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Physiological Adaptations to Prolonged Fasting and Apnea-Induced
Ischemia/Reperfusion in Northern Elephant Seals: Role of Oxidative Stress

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor
of Philosophy

in

Quantitative and Systems Biology

by

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2013

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2013

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Acknowledgements

Résumé

Education

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**Physiological Adaptations to Prolonged Fasting and Apnea-Induced
Ischemia/Reperfusion in Northern Elephant Seals: Role of Oxidative Stress**

José Pablo Vázquez-Medina
Doctor of Philosophy in Quantitative and Systems Biology
University of California, Merced, 2013
Chair: Dr. Rudy M. Ortiz

Abstract

While diving, seals are repeatedly exposed to hypoxemia and ischemia/reperfusion. While on land, seals experience sleep apnea and prolonged periods of absolute food and water deprivation. Prolonged fasting, sleep apnea, hypoxemia and ischemia/reperfusion increase oxidant production and oxidative stress in terrestrial mammals. The objectives of this project were to investigate if prolonged fasting and sleep apnea increase oxidative stress in elephant seals and to explore the adaptations seals evolved to cope with increased oxidant production. In the first chapter, we demonstrated that despite activating the renin-angiotensin system and increasing NADPH oxidase expression and activity, prolonged fasting does not increase local or systemic oxidative damage or inflammation. In the second chapter we showed that prolonged fasting increases systemic and local endogenous antioxidant defenses (glutathione and antioxidant enzymes), which likely contribute to the prevention of oxidative damage. The third chapter explored the physiological mechanisms leading to the up-regulation of the antioxidant system during prolonged fasting and demonstrated that systemic increases in the renin-angiotensin system can activate the redox-senstive transcription factor Nrf2 through stimulating the Smad pathway and increasing the expression of NADPH oxidase 4. The fourth chapter shows that rather than inducing local or systemic oxidative damage, repetitive sleep apnea bouts activate protective responses against hypoxia and oxidative stress in elephant seals by increasing the levels of Nrf2 and hypoxia inducible factor 1α (HIF-1α). This is the first work demonstrating the oxidant-mediated activation of hormetic responses against hypoxia and oxidative stress in a large, wild vertebrate. Our findings contribute to expanding our knowledge of the evolution of antioxidant defenses and adaptive responses to oxidative stress. Understanding the mechanisms that allow adapted mammals to avoid oxidative damage has the potential to advance our knowledge of oxidative stress-induced pathologies and to enhance the translative value of biomedical therapies in the long term.

Introductory Chapter. Coping with Physiological Oxidative Stress: A Review of Antioxidant Strategies in Seals*

Abstract

While diving, seals are exposed to apnea-induced hypoxemia and repetitive cycles of ischemia/reperfusion. While on land, seals experience sleep apnea, as well as prolonged periods of food and water deprivation. Prolonged fasting, sleep apnea, hypoxemia and ischemia/reperfusion increase oxidant production and oxidative stress in terrestrial mammals. In seals, however, neither prolonged fasting nor apnea-induced hypoxemia or ischemia/reperfusion increase systemic or local oxidative damage. The strategies seals evolved to cope with increased oxidant production are reviewed in the present manuscript. Among these strategies, high antioxidant capacity and the oxidant-mediated activation of hormetic responses against hypoxia and oxidative stress are discussed. In addition to expanding our knowledge of the evolution of antioxidant defenses and adaptive responses to oxidative stress, understanding the mechanisms that allow adapted mammals to avoid oxidative damage has the potential to advance our knowledge of oxidative stress-induced pathologies and to enhance the translative value of biomedical therapies in the long term.

Introduction

Oxidant formation and oxidative stress

Oxygen consumption by animal cells is essential for the production of the energy needed to maintain cellular functions and metabolic activity. Oxygen-mediated ATP production via the electron transport chain is, however, accompanied by the production of oxidants (25, 58). Under basal metabolic conditions, ~0.1% of the oxygen consumed undergoes an univalent reduction producing superoxide radical ($O_2^{\bullet-}$) (29). $O_2^{\bullet-}$ can spontaneously react with nitric oxide ($NO^{\bullet}$) generating peroxynitrite ($ONOO^-$) (3, 6, 76) or be converted by superoxide dismutases (SOD) into oxygen and hydrogen peroxide (H_2O_2) (62). H_2O_2 can diffuse across biological membranes or be decomposed to water and oxygen by catalase, glutathione peroxidases (GPx) and peroxiredoxins (Prx), in a series of reactions that prevent the formation of the highly reactive hydroxyl radical ($HO^{\bullet}$) (8, 51, 74).

Oxidants are not only formed as a by-product of oxygen metabolism in the electron transport chain. NAPDH oxidases (Nox), microsomal monooxygenases (cytochromes P450), xanthine oxidase (XO), nitric oxide synthases (NOS), lipoxygenases and cyclooxygenases produce $O_2^{\bullet-}$, H_2O_2, $NO^{\bullet}$ or hydroperoxides under physiological conditions. The autoxodiation of many biologically important molecules and the electron delocalization that takes place in the reactions of hemo-proteins, result in the production of oxidants as well (33). Under several pathological conditions (e.g. cardiovascular and metabolic diseases) oxidant production increases altering the balance between oxidants and antioxidants and thus promoting oxidative stress (84). Oxidative stress causes the formation of oxidatively-modified lipids, proteins, and nucleic acids and the disruption of redox signaling and control (47).

Potential sources of oxidative stress in seals

Apnea-induced hypoxemia and ischemia/reperfusion

Seals are routinely exposed to breath-holding (apnea) bouts while diving and sleeping (18, 54, 78). Apnea in seals is characterized by cardiovascular adjustments (reduction in cardiac output, bradycardia and peripheral vasoconstriction) that allow the maximum utilization of the oxygen stores, but simultaneously result in the depletion of blood oxygen content and in the redistribution of blood flow towards obligatory oxygen-dependent tissues, exposing seals to ischemia and hypoxemia (12, 17, 53, 63, 90). At the end of an apnea bout, perfusion of ischemic tissues can potentially increase oxidant production and oxidative stress (19, 111). In terrestrial mammals, hypoxemia increases electron leak from complexes I and III of the respiratory chain, resulting oxidant production (41, 92). Perfusion of ischemic tissues exacerbates oxidant production and oxidative damage promoting reperfusion injury (14, 61). During ischemia, XO is activated and the ATP degradation product, hypoxanthine (HX), accumulates. During reperfusion, XO hydroxylases HX generating xanthine, $O_2^{\bullet-}$ and H_2O_2 (75). Seal tissues do not possess higher levels of oxidative damage than terrestrial mammal tissues despite being chronically exposed to apnea-induced hypoxemia and ischemia/reperfusion (104, 108, 111). These observations suggest that seals either avoid apnea-induced oxidant

generation or that seals can efficiently cope with increases in oxidant production without experiencing oxidative damage.

Prolonged food and water deprivation

Along with being exposed to intermittent hypoxemia and chronic cycles of ischemia/reperfusion, phocid seals also experience prolonged periods of absolute food and water deprivation (fasting). Spontaneous long-term fasting is an integral part of the life history of phocid seals. Seals undergo prolonged fasting annually while breeding, molting and weaning (11). Prolonged fasting activates the hypothalamic–pituitary–adrenal axis (HPA) leading to alterations in fluid balance and cardio-respiratory function (65, 80). In the northern elephant seal, along with activating the HPA axis, prolonged fasting stimulates the renin-angiotensin system (RAS) and promotes insulin resistance (28, 68, 71, 73, 106, 107). In humans, rats and mice, prolonged fasting, chronic HPA and RAS activation, and insulin resistance, increase oxidative damage by activating Nox proteins, increasing mitochondrial oxidant generation and depleting antioxidants (13, 16, 21, 79, 87-89, 95). The fact that prolonged fasting does not increase local or systemic oxidative damage in elephant seals suggests that seals are adapted to tolerate fasting-induced oxidant production (99, 102).

Aging and postnatal maturation

Oxidant production and oxidative damage accumulation increase with age contributing to senescence and physiological aging (23, 85). Old Weddell seals experience muscular senescence but maintain muscle contractile ability and foraging capacity suggesting either that seals efficiently cope with age-associated oxidant production or that senescence in seals is not mediated by increased oxidative stress (37, 38, 40). The transition from a terrestrial to an aquatic environment during post-natal development also increases oxidant production without increasing oxidative damage in seals (100). Maturation-related increases in antioxidant capacity likely help seals to counteract age-related increases in oxidant production avoiding oxidative damage (101). The link between age- and dive-associated oxidant production and oxidative stress has only recently been explored (38, 39, 100, 101), but undoubtedly warrants further investigation.

Diving *vs* non-diving endotherms. Insights from comparative and *in vitro* studies
Oxidant production

The real-time *in vivo* measurement of oxidants is challenging in whole-animal vertebrate systems because most oxidant species are highly reactive and have a short half-life. Studies using *in vitro* approaches have demonstrated that seal heart and kidney accumulate HX after experimental ischemia (19, 20). Moreover, the tissue capacity to produce $O_2^{\bullet-}$ is higher in seal than in pig heart, kidney and skeletal muscle under basal conditions, and in response to an oxidant-generating system (xanthine + XO) (111). The production of $O_2^{\bullet-}$ is also higher, under basal conditions, in the liver and muscle of emperor penguins, another diving, endothermic vertebrate, than in those tissues of chickens and several non-diving marine birds (brown noddies, petrels, frigate birds, red-

billed tropic birds, boobies and shearwaters) (112). These findings suggest that avoiding oxidant production is not the main mechanism used by diving, endothermic vertebrates to cope with ischemia/reperfusion (111, 112).

Oxidative damage

Despite chronic exposure to prolonged fasting, hypoxemia and ischemia/reperfusion, seal tissues do not have higher levels of lipid peroxidation or protein oxidation products (TBARS, protein carbonyls) than pig tissues (104, 111). The intracellular content of TBARS is also lower in the red blood cells (RBCs) of a group of marine mammals (elephant seals, manatees, minke whales, and stripped and franciscana dolphins) than in the RBCs of wild, terrestrial mammals (raccoons, deer, anteaters, monkeys, and ferrets), and in the liver and muscle of emperor penguins than in the liver or muscle of chickens and non-diving, marine birds (108, 112). Although TBARS measurements alone are not enough to conclusively determine the absence of oxidative damage in penguin tissues and marine mammal organs and RBCs (34), taken together, the available data suggest that diving birds and mammals have the ability to cope with increased oxidant production without experiencing oxidative damage.

Antioxidant defenses

An enhanced antioxidant capacity appears to be a mechanism by which diving birds and mammals cope with increased oxidant production (10, 15, 19, 39, 59, 111). High constitutive activity and content of endogenous antioxidants has also been found in other animal species chronically exposed to variations in oxygen availability to their tissues due to factors such as environmental oxygen lack, extracellular freezing, or apneic breathing patterns in arrested metabolic states (36, 91). Penguins and seals possess higher concentrations and activities of enzymatic and non-enzymatic antioxidants than terrestrial birds and mammals. Plasma glutathione (GSH) levels are 2-3-fold higher in Weddell and harbor seals than in humans (66). Intracellular GSH content in RBCs is 2-fold higher in marine mammals than in wild, terrestrial mammals (108). Comparing ringed seals to pigs, the concentrations of GSH are 20-, 6-, 2- and 3-fold higher in heart, skeletal muscle, kidneys and lungs, respectively (Table 1) (104). The concentrations of several exogenous low molecular weight antioxidants (vitamins, carotenoids) are also higher in diving than in non-diving mammals and birds. Plasma content of α-tocopherol is higher in dolphins than in dogs or cows (49). Plasma scavenging capacity against peroxyl radical is higher in emperor and Adélie penguins than in polar skuas or snow petrels (15). Similarly, the activities of the antioxidant enzymes SOD, catalase, GPx, glutathione S-transferase (GST) and glutathione disulphide reductase (GR) are higher in RBCs of marine than of terrestrial mammals (108) while catalase, GPx and GST activities are higher in the liver and muscle of emperor penguins than of chickens and non-diving, marine birds (112). The activities of SOD and GST are higher in heart and lung of seals than of pigs whereas catalase activity is higher in the liver of seals than of pigs (Table 1) (103). The activity of GPx is also higher in heart, lung and skeletal muscle of seals than of pigs while the activities of GR and glucose-6-phosphate dehydrogenase (G6PDH), two key enzymes that maintain intracellular GSH, are higher in heart, kidney, liver, lung and skeletal

muscle of seals than of pigs (Table 1) (103, 104). Collectively, these findings suggest that possessing increased antioxidant protection is essential for diving vertebrates.

Table 1. Antioxidant enzyme activities and glutathione content in ringed seal (*Phoca hispida*) and domestic pig (*Sus scrofa*) tissues.

	SOD (U mg protein)	Catalase (U mg protein)	GPx (mU mg protein)	GST (mU mg protein)	GR (mU mg protein)	G6PDH (mU mg protein)	GSH-Eq (nmol g wt tissue)
Seal							
Heart	77 ± 25*	$1,956 \pm 581$*	15 ± 1*	13 ± 3*	7 ± 2*	15 ± 3*	$2,918 \pm 227$*
Kidney	55 ± 10	$8,245 \pm 960$	17 ± 2	7 ± 1	5 ± 1*	121 ± 42*	$1,121 \pm 19$*
Liver	71 ± 36	$19,196 \pm 4,52$*	33 ± 8	35 ± 5*	3 ± 0.6*	134 ± 27*	327 ± 4
Lung	78 ± 20*	$1,614 \pm 289$*	47 ± 10*	3 ± 1	6 ± 2*	67 ± 17*	830 ± 5*
Muscle	14 ± 3	891 ± 268*	4 ± 0.8*	0.9 ± 0.2	4 ± 1*	71 ± 29*	742 ± 67*
Pig							
Heart	26 ± 8	$7,154 \pm 1,900$	8 ± 0.9	4 ± 1	0.6 ± 0.1	2 ± 0.7	117 ± 0.04
Kidney	38 ± 7	$7,717 \pm 1,176$	36 ± 13	5 ± 0.8	0.5 ± 0.1	3 ± 0.5	490 ± 142
Liver	24 ± 3	$11,004 \pm 3,585$	25 ± 3	70 ± 12	0.5 ± 0.1	2 ± 0.4	451 ± 33
Lung	8 ± 2	$3,644 \pm 630$	16 ± 2	2 ± 0.7	0.8 ± 0.3	1 ± 0.5	262 ± 65
Muscle	15 ± 4	$3,462 \pm 848$	2 ± 0.26	0.9 ± 0.2	0.4 ± 0.1	4 ± 0.5	124 ± 7

Data are presented as mean $\pm$ SEM. Data from Vázquez-Medina et al. (2006, 2007)

SOD superoxide dismutase, *GPx* glutathione peroxidase, *GST* glutathione *S*-transferase, *GR* glutathione disulphide reductase, *G6PDH* glucose-6-phosphate dehydrogenase, *GSH-Eq* total glutathione

* Significantly different from pigs ($p < 0.05$)

Hypoxic and redox signaling

While oxidant production can be potentially damaging, it is also needed for the regulation of several adaptive processes (94). H_2O_2 is a second messengers that participates in redox reactions to regulate signal transduction by stimulating calcium-dependent pathways, protein phosphorylation and transcription factor activation (26, 94). Similarly, oxygen sensing and redox signaling are essential for mediating the physiological and pathophysiological responses to hypoxia (9). *In vitro* and *ex vivo* studies comparing seals and non-diving mammals have demonstrated that the seal brain possesses hypoxia tolerance and antioxidant protection due to an increased content and unique localization of neuroglobin in tissue (24, 64, 77, 109). Neuroglobin enhances oxygen extraction and intracellular diffusion and protects against oxidative stress by activating phosphoinositide-3 kinase and by opening the mitochondrial K_{ATP} channel (1, 57, 93). Another primary regulator of the adaptive response to hypoxia is the Hypoxia-Inducible Factor 1 (HIF-1) (81, 82). HIF-1 genes in the ringed seal are similar to those in terrestrial mammals, but in contrast to what has been observed in terrestrial mammals (43), HIF-1 proteins are constitutively expressed in several tissues of the ringed seal, and their levels are correlated to reduced levels of protein oxidation suggesting that HIF-1 may potentially mediate hypoxia and antioxidant protection in seal tissues (45, 46).

In vivo **studies**
Adaptive responses to apnea in seals

Several studies have been conducted to address the central question of how seals cope with apnea-induced oxidant production. The role of GSH, a primary antioxidant

with a key role in redox signaling (for a recent review see (27), in the protection against apnea-induced oxidant production, was first discovered in a study showing that plasma GSH levels in Weddell seals decreased during forced diving and rose above resting levels at the end of the submersions (66). In another study, $NO^{\bullet}$ was not detected in the exhaled gas of Weddell seals breathing through an isolated hole after freely diving under the sea ice (22). This finding suggests that the absence of $NO^{\bullet}$ may be an adaptive strategy to avoid $ONOO^{-}$ and $HO^{\bullet}$ formation after an event of ischemia/reperfusion that can potentially increase $O_2^{\bullet-}$ production (3, 6, 31, 76). More recently, several aspects of oxidant and antioxidant metabolism were evaluated in elephant seals exposed to rest- and voluntary submersion-associated apneas. Plasma XO activity, xanthine and HX levels, but not systemic oxidative damage (F_2-isoprostanes, nitrotyrosine, 4-hydroxyonenal or protein carbonyls), increased in response to apnea (105). Moreover, XO protein expression increased in the skeletal muscle after repetitive apnea bouts, along with an increase in the nuclear content of NF-E2-related factor 2 (Nrf2) and HIF-1α, and the protein expression of Cu,ZnSOD, catalase and myoglobin (Mb) (105). These findings suggest that H_2O_2 produced by XO can potentially mediate the adaptive response to oxidative stress during apnea in seals since Nrf2, the redox sensitive transcription factor that regulates antioxidant gene expression, translocates into the nucleus in response to increased intracellular H_2O_2 production and XO produces mainly H_2O_2 (44, 50, 52). These findings also suggest that repetitive apneas stimulate the adaptive response to hypoxia in seals and that HIF-1α and Mb potentially contribute to seal's tolerance to hypoxia (45, 46, 48, 67, 105). Collectively, these findings support the idea that apnea-induced oxidant production mediates the preconditioning of seal tissues (111) since oxidants are required for the activation of protective pathways against reperfusion injury (Figure 1) (2, 5, 30, 32, 56, 83, 110, 113).
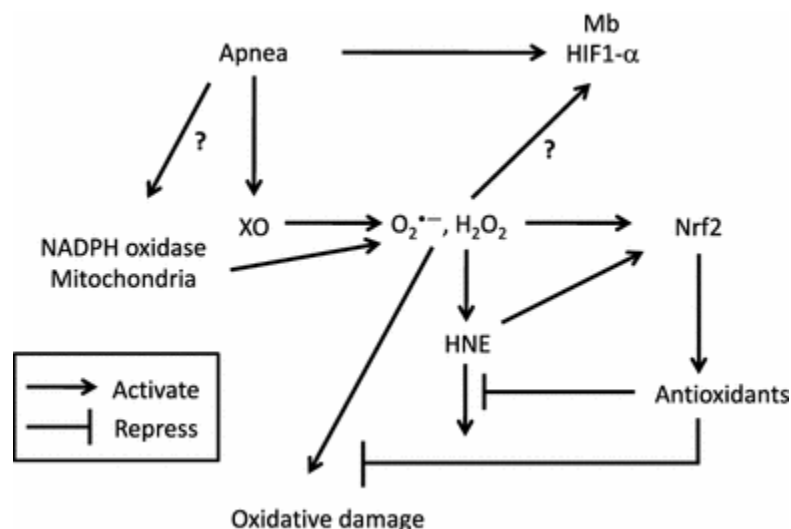
Figure 1. Apnea stimulates adaptive responses to hypoxia and oxidative stress in seals. Schematic representation of the proposed mechanisms leading to the activation of protective responses against oxidative stress and hypoxia in the skeletal muscle of

elephant seals. H_2O_2 = hydrogen peroxide. HIF-1α= hypoxia inducible factor 1α. HNE = 4-hydroxynonenal. Mb = myoglobin. Nrf2 = NF-E2-related factor 2. $O_2^{\bullet-}$ = superoxide radical. XO = xanthine oxidase.

Oxidant and antioxidant metabolism during prolonged fasting in elephant seals

      Oxidant and antioxidant metabolism has been studied in northern elephant seals during their natural fasting periods associated with breeding, molting and weaning. Prolonged fasting in the northern elephant seal promotes insulin resistance (28, 42, 106, 107), activates RAS (68, 73) and the HPA axis (69, 70, 72), and increases NADPH oxidase 4 (Nox4) and XO activity and protein expression (86, 99). Prolonged fasting, however, is not associated with increased oxidative damage in this species. Systemic (F_2-isoprostanes, plasma nitrotyrosine, C-reactive protein) and muscle markers of oxidative damage (TBARS, 4-hydroxynonenal, protein carbonyls, nitrotyrosine) remain unchanged after two months of absolute fasting in weaned pups (99). Increased activity and protein expression of several antioxidant enzymes (SOD, catalase, GPx, Prx, GST, GR, G6PDH, glutamate-cysteine ligase =GCL, γ-glutamyl-transpeptidase: GTT), as well as increased GSH, likely contribute to the prevention of fasting-associated oxidative damage in elephant seal pups (99, 102). Increased plasma content of water-soluble vitamins in pups and lactating females (7), and the maintenance of elevated levels of high-density lipoproteins in breeding and molting adult males (97), may also contribute to counteract fasting-induced oxidant production in elephant seals. The understanding of how the antioxidant system of the northern elephant seal is up-regulated in response to prolonged fasting remains elusive, but preliminary studies from our laboratories, along with the present findings, suggest that angiotensin II stimulates Nox4, and that Nox4 may mediate an hormetic response by activating Nrf2 (Figure 2; see chapter 3). Hormesis is defined as an adaptive response to a moderate stress (60). Since H_2O_2 activates Nrf2 (52, 98), and Nox4 constitutively produces H_2O_2 (96), it is possible that an increase in Nox4 expression mediates an adaptive response to fasting-induced oxidant production in elephant seals.
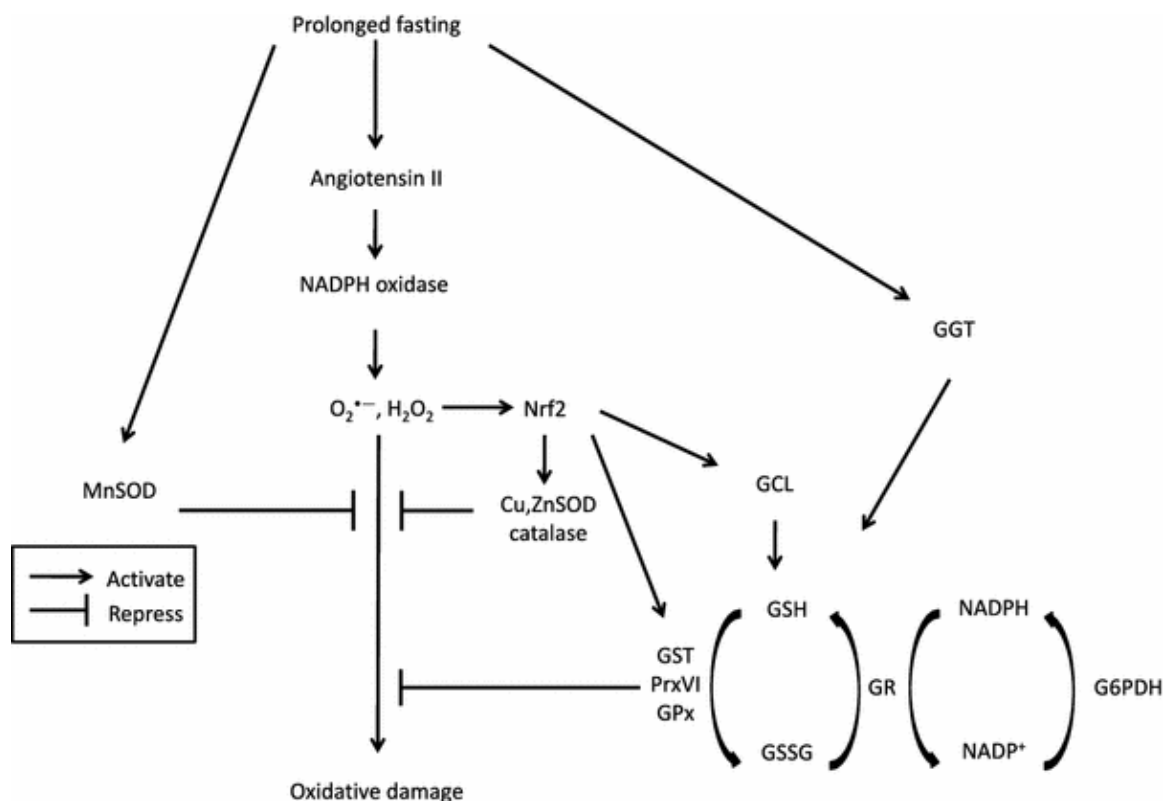
Figure 2. Prolonged fasting up-regulates the antioxidant system of the northern elephant seal. Schematic representation of the proposed mechanisms leading to the up-regulation of the antioxidant system in response to prolonged fasting in elephant seals. Cu,ZnSOD = copper and zinc-dependent superoxide dismutase. G6PDH = glucose-6-phosphate dehydrogenase. GCL = glutamate-cysteine ligase. GGT = γ-glutamyl transpeptidase. GSH = glutathione. GSSG = glutathione disulphide. GR = glutathione disulphide reductase. GPx = glutathione peroxidase. GST = glutathione S-transferase. H2O2 = hydrogen peroxide. MnSOD = manganese-dependent superoxide dismutase. Nrf2 = NF-E2-related factor 2. $O_2^{\bullet-}$ = superoxide radical. PrxVI = 1-cys peroxiredoxin.

Aging, maturation and oxidative stress in Weddell and hooded seals

According to the free radical theories of aging, the production of oxidants and the accumulation of oxidative damage are factors that mediate senescence and physiological aging (4, 23, 35, 85). Based on those theories, diving vertebrates may be particularly susceptible to oxidative stress, cellular dysfunction and senescence due to chronic exposure to diving-induced hypoxemia, ischemia/reperfusion and exercise during foraging. Unfortunately, aging or senescence studies in seals or any other diving, endothermic vertebrate are scant. Muscular senescence has been documented in Weddell seals in which collagen content is higher in *longissimus dorsi* and *pectoralis* muscles of old seals (+ 17 years old) compared to young adults (9-16 yr). In addition, a shift of the collagen isoform profile from Type III to the stiffer Type I occur with age in both muscles indicating that old seals experience muscular senescence (38). Consistent dive

durations throughout adulthood, however, imply unchanged swimming and foraging capacity, suggesting either that seals evolved mechanisms to cope with age-derived oxidant production or that senescence and physiological aging in seals are not mediated by oxidative stress (37-40, 55).

Post-natal maturation is another potential source of oxidative stress in phocid seals due to their transition from a terrestrial to an aquatic environment and the concomitant beginning of their diving lifestyle. Tissue capacity to produce $O_2^{\bullet-}$, but not lipid peroxidation (TBARS), protein carbonyls or oxidatively-modified DNA (8-oxo-7,8-dihydro-2'-deoxyguanosine) levels are higher in the skeletal muscle (*longissimus dorsi*) of adult hooded seals than of newborn or weaned pups (100). Maturation in hooded seals also increases SOD, GPx and thioredoxin 1 activities, MnSOD, PrxVI and glutaredoxin 1 protein expression, as well as GSH levels, suggesting that the antioxidant system of the hooded seal develops with age progression (100, 101). Interestingly, neither Nrf2 mRNA nor protein expression (in whole extracts or nuclear fractions) are increased in adults compared to pups suggesting that maturation in seals does not induce an acute adaptive response to oxidative stress. The later also suggests that age-related increases in oxidant production are efficiently counteracted by appropriately elevated antioxidant levels in seals and that Nrf2 activation may only increase in response to a particular extended diving episode or repetitive apneas in diving mammals (105).

Conclusions and future directions

The life history of seals is characterized by extreme behaviors that expose them to potential increases in oxidant production and oxidative stress. Seals, however, have evolved mechanisms that allow them cope with prolonged fasting, hypoxemia and ischemia/reperfusion without experiencing oxidative damage. Elevated levels of endogenous antioxidants likely help seals to counteract increases in fasting-, apnea- and age-derived oxidant production. The control and regulation of the adaptive responses to oxidative stress in seals remain elusive, but initial studies have shown that ischemic preconditioning and physiological oxidant production mediate hormetic responses that stimulate the antioxidant system of developing, fasting and diving seals. More thorough investigations along these lines not only can enhance our appreciation for the evolution of such mechanisms, but also have the potential to provide valuable insight to the contribution of oxidative stress to a number of human pathologies.

Acknowledgements

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Chapter I. Prolonged Fasting Does Not Increase Oxidative Damage or Inflammation in Postweaned Northern Elephant Seals*

Abstract

Elephant seals naturally undergo prolonged periods of absolute food and water deprivation (fasting). Prolonged food deprivation in terrestrial mammals increases oxidant production, oxidative damage and inflammation that can be induced by an increase in the renin-angiotensin system (RAS). To test the hypothesis that prolonged fasting in elephant seals does not increase oxidative stress or inflammation, blood samples and muscle biopsies were collected from early (2-3 wk post-weaning) and late (7-8 wk post-weaning) fasted seals. Plasma levels of oxidative damage, inflammatory markers and plasma renin activity (PRA), along with muscle levels of lipid and protein oxidation, were compared between early and late fasting periods. Protein expression of angiotensin receptor 1 (AT_1), NADPH oxidase 4 (Nox4) and antioxidant enzymes (CuZn- and Mn-superoxide dismutases, glutathione peroxidase, and catalase) was analyzed in skeletal muscle. Fasting induced a 2.5-fold increase in PRA, a 70% increase in AT_1, a 2-fold increase in Nox4 and a 70% increase in NADPH oxidase activity. In contrast, neither tissue nor systemic indices of oxidative damage or inflammation increased with fasting. Furthermore, muscle antioxidant enzymes expression increased 40-60% with fasting, in parallel with an increase in muscle and red blood cell antioxidant enzyme activities. Results suggest that despite the observed increases in RAS and Nox4, an increase in antioxidant enzymes appear to be sufficient to suppress systemic and tissue indices of oxidative damage and inflammation in fasted seals. The present study highlights the importance of antioxidant capacity in mammals during chronic stressors to help avoid deleterious systemic consequences.

Introduction

Food deprivation is a stressful physiological condition that activates the hypothalamic-pituitary-adrenal axis, increasing the release of adrenocorticotropic hormone and glucocorticoids, and leading to subsequent alterations in fluid balance (37), cardio-respiratory function (51) and oxidant production (10, 33). While moderate caloric restriction has beneficial effects on animal health (2, 8, 30), prolonged food deprivation leads to the depletion of antioxidant stores, and to an increase in oxidant generation and oxidative damage in a variety of animals including humans (13, 14, 17, 33, 50, 52, 53, 56, 59).

The harmful effects of prolonged food deprivation are consistent among various vertebrate species (34, 47). Prolonged food deprivation in rats increases hepatic mitochondrial oxidant production, protein oxidative damage, lipid peroxidation and lipoperoxidation-derived protein modifications (52). Food deprivation also increases tumor necrosis factor-α (TNF-α) activity in rat adipose tissue (63) and oxidatively-modified DNA content in peripheral blood mononuclear cells (53). Likewise, prolonged food deprivation increases myocardial hydrogen peroxide production and hepatic lipid peroxidation, and decreases liver and muscle glutathione (GSH) content and antioxidant enzyme activities (11, 13, 17, 24).

Northern elephant seals (*Mirounga angustirostris* Gill, 1866) maintain electrolyte and fluid homeostasis during prolonged food and water deprivation (fasting) by activating the renin-angiotensin system (RAS) (39, 44). In humans and rats, the chronic activation of RAS is associated with inflammatory disease, hypertension, heart and kidney disease, endothelial dysfunction, and insulin resistance (9, 23, 29, 31, 40, 60). RAS increases oxidant production by increasing the expression/activity of NADPH oxidases. Oxidants generated in high levels react with lipids, proteins, or nucleic acids, in a variety of tissues and cells leading to oxidative damage and inflammation (4, 5, 25, 26, 48).

Northern elephant seals naturally experience prolonged periods (up to 3 months) of absolute food and water deprivation while breeding, nursing, molting or weaning, with no apparent detrimental effects (12, 38, 43). Unlike hibernators, elephant seals remain normothermic during their post-weaning fasting with relatively high metabolic rates compared with mammals of similar size (12, 49). Because prolonged fasting is a natural component of the elephant seal's life history, we hypothesized that is not associated with increased oxidative damage or inflammation despite an increase in RAS. In order to test our hypothesis, we compared systemic and tissue indices of oxidative stress and inflammation, along with selected RAS components, between early- and late-fasted northern elephant seal pups, in an effort to contribute to the elucidation of the mechanisms evolved by these animals to cope with this potentially detrimental behavior. Elephant seals have clearly evolved robust physiological mechanisms that have allowed them to adapt to extreme behaviors or environmental conditions making them an ideal and interesting model to study the physiological mechanisms by which mammals contend with the detrimental effects of fasting-induced oxidative stress.

Methods

      All procedures were reviewed and approved by the Institutional Animal Care and Use Committee's of both the University of California Merced and Sonoma State University. All work was carried out under the National Marine Fisheries Service marine mammal permit # 87-1743.

Animals and sample collection

      Post-weaned northern elephant seal pups of known age from Año Nuevo State Reserve (30 km north of Santa Cruz, CA, USA) were sampled at two periods: Early (2-3 wk post-weaning; n = 10, 5 males, 5 females) and Late (7-8 wk post-weaning; n = 9, 5 males, 4 females). Each sampling group was independent, but because their age was known with relative accuracy (within 2-3 days of weaning), they represented Early and Late fasting. Pups were weighed at weaning and the percentage of body mass loss over the fasting period was calculated. The day of sampling, animals were initially sedated with 1 mg/kg Telazol (tiletamine/zolazepam HCl, Fort Dodge Labs, Ft Dodge, IA, USA) administrated intramuscularly. Once immobilized, a 16 gauge, 3.5 inch spinal needle was inserted into the extradural spinal vein, and blood samples were collected in pre-chilled EDTA-treated vacutainer sample tubes. Immobilization was maintained with a 100 mg bolus of intravenous injections of ketamine as needed. Muscle biopsies were collected by first cleaning a small region in the flank of the animal near the hind flipper, with alternating wipes of isopropyl alcohol and betadine, followed by a subQ injection of 2-3 ml of lidocaine (Henry Schein, Melville, NY, USA). A small (<1.5 cm) incision was made using a sterile scalpel, and a muscle biopsy (ca. 50 mg) was collected with a sterile biopsy punch needle (Henry Schein). Biopsies were rinsed with ice-cold PBS, placed in cryogenic vials, frozen by immersion in liquid nitrogen, and stored at -80°C until analyzed. Blood samples were centrifuged for 15 min at 3000 x g at 4°C, plasma was transferred to cryo-vials, snap-frozen and stored at -80°C. Red blood cells (RBC) were packed by adding 2 volumes of 0.9% saline solution and centrifuged for 10 min at 3000 x g. Buffy coat was discarded and the RBC pellet was lysed by vortexing in 4 volumes of HPLC water for 1 min. Lysed RBCs were centrifuged and the supernatant aliquoted into separate cryovials, snap-frozen and stored at -80 °C. Frozen tissue samples were homogenized in 2 volumes of 50mM potassium phosphate buffer containing 1mM EDTA, 1% Triton X-100, 1% PSMF and 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Total protein content in tissue samples was measured by using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Western Blot

      Thirty µg of total protein were resolved in 4-15% Tris-HCl SDS gradient gels. Proteins were electroblotted using the Bio-Rad Trans Blot SD semi-dry cell onto 0.45 µm nitrocellulose membranes. Membranes were blocked with 3% bovine serum albumin in PBS containing 0.05% of Tween 20, and incubated overnight with primary antibodies against NADPH oxidase 4 (Nox4), angiotensin receptor type 1 (AT$_1$), catalase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CuZn-superoxide dismutase (CuZnSOD), Mn-superoxide dismutase (MnSOD), glutathione peroxidase (GPx) or actin (Assay

designs, Ann Arbor, MI, USA) diluted 1:500 to 1:5000. Membranes were washed, incubated with HRP-conjugated secondary antibodies (Pierce, Rockford, IL, USA), re-washed, and developed using the Immun-Star Western C kit (Bio-Rad). Blots were visualized using a Chemi-Doc XRS system (Bio-Rad) and quantified by using Bio-Rad's Quantity One software.

Inflammatory markers and oxidative damage

Plasma TNF-α levels were measured using a commercially available EIA kit (Cayman Chemical, Ann Arbor, MI, USA). Plasma C-reactive protein (hs-CRP) concentration was quantified by using a Tina-quant high sensitivity assay (Roche Diagnostics, Indianapolis, IN). Circulating levels of 8-isoprostanes (8-iso-PGF$_{2\alpha}$) were quantified by GC-MS as previously described (32, 35). Muscle lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS) content using a commercially available kit (Cayman Chemical). Relative 4-hydroxynonenal (4HNE) concentration in muscle samples, as well as plasma and muscle levels of total nitrotyrosine (NT) were measured by slot blot. Thirty μg of total protein (for tissue samples) or 25 uL of plasma were loaded onto 0.45 μm nitrocellulose membranes using the Bio-Dot SF microfiltration apparatus (Bio-Rad). Membranes were blocked, probed with antibodies against 4HNE (Calbiochem, San Diego, CA, USA) and NT (Cayman Chemical), and developed as described above. Protein carbonyl content in muscle samples was evaluated by using an Oxyblot™ Protein Oxidation Detection Kit (Millipore, Temecula, CA, USA). Fifteen μg of total protein were treated and loaded onto membranes by using the Bio-Dot SF. Membranes were blocked, probed and developed as described above.

Enzyme activities

Plasma renin activity (PRA) was measured using a commercially available RIA kit (DiaSorin Inc., Stillwater, MN, USA), previously validated for its use with northern elephant seal plasma (39, 43, 45). Total SOD and GPx activities were measured in tissue extracts and RBC lysates with commercially available kits (Cayman Chemical). Catalase activity was measured by monitoring the removal of 10 mM H_2O_2 at 240 nm as described previously (57). NADPH oxidase activity was measured by using the lucigenin-enhanced chemiluminescence (CL) assay (18). Homogenates were incubated in the dark with 100μM NAPDH and 5μM lucigenin in PBS. Relative CL was measured for 10 min in a luminometer (Berthold, Oak Ridge, TN, USA). Basal activity was measured in the absence of NADPH. A buffer blank was subtracted from each reading. NADPH oxidase activity was expressed as RLU mg protein^{-1}.

Statistics

Means were compared between Early and Late fasting groups by two-sample t-tests with Bonferroni adjustment. Means ($\pm$ SEM) were considered significantly different at $p < 0.05$. Statistical analyses were performed with the SYSTAT© 11.0 software (SPSS, Richmond, CA, USA)

Results

Prolonged fasting induces loss of body mass

Body mass loss in elephant seals was calculated in order to evaluate the effects of prolonged fasting on elephant seal's overall body composition. Elephant seal's average body mass at weaning was 124 ± 6 kg and at Late sampling 92 ± 5 kg. Elephant seals lost $26 \pm 2\%$ ($p < 0.001$) of their body mass during the course of fasting (ca. 8 wk). In contrast, muscle total protein content did not change with fasting (Early: 4.2 ± 0.5 vs Late: 4.5 ± 0.6 mg ml^{-1}), suggesting that despite losing nearly one-quarter of their body mass, elephant seals possess robust physiological mechanisms to tolerate this potentially detrimental condition.

Prolonged fasting activates RAS

PRA and AT_1 protein expression were measured to evaluate the effects of prolonged fasting on RAS activation in elephant seals. Fasting increased PRA nearly 2.5-fold (Early: 2.8 ± 0.5 vs Late: 7.1 ± 1.1 ng Ang I ml^{-1} h^{-1}, $p < 0.01$) and muscle AT_1 protein expression by 77% (Early: 100 ± 13 vs Late: 177 ± 19, $p < 0.05$) (Figure 1), indicating that RAS is activated as a response to prolonged fasting in elephant seals.
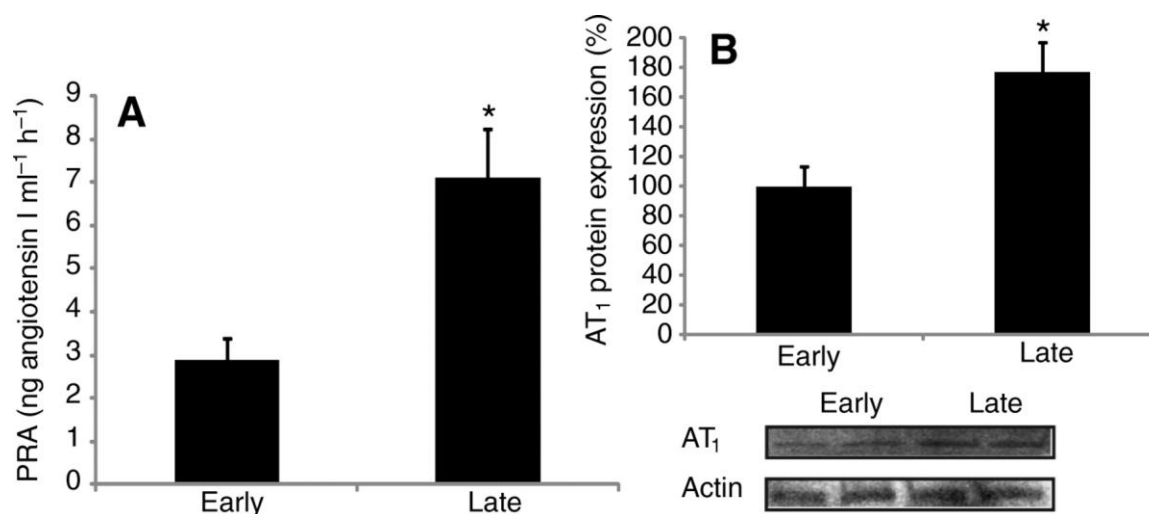
Figure 1. Prolonged fasting activates the renin-angiotensin system in northern elephant seal pups. Mean ($\pm$ SEM) **A)** plasma renin activity (PRA) and **B)** muscle AT_1 protein expression (as percent change from Early) between Early (2-3 wks postweaning) and Late (7-8 wks postweaning) fasting periods. * $p < 0.05$

Prolonged fasting increases Nox4 protein expression and NADPH oxidase activity

Nox4 muscle protein expression and NADPH oxidase activity were measured to evaluate whether prolonged fasting stimulates ROS-producing proteins in elephant seals. Fasting induced a 2-fold increase in Nox4 muscle protein expression (Early: 100 ± 8 vs Late: 201 ± 26, $p < 0.01$) and a 74% increase in NADPH oxidase activity (Early: 686 ± 31 vs Late: 1194 ± 172 RLU mg protein^{-1}, $p < 0.05$) (Figure 2), suggesting that prolonged

fasting increases oxidant production and can potentially contribute to increased oxidative damage and inflammation.
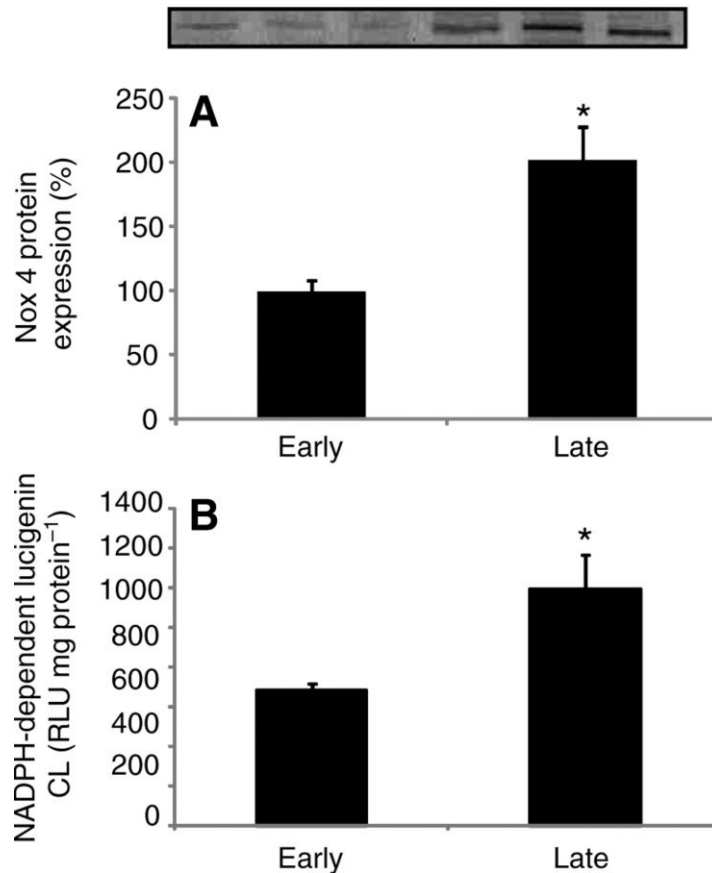
Figure 2. Nox4 protein expression and NADPH oxidase activity increase with fasting in northern elephant seal pups. Mean (± SEM) **A)** muscle Nox4 protein content (as a percent change from Early) and **B)** muscle NADPH oxidase activity between Early (2-3 wks postweaning) and Late (7-8 wks postweaning) fasting periods. * $p < 0.05$

Neither oxidative damage nor inflammation increases with prolonged fasting

Circulating levels of 8-iso-$PGF_{2\alpha}$, NT, TNF-α and hs-CRP, along with muscle levels of 4HNE, TBARS, NT, and protein carbonyls were measured to evaluate the effects of prolonged fasting on local and systemic indices of oxidative damage and inflammation in elephant seals. None of this suite of markers of oxidative damage and inflammation in plasma or muscle increased over the course of fasting (Table 1, Figure 3). Moreover, muscle protein carbonyls (Early: 100 ± 3 vs Late: 81 ± 4) and NT levels (Early: 100 ± 1 vs Late: 95 ± 1) decreased ($p < 0.05$) with fasting (Figure 3). These results show that despite the fasting-induced activation of RAS and Nox4, neither systemic nor local oxidative damage or inflammation increased with fasting, suggesting that these animals have evolved robust physiological mechanisms to avoid oxidative stress and inflammation during this potentially detrimental condition.

Table 1. Means (± SEM) of plasma markers of inflammation and oxidative damage between Early (2-3 wks postweaning) and Late (7-8 wks postweaning) fasting periods.

	Early	Late
hs-CRP ($\mu g\ ml^{-1}$)	0.35 ± 0.10	0.33 ± 0.08
TNF-α (pg ml^{-1})	20 ± 4	35 ± 8
8-iso-PGF$_{2\alpha}$ (pg ml^{-1})	88 ± 6	83 ± 5

hs-CRP = high sensitivity C-reactive protein; TNF-α = tumor necrosis factor-α; 8-iso-PGF$_{2\alpha}$ = 8-isoprostane PGF$_{2\alpha}$ (15-F$_{2t}$-IsoP).
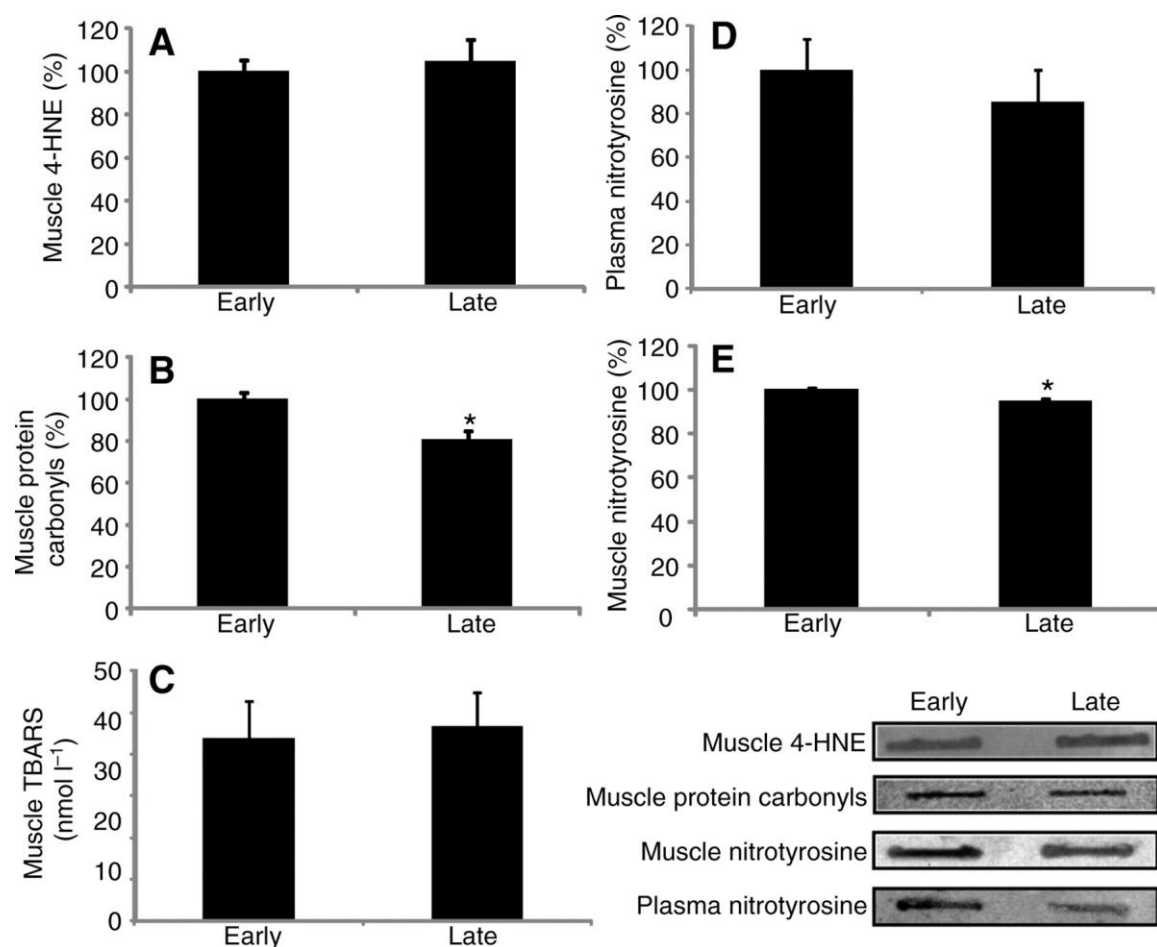
Figure 3. Neither systemic nor muscle levels of oxidative damage increase with fasting in northern elephant seal pups. Mean (± SEM) as percent change from Early of **A)** muscle 4-hydroxynonenal (4HNE), **B)** muscle protein carbonyls, **C)** muscle nitrotyrosine (NT), **D)** plasma nitrotyrosine and muscle thiobarbituric acid reactive substances (TBARS) between Early (2-3 wks postweaning) and Late (7-8 wks postweaning) fasting periods. * $p < 0.05$

Prolonged fasting increases antioxidant enzymes

In order to explore the mechanisms elephant seals have evolved to cope with fasting-induced RAS and NADPH oxidase activation, we compared muscle protein expression of the antioxidant enzymes CuZnSOD, MnSOD, GPx, and catalase, as well muscle and RBC SOD, catalase and GPx activities, between early and late fasted seals. CuZnSOD (Early: 100 ± 9 vs Late: 158 ± 17), MnSOD (Early: 100 ± 9 vs Late: 140 ± 8), GPx (Early: 100 ± 7 vs Late: 151 ± 14) and catalase (Early: 100 ± 10 vs Late: 142 ± 2) protein expression increased 40-60% ($p < 0.05$) with fasting (Figure 4). Similarly, the activities of muscle catalase (Early: 29 ± 4 vs Late: 69 ± 15 U mg protein^{-1}) and GPx (Early: 6.9 ± 1.4 vs Late: 10.7 ± 0.5 U mg protein^{-1}), and RBC SOD (Early: 839 ± 16 vs Late: 920 ± 24 U mL^{-1}), catalase (Early: 5192 ± 753 vs Late: 8185 ± 973 U mL^{-1}) and GPx (Early: 430 ± 90 vs Late: 935 ± 149 U mL^{-1}) increased ($p < 0.05$) (Figure 5) suggesting that prolonged fasting stimulates the enzymatic antioxidant system of the elephant seal, which likely contributes to the suppression of oxidative stress and inflammation, despite the increases in RAS and NADPH oxidase.
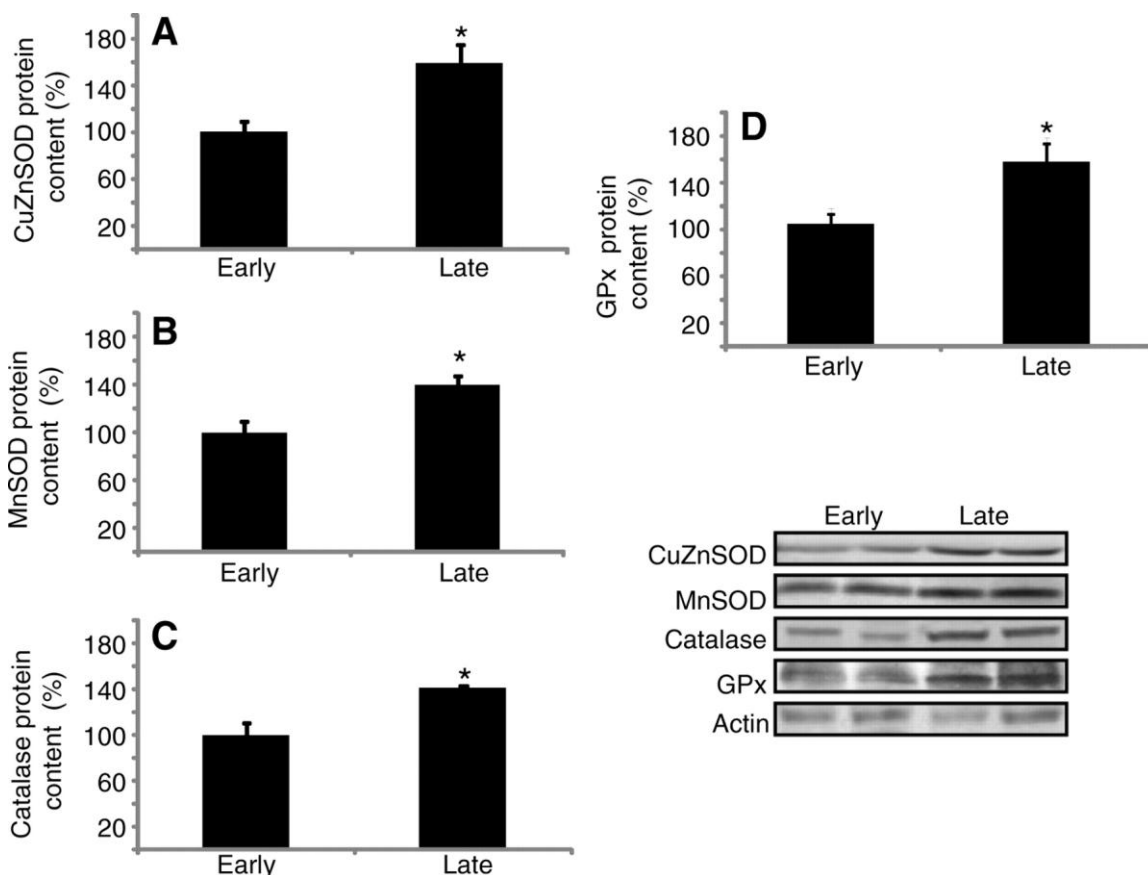
Figure 4. Expression of antioxidant enzymes increases with fasting in northern elephant seals. Mean ($\pm$ SEM) content as percent change from Early of **A)** copper-zinc superoxide dismutase (CuZnSOD), **B)** manganese superoxide dismutase (MnSOD), **C)** catalase, and **D)** glutathione peroxidases (GPx) between Early (2-3 wks postweaning) and Late (7-8 wks postweaning) fasting periods. * $p < 0.05$
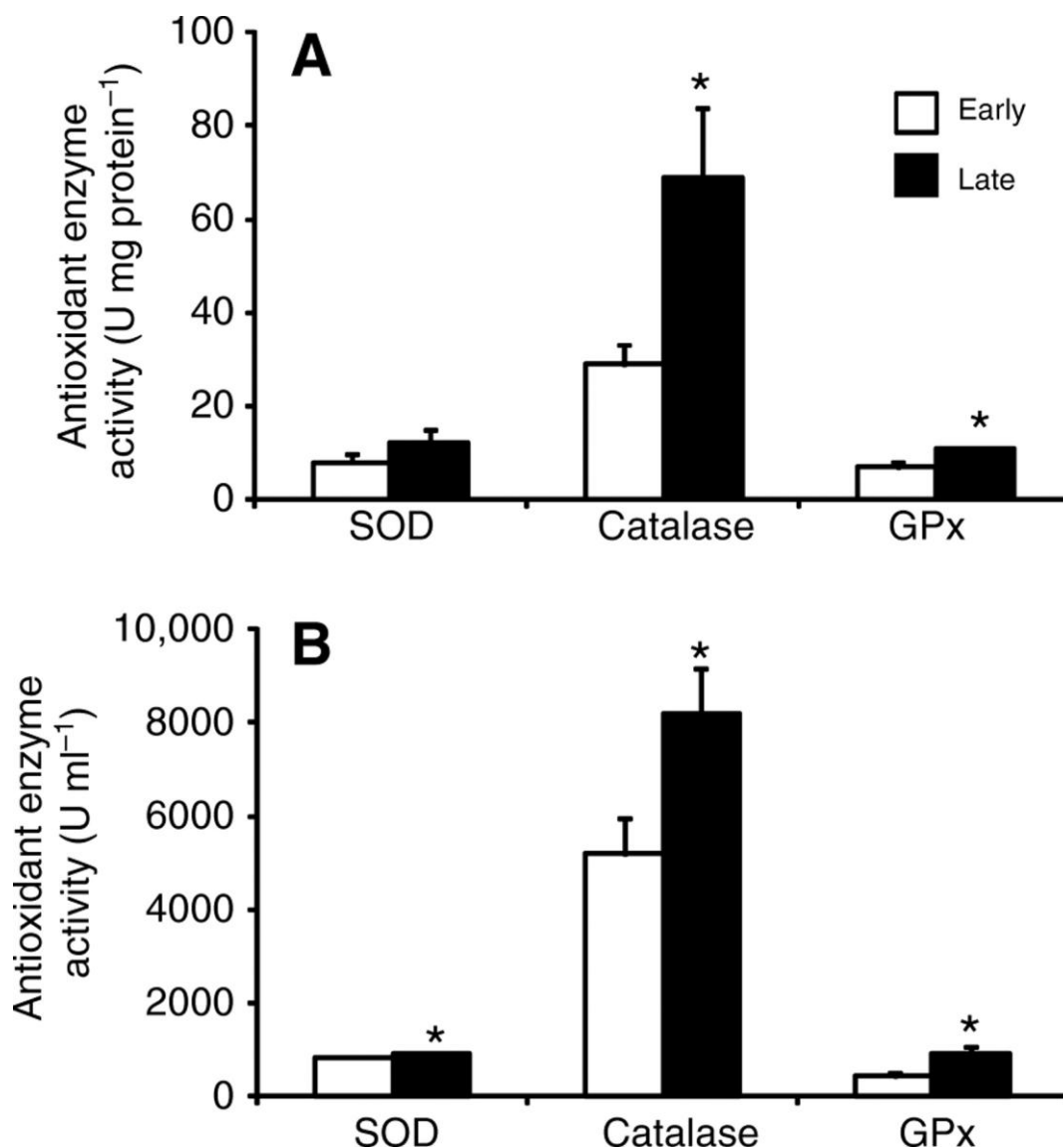
Figure 5. Activity of antioxidant enzymes increases with fasting in northern elephant seal pups. Mean (± SEM) activities of total superoxide dismutase (SOD), catalase, and glutathione peroxidases (GPx) in **A)** muscle and **B)** red blood cells (RBC) between Early (2-3 wks postweaning) and Late (7-8 wks postweaning) fasting periods. * $p < 0.05$

Discussion

Prolonged food deprivation is a potentially detrimental behavior, which stimulates oxidant production and oxidative damage that, when chronically elevated, can contribute to a host of complications including cardiovascular, renal and hepatic diseases, and multi-organ failure (9, 23, 29, 31). The present study demonstrates that some mammals are uniquely adapted to counteract the detrimental effects of extreme behaviors or environmental conditions such as prolonged food and water deprivation. Our results show that while prolonged fasting increases the expression of the oxidant-producing protein

Nox4, along with NADPH oxidase activity, an increase in antioxidant enzymes is sufficient to avoid the oxidative damage and inflammation commonly associated with prolonged fasting in terrestrial mammals.

Prolonged food and water deprivation can be detrimental by promoting oxidative stress and inflammation via activation of RAS. While northern elephant seals have adapted to tolerate such a situation, this behavior is associated with a number of chronic metabolic adjustments that include the activation of RAS (39, 44). Elevated RAS increases oxidant production in rats and humans (4, 5, 25, 26, 48) and, thus, has been associated with increased concentrations of tissue and systemic markers of oxidative damage and inflammation (9, 23, 29, 31). Elevated RAS also increases Nox4 protein expression *in vitro* and *in vivo* in rats and humans (Wingler *et al.*, 2001; (27, 62). Otherwise, data on the effects of elevated RAS on oxidant-generating proteins during prolonged fasting are scarce. The present study demonstrates that prolonged fasting increases the expression of Nox4, along with NADPH oxidase activity, in the presence of increased PRA and AT_1, suggesting that elevated RAS may contribute to the generation of oxidants via Nox4. Alternatively, the previously observed increases in cortisol in fasting pups (41-43) may have contributed to the increase in Nox4 and NADPH oxidase activity since elevated glucocorticoids can increase oxidant production and oxidative damage as well (6, 33).

Neither local nor systemic oxidative damage nor inflammation indices increased with fasting in elephant seals, despite the increases in Nox4 and RAS, indicating that these animals have evolved robust physiological mechanisms to alleviate the potentially detrimental consequences of this extreme condition. Further elucidation of these mechanisms can provide significant advancement of our understanding of diseases related with oxidative stress and RAS.

The lack of increases in local and systemic indices of oxidative stress and inflammation despite the observed increases in Nox4 and RAS can be explained, at least in part, by the observed increases in antioxidant enzymes. It has been suggested that diving seals possess an enhanced antioxidant system that contributes to the protection against oxidants produced in response to recurrent episodes of diving-induced ischemia/reperfusion (15, 19, 21, 22). Higher activities of the antioxidant enzymes SOD, catalase, GPx, glutathione-S transferase and glutathione disulfide reductase, as well as higher GSH content have been found in seals than in terrestrial mammals (57, 58). In the present study, protein expression of CuZnSOD, MnSOD, catalase and GPx, as well as their activity levels increased with fasting, which likely contributed to the avoidance of oxidative damage and inflammation. These data suggest that fasting seals are responsive to increased antioxidant capacity, which contributes to their ability to avoid the detrimental effects of oxidants. Furthermore, the end of the fasting period in northern elephant seal pups immediately precedes the beginning of their diving lifestyle (28). Therefore, it is possible that the accumulation of antioxidants during this period prepares elephant seals to cope with the potential oxidant production associated with diving-induced ischemia/reperfusion. The increase in NADPH oxidase activity likely stimulated the up-regulation of antioxidant enzymes because oxidant production is one of the main factors that regulate antioxidant enzyme expression and activity (16, 46, 61).

Fasting seals utilize their fat stores as the primary source of energy, resulting in extensive lipid mobilization and increased concentrations of circulating non-esterified fatty acids (NEFA) (7, 20, 38, 42, 49). In humans, elevated NEFA concentrations are associated with higher content of circulating 8-iso-PGF$_{2\alpha}$ (54, 55), a widely used marker of systemic lipid peroxidation (35, 36). In rats, elevated RAS increases circulating levels of 8-iso-PGF$_{2\alpha}$ (3). In the present study, circulating concentrations of 8-iso-PGF$_{2\alpha}$ remained unchanged with fasting. Moreover, in contrast to the previously reported increases in protein oxidation/nitration induced by fasting and increased RAS activation in terrestrial mammals (9, 13, 52), muscle and plasma NT levels remained unchanged, and muscle protein carbonyls decreased, suggesting that fasting seals possess robust cellular mechanisms to protect lean tissue from the potentially damaging effects of oxidation associated with elevated RAS. The decrease in protein carbonyls is likely attributed to the fact that protein catabolism contributes to less than 4% of their average daily metabolic rate and that protein turnover decreases over the fast (1, 20).

In summary, our results show that despite the fasting-induced increases in RAS and Nox4, neither local nor systemic oxidative damage and inflammation indices were increased. The observed increases in antioxidant enzymes likely contributed to the suppression of oxidative damage and inflammation. Thus, the increased antioxidant response likely alleviated the potentially deleterious effects of fasting-derived oxidant production. Elucidating the mechanisms by which animals that evolved to be uniquely and naturally adapted to extreme environmental and behavioral conditions will prove to be fruitful as we strive to gain a better understanding of how oxidative stress and inflammation promote a number of patho-physiological conditions.

Acknowledgments

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Chapter II. Prolonged Fasting Increases Glutathione Biosynthesis in Postweaned Northern Elephant Seals*

Abstract

Northern elephant seals experience prolonged periods of absolute food and water deprivation (fasting) while breeding, molting or weaning. The postweaning fast in elephant seals is characterized by increases in the renin-angiotensin system, expression of the oxidant-producing protein, Nox4, and NADPH oxidase activity which, however, are not correlated with increased oxidative damage or inflammation. Glutathione (GSH) is a potent reductant and a cofactor for glutathione peroxidases (GPx), glutathione-S transferases (GST) and 1-cys peroxiredoxin (PrxVI) and thus, contributes to the removal of hydroperoxides, preventing oxidative damage. The effects of prolonged food deprivation on the GSH system are not well described in mammals. To test our hypothesis that GSH biosynthesis increases with fasting in postweaned elephant seals, we measured circulating and muscle GSH content at the early and late phases of elephant seal's postweaning fast along with the activity/protein content of glutamate-cysteine ligase (GCL; catalytic [GCLc] and modulatory [GCLm] subunits), γ-glutamyl transpeptidase (GGT), glutathione disulphide reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH), GST and PrxVI, as well as plasma changes in γ-glutamyl aminoacids, glutamate and glutamine. GSH increased 2 to 4-fold with fasting along with a 40-50% increase in the content of GCLm and GCLc, 75% increase in GGT activity, 2- to 2.5-fold increase in GR, G6PDH and GST activities and 30% increase in PrxVI content. Plasma levels of γ-glutamyl glutamine, γ-glutamyl isoleucine and γ-glutamyl methionine increased with fasting too, while glutamate and glutamine decreased. Results indicate that GSH biosynthesis increases with fasting and that GSH contributes to counteracting hydroperoxide production preventing oxidative damage in fasting seals.

Introduction

Northern elephant seals (*Mirounga angustirostris* Gill 1866) naturally experience prolonged periods of absolute food and water deprivation (fasting) while breeding, molting or weaning, without apparent detrimental effects (5, 28, 31). In terrestrial mammals, prolonged food deprivation is a stressful physiological condition that activates the hypothalamic-pituitary-adrenal axis, increases the release of adrenocorticotropic hormone and glucocorticoids, and leads to subsequent alterations in fluid balance and cardiorespiratory function (26, 34). Prolonged food deprivation also promotes production of superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and lipid hydroperoxides that contribute to oxidative damage, inflammation, and antioxidant depletion in a variety of terrestrial mammals including humans, rats and mice (4, 6, 12, 19, 24, 36, 37, 43).

Glutathione (GSH, γ-glutamyl-cysteinyl-glycine) is the most important non-enzymatic endogenous antioxidant in animal cells (10). It is synthesized from glutamate, cysteine and glycine in a two-step enzymatic process catalyzed by the enzymes glutamate-cysteine ligase (GCL) and glutathione synthase (13). The rate-limiting enzyme in GSH biosynthesis is GCL, which is composed of a catalytic heavy subunit (GCLc) and a light modifier subunit (GCLm) (13). GSH is used as a cofactor in glutathione peroxidases (GPx) and glutathione-S transferases (GST) catalytic reactions that yield glutathione disulphide (GSSG). GSH homeostasis is partially maintained by γ-glutamyl transpeptidase (GGT), which breaks down extracellular GSH providing cysteine, the rate-limiting substrate for intracellular *de novo* synthesis of GSH (46). Glutathione disulphide reductase (GR) restores GSH from GSSG at the expense of NADPH, which is recycled from $NADP^+$ by glucose-6-phosphate dehydrogenase (G6DPH) in an enzymatic process that maintains the reduced GSH pool (20, 25). GSH is also essential for the activity of 1-cys peroxiredoxin (peroxiredoxin VI, PrxVI), which, along with catalase, GPx and GST, protect cells against membrane oxidation since the GSH peroxidases and PrxVI eliminate H_2O_2, lipid and phospholipid hydroperoxides (23). Thus, they remove the H_2O_2 produced by dismutation of $O_2^{\bullet-}$ by superoxide dismutases (SOD) and the hydroperoxides produced by lipid peroxidation.

The effects of prolonged food deprivation on GSH metabolism have only been scarcely investigated. In humans, food deprivation depletes circulating GSH content after one week (24) while in rats, it decreases liver GSH content after 18 h (12). Beyond these few studies, the effects of prolonged food deprivation on GSH biosynthesis, recycling and the activity of the enzymes that regulate its content have not been examined. In postweaned northern elephant seals, prolonged fasting activates the renin-angiotensin system (RAS), which is known to stimulate pro-oxidant pathways, and increases the expression of the oxidant- producing protein, Nox4, as well as NADPH oxidase activity, without increasing oxidative damage or inflammation (32, 38). The postweaning fast in northern elephant seals also increases the activity and protein content of the antioxidant enzymes MnSOD, CuZnSOD, catalase and GPx suggesting that fasting seals are responsive to increased antioxidant capacity, which likely contributes to their ability to avoid the detrimental effects increased oxidant production (38). The associations among activated RAS, elevated antioxidant enzymes and the GSH system during prolonged food deprivation have not been examined. Because GSH is the most important non-enzymatic

endogenous antioxidant in animal cells and plays a major role in the cellular defense against H_2O_2, lipid and phospholipid hydroperoxides, we hypothesize that prolonged fasting upregulates the enzymes involved in GSH biosynthesis, recycling and utilization in postweaned northern elephant seals. To test our hypothesis, we measured circulating and muscle GSH content at the early and late phases of their natural postweaning fast along with activity and protein content of selected enzymes involved in GSH metabolism and glutathione-related metabolites in plasma, to better evaluate the effects of prolonged food deprivation on the GSH system in mammals.

Materials and methods

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee's of both University of California Merced (AUP08-0002) and Sonoma State University (2008-37). All work was realized under National Marine Fisheries Service marine mammal permit #87-1743.

Animal handling and sample collection

Seventeen northern elephant seal pups were sampled at Año Nuevo State Reserve at two periods during their natural postweaning fast: Early (1-2 wk n = 9, 5 males and 4 females) and Late (7-8 wk postweaning; n = 8, 4 males and 4 females). Each sampling group was independent, but, because their age was known with relative accuracy, they represented early and late fasting. Animals were sedated with 1 mg kg^{-1} Telazol (tiletamine/zolazepam HCl, Fort Dodge Labs, Ft Dodge, IA, USA) administrated intramuscularly. Once immobilized, a 16 gauge, 3.5 inch spinal needle was inserted into the extradural spinal vein. Blood samples were collected in pre-chilled EDTA-treated vacutainer sample tubes and placed on ice. Immobilization was maintained with 100 mg bolus of intravenous injections of ketamine as needed. Muscle biopsies were collected as described previously (38). Biopsies were rinsed with ice-cold PBS and divided in two pieces. One piece was placed in a cryogenic vial, frozen by immersion in liquid nitrogen, stored in dry ice and subsequently at -80°C until analyzed for enzyme activities or protein content. The other piece was placed in a microcentrifuge tube containing ice-cold PBS and minced on ice. PBS was removed and 0.45 mL of 10% perchloric acid (PCA) solution containing 7.5 nmols of γ-glutamyl glutamate (GGA) were added. Samples were kept in ice until they were homogenized at the nearby Long Marine Laboratory (UCSC). Homogenates were centrifuged for 20 min at 6000 x g at 4°C. Supernatants were removed, frozen by immersion in liquid nitrogen and stored at -80°C until analyzed for GSH content. Blood samples were centrifuged for 15 min at 3000 x g at 4°C, plasma was frozen in liquid nitrogen and stored at -80°C until assayed for GGT activity or glutathione-related metabolites. Red blood cells (RBC) lysates were prepared on site as described previously (38). RBC lysates were divided in two subsamples. One subsample was treated with 10% PCA containing 7.5 nmols of GGA and centrifuged 20 min at 6000 x g at 4°C. Clear supernatants were transferred to cryovials, frozen by immersion in liquid nitrogen and stored at -80°C until analyzed for GSH content. The other subsample was mixed with Drabkin's reagent, frozen by immersion in liquid nitrogen and stored at -80°C until analyzed for hemoglobin content. Frozen tissue samples were homogenized in

50 mM potassium phosphate buffer containing 1mM EDTA, 1% Triton X-100, 1% PMSF and 1% protease inhibitor cocktail (Sigma, Saint Lois, MO, USA) as described previously (38-41). Total protein content in tissue samples was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Hemoglobin content in RBC lysates was measured by the cyanomethemaglobin method using a commercially available kit (Cayman Chemical, Ann Arbor, MI, USA).

Glutathione measurement

Total glutathione equivalents (TGSH-eq) were measured by HPLC following the method of Fariss and Reed (9) and using GGA as internal standard in RBC lysates and muscle samples. Extracted samples were mixed with 100 mM iodoacetic acid. pH was adjusted to 8-9 using KOH (2 M)/KHCO3 (2.4 M). After 15 min of incubation in the dark at room temperature, 1% dinitrobenzene was added. Samples were vortexed and stored overnight at 4°C. L-lysine (1 M) was added and the precipitated salt was removed by centrifugation after incubating the samples at 4°C for 2 h. Supernatants were transferred to autosampler vials and analyzed by HPLC.

Western blot

Soluble proteins were extracted from untreated frozen muscle samples as described above. Twenty μg of total protein were resolved in 4-15% Tris-HCl gradient gels under denaturing conditions. Proteins were electroblotted using the Bio-Rad Trans Blot SD semi-dry cell (Bio-Rad Laboratories, Hercules, CA, USA) onto 0.45 μm nitrocellulose membranes. Membranes were blocked with 3% bovine serum albumin in PBS containing 0.05% of Tween 20, and incubated overnight with primary antibodies against mammalian GCLm, PrxVI, actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or GCLc (Liu et al. 1998) diluted 1:500 to 1:3000. Membranes were washed, incubated with HRP-conjugated secondary antibodies (Pierce, Rockford, IL, USA), re-washed, and developed by using the Immun-Star Western C kit (Bio-Rad). Blots were visualized using a Chemi-Doc XRS system (Bio-Rad) and quantified by using Bio-Rad's Quantity One software.

Enzyme activities

GR, GST and and G6PDH activities were measured in muscle tissue extracts using commercially available kits (Cayman Chemical; Biovision Research Products, Mountain View, CA, USA). Results were expressed in mU of enzyme per mg of protein as described previously (40, 41). GGT activity was measured in plasma using a commercially available kit (Bioo Scientific, Austin, TX, USA) and expressed in U per L.

Metabolomics

Fasting-induced changes in glutathione-related metabolites (γ-glutamyl amino acids, glutamate and glutamine) in plasma were assessed using a metabolomics approach. Samples were compared between early and late fasting periods in postweaned pups and opportunistically, in lactating females (day 5 and day 22 post-partum, n = 10). Metabolite analysis was performed by Metabolon, Inc. (Raleigh, NC, USA). Samples were divided

into two fractions for analysis by Liquid Chromatography/Mass Spectrometry-Mass Spectrometry (LC-MS, LC-MS2) and Gas Chromatography/Mass Spectroscopy (GC-MS). Mean ratios of the median scaled data from two samples for each seal were calculated.

Statistics

Means were compared between Early and Late fasting groups by two-sample t-tests with Bonferroni adjustment. Means (± s.e.m.) were considered significantly different at $p < 0.05$. For the metabolomics data, peak heights were scaled to the median of the early samples and comparisons with late samples was made using a paired t-test. Statistical analyses were performed with the SYSTAT© 11.0 software (SPSS, Richmond, CA).

Results

Glutathione content increases with prolonged fasting in elephant seals

Total glutathione content was measured in muscle and RBC lysates to evaluate whether this non-enzymatic antioxidant thiol increases with fasting in elephant seals and thus, has a potential participation in the cellular defenses against fasting-induced hydroperoxide production and oxidative damage. Fasting increased ($p < 0.05$) mean TGSH-Eq content 4-fold in muscle and over 2-fold in RBC (Figure 1).
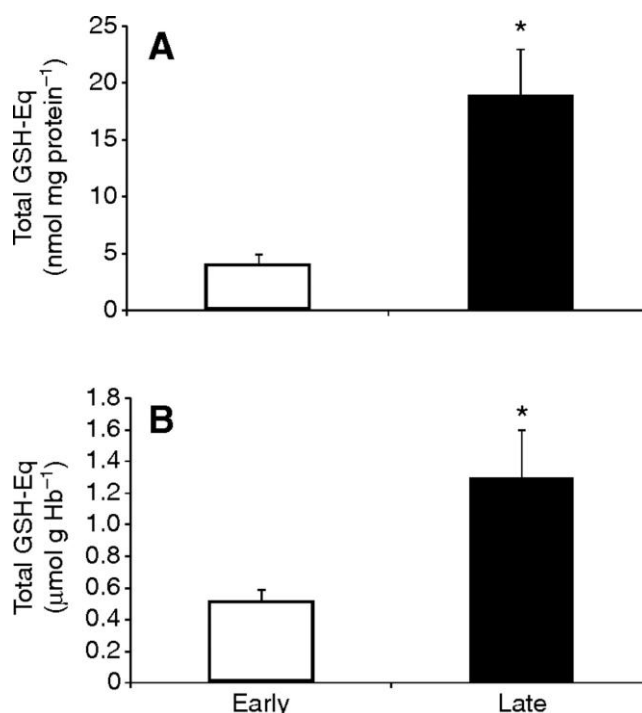
Figure 1. Mean (± SEM) total glutathione equivalents (TGSH-Eq) in northern elephant seals **A)** muscle and **B)** red blood cells (RBC) during Early (1-2 wk postweaning, n = 9) and Late (7-8 wk postweaning, n = 8) fasting. * denotes significant ($p < 0.05$) difference from Early.

Glutathione biosynthesis increases with prolonged fasting in postweaning elephant seals

GCLc and GCLm muscle protein expression was measured to evaluate whether prolonged fasting increases the levels of GCL, the rate-limiting enzyme in GSH biosynthesis. Plasma GGT activity was also measured to evaluate whether this enzyme, which breaks down extracellular GSH providing cysteine, the rate limiting substrate for intracellular GSH *de novo* synthesis, increases with fasting too. Fasting increased ($p < 0.05$) GCLc by 40%, GCLm by 50%, and plasma GGT activity by approximately 75% (Figure 2). In the same way, the metabolomics study revealed that the plasma content of several γ-glutamyl amino acids increased with fasting in pups but not in lactating females (Table 1). Likewise, plasma content of glutamate and glutamine decreased with fasting in the pups but remained unchanged in the females (Table 1) suggesting that GSH biosynthesis increases with fasting in postweaned pups but not in lactating female elephant seals.
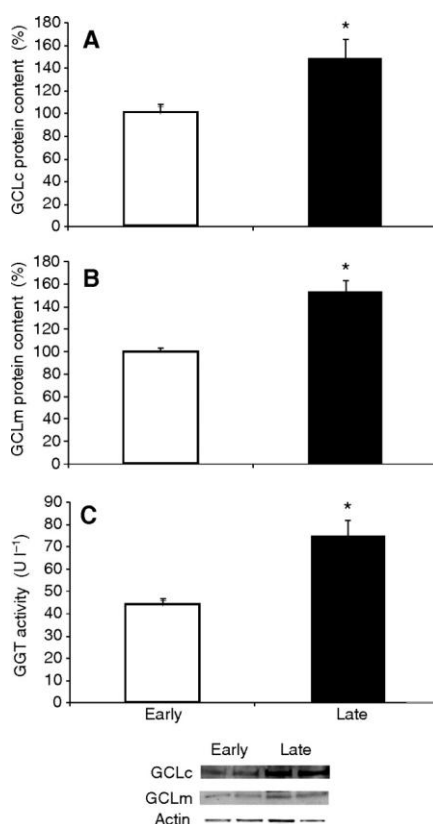
Figure 2. Mean (± SEM) protein expression of northern elephant seal muscle **A)** glutamate-cysteine ligase catalytic (GCLc) and **B)** modulatory (GCLm) subunits, and **C)** plasma γ-glutamyl transpeptidase (GGT) activity during Early (1-2 wk postweaning, n = 9) and Late (7-8 wk postweaning, n = 8) fasting. A representative western blot of GCLc and GCLm expression is shown. Results are expressed in % change from early for GCL protein expression and in U per L for GGT activity. One unit of GGT activity is defined as the amount of enzyme that catalyzes the transformation of one μmol of substrate per minute at 37°C. * denotes significant ($p < 0.05$) difference from Early.

Table 1. Changes in glutathione-related metabolites in plasma samples from postweaning northern elephant seal pups and fasting lactating females

Stage	Metabolite	P	Change
Postweaning pups	Glutamate	0.0001	↓
	Glutamine	0.01	↓
	γ-Glutamyl glutamine	0.01	↑
	γ-Glutamyl isoleucine	0.003	↑
	γ-Glutamyl methionine	0.02	↑
Lactating females	Glutamate	0.15	↔
	Glutamine	0.07	↔
	γ-Glutamyl glutamine	0.002	↓
	γ-Glutamyl isoleucine	0.046	↓
	γ-Glutamyl methionine	0.25	↔

↑, Increase; ↓, decrease; ↔, unchanged.

GR and G6PDH activities increase with prolonged fasting in elephant seals

Muscle GR and G6PDH activities were measured to evaluate whether GSSG recycling to GSH increases with fasting in elephant seal pups. Because the direct measurement of GSSG is not feasible in a field setting, the activity measurements of GR and G6PDH provide an indirect assessment of GSH recycling. Fasting increased (p < 0.05) mean GR and G6PDH activities 2- and 2.5-fold, respectively, (Figure 3) suggesting that GSSG recycling to GSH increases with fasting and that the reduced GSH pool is maintained in postweaned northern elephant seal pups.
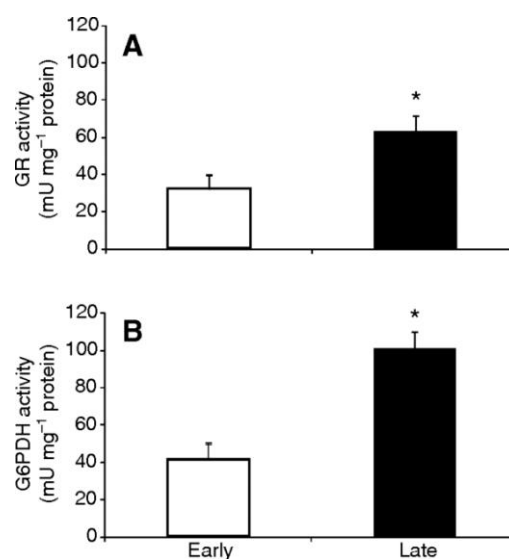
Figure 3. Mean (± SEM) activities of muscle **A)** GR and **B)** G6PDH during Early (1-2 wk postweaning, n = 9) and Late (7-8 wk postweaning, n = 8) fasting in northern elephant seals. One unit of GR is defined as the amount of enzyme that will cause the oxidation of 1 nmol of NADPH per minute at 25°C. One unit of G6PDH defines the amount of enzyme that catalyzes the conversion of 1 μmol of NAD^+ to NADH per minute at 37°C.* denotes significant (p < 0.05) difference from Early.

PrxVI expression and GST activity increase with prolonged fasting in elephant seals

Muscle PrxVI protein content and GST activity were measured to evaluate whether these peroxide-removing enzymes increase with fasting in elephant seals. Fasting increased ($p < 0.05$) mean PrxVI protein content by 30% and GST activity nearly 2-fold (Figure 4) suggesting that peroxide removal increase with fasting in postweaned northern elephant seals.
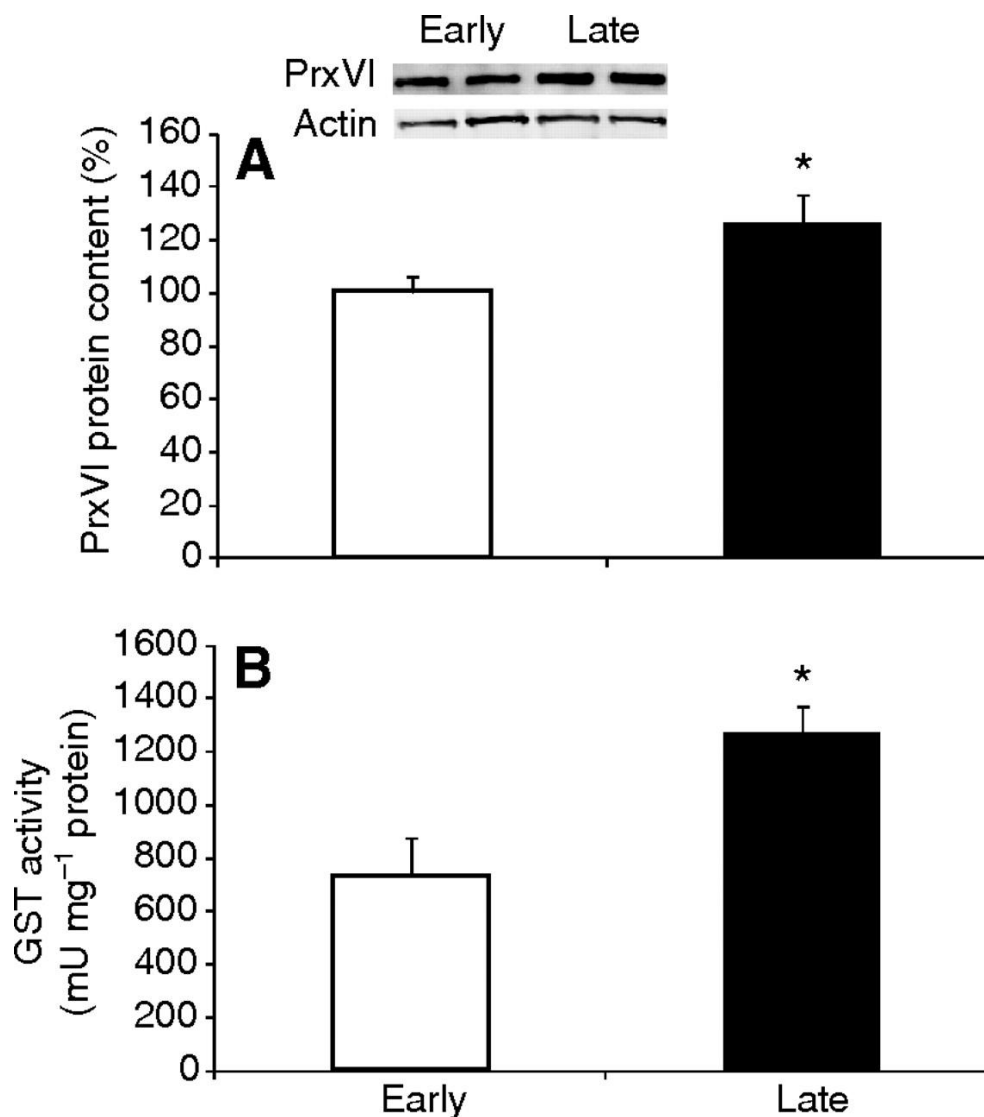
Figure 4. Mean (± SEM) muscle **A)** PrxVI protein expression and **B)** GST activity in northern elephant seal's muscle during Early (1-2 wk postweaning, n = 9) and Late (7-8 wk postweaning, n = 8) fasting in northern elephant seals. A representative western blot of PrxVI expression is shown. Changes in protein content are expressed as % change from early. One unit of GST is defined as the amount of enzyme needed to conjugate 1 nmol of chlorodinitrobenzene (CNDB) with GSH per minute at 25°C.* denotes significant ($p < 0.05$) difference from Early.

Discussion

Prolonged food and water deprivation is a potentially pathological condition that in most mammalian species increases $O_2^{\bullet-}$ and hydroperoxide production, oxidative damage and inflammation. In elephant seals, prolonged food and water deprivation stimulates RAS, up-regulates Nox4, and increases NADPH oxidase activity, circulating cortisol and non-esterified fatty acids content, without increasing local or systemic oxidative damage or inflammation (29-32, 38). This sequence of events suggests that elephant seals have evolved mechanisms that allow them to cope with conditions characterized by increased $O_2^{\bullet-}$ and hydroperoxide production. However, the contributing cellular responses that help alleviate the potential detriments associated with prolonged fasting are not well described in mammals.

We have shown that expression and activity of the antioxidant enzymes SOD, GPx and catalase increase with fasting in elephant seals, likely contributing to the alleviation of the fasting-induced RAS stimulation, Nox4 up-regulation and increased NADPH oxidase activity (38). In the present study we found that GSH biosynthesis along with GR, G6PDH, GST and PrxVI activity/protein content increase with fasting suggesting that GSH plays a key role in elephant seal's defense against the increased oxidant production associated with their natural, prolonged fast. GSH is crucial for the adaptive response against oxidative stress because it is a potent reductant and cofactor for GPx, GST and PrxVI catalytic reactions, and thus contributes significantly to the removal of hydroperoxides (10).

The observed increases in the expression of both GCLc and GCLm, along with the increase in circulating and tissue GSH-Eq indicate that GSH biosynthesis increases with fasting in postweaned northern elephant seals. Increased plasma GGT activity, increased plasma content of γ-glutamyl amino acids and decreased circulating content of glutamate and glutamine support this idea since GGT breaks down extracellular GSH conjugates into γ-glutamyl groups and cysteinylglycine-conjugates, providing cysteine, the rate limiting substrate for intracellular GSH synthesis (46). The GSH system has been proposed to be a key component of seal's protection against diving-induced ischemia/reperfusion (27, 41, 42), a condition that increases $O_2^{\bullet-}$ and hydroperoxide production (14, 17, 18, 40, 44, 45). Because the postweaning fast of elephant seals is the developmental stage that immediately precedes the beginning of their diving lifestyle (21), and is characterized by adjustments that potentially lead to increased oxidant production (31, 38) that can activate the cellular adaptive oxidative stress response (11, 35), it is likely an anticipatory mechanism that stimulates the seal's antioxidant system, thus preventing systemic oxidative damage. The observed differences in the circulating content of γ-glutamyl amino acids between fasting pups and adult lactating females also suggest that the enhancement of the seal's antioxidant system is a critical component of their postweaning development as they prepare to enter a diving lifestyle (38). Although the adult female's lactating fast is also followed by a feeding/diving phase, existing evidence suggests that the antioxidant system in pinnipeds develops with maturation in parallel with the development of their diving capacity (2, 3, 22, 39).

The observed increase in GR and G6PHD activities suggests that the recycling of GSSG to GSH is increased and the reduced GSH pool is maintained over the course of

elephant seal's postweaning fast (20, 25). The maintenance of the reduced GSH pool is crucial for cell survival during oxidative stress since the reactions of GSH with peroxides and reactive electrophiles, which are catalyzed by GPx, PrxVI, and GST, use reduced GSH and yield GSSG (20, 23, 25). The maintenance of the GSH pool is critical for providing available co-substrate to support the observed increase in GST and PrxVI. The fasting-related increases in GST activity, PrxVI expression, and GPx and catalase activities and protein content (38) suggest that there is an increase in the rate of the removal of H_2O_2, which is the principal substrate for those antioxidant enzymes and is also the main oxidant produced in response to ischemia/reperfusion due the hypoxanthine/xanthine oxidase pathway (1) that is activated after experimental ischemia in seal organs (8).

The postweaning fast in seals is associated with increases in RAS and Nox4 (29, 31, 38), which collectively have been shown to increase intracellular H_2O_2 production (7). The modulation of H_2O_2 levels by peroxide-removing enzymes is essential for the actions of H_2O_2 as a second messenger in the activation of the NF-E2-related factor 2 (Nrf2) pathway, which leads to the up-regulation of proteins involved in GSH synthesis, antioxidant defense and phase II detoxification, via the electrophile responsive element (15, 16, 33, 35). Thus, the observed increases in PrxVI and GST, along with the previously reported increases in catalase and GPx (38), suggest that H_2O_2 is actively modulated during the postweaning fast in elephant seals, which likely results in the up-regulation of enzymes involved in GSH biosynthesis such as GCLc, GCLm, GST and GR (Figure 5).
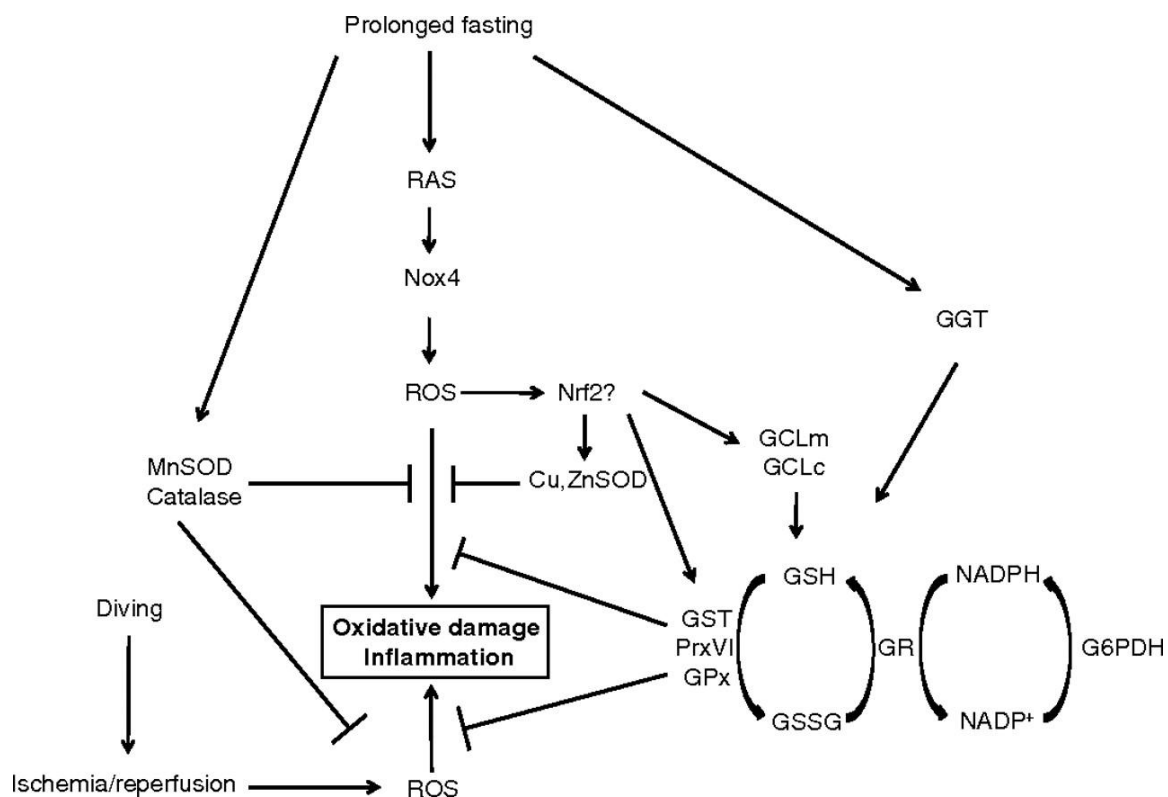
Figure 5. Schematic representation of proposed molecular mechanisms leading to increased glutathione biosynthesis and antioxidant enzymes up-regulation in response to prolonged fasting in elephant seals. Cu,ZnSOD = copper,zinc-dependent superoxide dismutase. G6PDH = glucose-6-phosphate dehydrogenase. GCLc = glutamate-cysteine ligase catalytic subunit. GCLm = glutamate-cysteine ligase modulatory subunit. GGT = γ-glutamyl transpeptidase. GSH = glutathione. GSSG = glutathione disulphide. GR = glutathione disulphide reductase. GPx = glutathione peroxidase. GST = glutathione S-transferase. MnSOD = manganese-dependent superoxide dismutase. Nox4 = NADPH oxidase 4. Nrf2 = NF-E2-related factor 2. PrxVI = 1-cys peroxiredoxin. RAS = renin-angiotensin system. ROS = reactive oxygen species.

In summary, our results show that GSH biosynthesis increases with fasting in postweaned northern elephant seals suggesting that the GSH system contributes to the cellular defense against the potential increases in fasting-derived oxidant production. Our results also suggest that fasting pups require this postweaning, developmental phase for the activation of their antioxidant systems. Increased protein content and activities of the enzymes associated with the GSH system contribute to the mechanisms that elephant seals evolved and permit them to tolerate potentially pathological conditions characterized by increased hydroperoxide production.

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Chapter III. Prolonged Fasting Activates Nrf2 in Postweaned Elephant Seals*

Abstract

Elephant seals naturally experience prolonged periods of absolute food and water deprivation (fasting). In humans, rats and mice, prolonged food deprivation activates the renin angiotensin system (RAS) and increases oxidative damage. In elephant seals, prolonged fasting activates RAS without increasing oxidative damage likely due to an increase in antioxidant defenses. The mechanism leading to the up-regulation of antioxidant defenses during prolonged fasting remains elusive. Therefore, we investigated if prolonged fasting activates the redox-sensitive transcription factor Nrf2, which controls the expression of antioxidant genes, and if such activation is potentially mediated by systemic increases in RAS. Blood and skeletal muscle samples were collected from seals fasting for 1, 3, 5 and 7 weeks. Nrf2 activity and nuclear content increased by 76% and 2.5 fold at week 7. Plasma angiotensin II (Ang II) and transforming growth factor β (TGF-β) were 50-fold and 3-fold higher at week 7 than at week 1. Smad2 phosphorylation, an effector of Ang II and TGF signaling, increased by 120% at week 7 and by 84% in response to intravenously infused Ang II. NADPH oxidase 4 (Nox4) mRNA expression, which is controlled by smad proteins, increased 5-fold at week 7, while Nox4 protein expression, which can activate Nrf2, was 2.5-fold higher at wk 7 than at wk 1. Results demonstrate that prolonged fasting activates Nrf2 in elephant seals and that RAS stimulation can potentially result in increased Nox4 through Smad phosphorylation. Results also suggest that Nox4 is essential to sustain the hormetic adaptive response to oxidative stress in fasting seals.

Introduction

Spontaneous long term fasting is an integral part of the life history of phocid seals (9). The northern elephant seal (*Mirounga angustirostris*) annually undergoes natural periods of prolonged fasting, while breeding, molting and weaning (30). Prolonged fasting in elephant seals is associated with a series of physiological changes that result in the activation of the hypothalamic–pituitary–adrenal axis (HPA) (37-39) and the renin angiotensin system (RAS) (36, 40), as well as in the onset of insulin resistant-like conditions (20, 65-67).

Prolonged fasting, insulin resistance, and chronic HPA and RAS activation, induce oxidative stress by activating NADPH oxidase (Nox) proteins, increasing mitochondrial oxidant generation and depleting antioxidants in humans, rats and mice (11, 14, 19, 43, 49, 51, 52, 56). In the northern elephant seal, prolonged fasting increases Nox4 and xanthine oxidase (XO) without increasing oxidative damage or inflammation (48, 59). Systemic and muscle markers of oxidative damage (F_2-isoprostanes, nitrotyrosine, C-reactive protein, TNF-α, 4-hydroxynonenal, protein carbonyls) remain unchanged after two months of absolute fasting in seal pups (59). Fasting-related increases in the activity and protein expression of several antioxidant enzymes and glutathione (GSH) levels (59, 61), as well as increased purine recycling (48), likely contribute to the prevention of oxidative damage in elephant seals.

How the antioxidant system is up-regulated in response to prolonged fasting in elephant seals remains elusive. The nuclear factor erythroid 2-related factor 2 (Nrf2) is a central regulator of the adaptive response to oxidative stress (26). Nrf2 induces the transcription of genes involved in antioxidant defense through its binding to the electrophilic responsive element (EpRE) (24). Nrf2 is a member of the basic leucine-zipper NF-E2 family that is bound to its repressor protein Keap1 under unaltered conditions (25). Binding of Nrf2 to Keap1 targets its ubiquitin conjugation and consequent proteosomal degradation (24). Increases in intracellular oxidant generation modify Cys273 and Cys288 residues in Keap1, inhibiting Nrf2 ubiquitination and promoting its nuclear translocation and binding to the EpRE (6, 27, 28, 72). The Nrf2 of the seal has high identity to the Nrf2 of other mammals, contains the conserved leucine zipper domain, key residues for nuclear export signal and Keap1-mediated degradation, and is expressed at mRNA and protein levels in seal muscle (60, 63).

Whether Nrf2 is activated in response to fasting in elephant seals, or any other mammal, has not been investigated. Therefore, the goal of the present study was to elucidate the role of Nrf2 in mediating the adaptive response to oxidative stress during prolonged fasting in a mammal adapted to cope with such condition, the northern elephant seal. We have previously shown that prolonged fasting increases Nox4 expression in the skeletal muscle of the elephant seal (59). Unlike other NADPH oxidases, Nox4 is independent of cytosolic activator subunits, and thus, is constitutively active (34, 35, 68). Nox4 is also uniquely localized in several subcellular compartments (1, 5, 55) and produces intracellular hydrogen peroxide (H_2O_2), a potent activator of Nrf2 (28), due to a particular property of its E-loop which contains a highly conserved histidine that serves as a source for protons to accelerate spontaneous dismutation of superoxide to H_2O_2 (57). Nox4 transcription is thought to be controlled by Smad proteins,

which act as transcription factors once they are phosphorylated in the transforming growth factor β (TGF-β) signaling cascade (42). Nox4 expression has also been shown to be regulated *in vivo* by angiotensin II (Ang II), in a TGF-β-independent manner, during acute stimulation (4, 15, 32, 69). We hypothesized that prolonged fasting activates Nrf2 in parallel with increasing Nox4 expression and circulating Ang II, and that the activation of the angiotensin receptor type 1 (AT1) increases Smad2 phosphorylation in the skeletal muscle of the elephant seal. The present study demonstrates that prolonged fasting stimulates the adaptive response to oxidative stress in elephant seals by activating Nrf2, suggests that systemic increases in RAS mediate such adaptive response and highlights the potential role of Nox4 in sustaining a hormetic protective response in fasting seals.

Methods

All procedures were reviewed and approved by the Institutional Animal Care and Use Committees of both The University of California Merced and Sonoma State University. All work was realized under the National Marine Fisheries Service marine mammal permit no. 87-1743.

Animal handling and sample collection

Twenty eight elephant seals pups of known age were sampled at Año Nuevo State Reserve (Pescadero, CA, USA), seven at a time, at four periods during their natural post-weaning fast (within 1, 3, 5 and 7 weeks postweaned). Pups were initially sedated with 1 mg kg^{-1} tiletamine hydrochloride and zolazepam hydrochloride (Telazol; Fort Dodge Animal Health, Fort Dodge, IA, USA). Once immobilized, a 16 gauge, 3.5 inch spinal needle was inserted into the extradural vein. Sedation was maintained with 100 mg bolus intravenous injections of Ketamine (Fort Dodge Animal Health) as needed. Blood samples were collected into pre-chilled EDTA-treated collection tubes containing 10 µL mL^{-1} protease inhibitor cocktail (PIC) and 0.005% BHT (Sigma, St. Lous MO, USA), and centrifuged on site before plasma was aliquoted into separate cryovials. Muscle biopsies (20-30 mg) were collected from a small region in the flank of the animal near the hind flipper as previously described (59, 61). Tissue samples were rinsed with ice-cold sterile saline solution and placed in cryogenic vials. Plasma and tissue samples were frozen by immersion in liquid nitrogen immediately after collection and stored at -80°C until analyzed.

Angiotensin II infusions

Fifteen additional seal pups (7 males, 8 females, 1-3 weeks postweaned) were randomly assigned to three experimental groups (n = 5 per group): 1) control, 2) Ang II (3.6 µg kg^{-1}) and 3) Ang II + angiotensin receptor type 1 (AT1) blocker (ARB; 10 µg olmesartan kg^{-1}). Animals were immobilized as describe in the animal handling section. Vehicle (sterile saline), Ang II (Sigma) or Ang II + ARB (olmesartan, Daiichi-Sankyo, Tokyo, Japan) were infused at a rate of 1 mL min^{-1} through the extradural spinal vein. Blood samples were collected at 0, 10, 30, 60 and 120 minutes post-infusion. Muscle biopsies were collected before and 1 h after the intravenous infusion. Blood and tissue samples were processed as described in the sample collection section.

Plasma analyses

Plasma Ang II was extracted using methanol and measured using a commercial RIA kit (Phoenix Pharmaceuticals Inc, Burlingame, CA, USA) previously validated for the northern elephant seal (70). Plasma aldosterone was also measured using a commercially available RIA kit (Siemens Medical Solutions, Los Angeles, CA, USA) that has been validated for elephant seals (40). Plasma TGF- β 1 was measured using a Mouse/Rat/Porcine/Canine Quantikine® immunoassay kit following manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Western blot

Frozen tissue samples were homogenized 1:20 (w/v) in RIPA (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS) buffer supplemented with a cocktail of protease and phosphatase inhibitors (Pierce) (crude extracts). Nuclear protein fractions were prepared from frozen tissue samples using the Pierce NE-PER nuclear extraction kit supplemented with protease and phosphatase inhibitors. Total protein content in crude extracts and nuclear fractions was measured using the Bio-Rad Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Twenty μg of crude extract or 10 ug of nuclear protein were mixed with Laemmli sample buffer, boiled and resolved in 4-12% Tris-glycine acrylamide gels under denaturing conditions. Proteins were electroblotted onto nitrocellulose membranes using a Bio-Rad Trans Blot Turbo transfer cell. Membranes were blocked with 3% bovine serum albumin 1h at room temperature and incubated overnight with antibodies against Smad (Cell Signaling Technology, Boston, MA, USA; Smad2: cat # 5339; Phospho-Smad2 Ser465/467, cat # 3104) and Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, cat # 8882). Nox4 protein expression was detected using monoclonal commercial antibodies (Epitomics Inc., Burlington, CA, USA, cat # 3174-1) raised against a peptide sequence within the NADPH binding domain of Nox4 that is unique among NADPH oxidases, but is conserved between mammalian protein sequences (31). Membranes were incubated with HRP-conjugated secondary antibodies (Pierce) and developed using Super Signal West Pico ECL substrate (Pierce). Blots were visualized using a Kodak Image Station 440 (Kodak, Rochester, NY, USA) and quantified using Kodak 1D 3.6 Image Analysis Software. Percent change from week 1 was calculated after band densities were normalized using actin (crude extracts) or TATA binding protein (nuclear fractions).

Nrf2 transcription factor activity

Binding of activated Nrf2 to the EpRE was measured in nuclear extracts using a TransAM® Nrf2 Transcription Factor kit (Active Motif, Carlsbad, CA, USA). Fifteen μg of nuclear protein were diluted in lysis buffer supplemented with protease and phosphatase inhibitors and incubated with immobilized oligonucleotides containing the EpRE consensus binding site (5′-GTCACAGTACTCAGCAGAATCTG-3′). The assay was performed following manufacturer's instructions.

RNA extraction, cDNA cloning and real-time quantitative PCR

Total RNA was isolated from frozen tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA integrity was confirmed by measuring the ratio of absorbance at 260 nm/280 nm and by 1% agarose gel electrophoresis. Genomic DNA was eliminated by digestion with DNase I (Roche, Indianapolis, IN, USA). First-strand cDNAs were reverse-transcribed from total DNA-free RNA using the QuantiTec Reverse Transcription kit (Qiagen, Valencia, CA, USA) and oligo-dT. Annealing and extension steps were performed at 42°C for 30 min and 95°C for 3 min.

A partial sequence encoding for elephant seal Nox4 was obtained using primers (1234Nox4F + 1469Nox4R, dogNox4F5 + esealNox4R5 and esealNox4F1 + dogNox4R1 (Table 1) designed based on published mammalian Nox4 sequences. For a 25 µL final volume reaction, 12.5 µL of Platinum PCR SuperMix (Invitrogen, Carlsbad, CA, USA), 3 µL of muscle cDNA and 1 µL (20 µM) of each primer were mixed and subjected to the following conditions: 94°C for 3 min for one cycle; 40 cycles of 94°C for 30 s, 55°C for 40 s and 68°C for 2 min; and to an overextension step of 68°C for 7 min. PCR fragments of 230 (esNox4a), 1200 (esNox4b) and 550 bp (esNox4c) were obtained and cloned using the pGEM-T Easy Vector System (Promega Corporation, Fitchburg, WI, USA). Sequences were identified as Nox4 by comparing them with GenBank data using the Blast algorithm. A partial sequence of 1730 bp (esNox4) that encodes for Nox4 was obtained by overlapping esNox4 -a, -b and -c sequences.

Nox4 mRNA expression was quantified using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal standard (GeneBank accession No. NM_002046). Nox4 and GAPDH transcripts were measured by quantitative reverse transcription PCR (qRT-PCR) using a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) as described previously (60). Positive and negative controls were included. Standard curves of Nox4 and GAPDH were run to determine amplification efficiency, which was 99.8% for Nox4 and 99.5% for GAPDH, using dilutions from 5×10^{-4} to 5×10^{-9} ng µl^{-1} of PCR fragments. Primer sequences used for qPCR (esealNox4Fw3 + esealNox4Rv1) are provided (Table 1).

Statistics

Means (± SEM) were compared by ANOVA with Fisher's Protected Least Significant Difference (PLSD) post-hoc test, and were considered significantly different at $p < 0.05$. For plasma aldosterone measurements means (± SEM) were compared by ANOVA adjusted for repeated measures and considered significant at $p < 0.05$. Statistical analyses were performed with the SYSTAT© 11.0 software (SPSS, Richmond, CA, USA).

Table 1. Primers used for cDNA cloning and qRT-PCR.

Primer name	Sequence (5'-3')
1234Nox4F	TTTGGAAGTCCATTTGAGGA
1469Nox4R	TCAGGTCTGTTTTCTTGCCA
dogNox4F5	GGCTCTCCCTGAATGTTTTGC
esealNox4R5	GTCATCCAGCAGGGTGTTGAG
esealNox4F1	GCTGGAGGCATTGGAGTAAC
dogNox4R1	TCTTTGGCATGACACAGCT
esealNox4Fw3	GGAAGTCCATTTGAGGAATCG
esealNox4Rv1	CTTCCGTTGGTTTGCAGAC

Results

Prolonged fasting increases circulating Ang II and TGF-β, and activates the Smad pathway

Plasma Ang II and TGF-β1 levels were measured to confirm that prolonged fasting activates RAS, and to test if fasting also increases TGF-β. Plasma Ang II increased in a time dependent manner over the course of the fast and was 50-fold higher at week 7 (257 ± 36 fmol mL^{-1}) than at week 1 (5 ± 1 fmol mL^{-1}) (Figure 1A). Plasma TGF-β1 concentration also increased over the course of the fast, from 2-fold higher at week 5 (10 ± 1 ng mL^{-1}) to 3-fold higher at week 7 (14 ± 6 ng mL^{-1}) compared to week 1 (4 ± 1 ng mL^{-1}) (Figure 1B). Phosphorylation of Smad2 was measured to assess its association with increased circulating Ang II and TGF-β1, potent activators of the Smad pathway. Smad 2 phosphorylation in skeletal muscle was 58% higher at week 5 and 120% higher at week 7 than at week 1 (Figure 2).
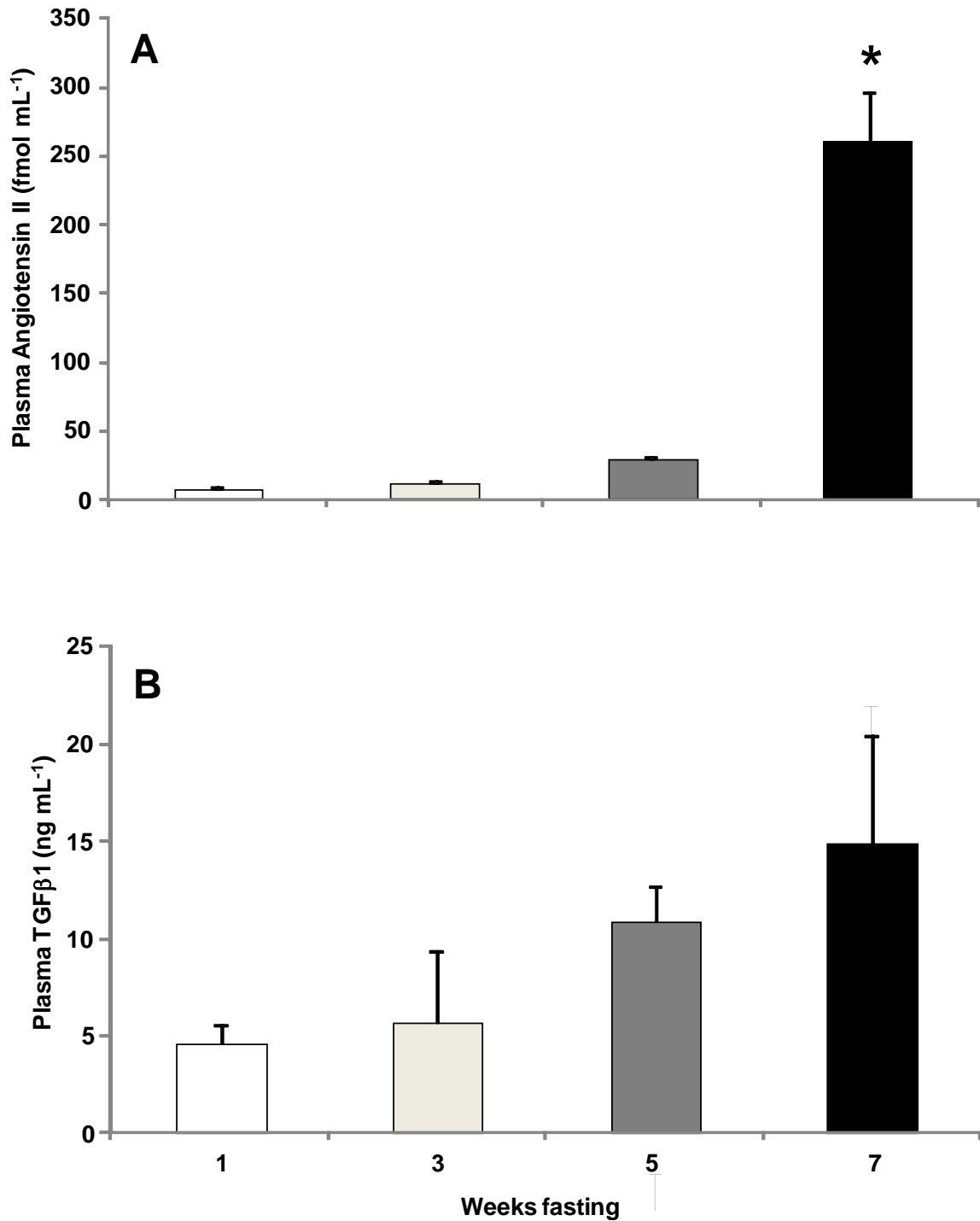
Figure 1. Prolonged fasting increases plasma Angiotensin II and TGF-β in elephant seal pups. Mean (± SEM) **A)** Ang II and **B)** TGF-β1 circulating levels in elephant seals during their postweaning fast. * = significantly different from week 1 (p< 0.05).
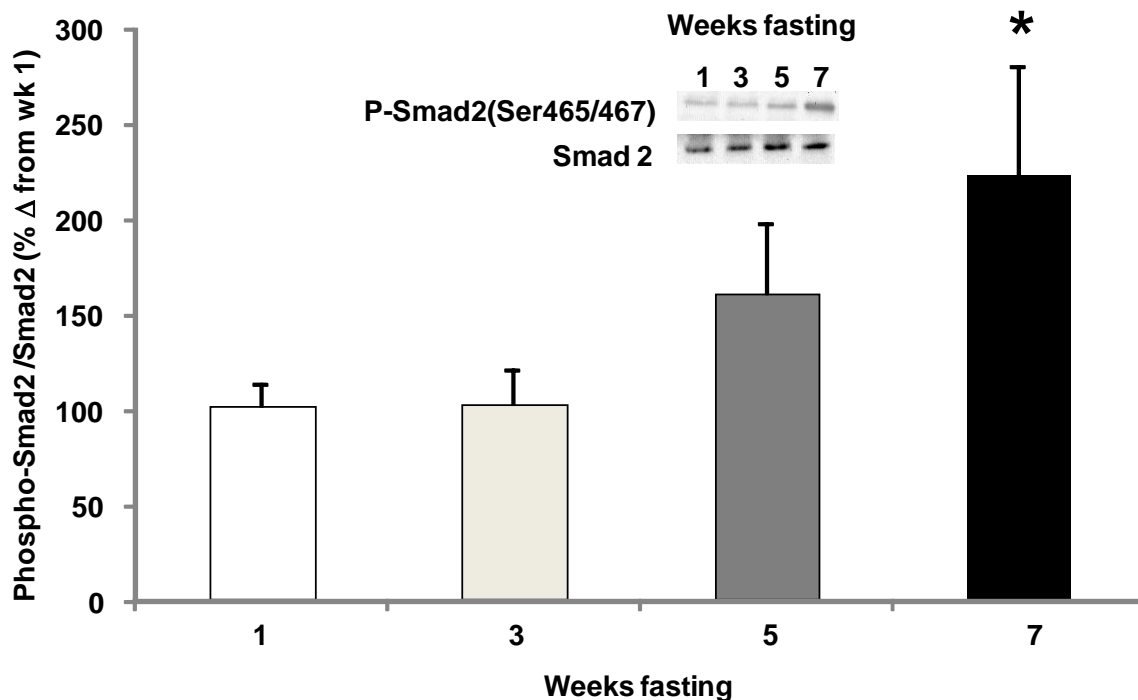
Figure 2. Prolonged fasting increases Smad activation in elephant seal pups. Mean (± SEM) Smad 2 phosphorylation (Ser465/67) levels in the skeletal muscle of the elephant seal during its postweaning fast. A representative blot is included. * = significantly different from week 1 (p< 0.05).

Acutely infused Ang II activates Smad2

To confirm the effects of the fasting-induced increase in plasma Ang II on the Smad pathway, Smad2 phosphorylation was measured after acute intravenous infusion of Ang II, with and without an ARB. The use of ARB helped confirm the contribution of Ang II to Smad phosphorylaton via AT1 activation. Plasma aldosterone concentration increased from 398 ± 101 pg mL^{-1} at time 0, to a maximum of 770 ± 172 pg mL^{-1} at 1 h, and decreased to 597 ± 53 pg mL^{-1} at 2h confirming the effectiveness of the Ang II infusion. The increase in circulating aldosterone in response to acutely infused Ang II was completely inhibited by the simultaneous infusion of the ARB (Figure 3A) confirming the effectiveness of the ARB dosage to block AT1. Smad2 phosphorylation increased by 84% in the Ang II infused animals (Figure 3B). Phosphorylated Smad2 levels did not change in the Ang II + ARB group (Figure 3B), suggesting that Ang II stimulates the Smad pathway by activating AT1.
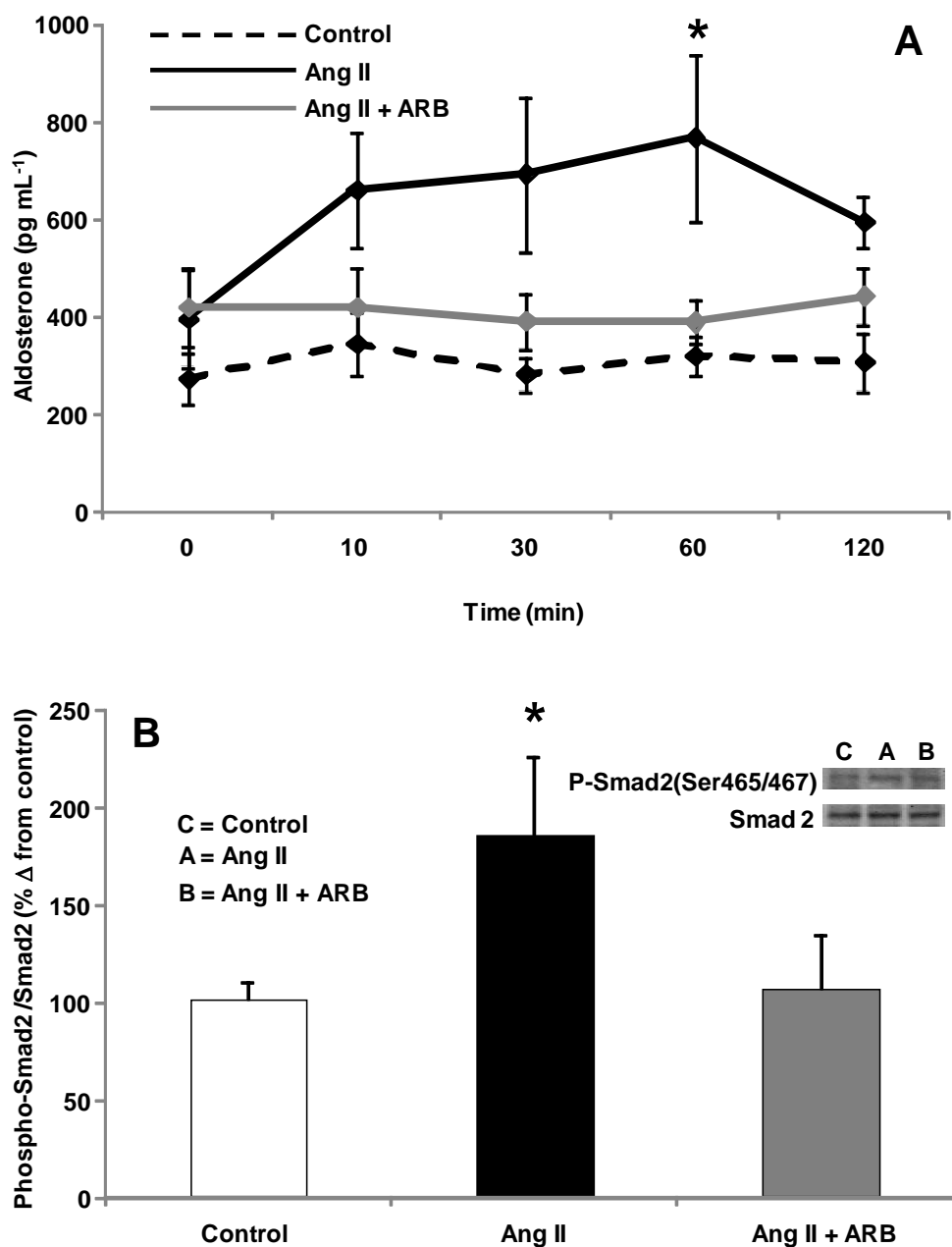
Figure 3. Acutely infused Ang II stimulates Smad activation in elephant seal pups. **A)** Circulating aldosterone levels in response to an acute intravenous infusion of Ang II in postweaned elephant seals. Ang II = angiotensin II infused, Ang II + ARB = Ang II + Angiotensin receptor type 1 blocker (Olmesartan, ARB) infused. **B)** Mean (± SEM) Smad 2 phosphorylation (Ser465/67) levels in the skeletal muscle of postweaned elephant seals in response to acutely intravenous infused Ang II. * = significantly different from week 1 ($p < 0.05$). See methods section for further details.

Prolonged fasting increases Nox4 expression

Nox4 was cloned and sequenced, and its expression was measured, at mRNA and protein levels, to test whether prolonged fasting, along with increasing Ang II and TGF-β, and activating Smad, increases Nox4. A partial sequence (95%) coding for Nox4 was obtained from the skeletal muscle of the elephant seal (GenBank accession No. JX310325). Partial seal Nox4 is 1,730 bp long and encodes a peptide of 544 amino acids with high identity to Nox4 from the giant panda (96%), human (91%), domestic dog (90%), rat (87%) and mouse (81%) (Figure 4A). Conserved regions that encode the unique functional domain of Nox4 and binding site for FAD and NADPH were found in the predicted Nox4 protein sequence of the elephant seal (Figure 4B). Expression of Nox4 mRNA increased 5-fold at week 7 (530 ± 80%) compared to week 1 (100 ± 9%) (Figure 5A) while Nox4 protein expression increased 2.5-fold at week 7 (270 ± 47%) compared to week 1 (100 ± 21%) (Figure 5B), suggesting that along with increasing systemic RAS and TGF-β prolonged fasting activates the Smad pathway and increases Nox4 in the skeletal muscle of the elephant seal.

Figure 4. The Nox4 of the elephant seal is a conserved enzyme that is distinct from other Nox homolog proteins. **A)** Multiple alignment of amino acid sequences of Nox4 proteins. Northern elephant seal (esNox4), domestic dog (XP_542262), giant panda (XP_002927888), rat (NP_445976) and human (AAF68973) predicted amino acid sequences are included in the analysis. ▼ indicates conserved histidine residues involved in heme iron binding. Black boxes represent predicted transmembrane alpha helices. The putative FAD and NADPH binding sites are indicated. **B)** Multiple alignment of amino acid sequences of several Nox homolog proteins. The northern elephant seal Nox4

(esNox4) and human Nox proteins (Hm.gp91phox, NP_000388; Hm.Nox1, CAI42336; Hm.Nox3, AAG17121; Hm.Nox4, AAF68973 and Hm.Nox5, AAG33638) amino acid sequences are included in the analysis. Black boxes represent identical amino acid residues between esNox4 and Hm.Nox4 but different other human Nox proteins. The putative FAD and NADPH binding sites are indicated.
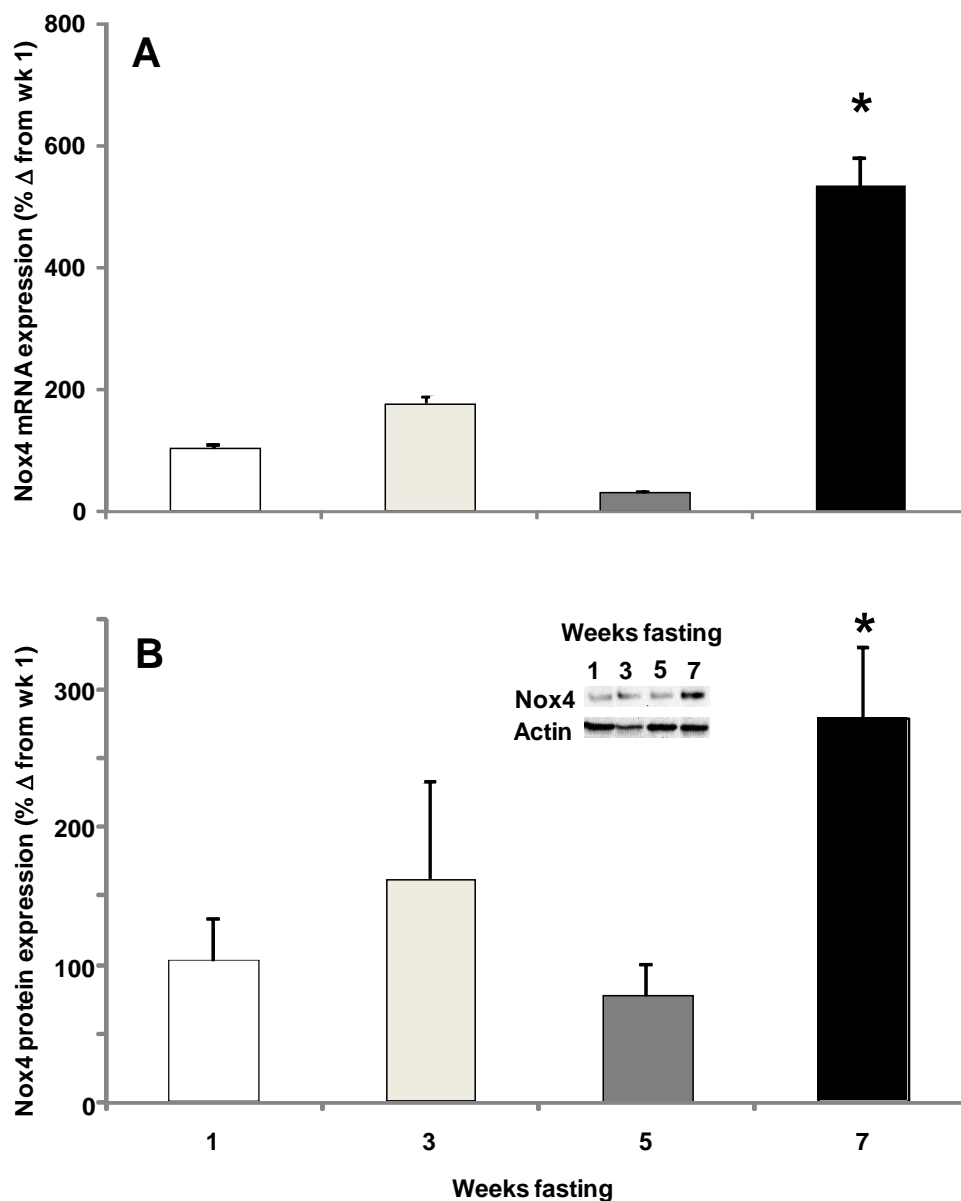
Figure 5. Prolonged fasting increases Nox4 expression in elephant seal pups. Mean (± SEM) Nox4 **A)** mRNA and **B)** protein expression during prolonged fasting in the skeletal muscle of postweaned elephant seals. A representative blot is included. * = significantly different from week 1 (p< 0.05).

Prolonged fasting activates Nrf2

Levels of Nrf2 in nuclear fractions prepared from skeletal muscle and Nrf2 activation were quantified to determine whether prolonged fasting activates the Nrf2/EpRE pathway, which can ultimately result in the previously reported increases in antioxidant enzymes and GSH levels (59, 61). Nuclear levels of Nrf2 increased with fasting and were 2-fold higher at week 7 than at week 1 (Figure 6A). Activation of Nrf2 increased 41% at week 5 and 76% at week 7 compared to week 1 (Figure 6B) suggesting that prolonged fasting stimulates the antioxidant system of the elephant seal by activating Nrf2 and that Nrf2 activation is likely mediated by increases in systemic RAS and Nox4 expression.

Figure 6. Prolonged fasting activates the Nrf2/EpR pathway in elephant seal pups. Mean (± SEM) **A)** Nrf2 levels in nuclear fractions prepared from skeletal muscle and **B)** Binding ability of activated Nrf2 to the EpR consensus binding site during prolonged fasting in postweaned elephant seals. * = significantly different from week 1 (p< 0.05).

Discussion

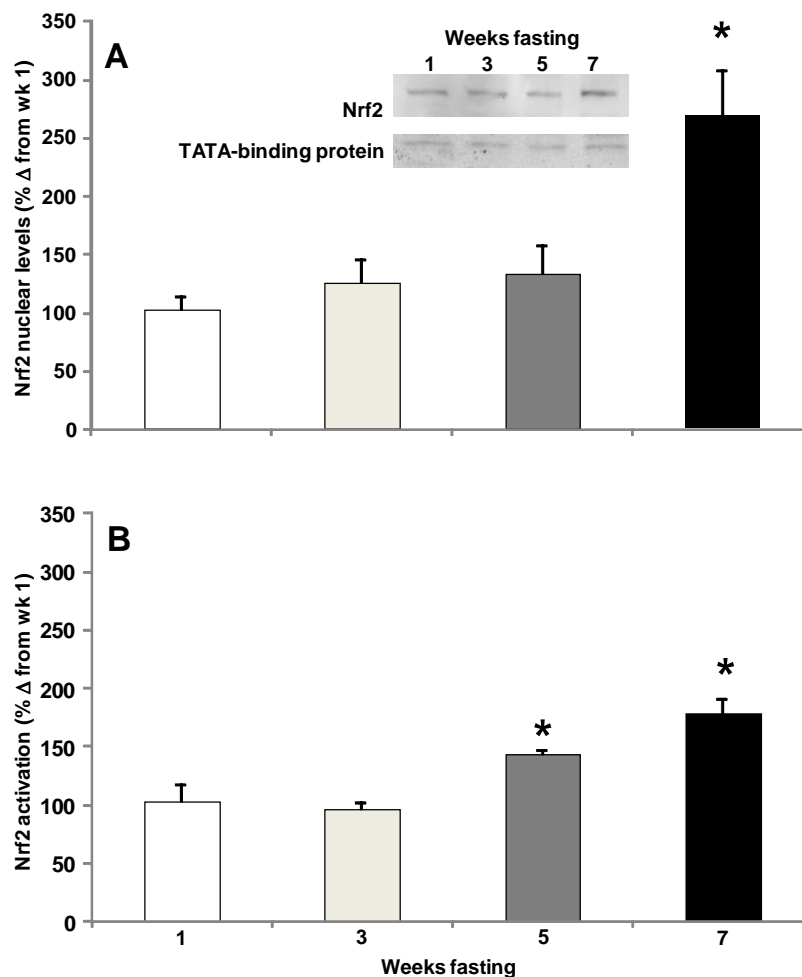Prolonged food deprivation increases oxidative stress in humans, rats and mice by activating Nox proteins, increasing mitochondrial oxidant generation and depleting antioxidants (16, 17, 21, 33, 41, 49, 51, 56, 64). In the northern elephant seal, however, prolonged fasting does not increase oxidative stress, likely due to increases in endogenous antioxidant defenses (59, 61). Results of the present study demonstrate that prolonged fasting stimulates the activation and nuclear accumulation of the redox-sensitive transcription factor, Nrf2, which can potentially increase the expression of antioxidant enzymes and glutathione levels. Prolonged fasting also increased plasma Ang II, activated Smad2 and increased Nox4 expression in skeletal muscle, suggesting that chronic increases in circulating Ang II stimulate Nox4, and ultimately increase Nrf2 activity through the activation of the Smad pathway. This was further confirmed by demonstrating that an acute infusion of Ang II increased Smad2 phosphorylation via AT1 activation.

Progressive increases in Nox4 expression in parallel with increased activation of Nrf2 suggest that Nox4 may be mediating a hormertic response that promotes the stimulation of the antioxidant system by activating Nrf2 (8, 29). Intracellular oxidants modify Nrf2, leading to its dissociation from Keap1 and its subsequent translocation into the nucleus where it binds to the EpRE (28, 72). Nox4 is constitutively active (22, 34, 47) and is uniquely localized to several intracellular membranes (1, 5, 22, 55). Endogenous, low levels of H_2O_2 derived from Nox4 have been recently suggested to control Nrf2 activity in endothelial cells and cardiomyocytes *in vivo* (7, 46); thus, increases in Nox4 may activate Nrf2, which up-regulates the expression of antioxidant enzymes during prolonged fasting in seals, as previously reported (59, 61).

TGF-β is a potent inducer of Nox4 expression via the Smad signaling pathway (15, 32, 53, 54). Ang II signaling downstream of AT1 can also control Nox4 expression *in vivo* and *in vitro* (2, 4, 69). Although Ang II up-regulates TGF-β via AT1 activation (44), it can also activate the Smad pathway independently of TGF-β (42, 45). Acutely infused Ang II increased, and ARB prevented, Smad phosphorylation, suggesting that AT1 activation may directly increase Nox4 expression (42). After two months of absolute fasting, however, when both circulating Ang II and TGF-β concentrations are increased, fasting-associated increases in Smad phosphorylation and Nox4 expression may be a consequence of the synergistic effects of chronic and progressive increases in circulating Ang II and TGF-β (50). Furthermore, the observed increases in TGF-β may itself be a consequence of increased Ang II (44).

An increase in GSH levels and antioxidant enzymes expression at the end of the fast (59, 61) may potentially ameliorate the oxidant generation derived from augmented XO and Nox expression and activity (48, 59), impaired insulin signaling (20, 65, 66), high rates of glucose auto-oxidation (12, 13, 23) and lipid oxidation (67), RAS activation (36, 39) and chronic HPA stimulation (37, 39). Increased antioxidant defenses at the end of the fast are also consistent with an increase in number and duration of sleep apnea bouts that normally last between 8 and 12 min and constitute 80% of the seals' time on land (3, 10), along with increases in the time spent submerged in near-shore waters (58). Furthermore, repetitive sleep apneas and voluntary submersions increase nuclear

accumulation of Nrf2 in the skeletal muscle of late-fasting elephant seal pups (63). Therefore, physiological adjustments associated with both prolonged fasting and breath-holding may stimulate Nrf2, ultimately preconditioning seal muscle to tolerate diving-induced ischemia/reperfusion, which follows immediately upon departure from the rookery (after fasting) and which has the potential to increase oxidant generation and oxidative stress (18, 62, 71).

In summary, results demonstrate that prolonged fasting activates Nrf2 and suggest that such activation is mediated by increased expression of Nox4. Furthermore, results suggest that Ang II stimulates Smad and thus, can potentially regulate Nox4 expression through AT1 activation. Finally, results suggest that physiological adjustments associated with prolonged fasting upregulate the antioxidant system of the elephant seal, conferring enhanced antioxidant protection to tolerate fasting-related oxidant production and diving-induced ischemia/reperfusion (Figure 7). The present study describes, for the first time, a potential mechanism for the regulation of the adaptive response to oxidative stress during food deprivation in mammals.
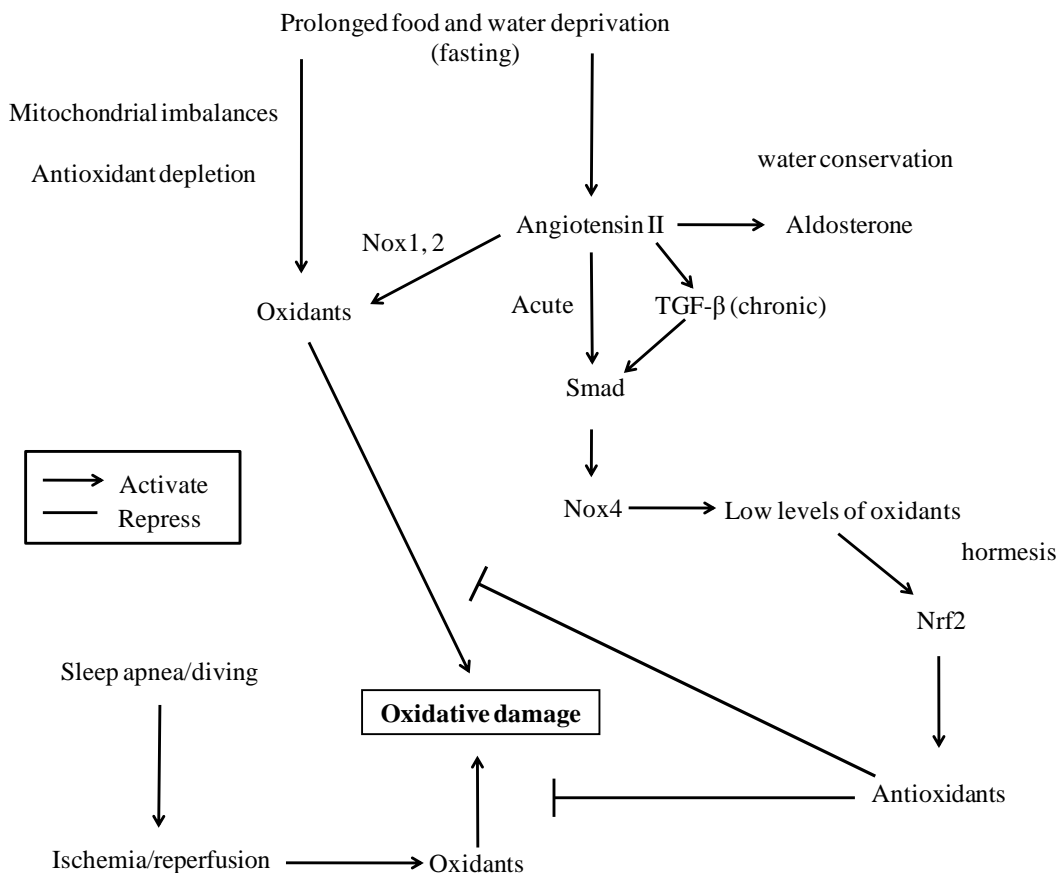

Figure 7. Schematic representation of the proposed mechanisms leading to the activation of the antioxidant system of the elephant seal during prolonged fasting.

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Chapter IV. Repetitive Apneas Stimulate the Adaptive Response to Oxidative Stress in Elephant Seal Pups*

Abstract

Extended breath-hold (apnea) bouts are routine during diving and sleeping in seals. These apneas result in oxygen store depletion and blood flow redistribution towards obligatory oxygen-dependent tissues, exposing seals to critical levels of ischemia and hypoxemia. The subsequent reperfusion/reoxygenation has the potential to increase oxidant production and thus, oxidative stress. The contributions of extended apnea to oxidative stress in adapted mammals are not well defined. To address the hypothesis that apnea in seals is not associated with increased oxidative damage, blood samples were collected from northern elephant seal pups (n = 6) during eupnea, rest- and voluntary submersion-associated apneas, and post-apnea (recovery). Plasma 4-hydroxynonenal (HNE), 8-isoprostanes (8-iso $PGF_{2\alpha}$), nitrotyrosine (NT), protein carbonyls, xanthine and hypoxanthine (HX) levels, along with xanthine oxidase (XO) activity, were measured. Protein content of XO, superoxide dismutase 1 (Cu,ZnSOD), catalase, myoglobin (Mb), as well as nuclear content of hypoxia inducible factor 1α (HIF-1α) and NF-E2-related factor 2 (Nrf2), were measured in muscle biopsies collected before and after the breath-hold trials. HNE, 8-iso $PGF_{2\alpha}$, NT, and protein carbonyl levels did not change among eupnea, apnea or recovery. XO activity, HX and xanthine concentrations were increased at the end of the apneas and during recovery. Muscle protein content of XO, CuZnSOD, catalase, Mb, HIF-1α and Nrf2 increased 25-70% after apnea. Results suggest that rather than inducing the damaging effects of hypoxemia and ischemia/reperfusion that have been reported in non-diving mammals, apnea in seals stimulates hormetic adaptive responses to oxidative stress and hypoxia, allowing these mammals to cope with the potentially detrimental effects associated with this condition.

Introduction

Seals are routinely exposed to breath-holding (apnea) bouts while diving and sleeping (11, 28, 44). Apnea in seals is characterized by a series cardiovascular adjustments (reduction in cardiac output, bradycardia, peripheral vasoconstriction) that allow seals to maximize the use of their oxygen stores but at the same time result in blood oxygen depletion and blood flow redistribution towards obligatory oxygen-dependent tissues, exposing seals to ischemia and hypoxemia (7, 10, 27, 49). At the end of an apnea, an increase in cardiac output and ventilation restore blood flow to tissues and blood oxygen content, presenting seals with potential increases in oxidant production and oxidative stress (12, 62).

Blood reoxygenation after hypoxemia and ischemia/reperfusion are conditions that in terrestrial mammals increase oxidant production and oxidative damage (32). During ischemia and hypoxemia, ATP degradation results in the accumulation of the purine nucleotides, xanthine and hypoxanthine (HX), and in the proteolytic conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO). During reperfusion/reoxygenation, XO reduces xanthine and HX generating superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (40). Despite routine and chronic exposure to cyclic bouts of apnea-induced hypoxemia and ischemia/reperfusion, seals do not exhibit higher levels of oxidative damage (ie, lipid peroxidation and protein carbonyls) in their tissues or red blood cells (RBCs) in comparison to terrestrial mammals (59, 60, 62). Interestingly, seal tissues do accumulate HX after *ex vivo* exposure to ischemia (12). Moreover, basal capacity to produce $O_2^{\bullet-}$ is higher in tissues of seals than in tissues of non-diving mammals and $O_2^{\bullet-}$ production, but not oxidative damage, is higher in seal than in terrestrial mammal tissues when exposed *in vitro* to an oxidant-generating system (xanthine + XO) (62). The latter suggests that seals, as well as other diving vertebrates (13, 53, 63), are constantly exposed to apnea-induced oxidant production, but possess the mechanisms to avoid oxidative damage. These mechanisms, however, are not well-defined in mammals adapted to tolerate repetitive and routine bouts of apnea.

An enhanced antioxidant capacity likely contributes to the seal's tolerance to apnea-induced hypoxemia and ischemia/reperfusion (17, 62). Plasma, tissues, and RBCs of seals possess higher basal activities of several antioxidant enzymes and higher glutathione (GSH) levels than those of terrestrial mammals (35, 58-60). Furthermore, the hypoxia-inducible factor 1 (HIF-1), a key transcriptional regulator of the adaptive response to hypoxia, and the NF-E2-related factor 2 (Nrf2), which controls the adaptive response to oxidative stress by up-regulating antioxidant genes in response to increased oxidant production, have also been implicated in seal's protection against apnea-induced hypoxemia and ischemia/reperfusion (22, 23, 56). No *in vivo* studies, however, have been conducted to elucidate the cellular and molecular responses that protect seals against apnea-induced hypoxemia and ischemia/reperfusion. The available data on the effects of submersion on antioxidant responses in seals is also scant, with results showing that blood GSH content in Weddell seals decreases during forced submersions and increases above pre-submersion levels during recovery (35).

In the present study, plasma levels of a suite of markers of oxidative damage, along with plasma XO activity and plasma concentrations of xanthine and HX, were

measured in seals before, during and after rest- and voluntary submersion-associated apneas to assess the impact of apnea on oxidant mechanisms. Muscle protein levels of catalase, HIF-1α myoglobin (Mb), Nrf2, superoxide dismutase 1 (Cu,ZnSOD) and XO, along with muscle levels of oxidative damage, were also measured before and after a series of repetitive apneas to further examine the cellular mechanisms that protect seals against apnea-induced hypoxemia and ischemia/reperfusion. We hypothesize that 1) apnea does not increase muscle or systemic oxidative damage in elephant seals and that 2) HIF-1α and Nrf2 nuclear content, antioxidant enzymes and Mb expression increase in the skeletal muscle of elephant seals after repetitive apneas.

Methods

All work was realized under the National Marine Fisheries Service marine mammal permit # 87-1743. All procedures were approved by the Institutional Animal Care and Use Committee's of the University of California Merced and Sonoma State University.

Animal handling

Eleven-week old (7 wk post-weaned, n = 6) northern elephant seal pups (*Mirounga angustirostris* Gill 1866) were captured without sedation by rolling them into a transport cage, one at a time, at Año Nuevo State Reserve (30 km north of Santa Cruz, CA) and transported to Sonoma State University (Ronhert Park, CA) where the rest- and voluntary submersion-associated apnea experiments were conducted. Upon arrival to the laboratory, animals were allowed to recover from transportation for 6 h. Animals were then sedated with 1 mg/kg tiletamine hydrochloride and zolazepam hydrochloride (telazol; Fort Dodge Animal Health, Fort Dodge, IA) administrated intramuscularly prior to venous catheterization. Immobilization was maintained with 100 mg intravenous injections of ketamine (Fort Dodge Animal Health) as needed. Sedation with telazol and ketamine does not activate the hypothalamic-pituitary-adrenal axis in elephant seals (8). With the exception of a different catheter tube, the catheterization procedure was similar to that previously reported for elephant seal pups (38). Eupenic samples (matching blood and muscle biopsies) were collected at this time, when the animals had been continuously breathing for at least two hours. Animals were allowed to recover from the catheterization overnight before the beginning of the apnea trials.

Rest- and voluntary submersion-associated apneas

Caged animals were allowed to sleep in a large quiet room for up to 6 h. For the purposes of this paper, "rest-associated apneas" were characterized by breath-holds with eyes closed, occasional facial twitching and lack of body movements (7, 41, 49). Following the rest-apnea trials, animals were then transferred to a shallow water tank and allowed to submerge freely for another 6 h. For the rest- and voluntary submersion-associated apnea studies, blood samples were collected at 1, 3, 7 and 9 min during breath-holds and at 1, 3 and 5 min after the bouts (recovery). Venous oxygen partial pressure (Po$_2$) was measured using a blood gas analyzer (Rapidlab 845, Bayer Diagnostic Division, Milan, Italy) to corroborate blood oxygen depletion during breath-holds (49).

Sample collection

Blood samples were collected from the extradural spinal vein into pre-chilled EDTA-treated collection tubes containing 10 μL mL^{-1} protease inhibitor cocktail and 0.005% BHT (Sigma, St. Lous MO). Samples were centrifuged for 15 min at 3000 x g at 4°C. Plasma was aliquoted into cryo-vials, frozen by immersion in liquid nitrogen and stored at -80°C. Muscle biopsies (ca. 10 mg) were collected during catheterization (eupnea) and immediately after the end of the submersion experiments (post-apnea), without sedation, using sterile 14G x 15 cm Tru-Cut biopsy needles (Cardinal Health, McGaw Park, IL). Samples were snap-frozen in liquid nitrogen and stored at -80°C.

Plasma measurements

Plasma levels of 8-isoprostanes (8-isoPGF$_{2\alpha}$) were measured using a competitive EIA assay kit (Cayman Chemical, Ann Arbor, MI). Samples were extracted using ethanol, 30% acetic acid and C-18 solid phase extraction cartridges. Plasma and muscle levels of nitrotyrosine (NT) and 4-hydroxynonenal (HNE), as well as plasma carbonyls, were also measured using commercially available ELISA kits (Cell BioLabs, San Diego, CA) (54). Plasma XO activity, xanthine and HX concentrations were measured using an Amplex® Red assay kit (Molecular Probes, Eugene, OR).

Western blot

Nuclear and cytosolic protein fractions were prepared from frozen tissue samples using the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL). Total protein content in both fractions was measured using the Bio-Rad Bradford protein assay (Bio-Rad Laboratories). Twenty μg of cytosolic or 10 μg of nuclear protein were mixed with Laemmli sample buffer, boiled and resolved in 4-15% Tris-HCl gradient gels. Proteins were electroblotted for 25 min at 25 V using a Bio-Rad Trans Blot SD semi-dry cell or overnight at 34 V using the Bio-Rad Mini Protean transfer apparatus (for XO analyses) onto 0.45 μm nitrocellulose membranes. Membranes were blocked one hour at room temperature and incubated overnight with primary antibodies against Cu,Zn SOD (Assay Designs, Ann Arbor, Michigan), Mb (Abcam, Cambridge, MA), catalase, HIF-1α, Nrf2 or XO (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 to 1:4000. Membranes were washed, incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5000, re-washed, and developed with the Immun-Star Western C kit (Bio-Rad). Blots were visualized using a Chemi-Doc XRS system (Bio-Rad) and quantified by using Bio-Rad's Quantity One software. Percent change from eupnea was calculated after band densities were normalized against actin (cytolosic proteins) or lamin (transcription factors).

Statistics

Results from different time points were analyzed using general linear mixed models with individual seal as a random effect subject term. Means of eupnea and post-apnea muscle protein measurements were compared using paired t-tests. Means (± SEM) were considered significantly different at $p < 0.05$. Statistical analyses were performed with the SYSTAT© 11.0 software (SPSS, Richmond, CA, USA).

Results

Partial pressure of O_2

Venous Po_2 was measured during rest- and voluntary submersion-associated apneas to corroborate blood oxygen depletion during breath-holds. Venous Po_2 decreased ($p < 0.05$) from 51 ± 2 mm Hg at 1 min to 30 ± 2 mm Hg at 7 min during rest-associated apneas and from 52 ± 4 mm Hg at 1 min to 35 ± 1 mm Hg at 9 min during voluntary submersion-associated apneas (Figure 1).
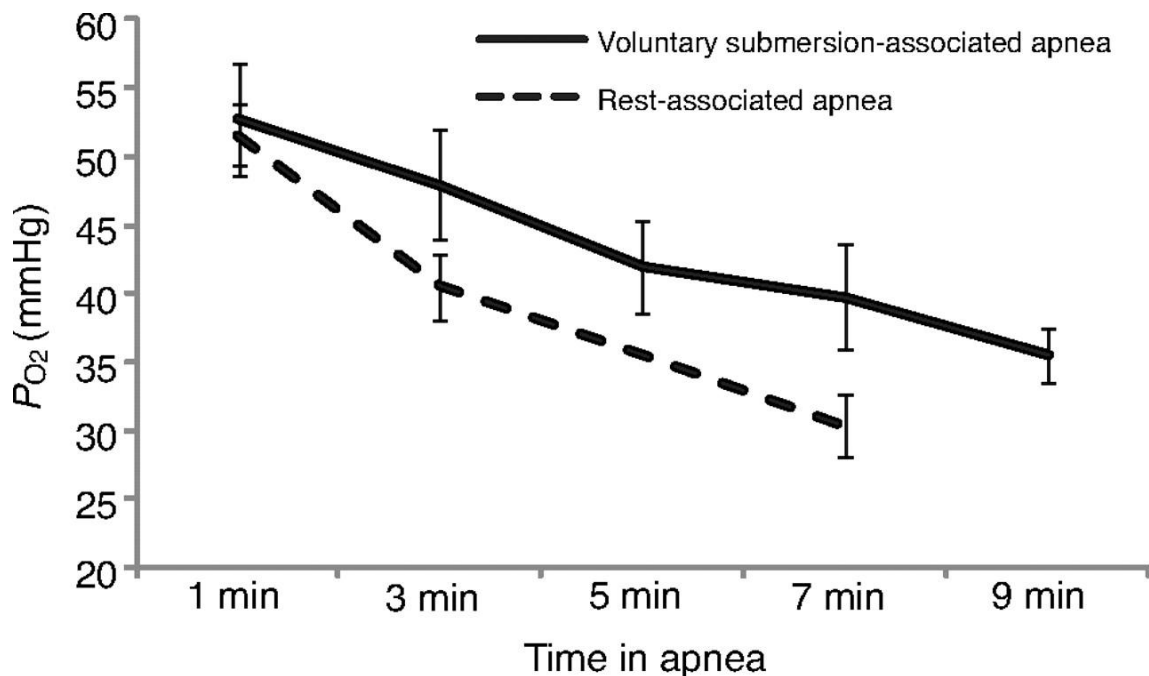

Figure 1. Rest- and voluntary submersion-associated apneas promote blood oxygen depletion in elephant seal pups. Mean $\pm$ (SEM) venous oxygen partial pressure (Po_2) values (mm Hg) during rest- and voluntary submersion-associated apneas.

Purine metabolism

Plasma content of xanthine and HX was measured before, during and after rest- and voluntary submersion-associated apneas to test whether apnea increases the circulating content of these ATP degradation products. HX content was higher ($p < 0.05$) during the recovery period (190 ± 20 mM) than at the end (60 ± 7 mM at 7 min) of the rest-associated apneas (Figure 2A). HX circulating concentration was also higher ($p < 0.05$) at the end (5 and 7 min) than at the beginning (1 and 3 min) of the voluntary submersions (170 ± 20 and 150 ± 20 vs 60 ± 20 and 60 ± 7 mM, respectively) and returned to eupnea levels (120 ± 10 mM) during recovery (110 ± 10 mM) (Figure 2A). Xanthine circulating content was higher ($p < 0.05$) during the recovery period (500 ± 50 mM) than at the end of the rest-associated apneas (230 ± 40 mM at 5 min and 260 ± 30 mM at 7 min). Plasma content of xanthine also decreased at the beginning of the

voluntary submersions (120 ± 40 mM) and gradually increased until it reached eupnea levels (340 ± 50 mM) at 5 min (380 ± 70 mM) ($p < 0.05$) (Figure 2B).

Plasma XO activity and muscle protein expression was measured to test whether this enzyme, which is responsible for $O_2^{\bullet-}$ and H_2O_2 production upon reperfusion, increases in response to apnea. Plasma XO activity did not change during or after rest-associated apneas but was decreased ($p < 0.01$) during the beginning of the voluntary submersions (0.5 ± 0.06 mU mL^{-1} at 1 min and 0.7 ± 0.1 mU mL^{-1} at 3 min) and increased gradually to eupnea levels (1.5 ± 0.1 mU mL^{-1}) at the end of the submersions (1.4 ± 0.1 mU mL^{-1} at 7 min and 1.6 ± 0.3 mU mL^{-1} at 9 min) and during recovery (1.4 ± 0.1 mU mL^{-1}) (Figure 2C). Mean muscle XO protein content increased ($p < 0.05$) 25% after apnea (Figure 2D). Taken together, these results suggest that rest- and voluntary submersion-associated apneas can potentially increase oxidant production in elephant seals due to the HX/XO pathway.
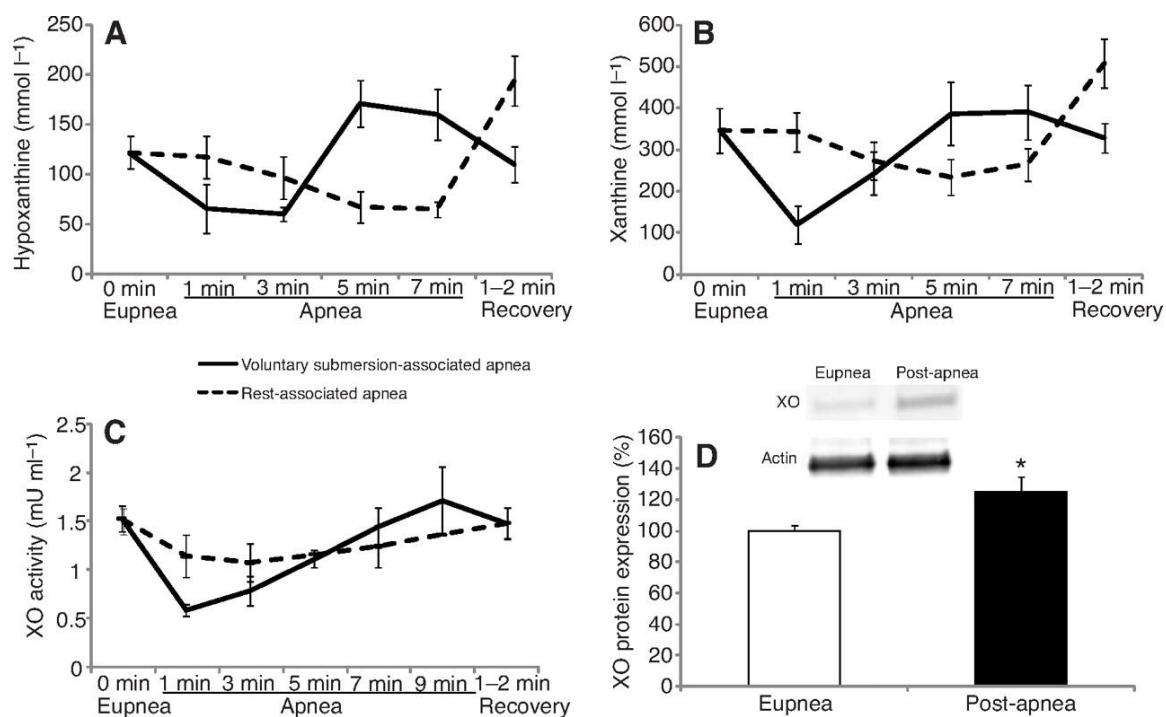

Figure 2. Rest- and voluntary submersion-associated apneas increase hypoxanthine, xanthine and xanthine oxidase in elephant seals. Mean ± (SEM) **A)** plasma hypoxanthine (HX) **B)** plasma xanthine concentration, **C)** plasma xanthine oxidase (XO) activity before, during and after rest- and voluntary submersion-associated apneas, **D)** XO muscle protein expression before (eupnea) and after repetitive apneas in elephant seal pups. * denotes significant ($p < 0.05$) difference from eupnea.

Oxidative damage

Plasma content of 8-isoPGF$_{2\alpha}$, HNE, NT, and protein carbonyls, along with muscle levels of HNE and NT were measured to test whether apnea increases oxidative

damage in elephant seal pups. No differences in the plasma or the muscle levels of 8-isoPGF$_{2\alpha}$, NT or protein carbonyls increased in response to rest- or voluntary submersion-associated apneas (Figures 3A, 3B and Table 1). Only muscle HNE levels increased ($p < 0.05$) after repetitive apneas (0.69 ± 0.06 µg mL^{-1} vs 0.96 ± 0.07 µg mL^{-1}) suggesting that elephant seals do not suffer the detrimental effects of apnea-induced hypoxemia and ischemia/reperfusion that have been reported in terrestrial mammals.
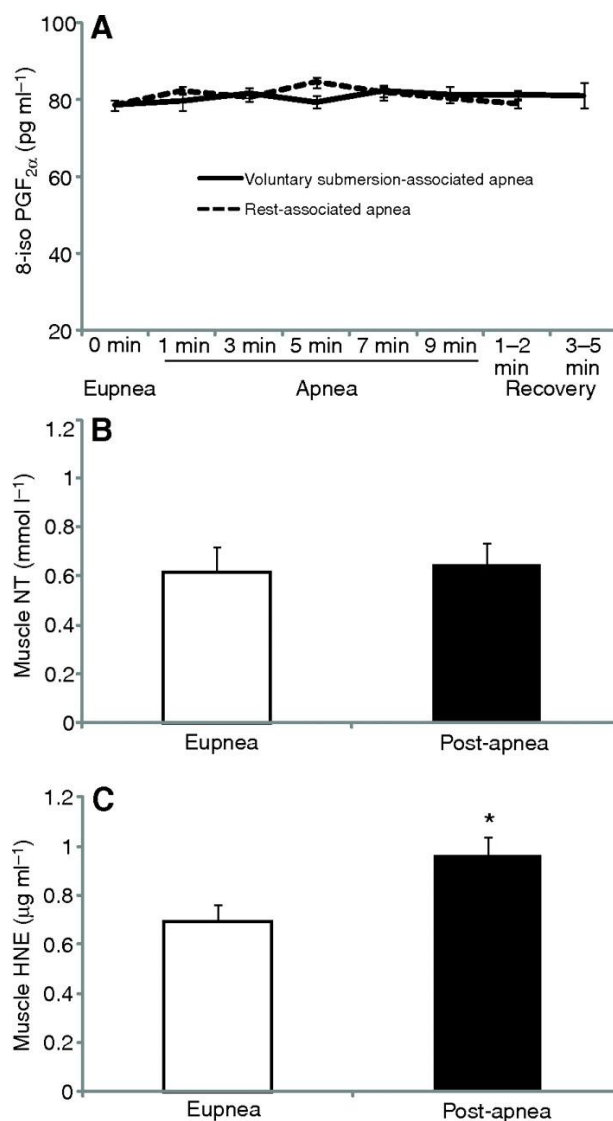
Figure 3. Rest- and voluntary submersion-associated apneas do not induce oxidative damage in elephant seals. Mean $\pm$ (SEM) **A)** plasma concentration of 8-isoPGF$_{2}$ before, during and after rest- and voluntary submersion-associated apneas, **B)** muscle nitrotyrosine (NT) concentration, and **C)** muscle 4-hydroxynonenal (HNE) concentration before (eupnea) and after repetitive apneas in elephant seal pups. * denotes significant ($p < 0.05$) difference from eupnea.

Table 1. Mean (±s.e.m.) plasma levels of oxidative damage markers before, during and after rest- and voluntary submersion-associated apnea in elephant seals

	HNE ($\mu g\,ml^{-1}$)	NT ($nmol\,l^{-1}$)	Plasma carbonyls ($nmol\,mg^{-1}$ protein)
Eupnea	2.2±0.08	1.4±0.10	0.37±0.02
Rest-associated apnea			
1 min	2.1±0.13	1.5±0.07	0.34±0.03
7 min	1.8±0.19	1.4±0.10	0.35±0.02
Recovery (1–2 min)	2.3±0.13	1.4±0.07	0.31±0.04
Voluntary submersion-associated apnea			
1 min	1.9±0.09	1.4±0.05	0.31±0.02
7 min	2.0±0.09	1.4±0.06	0.34±0.03
Recovery (1–2 min)	1.7±0.05	1.5±0.09	0.33±0.06

HNE, 4-hydroxynonenal; NT, nitrotyrosine.

Muscle adaptive responses to apnea

HIF-1α and Nrf2 nuclear content along with antioxidant enzymes (Cu,ZnSOD and catalase) and Mb protein expression were measured before and after repetitive apneas in muscle of northern elephant seal pups, to test whether apnea up-regulates these key proteins involved in the adaptive responses to hypoxia and oxidative stress. Muscle protein expression of Cu,Zn SOD and catalase increased ($p < 0.05$) 84% and 48%, respectively (Figures 4A and 4B), while nuclear content of Nrf2 increased ($p < 0.05$) 56% (Figure 4C) after apnea. The nuclear content of HIF-1α and the protein expression of Mb also increased ($p < 0.05$) 68% and 60% respectively (Figures 5A, 5B), suggesting that apnea in seals activates the adaptive responses to hypoxia and oxidative stress, and that HIF-1α Mb and the antioxidant system, play a role in seal's tolerance against apnea-induced hypoxemia and ischemia/reperfusion.

Figure 4. Rest- and voluntary submersion-associated apneas activate the adaptive response to oxidative stress in elephant seals. Mean ± (SEM) muscle protein expression of **A)** superoxide dismutase 1 (Cu,ZnSOD) and **B)** catalase before (eupnea) and after repetitive apneas in elephant seal pups. **C)** Nuclear factor erythroid 2-related factor 2 (Nrf2) nuclear content before (eupnea) and after repetitive apneas in elephant seal pups. **D)** Representative western blot analyses of Nrf2, Cu,ZnSOD and catalase protein expression in northern elephant seal pups. * denotes significant (p < 0.05) difference from eupnea.
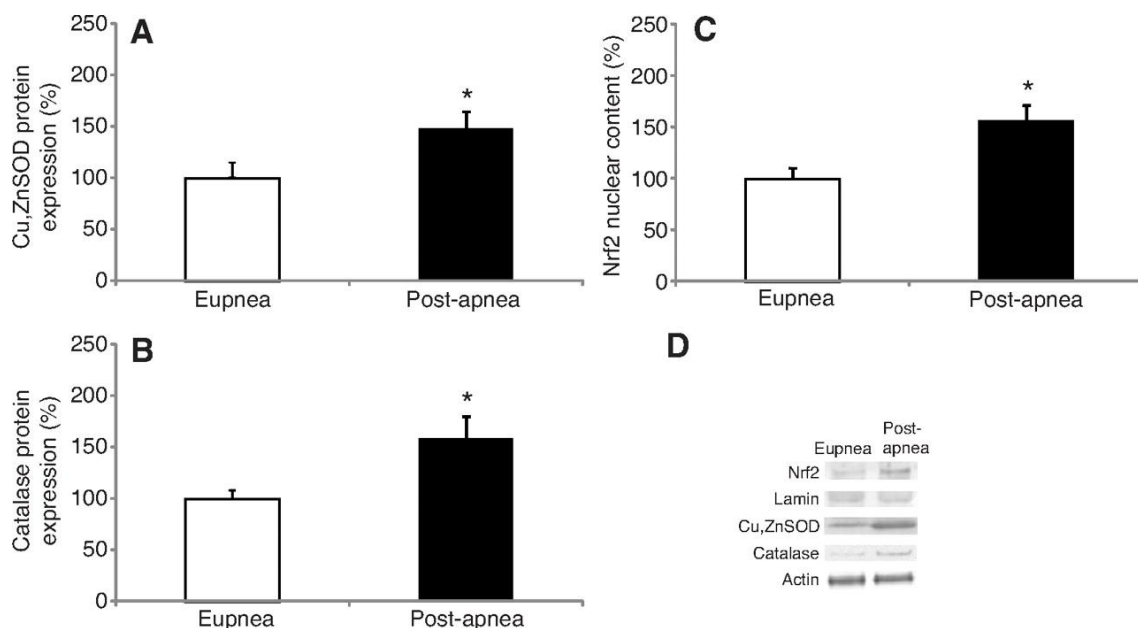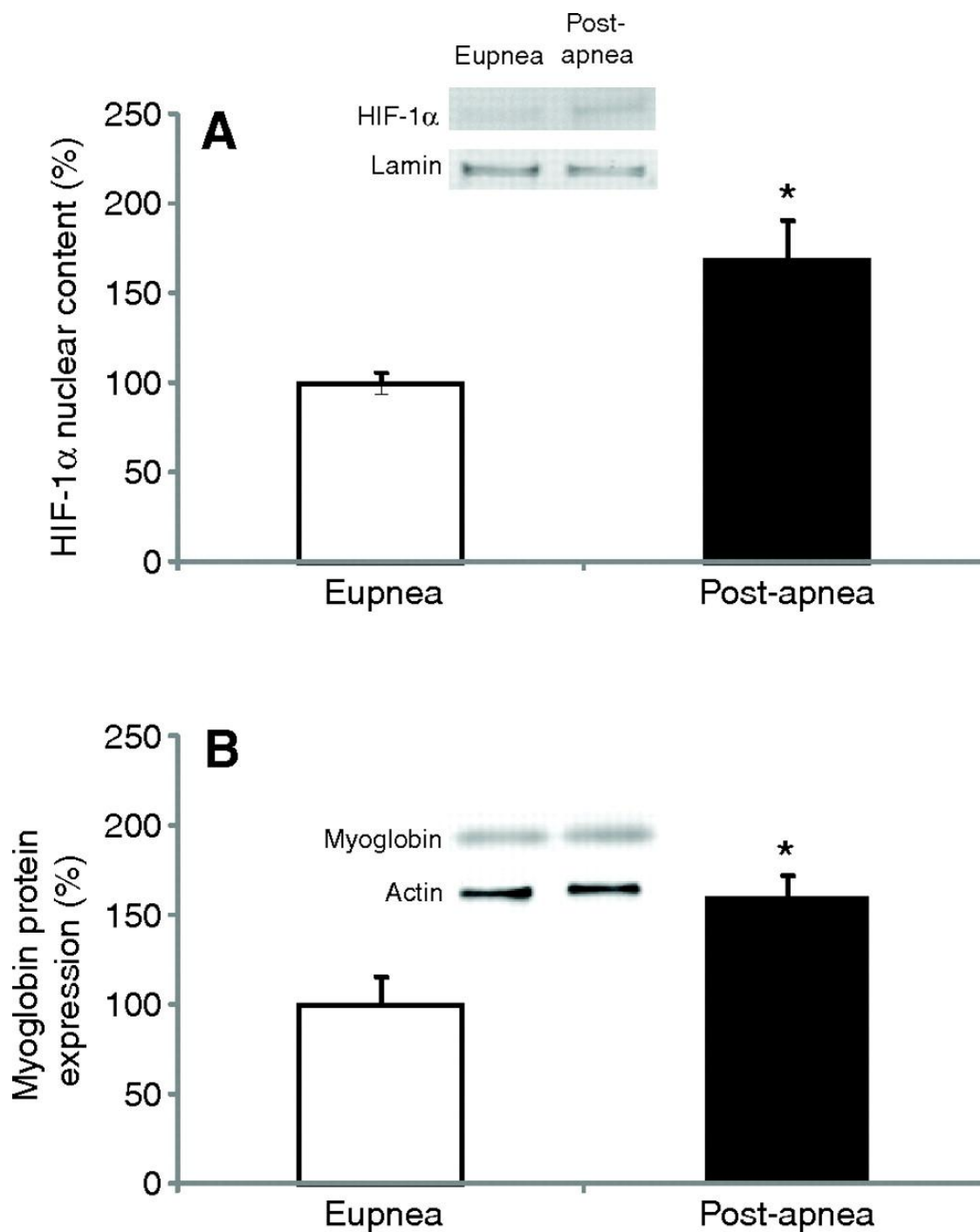
Figure 5. Rest- and voluntary submersion-associated apneas activate the adaptive response to hypoxia in elephant seals. Mean ± (SEM) **A)** nuclear content of hypoxia inducible factor 1α (HIF-1α) and **B)** protein expression of myoglobin (Mb) in skeletal muscle of elephant seal pups before (eupnea) and after repetitive apneas. * denotes significant (p < 0.05) difference from eupnea.

Discussion

Blood reoxygenation after hypoxemia and ischemia/reperfusion are critical conditions that in terrestrial mammals increase oxidant production and oxidative damage (9, 32). Seals, however, naturally experience repetitive episodes of hypoxemia and ischemia/reperfusion associated with breath-holding during diving and sleeping (11, 28, 44). The present study demonstrates that despite inducing hypoxemia and increasing xanthine, HX and XO, apnea does not increase systemic oxidative damage or muscle protein nitration in elephant seals. Moreover, repetitive apneas stimulate protective responses against hypoxia and oxidative stress in the skeletal muscle of elephant seals.

The observed differences in the trends of circulating xanthine, HX and XO during rest-associated apneas and voluntary submersions may be due differences in the degreee of the diving reponse observed during bouts of breath-holding with or without face immersion in the water. It's been consistently found that breath-holding with face immersion produces a more profound response (greater bradycardia and hypometabolism) than breath-holding alone (for a complete review see (10). Also, the decrease in circulating xanthine, HX and XO activity at the first minute of the voluntary submersions can be explained by the fact that submersion produces an immediate and profound splenic contraction without any further significant decrease in splenic volume after minute 2 resulting in higher hematocrit in venous ciruclation at the beginning of a submersion than during eupnea (51).

While routinely diving at sea (>10 min) results in extreme hypoxemia with near total blood oxygen depletion (venous Po_2 values = 2 mm Hg; arterial Po_2 values = 12 mm Hg) (33), during rest-associated apneas, venous Po_2 values in juvenile elephant seals decline exponentially but only to about 21·mm Hg after 9 min (49). Furthermore, the decline in muscle blood flow during rest-associated apneas is gradual, suggesting that muscle ischemia during this condition is incomplete (42). These considerations, along with the lack of lactate accumulation, suggest that aerobic metabolism is maintained at low oxygen tensions during rest-associated apneas in elephant seals (49), and partially explain the lack of systemic oxidative damage and muscle protein nitration, despite the observed increases in xanthine, HX and XO, in response to rest- and voluntary submersion-associated apneas in elephant seal pups.

In contrast to extended ischemia, which promotes necrosis and triggers oxidant-mediated tissue damage upon reperfusion (15), short ischemia/reperfusion intervals (preconditioning) attenuate reperfusion injury in myocardium and skeletal muscle (34, 36, 39). Small rates of oxidant generation during preconditioning are needed for the activation of protective pathways upon reperfusion (1). XO-derived H_2O_2 has been shown to increase superoxide dismutase (SOD) and glutathione peroxidase mRNA, protein content and activity (14, 61, 65), as well as Nrf2 activation (30, 52). The present study shows that repetitive exposure to breath-holds in seals increases muscle XO protein expression along with an increase in Nrf2 nuclear content, SOD1 and catalase protein expression, without increasing muscle NT or circulating 8-isoPGF$_{2\alpha}$, HNE, NT, or protein carbonyls, suggesting that XO-derived oxidant production does not induce oxidative damage, and rather contributes to the activation of the protective response against oxidative stress. The latter is consistent with the observed increases in plasma

xanthine, HX and XO activity at the end of the apneas and during recovery, with the previously reported increases in HX after *ex vivo* exposure to ischemia in seal heart and kidney (12), and with the increased content of muscle HNE after repetitive apneas, since HNE also contributes to the activation of the Nrf2-mediated oxidative stress response (48, 50).

Nrf2 induces the transcription of genes involved in antioxidant defense such as SOD1 and catalase (18, 21). This transcription factor is, under unaltered conditions, bound to its repressor protein Keap1 in the cytosol, which targets its continuous proteosomal degradation (19). Oxidants and electrophiles, such as H_2O_2 and HNE, modify Keap1 promoting Nrf2 activation and nuclear accumulation (4, 25, 26, 64). The observed increases in XO, HNE, Nrf2, SOD1 and catalase suggest that repetitive breath-holds stimulate the hormetic response against oxidative stress in elephant seals, likely contributing to the up-regulation of their antioxidant system and likely preparing them to cope with extended ischemia and hypoxemia while at sea.

While diving, elephant seals remain submerged 90% of the time (29). When resting on land, apnea bouts comprise up to 60% of their time sleeping (3). Although bouts of sleep apnea occur in all elephant seals regardless of age, weaned pups increase the number and duration of apneas at the end of their postweaning fast, when seals are learning to swim and dive, and just prior to initiating their diving lifestyle (3). Elephant seal pups also increase their antioxidant defenses at the end of their postweaning fast suggesting that the activation of the antioxidant system in these mammals is an essential part of their developmental process and prepares them to dive, which is the next step in their life history (54, 57). The present results suggest that, since apnea stimulates the adaptive response to oxidative stress, and the number and duration of apneas increase with development in elephant seal pups, apnea is essential to prime the seal's antioxidant mechanisms that allow them to cope with the subsequent diving-induced hypoxemia and ischemia/reperfusion/reoxygenation while at sea.

Maturation-related increases in antioxidant capacity have also been documented in the deep-diving hooded seal (55, 56), in which oxygen stores (hemoglobin and Mb content), acid buffering capacity, and aerobic and anaerobic enzyme activities, also increase with maturation in the skeletal muscle (5, 6, 31). In the present study, repetitive apneas increased Mb expression and HIF-1α nuclear content in the skeletal muscle of elephant seals. Under normoxia, HIF-1α is hydroxylated at proline and asparagine residues, which allows its interaction with the von Hippel-Lindau protein, causing HIF-1α ubiquitination and its subsequent proteosomal degradation (20). Under hypoxia, hydroxylation of HIF-1α is prevented causing its interaction with a constitutive protein (HIF-1α) to form a dimer (HIF-1), which translocates into the nucleus up-regulating the expression of genes involved in angiogenesis, erythropoiesis, energy metabolism, apoptosis, heat shock and cell proliferation in response to hypoxia (45, 46). The role of HIF-1α as a physiological mediator of the protective effects of ischemic preconditioning has been extensively demonstrated (2, 16, 47). The observed increases in HIF-1α nuclear content after repetitive breath-holds suggest that rest-and voluntary submersion-associated apneas also contribute to the preconditioning of the seal's muscle by activating the HIF-1-mediated adaptive response to hypoxia (22, 23, 62). The observed increase in

Mb protein content further supports the later idea since Mb expression is up-regulated in response to hypoxia in mammalian tissues and cells (24), and without physical activity in vertebrates adapted to dive, such as penguins (43) and seals (37).

In summary, results of the present study demonstrate that rather than causing systemic oxidative damage and despite inducing hypoxemia and increasing xanthine, HX and XO, rest- and voluntary submersion-associated apneas stimulate adaptive responses to hypoxia and oxidative stress in elephant seals by increasing HIF-1α, Mb, Nrf2, SOD1 and catalase (Figure 6). Thus, experiencing repetitive breath-holds while on land appears to precondition developing elephant seal pups by up-regulating their antioxidant system, HIF-1α and Mb content, and thus preparing them to tolerate the repetitive bouts of extended hypoxemia and ischemia/reperfusion/reoxygenation associated with their ensuing diving lifestyle. Understanding the mechanisms that allow adapted mammals to naturally avoid oxidative damage has the potential to advance therapeutic approaches for oxidative stress-induced pathologies in the long term.
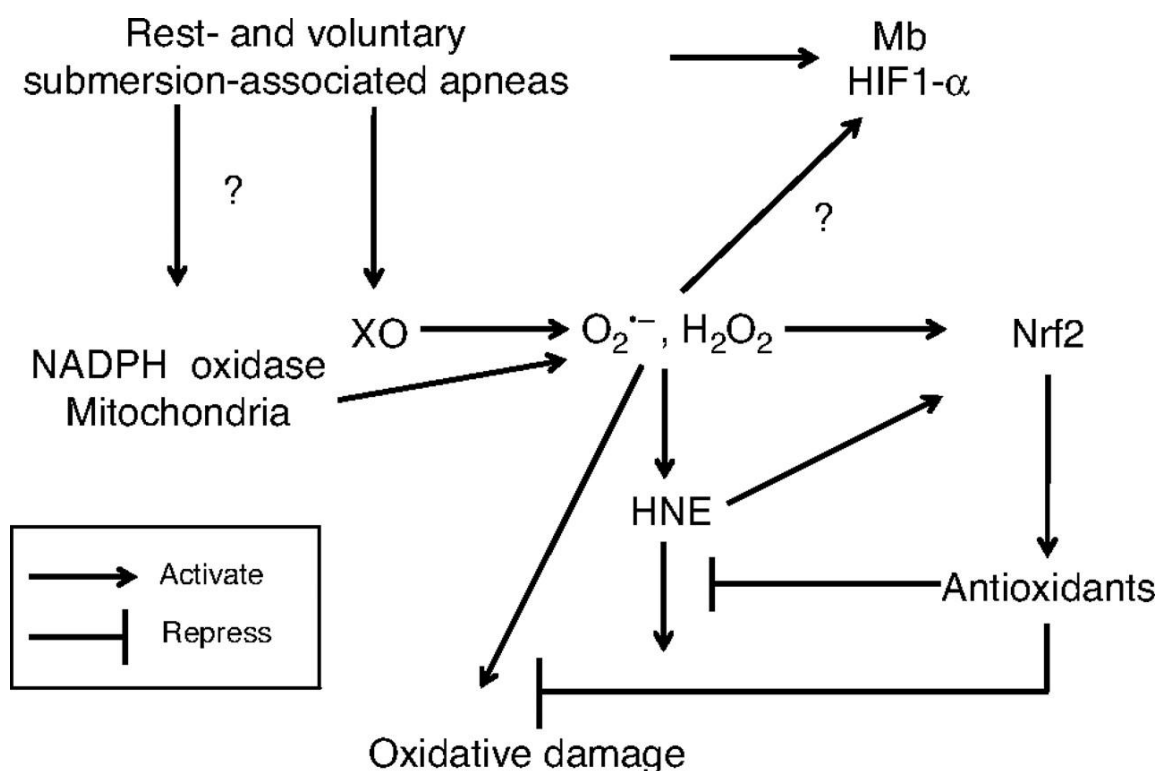
Figure 6. A schematic representation of the proposed mechanisms leading to the activation of the protective responses against oxidative stress and hypoxia in elephant seal pups exposed to repetitive rest- and voluntary submersion-associated apneas. H_2O_2 = hydrogen peroxide, HIF-1α = hypoxia inducible factor 1α HNE = 4-hydroxynonenal, Mb = myoglobin, Nrf2 = NF-E2-related factor 2, $O_2^{\bullet-}$ = superoxide radical, XO = xanthine oxidase.

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Conclusions and Future Directions

Extreme behaviors such as extended food deprivation and apneustic breathing are natural components of the life history of the northern elephant seal. In terrestrial mammals, these potentially detrimental conditions are commonly associated with increased oxidant production, antioxidant depletion and oxidative stress. In the northern elephant seal, however, neither prolonged fasting nor apneustic breathing increase oxidative stress. This work examined the physiological mechanisms northern elephant seals evolved to avoid the oxidative stress associated with these conditions.

Early studies have shown that the content and activity of endogenous antioxidants are higher in seals than in terrestrial mammals, which can help to counteract increases in oxidant generation. Our results show that the antioxidant system of the elephant seal is tightly regulated in response to prolonged fasting and repetitive apneas, and that oxidants derived from such conditions may play a role in triggering adaptive responses.

Although we demonstrated the role of Ang II in stimulating the antioxidant system of the elephant seal by activating Nrf2 during prolonged fasting, the potential role of other hormones in eliciting similar responses remains elusive. Several studies have shown that circulating cortisol increases with fasting. The physiological effects of cortisol are mediated by the glucocorticoid receptor (GR). Upon glucocorticoid binding, GR translocates into the nucleus where it regulates several targets such as the redox-sensitive transcription factors nuclear factor-kB and activator protein-1. We recently established a pharmacological system that will allow us to antagonize GR and to manipulate cortisol and GR levels in elephant seals. Thus, the use of this system may prove to be fruitful in the elucidation of the potential role of cortisol in regulating redox-mediated adaptive responses during extended food deprivation in a large mammal.

How the antioxidant system of the elephant seal is activated during free diving also remains elusive. While sleep apneas generate mild hypoxemia and incomplete ischemia/reperfusion that likely result in a preconditioning response, diving at sea involves extreme hypoxemia and severe ischemia/reperfusion that may promote different regulatory mechanisms. Logistic limitations in conducting biochemical and molecular studies on animals diving at sea currently prevent us from elucidating these mechanisms.

The role of oxidative stress in the aging process of large long-lived mammals such as the elephant seal also remains under investigation. Based on classic free radical theories, which state that oxidant production and oxidative damage accumulation increase with age, contributing to senescence and cellular dysfunction; diving vertebrates may be particularly susceptible to aging due to chronic exposure to diving-induced hypoxemia and ischemia/reperfusion. A few studies conducted in old seals suggest either that they efficiently cope with age-associated increases in oxidant generation or that senescence in seals is not mediated by increased oxidative stress. The aging process in long-lived diving vertebrates undoubtedly warrants further investigation.

As the field of redox biology moves forward, the incorporation of new concepts and views increases our understanding of the molecular processes driving physiology and disease. In the same way, the use of natural models of tolerance against pathological conditions can provide insight to the contributions of redox signaling and oxidative stress

to a number of pathologies. The elucidation of the mechanisms seals evolved to cope with behaviors associated with increased oxidant generation can therefore enhance our appreciation for the evolution of such mechanisms, but also have the potential to contribute to our understanding of mammalian adaptive responses to such conditions.