blood from diving mammals was compared with that obtained from non-diving mammals, the results indicated that diving mammals have generally higher antioxidant capacity compared to non-diving mammals (Figs. 1 and 2; Table 1), suggesting that the antioxidant status of diving mammals is constitutively high in relation to terrestrial mammals.

The intraerythrocytic activities of catalase and glutathione *S*-transferase did not show significant differences between the groups (Fig. 1b, d), while the intraerythrocytic enzymatic activities of superoxide dismutase, glutathione reductase, and glutathione peroxidase (Fig. 2a, c and d) were higher in diving mammals compared to terrestrial mammals. TBARS concentrations in plasma showed higher values in non-diving mammals compared to diving mammals (Fig. 1a). Probably as a consequence, the TBARS concentrations found in plasma were in agreement with such improved AD status, revealing a lower oxidative stress condition in diving mammals.

The contents of reduced glutathione found in the red cells of diving mammals were higher than in non-diving mammals (Fig. 1c), while total

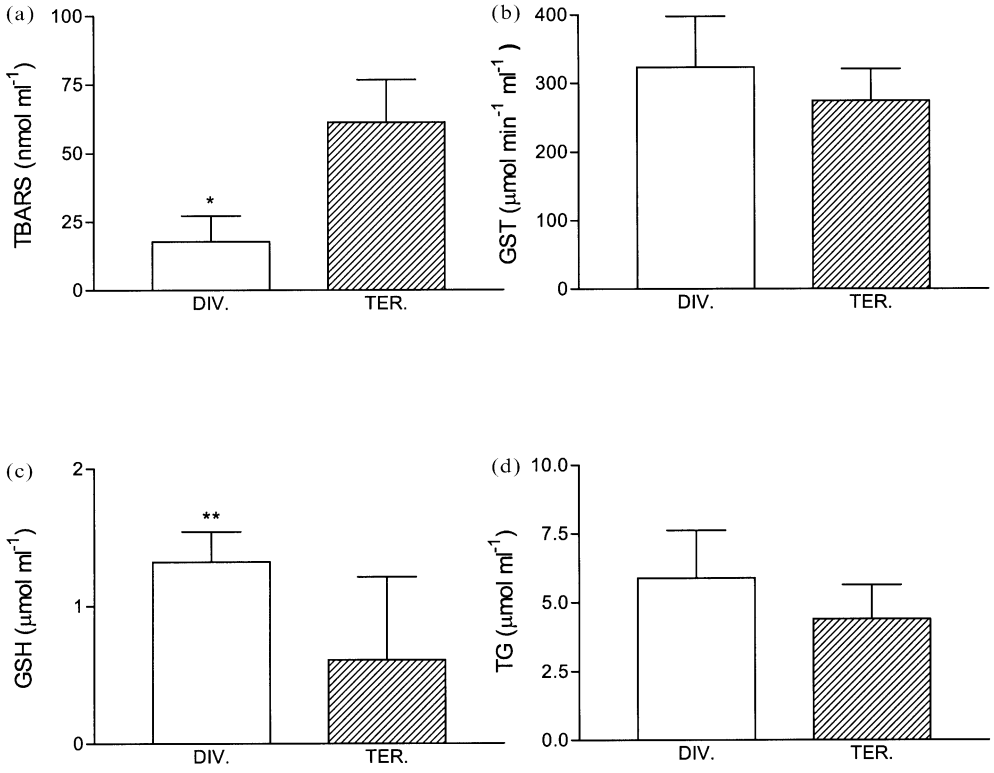

Fig. 1. (a) Plasma TBARS concentrations; (b) erythrocyte glutathione *S*-transferase (GST) activities; (c) blood reduced glutathione (GSH) concentrations; and (d) blood total glutathione concentrations (TG) of terrestrial (TER.; $n=5$) and diving (DIV.; $n=5$) mammalian species. Values represent Means ± S.E.M. Asterisks indicate significant differences ($*P<0.05$; $**P<0.01$) between the two groups.

glutathione contents were not significantly different (Fig. 1d). These values were in the range to those reported in the literature for terrestrial mammals (Wilhelm Filho et al., 2000). Glutathione is ubiquitously present in animals in relatively high concentrations (Kosower and Kosower, 1978; Härdig and Höglund, 1983; Meister and Anderson, 1983; Wilhelm Filho et al., 1996, 2000). This tripeptide is one of the most important non-enzymatic AD and is often considered as a first line of defense due to its ability to be readily and continuously recovered by the GSH cycle (Kosower and Kosower, 1978; Meister and Anderson, 1983). It may also play an important role in diving. Furthermore, GSH red cell contents are approximately equimolar in relation to hemoglobin in vertebrates (Wilhelm Filho et al., 2000), and they seem to prevent hemoglobin oxidation and can scavenge superoxide anions derived from its oxidation (Meister and Anderson, 1983).

Few other studies have reported a similar trend. Avian divers such as penguins show enhanced scavenging capacity when compared to other polar birds (Corsolini et al., 2001), whilst another dolphin species exhibited the highest non-enzymatic antioxidant capacity among other mammalian and avian polar species studied (Ninfali and Aluigi, 1998). Furthermore, post-ischemic hearts from seals showed higher SOD activities than those of pigs, and such greater protection against ROS generation was attributable to the ischemia–reperfusion process that characterizes diving mammals (Elsner et al., 1998).

The few data available in related studies are well in line with those obtained in the present study and may be explained as follows. To avoid sudden exposure of tissues to drastic changes in oxygen levels and therefore, to avoid oxidative stress related to antioxidant depletion and ROS production caused by ischemia–reperfusion processes (Halliwell and Gutteridge, 1999), diving mammals require a higher AD system than non-diving mammals. These data are in agreement with AD short-term adaptations related to animals that

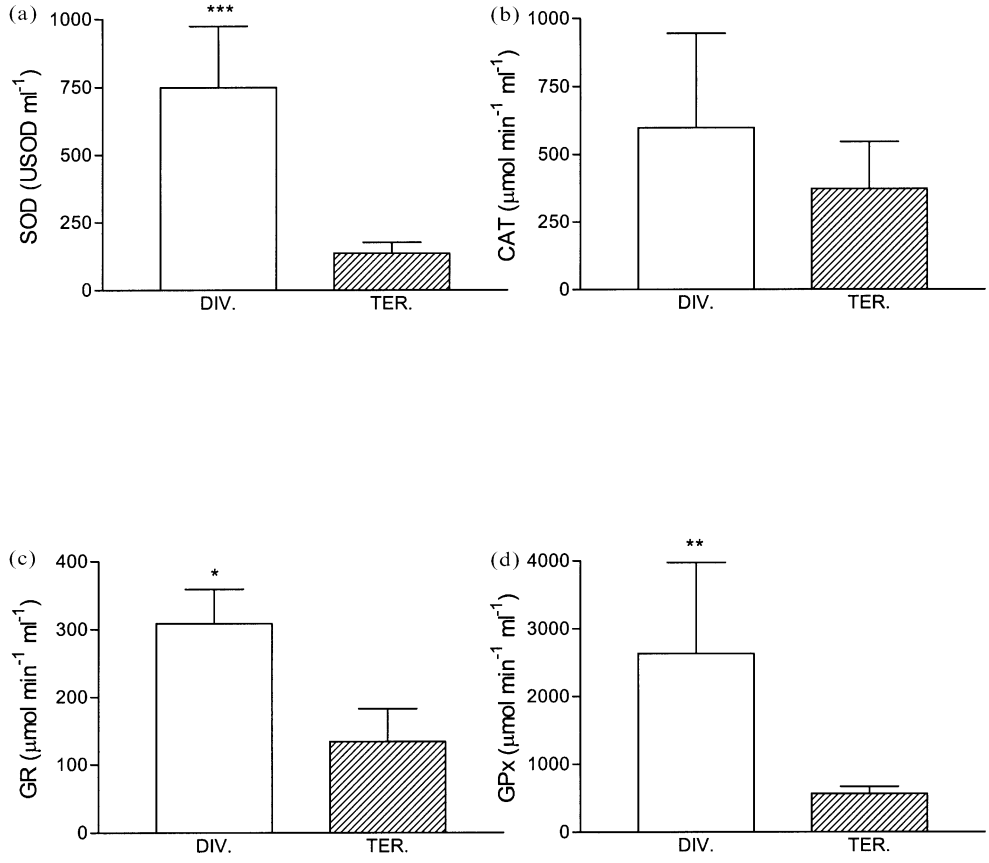

Fig. 2. (a) Erythrocyte superoxide dismutase activities; (b) erythrocyte catalase activities (CAT); (c) erythrocyte glutathione reductase activities (GR); and (d) erythrocyte glutathione peroxidase activities (GP*x*) of terrestrial ($n=5$) and diving ($n=5$) mammalian species. Values represent means ± S.E.M. Asterisks indicate significant differences (*$P<0.05$; **$P<0.01$; ***$P<0.001$) between the two groups.

experience large daily changes in oxygen consumption (Wilhelm Filho et al., 2000), like fish that experience large diurnal changes in temperature and metabolism (Wilhelm Filho et al., 1993; Wilhelm Filho et al., unpublished data), and microchiropteran bats from southern Brazil that experience large daily decreases in their metabolic activities during torpor (Wilhelm Filho et al., 1996). Interestingly, extended dives of the Weddell seal can elicit metabolic depression (Berta and Sumich, 1999) decreasing oxygen consumption and consequently ROS generation during these circumstances.

This short-term and preventative antioxidant strategy of maintaining high constitutive AD levels is similar to the long-term anticipation strategy displayed by some vertebrates and also invertebrates when experiencing oxygen changes due to anoxic–aerobic transition related to arrested states (Storey, 1996). Therefore, in hibernating mammals (Buzádzic et al., 1990; Toien et al., 2001), freeze-tolerant frogs, dehydration-tolerant frogs, and anoxia-tolerant snakes, frogs, and turtles (Storey, 1996), antioxidants are activated in preparation for ROS over-generation following reoxygenation just before arousal, thawing or rehydration, i.e. when aerobic metabolism resumes (Storey, 1996; Willmore and Storey, 1997).

This behaviour resembles the so-called ischemia–reperfusion process following cardio- and cerebro-vascular pathological events such as heart attack and stroke (Halliwell and Gutteridge, 1999). The main difference between these two conditions is that animals adapted to environmental changes are able to routinely cope with the oxidative challenge in nature, while in pathological events the tissues are usually unable to compensate for the sudden changes in ROS generation. In sum-

Table 1
Individual values of plasma TBARS, blood reduced glutathione (GSH), and total glutathione (TG) concentrations, and enzymatic activities of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione *S*-transferase (GST) in red blood cells of terrestrial and diving mammalian species

	GSH mM	TG mM	TBARS nmol ml^{-1}	CAT μmol ml^{-1} min^{-1}	SOD USOD ml^{-1}	GR μmol ml^{-1} min^{-1}	GPX μmol $ml^{-1}min^{-1}$	GST μmol ml^{-1} min^{-1}
Terrestrial mammals								
Procyon cancrivorous	0.42	0.60	43.82	24.92	50.00	33.11	293.38	142.95
Tamandua tetradactyla	0.65	3.35	45.00	854.85	62.50	30.80	293.62	228.05
Grison vittatus	0.68	4.91	53.43	58.87	254.24	180.9	656.64	280.49
Cebus apella	0.74	8.25	22.87	719.63	108.72	135.87	613.31	426.56
Ozotocerus besoarticus	0.56	4.89	140.85	204.00	205.49	290.15	827.72	292.19
Means $\pm$ S.E.M.	0.61 $\pm$ 0.05	4.40 $\pm$ 1.24	61.20 $\pm$ 15.54	372.45 $\pm$ 173.32	136.19 $\pm$ 40.15	117.97 $\pm$ 36.94	536.93 $\pm$ 105.47	274.05 $\pm$ 46.25
Diving mammals								
Balaenoptera acutorostrata	1.77	5.94	4.71	460.9	666.7	389.52	922.57	276.04
Stenella clymene	1.54	5.81	4.17	152.9	347.83	113.12	1576.73	139.06
Pontoporia blainvillei	0.91	3.14	7.80	392.74	1000.01	341.27	7870.13	344.79
Trichechus manatus manatus	0.68	12.23	19.27	1945.25	253.7	390.67	1629.06	588.97
Mirounga leonina	1.68	2.35	57.72	35.04	1481.48	380.27	1722.41	267.29
Means $\pm$ S.E.M.	1.32 $\pm$ 0.22	5.89 $\pm$ 1.74	18.74 $\pm$ 10.15	597.37 $\pm$ 346.70	749.95 $\pm$ 225.62	306.04 $\pm$ 58.43	2744.18 $\pm$ 1292.77	323.23 $\pm$ 74.48

mary, animals that routinely face high changes in oxygen availability or consumption seem to have a general strategy to prevent oxidative damage by having either appropiate high constitutive AD, or the ability to adjust their AD according to the metabolic changes associated with their ability to undergo arrested states.

Ongoing investigations in our laboratory suggest that the antioxidant status is probably directly related to the diving capacity of diving mammals. Further studies in diving mammals and other diving vertebrates may contribute to the elucidation of their extraordinary ability to cope with the continuous exposure to changing oxygen availability and to ischemia–reperfusion events, especially considering the consequent involvements of ROS and AD in such processes.

Acknowledgments

This work would not have been accomplished without the invaluable efforts to obtain blood samples from the animals here studied. We are deeply grateful to the personnel from CETRAS, Santa Catarina, Brazil (blood samples from terrestrial mammals), to Dr Milton C.C. Marcondes and Dr Fábia Luna (Projeto Peixe-boi, blood samples from manatee), to Dr Claudio Campagna from Centro Nacional Patagônico, Argentina (samples from elephant seals), and to the students of the Laboratório de Mamíferos Aquáticos (LAMAQ, Universidade Federal de Santa Catarina, Brazil; samples from dead diving mammals). We also thank Dr Gareth Cuttle (Universidade Federal de Santa Catarina) for English revision.

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