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Prolonged fasting activates hypoxia inducible factor -1 α , -2 α and -3 α in a tissue-specific manner in northern elephant seal pups

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Abstract

Hypoxia inducible factors (HIFs) are important regulators of energy homeostasis and cellular adaptation to low oxygen conditions. Northern elephant seals are naturally adapted to prolonged periods (1–2 months) of food deprivation (fasting) that result in metabolic changes that may activate HIF-1. However, the effects of prolonged fasting on HIFs are not well defined. We obtained the full-length cDNAs of HIF-1 α and HIF-2 α , and partial cDNA of HIF-3 α in northern elephant seal pups. We also measured mRNA and nuclear protein content of HIF-1 α , -2 α , -3 α in muscle and adipose during prolonged fasting (1, 3, 5 & 7 wks), along with mRNA expression of HIF-mediated genes, LDH and VEGF. HIF-1 α , -2 α and -3 α are 2595, 2852 and 1842 bp and encode proteins of 823, 864 and 586 amino acid residues with conserved domains needed for their function (bHLH and PAS) and regulation (ODD and TAD). HIF-1 α and -2 α mRNA expression increased 3- to 5-fold after 7 weeks of fasting in adipose and muscle, whereas HIF-3 α increased 5-fold after 7 weeks of fasting in adipose. HIF-2 α protein expression was detected in nuclear fractions from adipose and muscle, increasing approximately 2-fold, respectively with fasting. Expression of VEGF increased 3-fold after 7 weeks in adipose and muscle, whereas LDH mRNA expression increased 12-fold after 7 weeks in adipose. While the 3 HIF α genes are expressed in muscle and adipose, only HIF-2 α protein was detectable in the nucleus suggesting that HIF-2 α may contribute more significantly in the up-regulation of genes involved in the metabolic adaption during fasting in the elephant seal.

Keywords

hypoxia inducible factor; northern elephant seal; gene expression; prolonged fasting; nuclear protein

1. Introduction

Hypoxia inducible factors (HIFs) are transcriptional activators that regulate cellular oxygen homeostasis by regulating genes involved in erythropoiesis, angiogenesis, and glucose

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metabolism (Semenza 1994; Semenza et al. 1994; Semenza et al. 1996; Semenza et al. 1997). HIFs are heterodimeric proteins composed of a regulatory α subunit (HIF- α) and a constitutive β subunit (HIF- β), which is also known as aryl-hydrocarbon receptor nuclear translocator (ARNT) (Wenger, Gassmann 1997; Semenza 1998). Three isoforms of the HIF- α subunit (HIF-1 α , HIF-2 α [also called endothelial PAS domain protein 1] and HIF-3 α) and three paralogues of the HIF- β subunit (ARNT1, ARNT2 and ARNT3) have been characterized in mammals (Zagorska, Dulak 2004). Both HIF- α and HIF- β belong to the basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) family of transcription factors. These transcription factors contain the bHLH domain for DNA binding and two PAS domains for target gene specificity, dimerization and transactivation (Wang et al. 1995). HIF- α is regulated at the post-translational level by proteosomal degradation via an ubiquitin-dependent pathway. This pathway involves the hydroxylation of specific proline residues (Pro402/564) within the oxygen-dependent degradation domain (ODD) by prolyl hydroxylases (PHDs) under aerobic conditions (Bruick, McKnight 2001; Epstein et al. 2001). During hypoxia, prolyl hydroxylation does not occur, therefore HIF- α is rapidly stabilized and translocated into the nucleus where it dimerizes with HIF- β and binds to hypoxia-responsive elements (HREs) on hypoxia sensitive genes (Semenza et al. 1994; Jewell et al. 2001; Comerford et al. 2002; Nordal et al. 2004).

HIF- α genes are expressed constitutively in a tissue-specific manner under normoxia and hypoxia conditions in mammals (Heidbreder et al. 2003; Wiesener et al. 2003; Zhao et al. 2004; Law et al. 2006), fish (Soitamo et al. 2001; Law et al. 2006; Shen et al. 2010; Chen et al. 2012) and crustaceans (Li, Brouwer 2007; Soñanez-Organis et al. 2009). In rat, HIF- α mRNA isoforms are expressed higher in brain and lung compared to heart, liver and kidney, implicating an organ-specific priority. HIF-1 α and HIF-4 α expression in grass carp exposed to normoxia and hypoxia was constitutively and also tissue-specific (Law et al., 2006). Recurrent apneas (breath-holding episodes) (Prabhakar et al. 2009; Prabhakar et al. 2010), which cause periodic decreases in arterial blood O₂ or intermittent hypoxia (IH), are implicated in the induction of cellular HIF- α accumulation in mammals.

Northern elephant seals are naturally adapted to tolerate prolonged fasting, which is characterized by increased: 1) number and duration of sleep apneas, and 2) time spent submerged in near-shore waters (Castellini et al. 1988; Blackwell, Le Boeuf 1993; Castellini et al. 1994); all of which can contribute to the stimulation of HIF in mammals. However, the effects of prolonged food deprivation on HIF expression and regulation in mammals are not well defined. To assess the impact of prolonged fasting on adipose and muscle HIF in northern elephant seals, we 1) identified and characterized three distinct HIF- α cDNAs (*esHIF-1 α* , *esHIF-2* and *esHIF-3 α*), 2) quantified the mRNA expression and nuclear accumulation of each HIF- α isoforms, and 3) quantified the mRNA levels of the HIF target genes, LDH and VEGF. Because increased plasma Ang II and apneas occur simultaneously during prolonged fasting, we hypothesized that prolonged fasting up-regulates adipose and muscle HIF- α isoforms in elephant seals and that cDNA of HIF- α isoforms have high identity to their homologues of mammals. The elephant seal's ability to tolerate prolonged fasting without deleterious consequences makes it an interesting model to understand the molecular and physiological mechanisms that allow adapted mammals to cope prolonged food deprivation and the associated characteristics (ie, excessive sleep apneas).

2. Material and methods

Institutional Animal Care and Use Committees of the University of California, Merced, and Sonoma State University reviewed and approved all methods. All work was realized under the National Marine Fisheries Service marine mammal permit # 87-1743.

2.1. Animals and sample collection

Sixteen elephant seal pups of known age were sampled at Año Nuevo State Reserve, CA, seven at a time, at four periods during their natural post-weaning fast (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 inch spinal needle was inserted into the extradural spinal vein. Sedation was maintained with 100 mg bolus intravenous injections of ketamine (Fort Dodge Animal Health) as needed. Adipose and muscle biopsies (20–40 mg) were collected as described previously (Vázquez-Medina et al. 2010). Briefly, a small region in the flank of the animal near the hind flipper is cleansed with alternating wipes of isopropyl alcohol and betadine and 2–3 mL of lidocaine (Henry Schein, Melville, NY) is injected subcutaneously with a 21 ga. needle. Next, a small (< 1.5 cm) incision is made and, a muscle and adipose biopsy was collected with a sterile biopsy punch needle (Henry Schein). Finally, tissue samples were frozen by immersion in liquid nitrogen immediately after collection and stored at –80°C until analyzed.

2.2. HIF-1α, -2α, & -3α cDNA cloning

The complete cDNA sequences for each HIF-α isoform were obtained using primers designed based on their respective homolog nucleotide sequences from mammals using Primer3 software (<http://frodo.wi.mit.edu>) (Rozen, Skaletsky 2000) (Table 1). Internal PCR fragments of HIF-1α, -2α, and -3α were obtained using the following primers: **1)** for HIF-1α, phocaHIF1aFw1 + phocaHIFaRv1, phocaHIF1aFw2 + phocaHIF1aRv2, eseaHIF1aFw1 + dogHIF1a-R4 and dogHIF1a-F4 + eseaHIF1a-R2, **2)** for HIF-2α, HIF2aFw3 + HIF2aRv1, HIF2aFw4 + HIF2aRv5 and HIF2aFw2 + eseaHIF2aRv1, and **3)** for HIF-3α, HIF3aFw4 + HIF3aRv1 and HIF3aF3 + HIF3aRv4. To obtain a 30 μL final reaction volume, 15 μL of Platinum PCR SuperMix (Invitrogen, Carlsbad, CA, USA), 3 μL of muscle cDNA (equivalent to 150 ng of total RNA) and 1 μL (20 μM) of each primer were mixed and subjected to the following conditions: 94°C for 3min for one cycle, 40 cycles of 94°C for 30s, 55°C for 40s and 68°C for 1–2 min, and an overextension step of 68°C for 7min. PCR fragments of ~200 to ~1500 bp were obtained, sequenced and identified as HIF-1α, -2α and -3α, respectively, by comparing them with GenBank data using the BLAST algorithm (Altschull et al. 1990).

The 5' and 3' ends of each HIF-α isoform were obtained using primers designed on their respective untranslated regions (UTRs) of each homolog nucleotide sequence of HIF-α. The following primers were used to obtain the 5' and 3' ends from: **1)** HIF-1α, dogHIF1a-F3 + phocaHIF1a-R1 and phocaHIF1a-F2 + dogHIF1a-R5, respectively, **2)** HIF-2α, HIF2aFw1 + esHIF2aRv4 and eseaHIF2aFw1 + CDSIII, respectively, and **3)** HIF-3α, HIF3aFw5 + esHIF3aRv3 and HIF3aFw2, respectively. To obtain a 25 μL final reaction volume, 12.5 μL of Platinum PCR SuperMix (Invitrogen, Carlsbad, CA, USA), 3 μL of muscle cDNA (equivalent to 150 ng of total RNA) and 1 μL (20 μM) of each primer were mixed and subjected to the following conditions: 94°C for 3min for one cycle, 40 cycles of 94°C for 30s, 55°C for 40s and 68°C for 1 min, and an overextension step of 68°C for 7min. PCR products of ~500 bp (for the 5' and 3' end) were obtained, cloned on pGEM-T Easy Vector System (Promega, San Luis Obispo, CA, USA) and sequenced. The full-length cDNA sequences for HIF-1α (*esHIF-1a*) and HIF-2α (*esHIF-2a*), and partial cDNA sequence for HIF-3α (*esHIF-3a*) were obtained by overlapping their respective PCR fragments. The predicted HIF-α amino acid sequences were obtained using a translation web site (<http://arbl.cvmbs.colostate.edu/molkit/translate/>), aligned with other HIF-α using Clustal W (Thompson, Higgins, Gibson 1994) and compared with protein databases using BLAST (Altschull et al. 1990).

HIF-1 α , -2 α & -3 α phylogenetic analysis—A phylogenetic analysis based on 36 HIF- α amino acid sequences (Table 2, including *esHIF-1 α* , *esHIF-2 α* and *esHIF-3 α*) was performed with MEGA software version 5 (Tamura et al. 2007). The analyses were subjected to the Neighbor-joining method, the Jones-Taylor-Thornton model for substitution, and the bootstrap method supported by 2000 replicates to test phylogeny.

2.3. Quantification of HIF- α isoforms, LDH and VEGF mRNA expression

Total RNA was isolated individually from adipose and muscle samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer instructions. RNA integrity was confirmed by measuring the absorbance at 260 nm/280 nm and by 1% agarose gel electrophoresis (Sambrook, Russell 2001). Contamination of genomic DNA in total RNA was eliminated by digestion with DNase I (Roche, Indianapolis, IN, USA), as specified by the manufacturer. Separate cDNAs from each tissue were synthesized from total DNase-free RNA (1 μ g) using oligo-dT and the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA).

Specific primers for each *esHIF- α* isoform were designed and validated by PCR using specific clones from each HIF- α subunit, whereas LDH and VEGF primers were designed based on homologous mammalian nucleotide sequences. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard to normalize the expression of each *esHIF- α* isoform. Isoforms of *esHIF- α* , LDH, VEGF and GAPDH mRNA expressions were measured by quantitative RT-qPCR using *esHIF1aFw1* + *esHIF1aRv1*, *esealHIF2aFw1* + *esealHIF2aRv1*, *esealHIF3aFw1* + *esealHIF3aRv1*, *LDHFw2* + *LDHRv2*, *VEGF-Fw1* + *VEGF-Rv1* and *GAPDHFw* + *GAPDHRv* primers, respectively. The PCR reactions of each tissue sample were run on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) in a final volume of 20 μ L containing 10 μ L of SYBR Green PCR Master Mix (Applied Biosystems), 6 μ L of H₂O, 0.5 μ L of each primer (20 μ M) and 3 μ L of cDNA (equivalent to 150 ng of total RNA). After an initial denaturing step at 94°C for 5min, amplifications were performed for 40 cycles at 94°C for 30 s, 60°C for 30 s and a final step of 30 s at 72°C, with a single fluorescence measurement and a final melting curve program decreasing 0.3°C each 20 s from 95 to 60°C. Positive and negative controls were included. Standard curves for each *esHIF- α* isoform and GAPDH were run to determine the efficiency of amplification using dilutions from 5E⁴-to 5E⁸-ng μ L⁻¹ of PCR fragment. For each measurement, expression levels (ng μ L⁻¹) were normalized to the expression of GAPDH. The use of GAPDH to normalize the expressions of the genes of interest was appropriate here because we did not detect any changes in the expression of GAPDH with fasting duration.

2.4. HIF-1 α , -2 α , & -3 α protein expression

Nuclear protein fractions were prepared from frozen adipose and muscle samples using the NE-PER nuclear protein extraction kit (Pierce, Rockford, IL, USA). Total protein content was measured using the Bio-Rad Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Ten micrograms of protein were mixed with Laemmli sample buffer, boiled and resolved in 4–15% Tris- HCl gradient gels. Proteins were electroblotted onto 0.45 μ m nitrocellulose membranes using the Trans-Blot Turbo transfer system (Bio-Rad Laboratories). Membranes were blocked 1h at room temperature and incubated overnight with primary antibodies against HIF-1 α , -2 α , and -3 α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100. Membranes were washed, incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) diluted 1:20000, re-washed and developed with the Immun-Star WesternCTM kit (Bio-Rad Laboratories). Blots were visualized using a Chemi-Doc XRS system (Bio-Rad Laboratories) and quantified by using Bio-Rad's

Quantity One software. Percent change from 1 week was calculated after band densities were normalized against TATA binding protein (TBP).

2.5. Statistics

Differences among of HIF-1 α , -2 α and -3 α mRNA and protein levels across the fast were detected by one-way ANOVA with Bonferroni post hoc tests. Means ($\pm$ s.e.m.) were considered statistically different at $P < 0.05$. Statistical analyses were performed using STATISTICA 8 software (StatSoft Inc., Tulsa, OK, USA).

3.1. Results

3.1. Characterization of elephant seal HIF- α isoforms

The full-length of HIF-1 α (*esHIF-1 α* ; GenBank JX310322) and HIF-2 α (*esHIF-2 α* ; GenBank JX310323), and partial HIF-3 α (*esHIF-3 α* ; GenBank JX310324) cDNA sequences for *M. angustirostris* were obtained from seal muscle. The *esHIF-1 α* sequence is 2595 bp long with a 2469 bp open reading frame (ORF) and encodes a predicted protein of 823 amino acid residues and a calculated molecular weight of 92 kDa. The translated *esHIF-1 α* exhibited high identity to HIF-1 α from the giant panda (98%), the domestic dog (98%), the bovine (97%), the goat (97%) and the human (94%) (Fig. 1A). The *esHIF-2 α* sequence is 2852 bp long with a 2679 bp ORF and encode a predicted protein of 864 amino acid residues and a calculated molecular weight of 95.6 kDa. The predicted protein *esHIF-2 α* has high identity to HIF-2 α from the giant panda (94%), the domestic dog (93%), the bovine (87%), the pig (88%) and the human (87%) (Fig. 1B). The partial *esHIF-3 α* fragment sequenced is 1805 bp and encodes a predicted protein fragment of 601 amino acid residues. The translated *esHIF-3 α* fragment has high identity to HIF-3 α from the giant panda (89%), the domestic dog (88%), the bovine (85%), the pig (84%) and the human (84%) (Fig 1C).

The three *esHIF- α* isoforms have low homology among their full-length sequences: *esHIF-1 α* has overall identity of 44% and 39% with *esHIF-2 α* and *esHIF-3 α* , respectively. In contrast, the identity of the three *esHIF- α* isoforms is much higher within most of the different functional domains derived from their human counterparts. The bHLH domain of *esHIF-1 α* (positions 15–69) is 83% and 76% identical with *esHIF-2 α* (positions 10–69) and *esHIF-3 α* (positions 1–56), respectively. The PAS-A (positions 96–150) and PAS-B (positions 247–300) domains of *esHIF-1 α* have identities with the corresponding domains of *esHIF-2 α* (positions 81–151 and 231–300) and *esHIF-3 α* (positions 69–135 and 214–286) of 73% and 69% and of 72% and 60%, respectively. The ODD (positions 399–598) and TAD (positions 782–823) domains of *esHIF-1 α* have lower identities with the corresponding domains of *esHIF-2 α* (positions 403–569 and 821–864) and *esHIF-3 α* (positions 378–482 and 586–601) of approximately 15–37% and of 50%, respectively. However, the proline (Pro) and asparagine (Asp) important for the HIF- α regulation under normoxic conditions are conserved in the corresponding ODD and TAD domain of each *esHIF- α* isoform (Fig. 1A, B and C).

3.2. Phylogenetic analyses demonstrate distinct clades for homologues of mammalian HIF-1

The amino acid sequences of HIF- α from other species based on the closest homologues derived from BLASTX searches were obtained for phylogenetic analysis. Multiple predicted amino acid sequence alignment demonstrated that *esHIF- α* deduced proteins were clustered with the homologues of other vertebrate species and constructed three distinct clades. The three *esHIF- α* proteins revealed higher identity with the homologues of other mammals,

especially *esHIF-1a* and *esHIF-2a* with the giant panda, and *esHIF-3a* with the horse (Fig. 2).

3.3. Prolonged fasting increases tissue-specific expression of HIF- α isoforms

esHIF- α isoforms mRNA expression levels were measured in adipose and muscle from elephant seal pups to determine their tissue-specific expressions in response to prolonged fasting. *esHIF-1a* and *esHIF-2a* transcripts increased ($P<0.05$) at each week across the fast over the 7 week measurement period in adipose and muscle, respectively (Fig. 3 and 4A). Similarly, *esHIF-1a* and *esHIF-2a* transcripts increased ($P<0.05$) 4-fold (Fig. 3) and 3.2-fold (Fig. 4A) in adipose and muscle, respectively, after 7 weeks of fasting, but did not change after 3 or 5 weeks compared to week 1. Also, *esHIF-1a* transcript decreased ($P<0.05$) 100-fold after 5 weeks of fasting compared to week 1. *esHIF-3a* transcripts decreased ($P<0.05$) 6.9-fold in adipose after 5 weeks and increased ($P<0.05$) 5-fold after 7 weeks compared to week 1 (Fig. 5). In muscle, HIF-3 α transcripts decreased ($P<0.05$) 5.7-fold and 190-fold after 3 and 5 weeks, respectively, and increased to basal levels after 7 weeks (Fig. 5).

3.4. Prolonged fasting increases nuclear protein content of HIF-2 α

The nuclear protein content of HIF- α isoforms was measured in adipose and muscle to assess the tissue-specific expressions to relate the changes in transcript and protein levels in response to prolonged fasting. Nuclear protein content of HIF-1 α and HIF-3 α were not detected in adipose and muscle at any measurement period across the 7 weeks of fasting. However, nuclear accumulation of HIF-2 α in adipose and muscle increased ($P<0.05$) at each week across the fast over the 7 weeks (Fig 4B).

3.5 Prolonged fasting increase tissue-specific expression of LDH and VEGF

The HIF target genes, *LDH* and *VEGF*, were measured in adipose and muscle to relate with the increase in nuclear protein content of HIF-2 during the fast (Fig. 6). *LDH* transcript increased ($P<0.05$) 6-fold and 12-fold in adipose after 3 and 7 weeks, respectively, but did no change after 5 weeks compared to 1 week. In muscle, *LDH* transcript decreased ($P<0.05$) 6.8-fold and 2-fold after 5 and 7 weeks, respectively, compared to 1 week (Fig. 6A). *VEGF* transcripts increased ($P<0.05$) 4-fold at each week across the fast over the 7 weeks in adipose, whereas in muscle, they increased ($P<0.05$) 2.5-fold after 7 weeks compared to 1 week (Fig. 6B).

4. Discussion

The full-length cDNA sequences of three HIF- α isoforms, *esHIF-1a*, *esHIF-2a* and *esHIF-3a*, were identified and characterized from northern elephant seal muscle. The predicted amino acid sequences of each *esHIF- α* isoform are highly conserved compared to other vertebrate homologues (94–98% to HIF-1 α , 87–94% to HIF-2 α and 84–89% to HIF-3 α), and contain the typical domains of HIF- α proteins suggesting that the functional attributes of HIF proteins are evolutionarily conserved among mammals despite the great variation in life histories and behaviors. Homology of the deduced amino acid sequences of each *esHIF- α* was relatively low (39–44%) between their full-length sequences, but relatively high in the bHLH (73–83%) and PAS (69–73%) domains. The bHLH domain contains approximately 60 amino acids that are responsible for DNA binding and dimerization (Voronova, Baltimore 1990; Morgenstern, Atchley 1999), and the PAS domains contain 200–300 amino acids involved in ligand binding, dimerization and other biological activities (Taylor, Zhulin 1999). The degradation of HIF- α proteins under normoxic conditions is mediated mainly by post-translational hydroxylation of conserved prolines located in the ODD domain (Bruick, McKnight 2001; Epstein et al. 2001). The

three *esHIF-α* proteins contain the Pro residue in their respective ODD domain (residue 402, 405, and 439, respectively) suggesting that degradation of HIF in elephant seals is likely via post-translational hydroxylation as with other mammals. HIF-α stability is mediated by hydroxylation of conserved Pro and Asn residues present in their amino-terminal transactivation domain (N-TAD) and carboxyl-terminal transactivation domain (C-TAD), respectively (Jiang et al. 1997; Pugh et al. 1997; Bruick, McKnight 2002). Each *esHIF-α* predicted protein has this Pro (residue 567, 405 and 479, respectively) and Asn (residue 800, 841 and 588, respectively) in their respective N-TAD and C-TAD suggesting that mechanisms of stabilization and, thus, regulation of HIF-α in seals is consistent with other mammals. Finally, the nuclear localization signal (NLS) necessary for HIF-α accumulation and translocation to the nucleus under hypoxic conditions is present in the N-terminal of each *esHIF-α* protein (residues 17–33, 14–30 and 1–16, respectively). The typical domains of HIF-α proteins observed in *esHIF-α* predicted proteins here demonstrate that seals maintained the evolution of a similar functional adaptation to hypoxia as in other vertebrates. Furthermore, the phylogenetic tree constructed in the present study demonstrates that the HIF-α proteins were clustered with their respective protein homologues indicating that molecular evolution of HIF-α may be relatively slow among species.

The three *esHIF-α* mRNAs were detected and up-regulated in a tissue-specific manner across the 7 weeks of fasting. Several studies have demonstrated that HIF-α mRNAs levels are expressed constitutively in a tissue-specific manner under normoxia and hypoxia conditions in mammals (Heidbreder et al. 2003; Wiesener et al. 2003; Zhao et al. 2004; Law et al. 2006), fish (Soitamo et al. 2001; Law et al. 2006; Shen et al. 2010; Chen et al. 2012) and crustaceans (Li, Brouwer 2007; Soñanez-Organis et al. 2009). The mRNA expression level of HIF-1α was higher than that of HIF-2α and HIF-3α in adipose and muscle during the fast suggesting that HIF-1α is more sensitive to physiological changes associated with prolonged fasting than the other HIF-α isoforms. The observed tissue-specific increases in *esHIF-1α*, *-2α* and *-3α* mRNA levels demonstrate the responsiveness of these genes to prolonged fasting and suggest that HIF-α isoforms may contribute to the physiological regulation of metabolic functions in these tissues. Alternatively, because prolonged fasting is associated with a number of physiological and development changes occurring simultaneously that were not accounted for independently, it is possible that the changes in HIF-α isoforms were, at least in part, the result of developmental alterations. Regardless of the ultimate causative factor responsible for the up-regulation of HIF-α, this up-regulation may ultimately contribute to the preparation of the seals for their ensuing diving lifestyle, which will be associated with frequent ischemic-reperfusion events in peripheral tissues.

Adipose is a multifunctional tissue that participates in lipid metabolism and regulation of glucose homeostasis (Wronska, Kmiec 2012), whereas skeletal muscle is a major site for the oxidation of fatty acids and glucose, accounting for approximately 80% of insulin-stimulated glucose uptake (Sinacore, Gulve 1993; Rasmussen, Wolfe 1999). Another important distinction between adipose and muscle with respect to HIF-mediated functions may reside in the fact that muscle would be more susceptible to ischemia-related hypoxia because of the high vascularization of muscle compared to adipose. Conversely, because the metabolism of the elephant seal is primarily dependent on lipid oxidation (derived from adipose), adipose in prolong-fasted seals is metabolically active. These distinctions may help explain the variation in tissue expression of these genes during prolonged fasting.

The *HIF-α* genes are constitutively expressed and translated independently of altered cellular oxygen content, but regulated at the protein level by post-translational modifications that tags HIF-α for their rapid degradation (Ivan et al. 2001; Jaakkola et al. 2001; Yu et al. 2001). The nuclear protein content of HIF-2α increased serially in adipose and muscle over

the 7 weeks of fasting, whereas HIF-1 α and -3 α were not detected suggesting that HIF-2 α may have a greater and preferential contribution to the transcriptional regulation of genes involved in the maintenance of cellular energy homeostasis during prolonged fasting.

Elephant seals remain submerged 90% of the time while at sea (Le Boeuf et al. 1996), and up to 60% of their time on land is spent in sleep apneas (breath-hold episodes) (Castellini et al. 1988; Blackwell, Le Boeuf 1993; Castellini et al. 1994). At about week 7 of fasting (consistent with the timing of sample collection in the present study), weaned pups increase the number and duration of apneas, and spend an average of 12 h per day submerged in near-shore pools similar in duration to their terrestrial sleep apneas (Thorson, Le Boeuf 1994). Sleep apneas in seals cause periodic decreases in arterial blood O₂ or intermittent hypoxia (IH) (Ponganis et al. 2006; Stockard et al. 2007; Ponganis et al. 2008). Several studies have demonstrated that HIF-1 α functions as a physiological mediator of the protective effects of ischemic preconditioning (Bergeron et al. 2000; Semenza 2000; Grimm et al. 2005). Similarly, studies in seals suggest that apneas (resting- or submergence-induced) also contribute to preconditioning by activating the HIF-1- mediated adaptive response to hypoxia (Zenteno-Savín et al. 2002; Johnson, Zenteno-Savín 2004; Johnson, Zenteno-Savín 2005; Vázquez-Medina et al. 2011). Because the timing of the increase in the number and duration of sleep apneas in fasting seals coincides with the nuclear accumulation of HIF-2 α at week 7, the activation of HIF-2 may up-regulate genes involved in the adaptive response to sleep apneas during their post-weaning fast.

HIF-1 and HIF-2 regulate several other genes associated with maintenance of homeostasis during hypoxia including those encoding VEGF and enzymes associated with glucose transport and metabolism (ie, LDH) (Semenza, Prabhakar 2007). VEGF is involved in vascular development, angiogenesis and arteriogenesis (Crandall et al. 1997; Hausman, Richardson 2004; Cao 2010; Christiaens, Lijnen 2010), whereas LDH produces lactate from pyruvate in the last step of anaerobic glycolysis (Markert et al. 1975). The maturation of skeletal muscle in seal pups is important for the development of their diving capacity, and is characterized by an increase in oxygen storage capacity (myoglobin content), acid buffering capacity, and aerobic enzyme activities (Kanatous et al. 2002; Kanatous et al. 2008). The increase in mRNA levels of adipose and/or muscle VEGF along with the nuclear accumulation of HIF-2 α suggest that HIF-2 contributes to adipose metabolism and muscle development by up-regulation of VEGF during prolonged fasting in elephant seals.

Fatty acid oxidation of the extensive fat stores of elephant seal accounts for 90% – 95% of their metabolic expenditures (Ortiz et al. 1978; Castellini et al. 1987; Adams, Costa 1993) and contributes to the maintenance of their relatively high plasma glucose concentrations (Keith, Ortiz 1989; Champagne et al. 2012; Houser et al. 2012) during their fast. Furthermore, many of the metabolic features found in fasting elephant seals are consistent with HIF upregulation including high rates of glycolytic flux, lactate production and pyruvate cycling with low rates of glucose oxidation (Champagne et al. 2012; Houser et al. 2012). The decreased in mRNA level of muscle LDH after 5 and 7 weeks of fasting suggesting differential tissue-expression of LDH genes (-A, -B and -C) as is known in mammals (Ebert et al. 1996; McClelland, Brooks 2002). The increase in mRNA levels of adipose LDH is associated with the nuclear accumulation of HIF-2 α suggesting that HIF-2 contributes to glucose metabolism via the up-regulation of glycolytic genes such as *LDH*.

In summary, we identified and characterized three distinct HIF- α isoforms (*esHIF-1 α* , *esHIF-2 α* and *esHIF-3 α*) in the northern elephant seal. The comparison of *esHIF- α* isoforms with their respective protein homologues in other mammals indicates that the molecular structures of these proteins are highly conserved throughout evolution, likely conferring conservation of their cellular functions in elephant seals. The results demonstrate

that prolonged fasting up-regulates HIF-1 α , -2 α and -3 α in a tissue-specific manner in elephant seal pups. The increase in HIF-2 α mRNA and nuclear accumulation in adipose and muscle along with the increase in LDH and VEGF mRNA suggests that HIF-2 α , more so than the other two isoforms, contributes to metabolic adaptation to prolonged fasting. These features of HIF activation may provide one of the principal cellular mechanisms that elephant seals evolved to maintain their energetic metabolism as a component to their natural adaptation to prolonged fasting or to the metabolic alterations associated with sleep-induced apneas.

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Abbreviation list, GENE 38611

ARNT	Aryl-hydrocarbon receptor nuclear translocator
bHLH	Basic helix-loop-helix
C-TAD	Carboxyl-terminal transactivation domain
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HIFs	Hypoxia inducible factors
HREs	Hypoxia-responsive elements
IH	Intermittent hypoxia
LDH	Lactate deshydrogenase
NLS	Nuclear localization signal
N-TAD	Amino-terminal transactivation domain
ODD	Oxygen-dependent degradation domain
ORF	Open reading frame
PAS	Per-Arnt-Sim
PHDs	Prolyl hydroxylases
TAD	Transactivation domain
TBP	TATA binding protein
UTRs	Untranslated regions
VEGF	Vascular endothelial growth factor

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Highlights

- We characterized three hypoxia inducible factors -1 α , -2 α and -3 α
- Prolonged fasting up-regulate the mRNA expression of HIF-1 α , -2 α and -3 α
- Prolonged fasting increase the nuclear content of HIF-2 α in adipose and muscle

Figure 2.

Phylogenetic tree for HIF-1α, -2α and -3α from a selected list of animal species. Species and accession numbers of all used sequences are shown in Table 2. Each shaded box represents a distinct cluster on the tree. Numbers on the base of each node indicate the percentages of bootstrap support based on 1,000 bootstrap resamplings.

Figure 3. Mean ($\pm$ s.e.m.) mRNA levels of HIF-1 α in **A)** adipose and **B)** muscle from fasted elephant seal pups. Asterisks denote significant ($P < 0.05$) differences from week 1.

Figure 4. Mean ($\pm$ s.e.m.) mRNA levels of HIF-2 α in **A)** adipose and **B)** muscle, and the nuclear accumulation of HIF-2 α in **C)** adipose and **D)** muscle from fasted elephant seal pups. Asterisks denote significant ($P < 0.05$) differences from week 1. *Inserts:* A representative western blot of HIF-2 α nuclear accumulation.

Figure 5. Mean ($\pm$ s.e.m.) mRNA levels of HIF-3 α in **A)** adipose and **B)** muscle from fasted elephant seal pups. Asterisks denote significant ($P<0.05$) differences from week 1.

Figure 6. Mean ($\pm$ s.e.m.) mRNA levels of LDH in **A)** adipose and **B)** muscle, and of VEGF in **C)** adipose and **D)** muscle from fasted elephant seal pups. Asterisks denote significant ($P < 0.05$) differences from week 1.

Table 1

Primers used to obtain the cDNA sequences of *esHIF-1a*, *-2a* and *-3a*.

Primer Name	Nucleotide Sequences (5'-3')
HIF-1a	
phocaHIF1a-F1	GTGAGCTCGCATCTTGATAAGGC
phocaHIF1a-F2	GGATGCAAATCTAGTGAACAC
dogHIF1a-F3	CCGATTCGCCATGGAGGGC
dogHIF1a-F4	GATGCTTTAACTTTGCTGGCC
esealHIF1a-F1	GGTTCTCACAGATGATGGTGAC
phocaHIF1a-R1	GGATGAGTAAATCAAACACAC
phocaHIF1a-R2	GCTCTGAGTAATTCTTACCC
dogHIF1a-R4	GGTAATGAGCCACCAGTGTCC
dogHIF1a-R5	GCAGTATTGTAGCCAGGCTTC
dogHIF1a-R7	CACTACTTCGAATGTGCTTTGG
esealHIF1a-R1	CTGGTCAGTTGTGGTAATCC
esealHIF1a-R2	CTACATGCTAAATCAGAGGG
HIF-2a	
HIF2aFw3	GRTRTGAAACGRATGAAGAGC
HIF2aFw2	CCATGVCRARCATCTTCCAGCC
HIF2aFw4	CCTGGCCATCAGTTCCTGCG
esealHIF2aFw1	GTGGAAACGGATGAAGAGCC
esealHIF2aFw2	GTGAACTGCCCTCTGATGCC
HIF2aRv1	CCTGACMCCTTKTGAGCYCMTGG
HIF2aRv5	GCTCAGGCTCTATCTTCTTGC
esealHIF2aRv1	GCTGTAGTCCTGGTACGGAG
esealHIF2aRv2	CCTGACACCTTGTGAGCTCC
esealHIF2aRv3	CATGATGATGAGGCAGGAGAG
esealHIF2aRv4	CCAGAGCCACTTTTGAGACTC
esealHIF2aRv5	GATCATGTCGCCATCTTGGG
HIF-3a	
HIF3aFw2	CTTCCATGGCCTGTCRCCMCC
HIF3aFw3	CAGGAGACGGAGGTGCTGTAC
HIF3aFw4	GAGCAAGGGCCAGGCAGTAAC
esealHIF3aFw1	CTCTGGATCTGGAGATGCTGG
esealHIF3aFw2	GATGACTTCCAGCTCAACTCC
HIF3aRv1	CAGAAGGAACTCAGGCTGAG
HIF3aRv4	GTTACTGCCTGGCCCTTGCTC
esealHIF3aRv1	GAGCCAGGGTCTCTTCCGAG
esealHIF3aRv2	CTCTGGAAGGGCTGGAGCAGC
esealHIF3aRv3	GGATGGCTTCACAGATGAGC
esealHIF3aRv4	CTCATGTGTCCAGAGCAGTG
esealHIF3aRv5	GTGTCCGATGAGCTCAAGCTG

Primer Name	Nucleotide Sequences (5'-3')
LDHFw2	CTTAATGAAGGACTTGGCAG
LDHRv2	CTTCTCCCTCTTGCTGACG
VEGF-Fw1	ATGCGGATCAAACCTCACCAA
VEGF-Rv1	GTTCGTTTAACTCAAGCTGCC
GAPDHFw	CAGAACATCATCCCTGCCTC
GAPDHRv	CTGCTTCACCACCTTCTTGA

Table 2

List of amino acid sequences included in the phylogenetic analyses.

Organism	HIF- α type	GeneBank accession number
<i>Ailuropoda melanoleuca</i> (giant panda)	1,2,3	XP_002913080, XP_002912483, XP_002923099
<i>Bos Taurus</i> (cow)	1,2,3	NP_776764, DAA24674, NP_001098812
<i>Brucella melitensis</i> (bacterium)	*PDP	EEW88394
<i>Capra hircus</i> (goat)	1,2	AEW10558
<i>Canis lupus familiaris</i> (domestic dog)	1,2,3	XP_865513, XP_531807, XP_533636
<i>Equus caballus</i> (horse)	1,2,3	JX310322, JX310323, JX310324
<i>Gallus gallus</i> (domestic chicken)	1,2	NP_989628, NP_990138
<i>Homo sapiens</i> (human)	1,2,3	NP_001521, NP_001421, EAW57411
<i>Loxodonta africana</i> (African elephant)	2,3	XP_003417640, XP_003406518
<i>Meleagris gallopavo</i> (wild turkey)	1,2	XP_003206838, XP_003203937
<i>Mirounga angustirostris</i> (northern elephant seal)	1,2,3	XP_001493256, XP_001498492, XP_001500830
<i>Mus musculus</i> (house mouse)	1,2,3	AAC52730, AAB41496, NP_001156422
<i>Rattus norvegicus</i> (rat)	1,2,3	CAA70701, EDM02660, NP_071973
<i>Sus scrofa</i> (pig)	1,2,3	NP_001116596, NP_001090889, XP_003127298