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Hypoxia-Inducible Factor in Ringed Seal (*Phoca hispida*) Tissues

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Tissue hypoxia and ischemia–reperfusion pose a dangerous situation for oxidative stress. However, diving mammals and birds show pronounced resistance to oxidative injury under such conditions, which are a consequence of selective vasoconstriction during a dive. As the function of Hypoxia-Inducible Factor-1α (HIF-1α) in protection against and adaptation to hypoxia has been recognized in terrestrial animals, we have investigated the genomics and expression of this protein in ringed seal (*Phoca hispida*) in order to determine if it may play a protective role in this diving mammal. PCR studies using primers based on sequences from mouse HIF-1α exons 3, 4, 5, 6, 9, 10, 11, 12 and 15 showed that DNA from seal lung generated PCR products similar to those from mouse DNA. These studies have established that a putative HIF-1α gene exists in the seal genome that appears to have a similar but not identical sequence to the mouse gene. Seal lung and skeletal muscle tissues showed the highest relative levels of HIF-1α protein expression, with heart muscle showing significantly lower levels, and levels of HIF-1β protein expression paralleled this situation. Analysis of oxidized cellular protein levels indicated that seal lung and heart muscle had the lowest levels of oxidized proteins. Thus, as seal lung tissue had the highest level of HIF-1α protein expression and the second lowest level of protein oxidation, this suggests that HIF-1α expression may have an important protective effect in this tissue in diving mammals. Our results support the hypothesis that HIF-1α expression is dependent on both tissue-specific energy requirements and adequate metabolic supply-to-demand ratio. Combined, the evidence available suggests that diving mammals have an overall anticipatory response to avoid the ill effects of dive-associated ischemia–reperfusion which may involve the HIF-1 system.

Keywords: Hypoxia-inducible factor; Diving mammals; Hypoxia; Ischemia–reperfusion; Protein oxidation

INTRODUCTION

In a variety of animals, the intracellular expression of a protein known as Hypoxia-Inducible Factor-1α (HIF-1α) is increased under conditions of hypoxia.[1,2] Under normoxic conditions, this protein is hydroxylated at proline and asparagine residues,[3,4] which permits interaction with the von Hippel-Lindau tumor suppressor protein (pVHL), causing the polyubiquitination of HIF-1α and its subsequent degradation by intracellular proteases.[5,6] However, under hypoxic conditions, or when pVHL is not expressed[7] or is defective,[8] hydroxylation of HIF-1α is prevented and its intracellular level increases.[3] Under such circumstances, HIF-1α then interacts with a constitutive protein (HIF-1β) to form a dimer (HIF-1) which acts as a transcription factor whose binding to a hypoxia response element (HRE) associated with a number of genes causes up-regulation of gene expression.[1,9] The genes that appear to be controlled in this fashion include some of those controlling angiogenesis, erythropoiesis, energy metabolism, apoptosis, heat shock and cell proliferative responses to hypoxia.[1,9,10] Thus, HIF is believed to play important roles in the pathophysiology of pre-eclampsia, intrauterine growth retardation, hypoxia-mediated pulmonary hypertension and cancer.[11–14] HIF also promotes neovascularization in response to myocardial ischemia by activating transcription of the gene encoding vascular

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endothelial growth factor, and may also mediate the protective response to cerebral ischemia known as late-phase pre-conditioning.[15]

Oxidative stress is a particular problem in lung tissue,[16] and it appears that this tissue may have unique mechanisms of defense in addition to those seen in other tissues.[17] Another tissue that is of considerable interest in terms of oxidative stress is the myocardium, because oxidative stress has been correlated with cardiac hypertrophy and heart failure,[18] and evidence for an increased production of the hydroxyl radical originating from the superoxide and peroxide radicals in the failing myocardium has been obtained.[19] Ischemia–reperfusion, a phenomenon that occurs spontaneously and post-operatively after cardiac surgery, is known to be a situation that exacerbates production of reactive oxygen species (ROS),[20] and studies have reported the production of ROS in the reperfused heart,[21] with protection of the myocardium in the presence of ROS scavengers.[22] The physiological consequence of ROS generation during reperfusion after brief ischemia is the reversible post-ischemic myocardial dysfunction known as myocardial stunning.[23]

In diving mammals and birds, muscle tissues become hypoxic during diving as perfusion to vascular beds is restricted in order to maintain adequate circulatory oxygenation of the CNS.[24] Resistance to breath-hold diving in these species depends on a number of cardiovascular adaptations, including a large storage of O_2 in blood and tissues and redistribution of blood flow; the latter resulting in regional ischemia followed by reperfusion promptly upon emersion. This dive-associated ischemia–reperfusion and tissue hypoxia poses a large potential for oxidative stress and production of ROS. However, these animals apparently suffer no ill effects from their frequent diving episodes, suggesting the presence of specialized protective mechanisms. Elevated levels of antioxidants have been reported in diving birds and mammals,[25,26] and some of the antioxidant defense mechanisms in tissues of a diving mammal, the ringed seal (*Phoca hispida*), have been investigated in response to experimental ischemia and simulated oxidative stress.[27] However, no previous work has been performed on HIF-1 in diving seals or other warm-blooded diving animals. Recent studies[28] have identified HIF-1α in rainbow trout, a freshwater fish that undergoes exposure to wide fluctuations in dissolved oxygen content in its environment. Trout HIF-1α had considerable (61%) sequence similarity to human HIF-1α, although there were major differences in the C-terminal sequences of the proteins. An interesting and physiologically relevant finding was that trout HIF-1α protein expression occurred at considerably higher pO_2 levels than did terrestrial mammal HIF-1α, indicating that HIF-1α protein is expressed at normoxic venous oxygen tension.

Our studies were undertaken to determine if HIF-1 proteins exist and are expressed in diving mammals, and to establish if the putative HIF-1 system in these specialized animals has features that may protect against the repeated ischemia–reperfusion situations that are part of their lifestyle. In the present report, we establish that the ringed seal genome does contain an HIF-1α gene that is similar to that found in terrestrial animals, and that the HIF-1α and HIF-1β proteins are constitutively expressed in certain seal tissues. Our results also suggest that, in some cases, the seal tissue expression pattern of HIF-1α protein may be related to diminished oxidative damage in that tissue.

MATERIALS AND METHODS

Seal Tissue Collection

Tissues (heart, kidney, liver, skeletal muscle, lung) from 12 ringed seals were obtained incidental to subsistence hunting through collaboration with the North Slope Borough Department of Wildlife Management and Inupiat Eskimo hunters near Barrow, AK. Tissue samples were excised immediately following death of the animal and 1–2 g samples were placed in plastic vials and placed in liquid nitrogen, and subsequently stored at −20°C.

DNA Preparation

DNA was prepared from mouse heart and liver, as well as from seal heart, kidney, liver, skeletal muscle and lung using the Qiagen DNeasy kit according to the protocol for animal tissue extraction without RNase treatment. Following elution of the DNA from the spin columns, 100 μl aliquots of each preparation were then stored at −80°C. Samples were analyzed by electrophoresis in 1% agarose in the presence of 0.1 μg/ml ethidium bromide.

PCR Primers

The primers were custom synthesized by IDT (Coralville, IA) and were based on the sequences of HIF-1α exons in mouse, human, bovine and rat genomes. The primers were selected from exon sequences that showed very high sequence homologies and conformed to the general guidelines for primer design. Primer sets were designed to generate PCR products of less than 5 kb between exons 3 and 4, 5 and 6, 3 and 6, 9 and 10, 11 and 12, and 9–12 and a short (100 bp) region within exon 15. Table I shows the sequences of the primers that were synthesized and their relationship to exon number and base sequence in the mouse genome.

TABLE I PCR primer sequences used for seal HIF-1α gene analysis

Exon*	Bases*	Primer sequence (5′–3′)							
3	1773–1794	CAT	TTC	TGA	TAA	YGT	GAA	CAA	A
4	1953–1933	ATT	TCC	TCA	TGG	TCA	CAT	GGA	
5	4386–4406	CAG	AAT	GAA	GTG	CAC	CCT	AAC	
6	6091–6071	GGG	TTC	ACA	AAT	CAG	CAC	CAA	
9	13234–13254	GGT	ATT	ATT	CAG	CAC	GAC	TTG	
10	14280–14260	TTG	AAT	CTG	GGG	CAT	GGT	AAA	
11	15281–15301	GGT	CAA	TGW	ATT	CAA	GTT	GGA	
12	15846–15826	TTK	TCT	GTC	TGT	TCT	ATG	ACT	C
15	19109–19128	TAC	CAC	AGC	TGA	CCA	GTT	A	
15	19207–19189	CTT	GAT	CCA	AAG	CTC	TGA	G	

*The exon and base sequence numbers are from the mouse HIF-1 α sequence (Genbank Y09085). Some of the primers have mixed (wobble) base selections (K, W, Y) where the mouse, human (Genbank NM_001530), rat (Genbank NM_024359) and bovine (Genbank NM_174339) base sequences are not identical.

PCR Conditions

PCR was performed using the Roche Expand High Fidelity PCR System kit reagents and an Applied Biosystems GeneAmp PCR System 2400 thermocycler. For exon primer sets 3 + 4 and 11 + 12, a 55°C annealing temperature was used, for primer sets 3 + 6, 5 + 6, 9 + 10 and the two primers from exon 15, 57.8°C was used, and for primer set 9–12, 60°C was used. For primer sets 3 + 4, 5 + 6, 9 + 10 and 11 + 12, elongation was performed for an initial time of 1 min at 72°C, and for primer sets 3 + 6 and 9 + 12, elongation was performed for an initial time of 1 min at 68°C. In each elongation program, the time was constant for the first 10 cycles, and then for the next 20 cycles, 5 s was added successively to each elongation period.

Protein Detection Using Antibodies

The Pierce NE-PER Nuclear and Cytoplasmic Extraction kit was used to prepare nuclear and cytoplasmic fraction proteins from seal tissue samples employing recommended protease inhibitors. The presence of HIF proteins in the nuclear preparations was detected immunologically by dot blot and Western blot procedures using antibodies and a Luminol chemiluminescence detection kit purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For HIF-1α, the primary antibodies used were mouse monoclonal antibody HIF-1α (28 bases) and goat polyclonal HIF-1α (Y-15), and for HIF-1β protein, the primary antibody was goat polyclonal ARNT 1(C-19). The secondary antibodies used were anti-mouse and -goat HRP-labeled IgGs, and detection of the secondary antibodies was performed using the Luminol reagent. The presence of oxidized proteins in the cytoplasmic fractions was detected in dot blot assays using the Intergen Oxyblot Protein Oxidation kit and the Santa Cruz Luminol reagent. For these experiments, dot blots and electrotransfers were performed using Applied Biosystems ProBlott membrane. Visualization and quantitation of dot intensities were performed by densitometry using the Bio-Rad Quantity One software system. Analyses were performed on three different preparations in each case, and the Minitab 12.22 program was used for one-way ANOVA of the data to determine if significant differences between the data sets existed at the 95% confidence level ($P < 0.05$).

RESULTS

Seal DNA Preparation

Although DNA was obtained from all seal tissues, the best source in terms of quantity and quality (as evidenced from a lack of smearing on electrophoresis) was from lung tissue (Fig. 1), and seal lung DNA was used in subsequent experiments. As shown in Fig. 1, the approximate size of the sheared DNA fragments was 20 kb, which was identical to that obtained from mouse tissues using the same procedure.

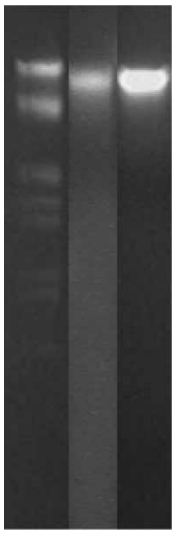

FIGURE 1 Electrophoresis of DNA preparations from mouse and ringed seal tissue. The left lane is the lambda *Eco* R1-*Hind* III molecular weight marker (Roche III), and the center and right lanes are DNA prepared from mouse liver and seal lung, respectively. In each preparation, the DNA has an approximate size of 20 kb.

PCR Analyses on Ringed Seal and Mouse Tissue DNA

Seal lung DNA isolated from five different animals was found to generate the same PCR products in each case. Figure 2 shows the results of representative PCR amplification when mouse liver DNA and one of the seal lung DNA preparations were used as the templates with primer sets based on the mouse HIF-1α exon sequences.

For the exon primer sets 9 + 10, 11 + 12, 9 + 12, and the two primers in exon 15, the polynucleotides generated from the seal lung template were of similar, if not identical, sizes to the products obtained from the mouse DNA template. With exon primer sets 5 + 6 and 3 + 6, there were large differences in the sizes of the products obtained from the mouse and seal DNA templates. The products obtained from the mouse templates were homogeneous in all cases, and with one exception, the PCR products obtained from the seal DNA template also appeared homogeneous on electrophoretic analysis. Heterogeneity was seen after PCR using the exon primer set 3 + 6, although the major band corresponded more closely to the size of the product obtained using the mouse template.

HIF-1 Protein Expression in Ringed Seal Tissues

Dot blot analysis (see Fig. 3 for a representative analysis) of tissue nuclear fractions revealed that in ringed seal kidney, HIF-1α protein was barely detectable, and that HIF-1β protein was not detectable. In seal liver, there was negligible expression of both proteins. Both proteins were detectable in seal lung, skeletal muscle and heart, with the highest expression levels (based on total protein) of both HIF-1α and -1β being found in seal lung with the next highest level of expression occurring in skeletal muscle. The levels of HIF-1α expression (based on the average of both antibody assays) in different tissues relative to lung (= 100%) were 41 ± 7% in skeletal muscle, 12 ± 3% in cardiac muscle, 6 ± 3% in kidney, with no expression in liver. The levels of HIF-1β expression in different tissues (relative to lung HIF-1α expression) were 52 ± 7% in lung, 24 ± 5% in skeletal muscle and 1 ± 0.6% in cardiac muscle. All of these values were significantly different from that of HIF-1α expression in lung.

Western blot analysis (Fig. 4) of the ringed seal lung cytoplasmic fraction revealed that for each

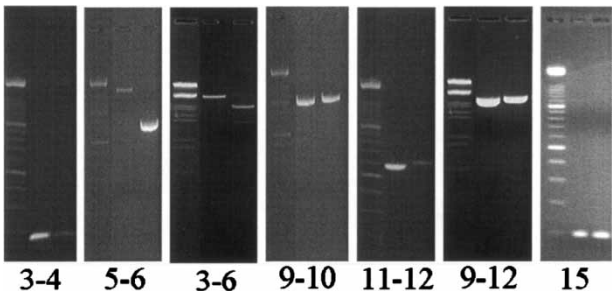

FIGURE 2 PCR products from mouse and ringed seal DNA preparations. The products were obtained by using primer sets based on mouse exon sequences, as described in the "Methods" section, and which are indicated under each gel picture. Each picture shows a base pair molecular weight standard on the left, with a 100 bp ladder (Roche XIV) being used for the 3–4, 5–6, 11–12 and 15 pictures and the lambda *Eco* R1-*Hind* III marker (Roche III) used in the 3–6 and 9–12 pictures. The central and right lanes show PCR products generated using mouse liver and seal lung DNA, respectively.

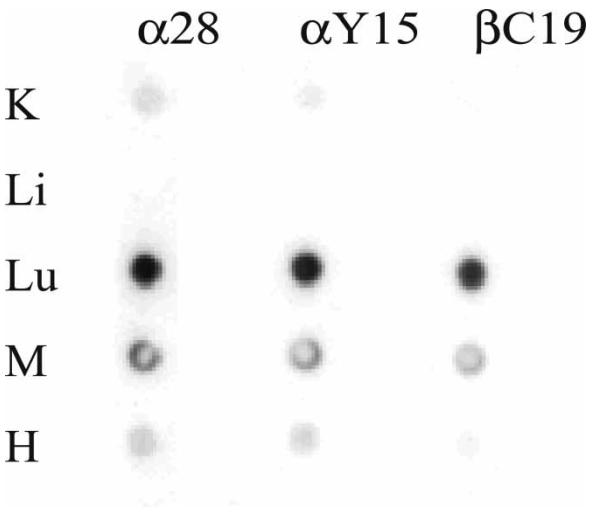

FIGURE 3 Dot blot analysis of HIF protein expression in ringed seal tissues. Nuclear protein fractions from kidney (K), liver (Li), lung (Lu), skeletal muscle (M) and heart (H) were prepared, and 5 μg protein samples were used in the assay with primary antibodies against mouse HIF-1α (α28), goat HIF-1α (αY15) and goat HIF-1β (βC19), as described in the "Methods" section.

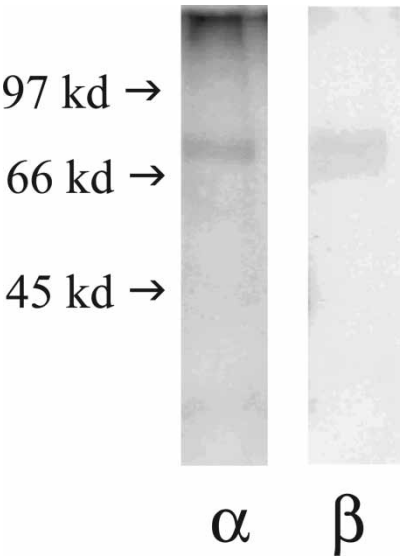

FIGURE 4 Western blot analysis of HIF-1 proteins in ringed seal lung nuclear protein fraction. Western blot analysis was performed as described in the "Methods" section, using primary antibodies against goat HIF-1α (α) and goat HIF-1β (β). The mobilities of molecular weight markers are indicated by arrows.

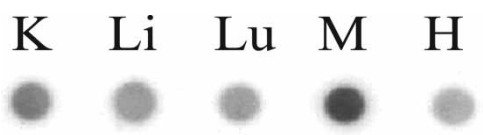

FIGURE 5 Dot blot analysis of oxidized proteins in ringed seal tissues. Cytoplasmic protein fraction samples (15 μg) from seal kidney (K), liver (Li), lung (Lu), skeletal muscle (M) and heart (H) were analyzed for oxidized protein as described in the "Methods" section.

protein, a single band was detectable, in addition to the material remaining at the sample well origin in the case of HIF-1α. The approximate molecular weight of each protein band detected by chemiluminescence was 84 kDa.

Protein Oxidation Status in Ringed Seal Tissues

Dot blot analysis was performed to detect oxidized protein in cytoplasmic fractions of seal tissues (see Fig. 5 for a representative analysis), and these results showed that skeletal muscle had the highest level of oxidized protein relative to total protein. Quantitation of levels of oxidized protein in other tissues (compared to skeletal muscle = 100%) were 73 ± 9% in kidney, 64 ± 9% in liver, 49 ± 5% in lung and 44 ± 7% in heart. All of these values were significantly different from that for skeletal muscle.

DISCUSSION

HIF-1α DNA, mRNA and protein studies have been previously reported for a diversity of species. Our studies on ringed seal HIF-1α genomic and proteomics were initiated to determine if there were differences in the seal HIF-1 system which could be related to the special physiological stresses associated with repetitive breath-hold diving in this species. Rat HIF-1α cDNA showed 96 and 90% identity to mouse and human HIF-1α, respectively,[29] and rainbow trout HIF-1α showed a 66% similarity to human and rainbow trout HIF-1α DNA sequences and 61% similarity human and mouse HIF-1α amino acid sequences.[28] These levels of homology in DNA sequences, even between fish and mammals, suggest that HIF-1α is a highly conserved gene.

The results of our PCR studies showed that DNA products were generated from all selected regions of the mouse HIF-1α gene by PCR using both mouse and seal DNA templates. The primer sequences selected spanned exons 3 and 4, 5 and 6, 9 and 10, and 11 and 12 in the mouse and human genes, larger spans between exons 3 and 6 and exons 9 and 12, and a small region (99 bases) in exon 15 which is involved in pVHL binding.[4] With the seal template, the products were of identical size between the exons 3 and 4 (about 200 bp) and with exon 15 primers (about 100 bp), but slightly different for all other exon pairings, viz. exons 5–6 (1.7 kb mouse; 0.7 kb seal), exons 3–6 (4.5 kb mouse; 2.5 kb seal), exons 9–10 (1 kb mouse; 1.1 kb seal), exons 11–12 (0.60 kb mouse; 0.62 kb seal), and exons 9–12 (3 kb mouse; 3.1 kb seal). These studies establish that a putative HIF-1α gene exists in the seal genome which appears to have a similar but not identical sequence to the mouse gene. There were some quite large differences in the sizes of analogous PCR products from the mouse and seal genomes, which must reflect differences in exon and/or intron sequences in parts of the gene. It will be of considerable interest to establish if such sequence differences have a physiological significance in the seal, particularly with respect to post-translational modification of HIF-1α protein and its protein–protein and protein–DNA interactions, as these are critical features in controlling its tissue levels and performance as a component of the HIF-1 transcription factor.[30,31]

HIF-1 protein expression studies in seal tissues revealed that proteins were detected by a mouse monoclonal antibody to human HIF-1α (residues 329–530), by a goat polyclonal antibody to the N-terminal sequence of human HIF-1α, and by a goat polyclonal to human HIF-1β carboxyl-terminal sequence. These results indicate that there must be close similarity between the seal protein sequences of the HIF-1 proteins and those of terrestrial mammals. Despite this similarity, the molecular weights of the protein bands detected (approximately 84 kDa for both HIF-1α and -1β) are smaller than those for HIF-1α (120 kDa) and HIF-1β (94 kDa) in terrestrial mammal tissues.[32] In rainbow trout, the sizes of both HIF-1α (766 residues compared to 826 residues in human HIF-1α) and HIF-1β (79 kDa) also appear to be smaller than those in terrestrial mammals.[28,29]

The analysis of HIF-1 protein expression in seal tissues revealed that there was either no or very little expression in kidney and liver, whereas in skeletal muscle, lung and heart, both proteins (HIF-1α and HIF-1β) were expressed, with lung and skeletal muscle showing the highest relative levels of expression. These situations contrast with those in terrestrial mammals and a fish (trout) where HIF-1α protein is generally not detectable in normoxic tissues but is expressed at lowered oxygen tensions.[1,28,33] Specifically, in mouse, although detectable levels of HIF-1α were found in skeletal muscle, as was found in seal; in contrast to the situation in seal, only very low levels of the protein were found in mouse heart muscle and no detectable amounts were found in lung tissue.[33] Additionally, HIF-1β protein is constitutively expressed in terrestrial mammalian tissues,[1] although its levels may increase with HIF-1α expression.[33] In contrast, our

data shows that in seal, HIF-1β protein is not detectable in tissues where HIF-1α is not expressed.

The above differences may be indicative of different structures and/or functions of the HIF-1 proteins in seal tissue. In this context, it should be noted that the degree of vasoconstriction in seal tissues during a dive is not homogeneous, and while the kidney undergoes persistent ischemia, the heart is intermittently perfused.[34–36] Thus, it is possible that HIF-1α is over-expressed in tissues where perfusion is insufficient to meet local metabolic requirements. Hypoxia-tolerance in seal heart has previously been ascribed, in part, to enhanced glycolytic energy sources and modulation of adrenergic activity.[37] Given that HIF-1 target genes include glucose transporters and glycolytic enzymes,[30] constitutive expression of HIF-1α in seal tissues such as heart could potentially contribute to the observed oxidative and glycolytic metabolism. The suggestions[31,33] that the basal expression of HIF-1α detected in mice contributes to the tissue-specific energy requirements also support this scenario. That HIF-1α is constitutively expressed in ringed seal tissues correlates with reports of higher hematocrit (from increased erythropoiesis) and a greater capillary density (from increased VEGF and angiogenesis), as well as enhanced oxidative and glycolytic metabolism, in marine mammals compared to terrestrial mammals.[30,37–39] This idea is also supported from studies[40] which showed that HIF-1α over-expression in HeLa cells under normoxic conditions allows HIF-1β recruitment and formation of a functional DNA-binding HIF-1 complex. It would be interesting to determine if constitutive expression of HIF-1α is a common feature of organisms adapted to frequent exposure to metabolic and/or environmental hypoxia, and if this expression suffices to allow for a functional HIF-1 system.

It is possible that the constitutive expression of HIF-1α in ringed seal tissues is part of the physiological adaptation to the progressive asphyxia (hypoxia, hypercapnia, acidosis) associated with diving.[24] Marine mammals are known to have higher antioxidant defenses compared to terrestrial mammals,[26,27] apparently as an anticipatory response to decrease oxidative stress induced by ischemia–reperfusion. This concept of preparedness for oxidative stress was first proposed for anoxia-tolerant frogs,[41] and has since been suggested for other hypoxia-tolerant species.[42] It is believed that hydroxyl radical concentrations contribute to controlling the activity of HIF-1α,[30] and that hydrogen peroxide is required for the binding of HIF-1 to DNA.[43] Although hydroxyl radical concentrations have not been directly measured in ringed seal, the elevated production of superoxide radical and increased level of superoxide dismutase activity found in these animals[27] suggest that elevated hydrogen peroxide and/or hydroxyl radical levels occur under normoxic conditions and that these species may play a role, via HIF-1, in the physiological adaptation to repetitive diving.

Our results lead to questions about how hypoxia-induced gene transcription is differentially regulated in ringed seal tissues. Although the control of HIF-1α expression levels through oxygen-dependent pVHL-modulated protein degradation is regarded as a major control mechanism,[5,6] it is now recognized that additional controls, some of which are unrelated to hypoxia, occur over HIF-1α expression. These include those involving the cyclic AMP response element-binding protein (CREB),[44] nitric oxide,[45] cytokines,[46] MAP kinase,[47] hormones[48,49] and insulin-like growth factors.[50] It has not been established which, if any, of the above mechanisms are operative in seal, but studies in our laboratories are currently in progress to determine the significance of the pVHL control system in seal tissues.

Because some seal tissues were found to express HIF-1α constitutively, we examined seal tissues to determine if HIF-1α expression protected tissue proteins from oxidation. In some cases, there appeared to be no obvious effect of HIF-1α protein expression in tissues on prevention of protein oxidation, as the tissue (skeletal muscle) with the second highest level of HIF-1α expression had the highest level of protein oxidation, and a tissue (liver) with a low level of HIF-1α expression had one of the lowest levels of protein oxidation. However, in the case of lung, the highest relative level of HIF-1α expression was seen and this tissue showed the second lowest relative level of protein oxidation. Both the extent and tissue distribution of protein oxidation correspond with our previous findings of elevated production of superoxide radical in ringed seal tissues, giving further support to our hypothesis that ringed seal tissues may exist in a more reducing environment due to a production of reducing equivalents with each dive.[27] Further studies will be needed to understand the integration of the oxygen sensing and the oxidative stress avoidance mechanisms in diving mammals.

In conclusion, we have found that ringed seal DNA served as a template to generate PCR products using primers based on HIF-1α mouse exon sequences. These products showed similar but not identical sequence to the mouse gene, as well as differences in size between the mouse and seal HIF-1α, which may reflect differences in exon and/or intron sequences. Constitutive expression of HIF-1α and HIF-1β was found in some seal tissues, suggesting that the presence of the HIF-1 dimer is dependent on both tissue-specific energy requirements and adequate metabolic supply-to-demand ratio. In comparisons of different seal tissues, there

appeared to be no direct correlation between the tissue level of HIF protein expression and protection of tissue proteins from oxidative damage. Combined, the evidence available suggests that diving mammals have an overall anticipatory response to avoid the ill effects of dive-associated ischemia-reperfusion which may involve the HIF system.

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