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RESEARCH ARTICLE

Prolonged fasting activates Nrf2 in post-weaned elephant seals

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SUMMARY

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 upregulation of antioxidant defenses during prolonged fasting remains elusive. Therefore, we investigated whether 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 167% at week 7. Plasma angiotensin II (Ang II) and transforming growth factor β (TGF-β) were 5000% and 250% higher at week 7 than at week 1. Phosphorylation of Smad2, 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 430% at week 7, while Nox4 protein expression, which can activate Nrf2, was 170% higher at week 7 than at week 1. These results demonstrate that prolonged fasting activates Nrf2 in elephant seals and that RAS stimulation can potentially result in increased Nox4 through Smad phosphorylation. The results also suggest that Nox4 is essential to sustain the hormetic adaptive response to oxidative stress in fasting seals.

Key words: antioxidants, hormesis, Nox4, oxidative stress, starvation, renin–angiotensin system.

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INTRODUCTION

Spontaneous long-term fasting is an integral part of the life history of phocid seals (Castellini and Rea, 1992). The northern elephant seal *Mirounga angustirostris* (Gill 1866) annually undergoes natural periods of prolonged fasting, while breeding, molting and weaning (Le Boeuf et al., 1973). 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) (Ortiz et al., 2003a; Ortiz et al., 2003b; Ortiz et al., 2001) and the renin–angiotensin system (RAS) (Ortiz et al., 2006; Ortiz et al., 2000), as well as in the onset of insulin resistance-like conditions (Fowler et al., 2008; Viscarra et al., 2011a; Viscarra et al., 2011b; Viscarra et al., 2012).

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 (Ceriello and Motz, 2004; Costantini et al., 2011; Evans et al., 2003; Romero and Reckelhoff, 1999; Sorensen et al., 2006; Rocha et al., 2008; Sowers, 2002; Szkudelski et al., 2004). In the northern elephant seal, prolonged fasting increases Nox4 and xanthine oxidase (XO) without increasing oxidative damage or inflammation (Soñanez-Organis et al., 2012; Vázquez-Medina et al., 2010). Systemic and muscle markers of oxidative damage (F_2-isoprostanes, nitrotyrosine, C-reactive protein, tumor necrosis factor α (TNF-α), 4-hydroxynonenal, protein carbonyls) remain unchanged after 2 months of absolute fasting in seal pups (Vázquez-Medina et al., 2010). Fasting-related increases in the activity and protein expression of several antioxidant enzymes and glutathione (GSH) levels (Vázquez-Medina et al., 2010; Vázquez-Medina et al., 2011b), as well as increased purine recycling (Soñanez-Organis et al., 2012), likely contribute to the prevention of oxidative damage in elephant seals.

How the antioxidant system is upregulated 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 (Jaiswal, 2004). Nrf2 induces the transcription of genes involved in antioxidant defense through its binding to the electrophilic responsive element (EpRE) (Itoh et al., 1997). Nrf2 is a member of the basic leucine-zipper NF-E2 family that is bound to its repressor protein Keap1 (kelch-like ECH-associated protein 1) under unaltered conditions (Itoh et al., 1995). Binding of Nrf2 to Keap1 targets it for ubiquitin conjugation and consequent proteosomal degradation (Itoh et al., 1997). 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 (Bloom and Jaiswal, 2003; Kobayashi et al., 2004; Kobayashi et al., 2006; Zhang and Hannink, 2003). 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 the mRNA and protein level in seal

muscle (Vázquez-Medina et al., 2011a; Vázquez-Medina et al., 2011c). Moreover, nuclear accumulation of Nrf2 increases in the skeletal muscle of the elephant seal in response to repetitive bouts of apnea-induced ischemia/reperfusion (Vázquez-Medina et al., 2011c), which are frequent at the end of the post-weaning fast (Thorson and Le Boeuf, 1994).

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 conditions, the northern elephant seal. We have previously shown that prolonged fasting increases Nox4 expression in the skeletal muscle of the elephant seal (Vázquez-Medina et al., 2010). Unlike other NADPH oxidases, Nox4 is independent of cytosolic activator subunits, and thus is constitutively active (Martyn et al., 2006; Nisimoto et al., 2010; von Löhneysen et al., 2012). Nox4 is also uniquely localized in several subcellular compartments (Anilkumar et al., 2008; Block et al., 2009; Sun et al., 2011) and produces intracellular hydrogen peroxide (H_2O_2), a potent activator of Nrf2 (Kobayashi et al., 2006), as a result of a particular property of its E-loop, which contains a highly conserved histidine that serves as a source of protons to accelerate spontaneous dismutation of superoxide to H_2O_2 (Takac et al., 2011). 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 (Rodríguez-Vita et al., 2005). 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 (Block et al., 2008; Cucoranu et al., 2005; Liu et al., 2010; Wingler et al., 2001). 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 an adaptive response and highlights the potential role of Nox4 in sustaining a hormetic protective response in fasting seals.

MATERIALS AND METHODS

Animal handling and sample collection

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.

Twenty-eight elephant seal 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 post-weaning). 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 in 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 Louis, 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 (Vázquez-Medina et al., 2010; Vázquez-Medina et al., 2011b). 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.

Ang II infusions

Fifteen additional seal pups (seven males, eight females, 1–3 weeks post-weaning) were randomly assigned to three experimental groups (N=5 per group): (1) control, (2) Ang II (3.6 µg kg^{-1}; Sigma) and (3) Ang II + AT1 blocker (ARB; 10 µg olmesartan kg^{-1}; donated by Daiichi-Sankyo, Tokyo, Japan to A.N.). Animals were immobilized as described above ('Animal handling and sample collection'). Vehicle (sterile saline), Ang II or Ang II + ARB was 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 min post-infusion. Muscle biopsies were collected before and 1 h after the intravenous infusion. Blood and tissue samples were processed as described above.

Plasma analyses

Plasma Ang II was extracted using methanol and measured using a commercial RIA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA) previously validated for the northern elephant seal (Zenteno-Savin and Castellini, 1998). 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 (Ortiz et al., 2000). Plasma TGF-β1 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 mmol l^{-1} Tris-HCl, 150 mmol l^{-1} NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) buffer supplemented with a cocktail of protease and phosphatase inhibitors (Pierce, Rockford, IL, USA) (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 micrograms of crude extract or 10 µg of nuclear protein was 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% BSA for 1 h at room temperature and incubated overnight with antibodies against Smad (Smad2: cat. no. 5339; Phospho-Smad2 Ser465/467, cat. no. 3104; Cell Signaling Technology, Boston, MA, USA) and Nrf2 (cat. no. 8882; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Nox4 protein expression was detected using monoclonal commercial antibodies (cat. no. 3174-1; Epitomics, Burlington, CA, USA) 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 (Lee et al., 2010). 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. The percentage 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); 15 µg of nuclear protein was 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 the 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 cDNA was 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 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), 3 µl of muscle cDNA and 1 µl (20 µmol l^{-1}) 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 a final extension step of 68°C for 7 min. PCR fragments of 230 bp (esNox4a), 1200 bp (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 codes for Nox4 was obtained by overlapping esNox4a, b and c sequences.

Nox4 and CuZnSOD mRNA expression were quantified using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal standard (GenBank 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 (Vázquez-Medina et al., 2011a). 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 and CuZnSODF2+CuZnSODR2) are provided in Table 1.

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
CuZnSODF2	CCTGGGCAATGTGACTGCTG
CuZnSODR2	ACACCACAAGCCAAACGACT

Statistics

Means (±s.e.m.) 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 (±s.e.m.) were compared by ANOVA adjusted for repeated measures and were considered significant at $P<0.05$. Statistical analyses were performed with the SYSTAT 11.0 software (SPSS, Richmond, CA, USA).

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 whether fasting also increases TGF-β. Plasma Ang II increased in a time-dependent manner over the course of the fast and was 500% higher at week 7 (257±36 fmol ml^{-1}) than at week 1 (5±1 fmol ml^{-1}) (Fig. 1A). Plasma TGF-β1 concentration also increased over the course of the fast, by 150% at week 5 (10±1 ng ml^{-1}) and by 250% at week 7 (14±6 ng ml^{-1}) compared with week 1 (4±1 ng ml^{-1}) (Fig. 1B). Phosphorylation of Smad2 was measured to assess its association

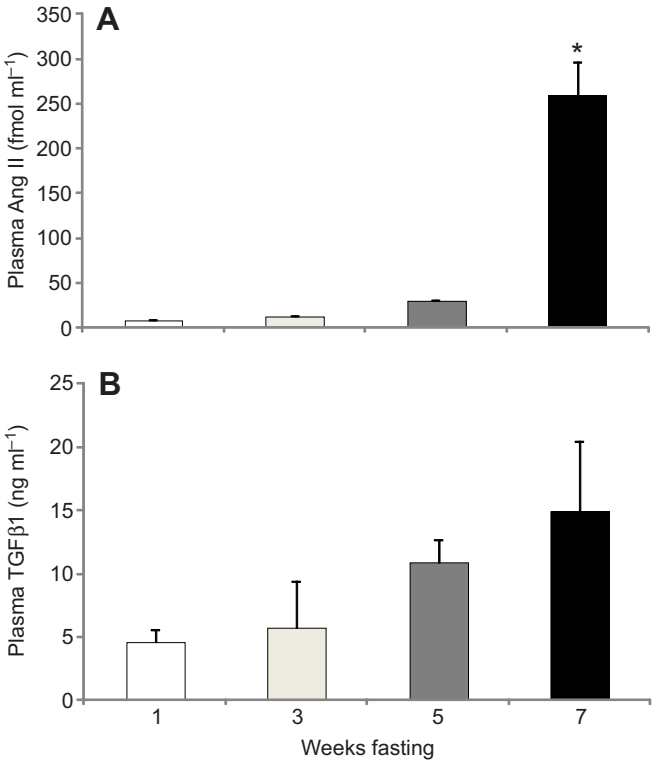

Fig. 1. Prolonged fasting increases plasma levels of angiotensin II (Ang II) and transforming growth factor β (TGF-β) in elephant seal pups. Mean and s.e.m. circulating levels of (A) Ang II and (B) TGF-β1 in elephant seals during their post-weaning fast are shown. *Significantly different from week 1 ($P<0.05$).

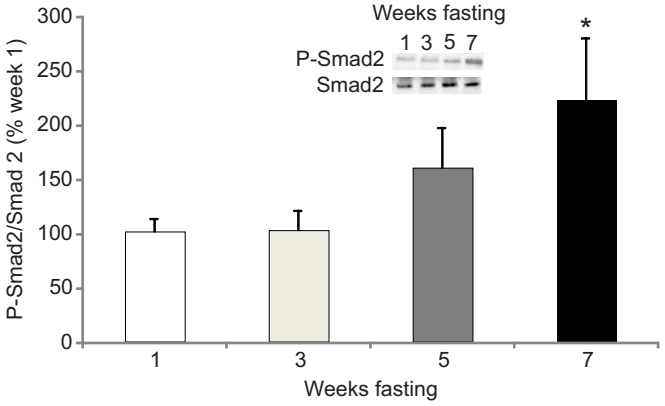

Fig. 2. Prolonged fasting increases Smad activation in elephant seal pups. Mean and s.e.m. Smad2 phosphorylation (Ser465/467) (phospho-Smad2, P-Smad2 levels) in the skeletal muscle of the elephant seal during its post-weaning fast. A representative blot is shown in the inset. *Significantly different from week 1 (P<0.05).

with increased circulating Ang II and TGF-β1, potent activators of the Smad pathway. Smad2 phosphorylation in skeletal muscle was 58% higher at week 5 and 120% higher at week 7 than at week 1 (Fig. 2).

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 ARB. The use of ARB helped confirm the contribution of Ang II to Smad phosphorylation *via* AT1 activation. In the presence of Ang II, plasma aldosterone concentration increased from 398±101 pg ml^{-1} at time 0, to a maximum of 770±172 pg ml^{-1} at 60 min, and decreased to 597±53 pg ml^{-1} at 120 min, confirming the effectiveness of Ang II infusion. The increase in circulating aldosterone in response to acutely infused Ang II was completely inhibited by the simultaneous infusion of ARB (Fig. 3A), confirming the effectiveness of the ARB dosage to block AT1. Smad2 phosphorylation increased by 84% in the Ang II-infused animals (Fig. 3B). Phosphorylated Smad2 levels did not change in the Ang II + ARB group (Fig. 3B), suggesting that Ang II stimulates the Smad pathway by activating AT1.

Prolonged fasting increases Nox4 expression

Nox4 was cloned and sequenced, and its expression was measured at the mRNA and protein level to test whether prolonged fasting, along with increasing Ang II and TGF-β, and activation of 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 1730 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%) (Fig. 4A). Conserved regions that encode the unique functional domain of Nox4 and the binding site for FAD and NADPH were found in the predicted Nox4 protein sequence of the elephant seal (Fig. 4B). Expression of Nox4 mRNA increased 430% at week 7 (530±80%) compared with week 1 (Fig. 5A) while Nox4 protein expression increased 170% at week 7 (270±47%) compared with week 1 (Fig. 5B), suggesting that along with increasing systemic RAS and TGF-β, prolonged fasting activates

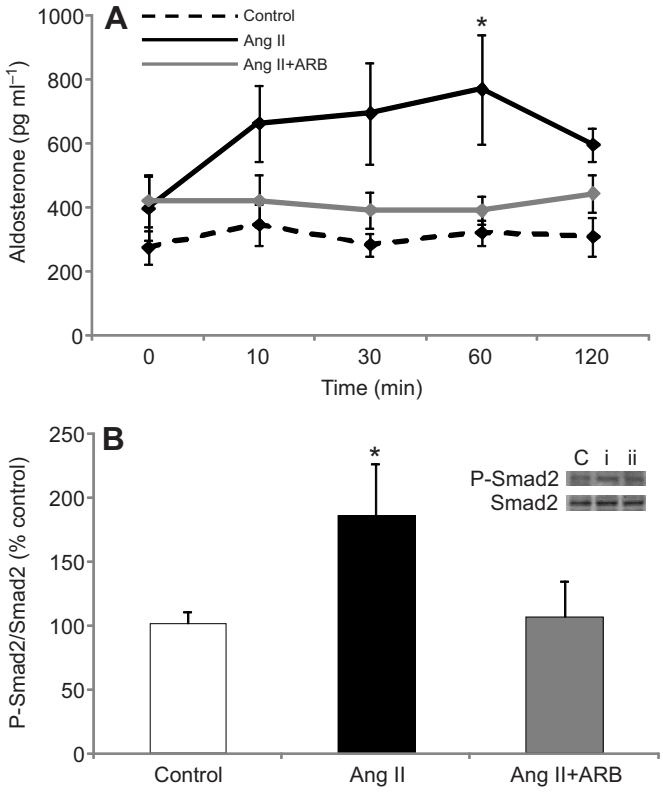

Fig. 3. Acute infusion of Ang II stimulates Smad activation in elephant seal pups. (A) Circulating aldosterone levels (mean ± s.e.m.) in response to an acute intravenous infusion of Ang II alone or with angiotensin receptor type 1 blocker (ARB) in post-weaned elephant seals. (B) Mean and s.e.m. phospho-Smad2 (Ser465/467) levels in the skeletal muscle of post-weaned elephant seals in response to acute intravenous infusion of Ang II/Ang II + ARB. A representative blot is shown in the inset (C, control; i, Ang II; ii, Ang II + ARB). *Significantly different from week 1 (P<0.05). See Materials and methods for further details.

the Smad pathway and increases Nox4 in the skeletal muscle of the elephant seal.

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 (Vázquez-Medina et al., 2010; Vázquez-Medina et al., 2011b). Nuclear levels of Nrf2 increased with fasting and were 167% higher at week 7 than at week 1 (Fig. 6A). Nrf2 transcriptional activity increased 41% at week 5 and 76% at week 7 compared with week 1 (Fig. 6B). The mRNA levels of CuZnSOD, an antioxidant gene regulated by Nrf2, also increased with fasting and were 174% higher at week 5 and 450% higher at week 7 than at week 1. Taken together, these results suggest 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.

DISCUSSION

Prolonged food deprivation increases oxidative stress in humans, rats and mice by activating Nox proteins, increasing mitochondrial oxidant

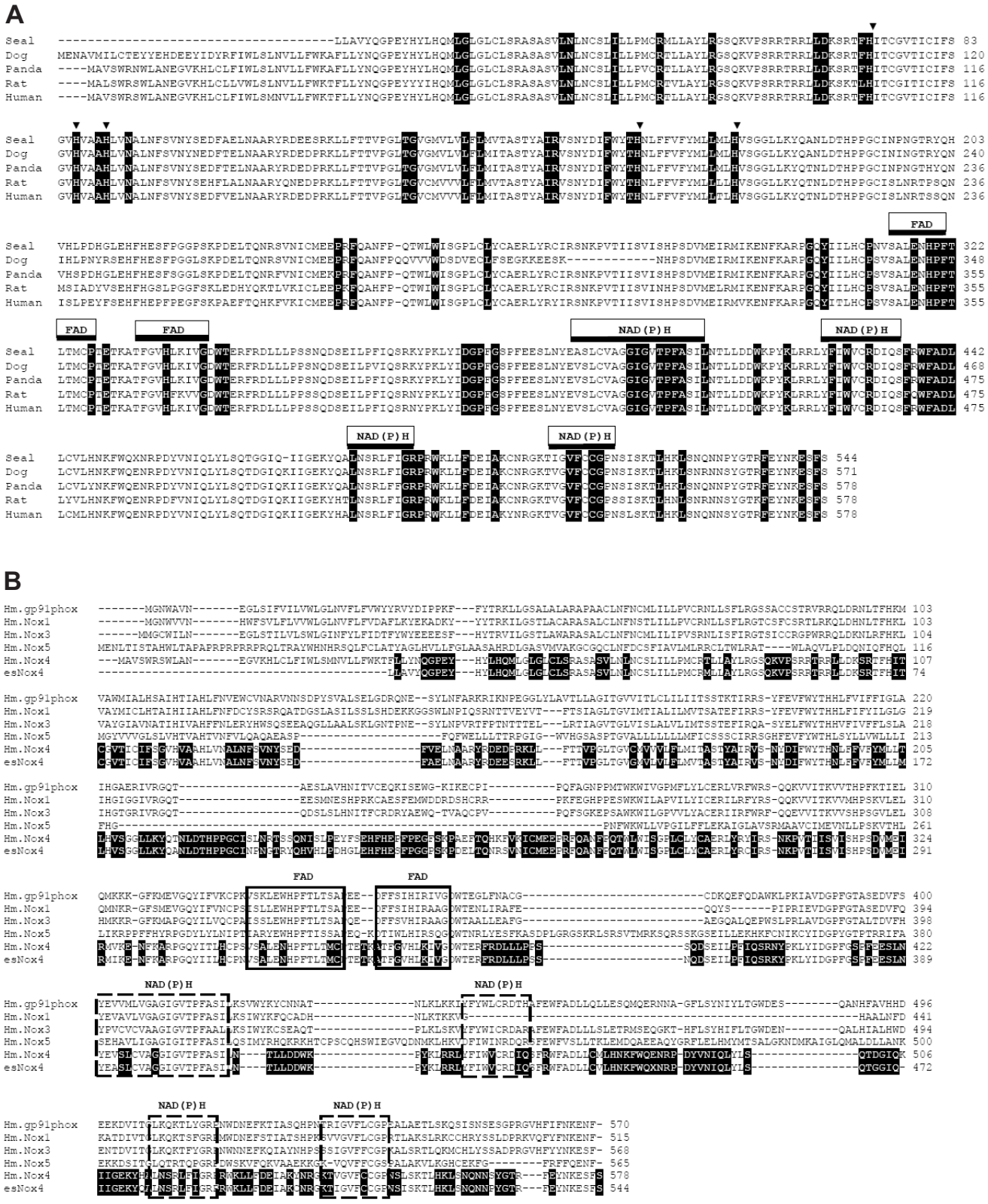

Fig. 4. Elephant seal Nox4 is a conserved enzyme that is distinct from other Nox homolog proteins. (A) Multiple alignment of amino acid sequences of Nox4 proteins. Northern elephant seal (esNox4), domestic dog (XP_542262), giant panda (XP_002927888), rat (NP_445976) and human (AAF68973) predicted amino acid sequences are included in the analysis. Black triangles indicate conserved histidine residues involved in heme–iron binding. Black boxes represent predicted transmembrane α-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 that differ from other human Nox proteins. The putative FAD and NADPH binding sites are indicated.

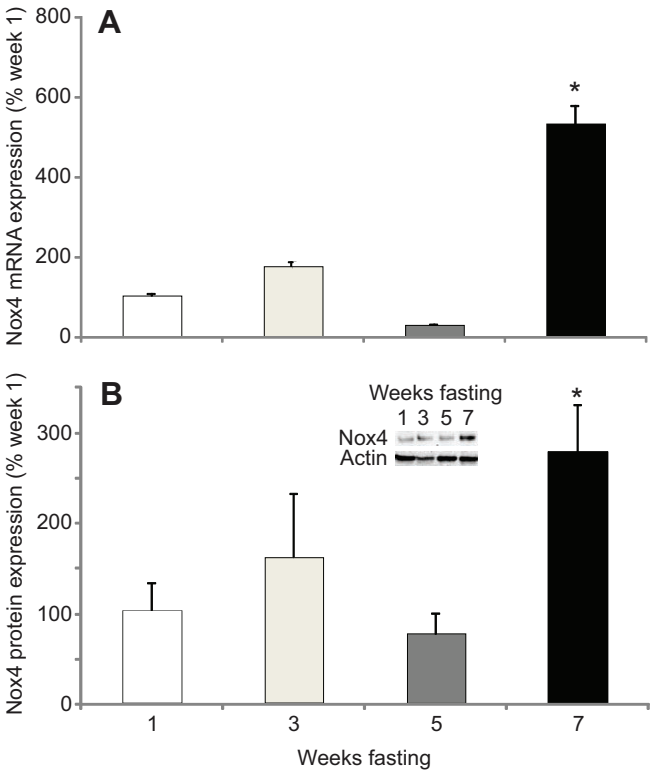

Fig. 5. Prolonged fasting increases Nox4 expression in elephant seal pups. Mean and s.e.m. Nox4 (A) mRNA and (B) protein expression during prolonged fasting in the skeletal muscle of post-weaned elephant seals. A representative blot is shown in the inset (band densities were normalized to actin). *Significantly different from week 1 (P<0.05).

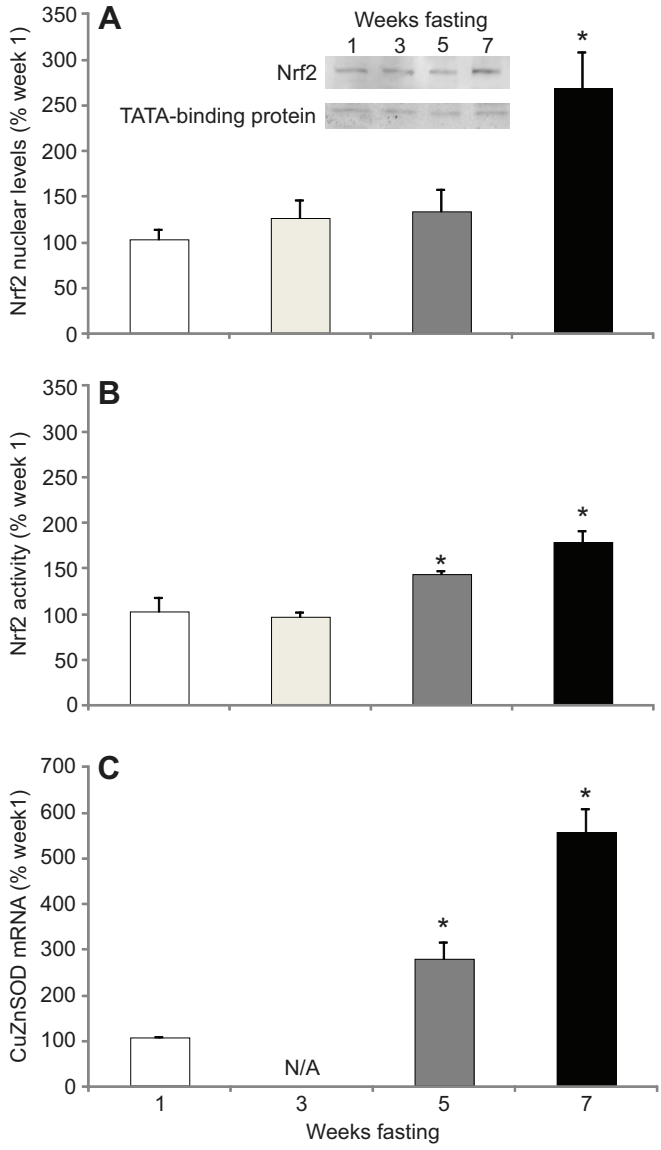

Fig. 6. Prolonged fasting activates the Nrf2/electrophilic responsive element (EpRE) pathway in elephant seal pups. Mean and s.e.m. (A) Nrf2 levels in nuclear fractions prepared from skeletal muscle, (B) binding ability of activated Nrf2 to the EpRE consensus binding site and (C) mRNA expression of CuZnSOD during prolonged fasting in post-weaned elephant seals. The inset in A shows a representative blot (band densities were normalized to TATA-binding protein). *Significantly different from week 1 (P<0.05).

generation and depleting antioxidants (Di Simplicio et al., 1997; Domenicali et al., 2001; Grattagliano et al., 2000; Mårtensson, 1986; Robinson et al., 1997; Sorensen et al., 2006; Rocha et al., 2008; Szkudelski et al., 2004; Vendemiale et al., 2001). In the northern elephant seal, however, prolonged fasting does not increase oxidative stress, likely due to increases in endogenous antioxidant defenses (Vázquez-Medina et al., 2010; Vázquez-Medina et al., 2011b). The 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 GSH 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 hormetic response that promotes stimulation of the antioxidant system by activating Nrf2 (Brigelius-Flohé and Flohé, 2011; Kobayashi and Yamamoto, 2005). Intracellular oxidants modify Nrf2, leading to its dissociation from Keap1 and its subsequent translocation into the nucleus, where it binds to EpRE (Kobayashi et al., 2006; Zhang and Hannink, 2003). Nox4 is constitutively active (Helmcke et al., 2009; Martyn et al., 2006; Serrander et al., 2007) and is uniquely localized to several intracellular membranes (Anilkumar et al., 2008; Block et al., 2009; Helmcke et al., 2009; Sun et al., 2011). Endogenous, low levels of H_2O_2 derived from Nox4 have recently been suggested to control Nrf2 activity in endothelial cells and cardiomyocytes *in vivo* (Brewer et al., 2011; Schröder et al., 2012); thus, increases in Nox4 may activate Nrf2, which upregulates the expression of antioxidant enzymes during prolonged fasting in seals, as previously reported (Vázquez-Medina et al., 2010; Vázquez-Medina et al., 2011b).

TGF-β is a potent inducer of Nox4 expression *via* the Smad signaling pathway (Cucoranu et al., 2005; Liu et al., 2010; Sturrock et al., 2006; Sturrock et al., 2007). Ang II signaling downstream of AT1 can also control Nox4 expression *in vivo* and *in vitro* (Arozal et al., 2010; Block et al., 2008; Wingler et al., 2001). Although Ang

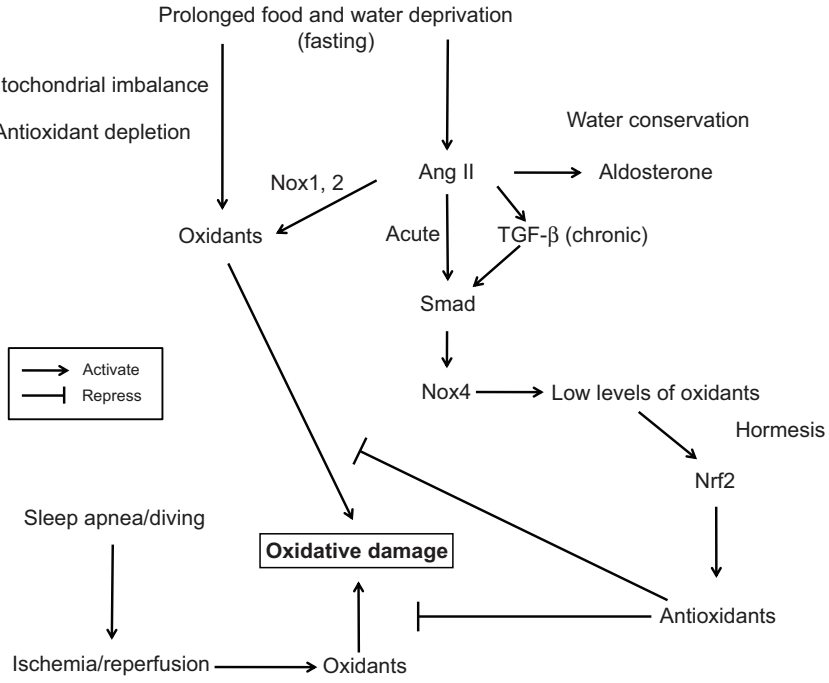

Fig. 7. Schematic representation of the proposed mechanisms leading to the activation of the elephant seal antioxidant system during prolonged fasting.

II upregulates TGF-β *via* AT1 activation (Rosenkranz, 2004), it can also activate the Smad pathway independently of TGF-β (Rodríguez-Vita et al., 2005; Ruiz-Ortega et al., 2007). Acutely infused Ang II increased, and ARB prevented, Smad phosphorylation, suggesting that AT1 activation may directly increase Nox4 expression (Rodríguez-Vita et al., 2005). After 2 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 (Sorescu, 2006). Furthermore, the observed increase in TGF-β may itself be a consequence of increased Ang II (Rosenkranz, 2004). An alternative explanation for the increased levels of Nox4 at the end of fasting is the activation of Nrf2 as Nox4 contains consensus sequences for EpRE in its promoter region and direct regulation of Nox4 by Nrf2 has been found *in vitro* during hyperoxia and laminar shear stress (Goettsch et al., 2011; Pendyala et al., 2011).

An increase in GSH levels and antioxidant enzyme expression at the end of the fast (Vázquez-Medina et al., 2010; Vázquez-Medina et al., 2011b) may potentially ameliorate the oxidant generation derived from augmented XO and Nox expression and activity (Soñanez-Organis et al., 2012; Vázquez-Medina et al., 2010), impaired insulin signaling (Fowler et al., 2008; Viscarra et al., 2011a; Viscarra et al., 2011b), high rates of glucose auto-oxidation (Champagne et al., 2005; Champagne et al., 2012; Houser et al., 2012) and lipid oxidation (Viscarra et al., 2012), RAS activation (Ortiz et al., 2006; Ortiz et al., 2001) and chronic HPA stimulation (Ortiz et al., 2003a; Ortiz et al., 2001). Increased antioxidant defenses at the end of the fast are also consistent with an increase in the number and duration of sleep apnea bouts that normally last between 8 and 12 min and constitute 80% of the seals' time on land (Blackwell and Boeuf, 1993; Castellini et al., 1994), along with increases in the time spent submerged in near-shore waters (Thorson and Le Boeuf, 1994). Furthermore, repetitive sleep apneas and voluntary submersions increase nuclear accumulation of Nrf2 in the skeletal muscle of late-fasting elephant seal pups (Vázquez-Medina et al., 2011c). 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 after departure from the rookery (after fasting) and which has the potential to increase oxidant generation and oxidative stress (Elsner et al., 1998; Vázquez-Medina et al., 2012; Zenteno-Savín et al., 2002).

In summary, our results demonstrate that prolonged fasting activates Nrf2 and suggest that such activation is mediated by increased expression of Nox4. Furthermore, our results suggest that Ang II stimulates Smad and thus can potentially regulate Nox4 expression through AT1 activation. Finally, our results suggest that physiological adjustments associated with prolonged fasting upregulate the antioxidant system of the elephant seal, conferring enhanced antioxidant protection and allowing them to tolerate fasting-related oxidant production and diving-induced ischemia/reperfusion (Fig. 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.

LIST OF ABBREVIATIONS

Ang II	angiotensin II
ARB	angiotensin receptor type 1 blocker
AT1	angiotensin receptor type 1
EpRE	electrophilic responsive element
GSH	glutathione
H_2O_2	hydrogen peroxide
HPA	hypothalamic–pituitary–adrenal axis
Keap1	kelch-like ECH-associated protein 1
Nox	NADPH oxidase
Nrf2	erythroid 2-related factor 2
RAS	renin–angiotensin system
TGF-β	transforming growth factor β
XO	xanthine oxidase

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AUTHOR CONTRIBUTIONS

J.P.V.-M., J.A.V., D.E.C. and R.M.O. designed the research. J.P.V.-M., R.R., J.A.V. and D.E.C. performed the animal experiments. J.P.V.-M., J.G.S.-O. and R.R. analyzed the samples and data. J.P.V.-M., J.G.S.-O., J.A.V., A.N. and R.M.O. interpreted the results. J.P.V.-M. wrote the original draft of the manuscript. J.P.V.-M., J.A.V., A.N., D.E.C. and R.M.O. edited and revised the manuscript. All authors approved the final version of manuscript for submission.

COMPETING INTERESTS

No competing interests declared.

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