Molecular cloning and expression of leptin in gray and harbor seal blubber, bone marrow, and lung and its potential role in marine mammal respiratory physiology

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hammond, john a., kimberley a. bennett, michael j. walton, and ailsa j. hall. molecular cloning and expression of leptin in gray and harbor seal blubber, bone marrow, and lung and its potential role in marine mammal respiratory physiology. am j physiol regul integr comp physiol 289: r545–r553, 2005. first published april 14, 2005; doi:10.1152/ajpregu.00203.2004.—leptin is a multifunctional hormone produced predominantly in adipocytes. it regulates energy balance through its impact on appetite and fat metabolism, and its concentration indicates the size of body fat reserves. leptin also plays a vital role in stretch-induced surfactant production during alveolar development in the fetus. the structure, expression pattern, and role of leptin have not previously been explored in marine mammals. phocid seals undergo cyclical changes in body composition as a result of prolonged fasting and intensive foraging bouts and experience rapid, dramatic, and repeated changes in lung volume during diving. here, we report the tissue-specific expression pattern of leptin in these animals. this is the first demonstration of leptin expression in the lung tissue of a mature mammal, in addition to its expression in the blubber and bone marrow, in common with other animals. we propose a role for leptin in seal pulmonary surfactant production, in addition to its likely role in long-term energy balance. we identify substitutions in the phocine leptin sequence in regions normally highly conserved between widely distinct vertebrate groups, and, using a purified seal leptin antiserum, we confirm the presence of the leptin protein in gray seal lung and serum fractions. finally, we report the substantial inadequacies of using heterologous antibodies to measure leptin in unextracted gray seal serum.

surfactant production; fat metabolism; parathyroid hormone-related protein; leptin antibody

however, the role of leptin in the regulation of energy balance in marine mammals is currently equivocal. phocid seals experience rapid and extensive fluctuations in fat deposition (during foraging) and fat utilization (during prolonged fasting when breeding and molting), resulting in radical changes in body composition (36). seals have a fat-based metabolism and store their reserves in a subcutaneous layer of blubber. these specializations are likely to result in rather different energy control mechanisms than is found in terrestrial mammals. unlike other mammals, there appears to be no correlation between fat mass and serum leptin in the seal species studied so far (16, 32). although this may reflect a fundamental difference in the role of leptin in energy balance between seals and terrestrial mammals, these studies used a commercially available leptin kit (multispecies kit xl-85k, linco research, st. charles, mo) to measure circulating leptin. although these studies demonstrated cross-reactivity between the anti-human antibody used and putative leptin in seal plasma samples, the results could only be expressed in units of human equivalent (16). although the leptin protein appears highly conserved among terrestrial vertebrates (43), it may be somewhat different in marine mammals. thus the lack of a relationship between fat stores and leptin in these animals may in fact be an artifact of inefficient detection of phocine leptin using an anti-human antibody.

leptin is also involved in various other aspects of hypothalamic-pituitary activity, such as the onset of puberty (1, 9). the ob gene is largely, although not exclusively, expressed in fat cells (14, 18). it is expressed in the placenta (27), where it is thought to be involved in placental and fetal growth regulation, and in the stomach (6), where it is implicated in the regulation of gastric function. bone marrow leptin expression is considered to be important in hematopoiesis and inflammation (24), and recent studies have also shown that leptin plays a key role in pulmonary surfactant production in the lung of the developing rat fetus (46).

here, we clone the leptin gene from harbor (phoca vitulina) and gray (halichoerus grypus) seals and investigate differences in tissue mRNA expression and leptin presence using a specifically designed seal leptin antiserum raised in rabbits against an amino acid sequence inferred from the cloned seal gene sequences. we also investigate the expression of parathyroid hormone-related protein (PTHrP) in lung tissue because of its important role in the leptin-signaling pathway for surfactant production in fetal lung (17). finally, we explore the
ability of presently available immunoassays to measure pho-
cicine leptin in gray seal blood samples.

METHODS

RT-PCR. Tissue samples were collected from freshly dead stranded animals washed ashore around the UK coast. Serum samples were collected from free-living animals under licence from the UK Home Office [Animal (Scientific Procedures) Act, 1986] with approval through the Ethical Review Process. Blubber and lung samples from two gray and two harbor seals were immediately stored in RNALater (Ambion, Cambridgeshire, UK) and kept at −20°C until RNA ex-
traction was performed, not more than 3 days after the samples were collected. The samples were homogenized in 3 ml of Tri-Reagent (Sigma, Poole, Dorset) before we proceeded with the extraction according to the manufacturer’s guidelines. After a washing pro-
dure, the RNA was dissolved in DEPC-treated water and quantified using a spectrophotometer at 260-nm absorption.

Reverse transcribed was performed with SuperScript II Moloney murine leukemia virus (Invitrogen, Paisley, UK) using 5 μg of denatured RNA (70°C for 10 min) from each of the blubber cores according to the manufacturer’s guidelines, with the inclusion of 40 U of RNase inhibitor. We performed RACE using advantage SMART RACE kit (BD Biosciences, Oxford, UK) in 10-

Partial purification of serum leptin. Serum proteins were fraction-
ated by gel filtration (50) on a Sephadex G100 column (Sigma; 16 ×
300 mm) that was equilibrated and eluted with 50 mM Tris, 1 mM EDTA, and 0.05% Triton X-100, pH 7.5 at 20°C, to minimize leptin binding effects. First, to ascertain where leptin eluted, 1 ml of seal serum was mixed with ~1,000 Bq of human 125I-labeled leptin [from the multispecies leptin radioimmunoassay (RIA) kit, Linco Research], which was then applied to the column, and 1.5-ml fractions of the eluate were collected. Protein was assayed in 25-μl aliquots of the fractions using the Lowry method (26), and counts per minute (cpm) of radioactivity in the remainder of the aliquot were measured for 1 min/fraction in a gamma counter. The peak of unbound leptin activity eluted after most serum proteins. Six milliliters of gray seal serum (without added radiolabeled leptin) were concentrated to ~1 ml in a vacuum desiccator over anhydrous silica gel for several hours at room temperature and then applied to and eluted from the column as described above. Fractions 29–31, 32–34, and 35–37 were combined to give three pooled fractions (I–III) for further testing. These samples were taken to dryness in a vacuum desiccator, and each was redis-
solved in 0.5 ml of elution buffer.

Immunoassays. The ability of two immunoassays to detect leptin in gray seal serum was tested; these were the commercially available multispecies leptin RIA kit (Linco Research) and an ultrasensitive canine leptin immunoenzymometric assay (IEMA), developed by Morin-
aga Institute of Biological Science (Yokohama, Japan). The protocol of the canine leptin IEMA was provided by the suppliers and was not modified for use with gray seal serum. To measure leptin concentra-
tions lower than the lowest standard concentration supplied (1 ng/ml), extra standards at the lower end of the range (0.1 and 0.5 ng/ml) were made up immediately before use, by dilution of the kit standards. Mean absorbance for duplicate wells or mean cpm for duplicate tubes were calculated. In the RIA, the mean cpm for nonspecific binding tubes was subtracted from the mean cpm from all other tubes, and the final mean cpm for each tube was expressed as a percentage of radiolabel bound to the antibody compared with the zero standard [%/(B/B0)]. Standard curves were constructed for each assay, and sample values were calculated from the equation of the line. Concen-
trations are expressed as nanograms per milliliter of human or canine equivalent. Assay coefficients of variation (CV) were calculated from replicate measurements of a single sample. Intra-assay CV for the RIA was calculated from 10 repeats of a single sample and three repeats of the human quality control (QC) on three occasions. Ten samples and the QCs measured on different days were used to calculate interassay CV. Intra-assay CV for the IEMA was calculated from three repeats of pooled serum within one assay run. There were insufficient anti-
body-coated plates and reagents to determine interassay CV.

In the RIA, six samples were sequentially diluted to 0.25 of their initial strength in assay buffer. For the IEMA, a sample pool was sequentially diluted in assay diluent (stripped canine serum) to 0.0625 initial strength and assayed on three occasions. Dilutions of pooled fractions I–III were also assayed in duplicate in the IEMA. Each dilution was measured in duplicate in the appropriate assay. For each dilution, the %/(B/B0) or absorbance values were plotted alongside values for the standard curve.

Protein separation and detection. Pooled fractions I and III, canine leptin, and broad-range SDS-PAGE markers (Bio-Rad, Hertfordshire, UK) were separated by SDS-PAGE (39) to resolve proteins of 1–100 kDa. Protein bands were detected by silver staining as follows. Gels were agitated in fixative 1 (40% methanol, 10% acetic acid) for 20 min at room temperature and in fixative 2 (40% ethanol) for 10 min. The gels were washed twice for 5 min in deionized water. Oxidizer (0.02% Na2S2O4) was added for 1 min, followed by a further two 20-s washes in deionized water. The gels were then agitated in chilled 0.1% AgNO3 for 20 min and washed again in deionized water before the addition of developer (0.05% CH2O: 3% Na2CO3) for 30 s. The gels were then covered in developer and agitated until the protein bands had developed sufficiently. Both development and stop solution (5% acetic acid) was added for 5 min.

Leptin expression RT-PCR. Tissue was obtained from a range of freshy dead animals. Tissues removed from these animals were stored at −70°C for <2 yr. These tissues from the gray seal were blubber (as described above), lung (adult male and juvenile female), spleen (juvenile female), liver (juvenile female), and total white blood cells (mixed sex adults). Samples from the harbor seal were blubber (as...
described above), lung (adult female), spleen (adult female and adult male), liver (adult female), brain (adult male), bronchial lymph node (adult male), bone marrow (juvenile female), heart (adult female), pancreas (adult female), and total white blood cells (mixed sex adults). Sample RNA was extracted and quantified in the same manner as described above. The amount of Tri-Reagent was varied depending on the size of the tissue sample. Reverse transcription was carried out as above, after incubation with DNase (Promega, Southampton, UK) to remove any genomic DNA carried over from the RNA extraction.

Primers were designed to anneal to both the harbor and gray seal leptin cDNA. A pair of primers was first designed to amplify a 550-bp fragment (5'-AGACATCCCTGGAAGAGAA-3' and 5'-TTGGGAAGAGACCTTTTGG-3'). A second sense primer was also synthesized to visualize low levels of expressed leptin mRNA and to confirm any negative results in a hemi-nested reaction amplifying a fragment (5'-ATGACATTTCACCACCGCAG-3'). PCR was carried out in the same manner as before using a final concentration of 1 μM of each primer over 30 cycles. Hemi-nested PCR was performed using 1 μl of template consisting of 2.5 μl of the first PCR reaction diluted 1:50 with tricine-EDTA buffer with the same concentration of primers over 30 cycles. All of the samples were subjected to PCR with primers for β-actin over 30 cycles to confirm the integrity of the cDNA and to provide a guide to the levels of leptin mRNA expression. PCR products were visualized on agarose gels containing ethidium bromide. Amplified DNA of the predicted size was extracted, cloned, and sequenced as above to confirm leptin cDNA; this was done for all tissues except the blubber because the presence of leptin in this tissue had already been established.

**Design and production of seal leptin antibody.** Using the same approach as described by Richards et al. (37), we derived a peptide sequence in the AB loop region of gray and harbor seal leptin from the coding sequence. This region contains a six-amino acid sequence that is totally conserved in all species, including seals, studied so far. An 18-amino acid peptide (CSRPRVAGLDFIPRVQ) was synthesized against which a polyclonal antiserum was produced by Cambridge Research Biochemicals by immunizing rabbits. The resulting crude antiserum were purified by affinity chromatography on thiopropyl Sepharose 6B derivatized with the antigen.

**Dot-blot analysis.** The ability of the anti-seal antibody to recognize leptin was qualitatively tested by dot blotting. Pooled serum fractions and III, unextracted gray seal serum, the seal leptin oligopeptide, and a human leptin standard (10 ng/ml, Linco Research) were bound onto nitrocellulose membrane (0.45-μm pore size). Samples of homogenized heart (as a negative control) and lung tissue from a freshly dead gray seal were also fractionated using the method described above for the partial purification of serum samples and the same fractions (fraction 29–31) that putatively contained the leptin protein were absorbed onto the membrane. After a drying period, the membrane was washed for 2 min using high-purity water; 15 ml of Tris-buffered saline Tween (TBST) with 3% skimmed milk were added, and this was incubated for 30 min. TEA-purified anti-seal antibody (2.5 μg/ml) was added and incubated for a further 75 min. The membrane was then washed in TBST; 10 ml of TBST with 3% skimmed milk and anti-rabbit peroxidase-labeled secondary antibody (1:10,000) were added and incubated for 75 min. The washed membrane was then visualized by adding 10 ml of 3,3',5,5'-tetramethylbenzidine.

**RESULTS**

**Degenerate PCR.** Using the leptin primer combinations, we amplified DNA of the predicted size from both gray and harbor seals. Successful primer combinations were different for each species. Gray seal cDNA fragments were amplified using the primer combinations S2/AS1 (367 bp) and S2/AS2 (182 bp). Harbor seal cDNA fragments were amplified using all the primer combinations. Both combinations of PTHrP primers amplified DNA of the predicted size from both species. The product from the primer combination amplifying the largest fragment for each gene was cloned, and five colonies were chosen for plasmid extraction and sequencing. Alignments with CLUSTAL W (44) from the Pôle BioInformatique Lyonnais web interface (http://npsa-pbil.ibcp.fr) and BLAST (4) were used to confirm that these gene fragments were homologous to the target sequences.

**RACE.** We used RACE to amplify DNA of the predicted size compared with other carnivore leptin sequences for both the 5' and 3' templates for both species. Construction of the
entire cDNA sequences revealed a high degree of identity to other leptin sequences. Gray seal leptin (AJ618982) consists of a 759-bp cDNA coding for a 167-amino acid open-reading frame. The signal peptide extends from amino acid 1 to amino acid 21 (Fig. 1), producing a mature peptide with a predicted molecular mass of 15.8 kDa. Gray seal leptin shows the highest level of identity with canine leptin (71.26%). Harbor seal leptin (AJ618981) is a 757-bp cDNA coding for a 167-amino acid open-reading frame. The signal peptide extends from amino acid 1 to amino acid 21 (Fig. 1), producing a mature peptide with a predicted molecular mass of 15.8 kDa. Harbor seal leptin also shows the highest level of identity with canine leptin (71.86%). The level of identity between the leptin sequences of these two closely related seal species is 98.2%. Both seal leptin sequences have a high amino acid conservation level shown by all members of this protein family (Fig. 2), with an overall identity of 76.74%. Of the amino acid substitutions present in each seal leptin that are conserved in other mammalian sequences, a majority are only seen in the seal sequences, although some appear to be a feature of carnivore species in general (Fig. 2). Some of these substitutions are within conserved regions (Fig. 3) that are predicted to make up the four α-helical structures of leptin (51).

PTHRP RACE cycling produced overlapping 5’ and 3’ DNA fragments in each species that were used to construct the cDNA sequences of gray seal PTHrP (AJ831410) and harbor seal PTHrP (AJ831411). The amino acid sequence shows 100% identity between these seal species with only two synonymous nucleotide changes in the coding region. This is a highly conserved protein; accordingly, the amino acid identities with canine and mouse are 97% and 86%, respectively.

Antibody cross-reactivity. RIA QC intra- and interassay CV was <14%. Sample intra- and interassay CV was 30–38% and 8–48%, respectively. The intra-assay CV was <6% in the IEMA. Parallelism of unextracted serum and fraction dilutions with the standard curves of the RIA and IEMA are shown in Fig. 4, A and B, respectively. It was difficult to establish for the RIA whether %B/B₀ values for sample dilutions were parallel to the standard curve because all values were close to the lower detection limits of the kit. Absorbance values for dilutions of pooled fraction I were parallel with the standard curve of the IEMA. Values for unextracted serum dilutions were not parallel with the standard curve, and the IEMA showed no reactivity to pooled fractions II and III.

**SDS-PAGE.** A protein band of ~16 kDa was visible in the lanes that contained canine leptin and pooled fraction I of the gray seal serum. Although a band of similar size can also be seen in lane D, pooled fraction III (Fig. 5), this fraction showed no cross-reaction with the anti-canine leptin antibody (Fig. 4B) or the anti-seal antibody (see Fig. 7).

**Leptin expression PCR.** β-Actin was successfully amplified from all of the templates (Fig. 6). After PCR, only blubber cDNA from both species amplified DNA bands of the correct size (Fig. 6). After hemi-nested PCR, both lung samples from each species and bone marrow from the harbor seal showed amplified DNA of the predicted size (Fig. 6). No bone marrow was available from the gray seal. Sequencing confirmed that this amplification was identical to blubber leptin cDNA. All other templates were negative.

**Dot-blot analysis.** The anti-seal antibody cross-reacted with the human leptin standard (Fig. 7E) but did cross-react with a protein in the lung fraction (Fig. 7F). A very weak reaction was also seen with the heart fraction, which was included as a negative control (Fig. 7G). However, the lung and heart tissue fractions are also likely to contain some residual leptin protein from cross-contamination with the blood remaining in these organs when they were sampled.
Leptin is expressed in the blubber of both gray and harbor seals. This is consistent with the findings in other mammalian species, including humans. Indeed, leptin gene expression and protein synthesis is maximal in the adipocytes (49), and this suggests that leptin in phocid seals functions in a similar manner to terrestrial species, as a major regulator of long-term energy balance (51). Low expression of leptin was seen in the bone marrow of the harbor seal. Leptin is secreted by bone marrow adipocytes in other species, including humans (23, 24), where it is thought to be important in hematopoiesis and inflammation. Some proinflammatory and hematopoietic cytokines inhibit the gene expression and secretion of leptin (25). In addition, leptin receptors are found on hematopoietic stem cells, and leptin provides a proliferative signal to hematopoietic cells (8), indicating its likely role in hematopoiesis. Phocid seals exhibit significant shortening of the long bones (particularly, the femur, tibia, and fibula) containing the bone marrow (10) and have dense bones in which the marrow is stored in a matrix. Despite these differences, it is likely that the bone marrow microenvironment is similar to that of terrestrial mammals. Our finding suggests that the role of leptin in hematopoiesis may be conserved in marine mammals.

The sequence differences between gray and harbor seal leptin are in three positions, two of which are in the mature protein after cleavage of the signal peptide, after residue 21 (Fig. 1). Both of these substitutions are either common or neutral in charge and unlikely to have an effect on the protein function. Leptin sequences from gray and harbor seals show the expected high degree of identity with other leptin sequences, with an overall identity of 76.74% (Fig. 2). Sequences from both the gray and harbor seal are most identical to the dog and the cat, respectively, species that are also carnivores. Chicken is the least identical leptin sequence to either seal species, but there is still a relatively high level of identity (58%) between these phylogenetically distant species. A comparison of both of the seal sequences with representative sequences from other vertebrates (Fig. 3) reveals that there are amino acid substitutions unique to the seal sequences in the most conserved regions of the four helices. It is unclear what overall effect this may have on the structure and function of the protein. Cys-117 and Cys-167 form a disulfide bond that is key to the structure of leptin; mutation of either inactivates the protein (56). These cysteine residues are present in both seal sequences, in common with all mammalian sequences. However, there is an extra cysteine at position 52 (Fig. 2) that may be significant for the tertiary structure of the protein. The only other leptin sequence to display an additional cysteine residue upstream of the key structural cysteine residues is from the chicken (Cys-21, Fig. 2). The potential implications of this...
cysteine are unclear. If we assume that the structure of leptin E-100 (56) applies to all of the leptin family members, then the helices A, B, C, and D (Fig. 3) face each other and the regions marked in bold form the hydrophobic cylindrical core. It is assumed that these residues are important in maintaining the structural integrity of this protein. Seal leptin shows some amino acid substitutions in these regions (Fig. 3). It is unlikely that these substitutions represent any changes in charge or function, as they are either equivalent or neutral in charge. Although Asp-85 and His-88, conserved in other leptin sequences but not in seals, could have a structural purpose, the amino acid changes to Ala and Arg, respectively, have little impact on the charge.

This study is the first to report the expression and presence of leptin in adult mammalian lung tissue. Both gray and harbor seals expressed leptin mRNA in the lung. In addition, the presence of the protein itself in partially purified gray seal lung and blood fractions was confirmed using a seal-specific antibody raised in rabbits against an 18-amino acid sequence of the AB loop. Although the leptin receptor has been found in the lung of many terrestrial species, including humans, leptin expression has only been reported in the lung of the rat fetus during alveolar development (45, 47). Torday et al. (47) demonstrated that leptin is a key component in alveolar development. Alveolar type II (ATII) cells secrete pulmonary surfactants such as surfactant phospholipid (largely dipalmitoylphosphatidylcholine) and surfactant proteins A, B, C, and D (22), and leptin mediates the paracrine effect of PTHrP on this synthesis. Pulmonary surfactants keep the alveoli and the small conducting airways open during expiration. Leptin expression in fetal rat lung occurs during the last third of gestation, during the phase of lung development when lipofibroblast (LF) and ATII cells mature and surfactant phospholipid synthesis is induced (47) between embryonic days 17 and 21. Leptin production then decreases from day 22 onward (47).
Upregulation of leptin mRNA occurs when rat fetal lung explant cultures are coincubated with leptin and PTHrP. Stimulation with both PTHrP and leptin also results in increased expression of surfactant protein B (45). Leptin is probably the low-molecular-weight soluble factor recognized as important in ATII cell differentiation by Smith in 1979 (40), then termed fibroblast pneumocyte factor.

Surfactant secretion occurs in response to stretch as the alveolar sacs distend with fluid. This mechanostimulation induces a paracrine signaling “loop” in which leptin, secreted from LF, interacts with PTHrP from ATII cells to promote ATII cell surfactant synthesis (17, 46). Stretch increases phospholipid synthesis by upregulating receptor-mediated mechanisms that synergize with the PTHrP and leptin target cell effects. The combination of stretch (6–9% radial elongation) and leptin treatment in fetal rat lung ATII cells stimulates saturated phosphatidylcholine synthesis by eightfold (compared with three- to fourfold by stretch alone) (45). Leptin expression in the lung has only been reported in fetal tissue and does not appear to be expressed by fully developed lung cells (17). However, ATII cells do secrete surfactant in response to mechanical forces in mature animals, without the leptin signaling pathway. Wyszogrodski et al. (55) found that increased ventilation in experimental animal models led to an increased usage of surfactant. Spontaneously breathing rabbits had an increased amount of phospholipid in bronchoalveolar lavages following hyperventilation (34); further in vitro and in vivo studies confirmed that hyperventilation through mechanical distension induces surfactant release (30, 53, 54). The presence of pulmonary surfactant in excess may be particularly important for seals, which repeatedly dive to forage. Phocid seals collapse their lungs during the initial period of a dive to avoid tissue nitrogen accumulation (12). They have compliant chest walls and possess structures that allow the alveoli to collapse first, followed by the terminal airways (21). Pulmonary surfactant integrity must be maintained during dives at depth for reflation of the lungs at the surface. It is possible that the additional stretch stimulation produced by the much greater distension and stretch of the ATII cells in marine compared with terrestrial mammals and the synergistic effects of leptin and stretch combined on surfactant secretion may be required because of the rapid turnover rate of surfactants in these species. The bronchoalveolar lavage fluid from nine species of pinniped was found to contain higher concentrations of phospholipid and more fluid phosphatidylcholine molecular species than terrestrial mammals (41).

Wirtz and Dobbs (54) suggested that breathing patterns may affect alveolar epithelial cell differentiation and surfactant synthesis and metabolism. Miller et al. (28) recently investigated the control of surfactant secretion in adult California sea lion (Zalophus californianus) lung cells. They tested the effect of a variety of agents that normally increase surfactant production (dexamethasone, triiodothyronine, isoproterenol, and carbamylcholine chloride) on phosphatidylcholine secretion in sea lion lung cells compared with sheep at different pressures. All compounds increased basal surfactant secretion in the sheep, but only isoproterenol increased secretion in the sea lions. They concluded that the increased surfactant production observed in seals is probably induced by mechanostimulation. This signaling pathway is switched off after a critical developmental stage in terrestrial mammals (48), presumably because they do not need to produce such large quantities of pulmonary surfactants after initial lung inflation at birth. It seems likely that seals retain the ability to secrete leptin from lung LFs throughout life to maintain surfactant production. Although the expression of PTHrP in the lung corresponds with this, adult ATII cells also release PTHrP, which independent of leptin production from LFs induces phosphatidylcholine secretion (17).

Qualitative confirmation of the presence of the leptin protein in the gray seal lung tissue was obtained following the design and production of an anti-seal antibody against a peptide in the AB loop of the leptin protein. The dot-blot analysis showed a clear reaction in the lung fraction and only a very weak reaction in the heart fraction. Although some blood contamination might account for the results found with the heart, the stronger reaction against the lung fraction would suggest that contamination by leptin from blood is unlikely to account for the observed result.

The presence of a protein of the same size as canine leptin (~16 kDa) in the pooled gray seal serum fraction and its reaction with the anti-seal antibody verified that gray seal serum contains a protein of the same molecular mass as other mammalian leptins. Accurate measurement of serum leptin in pinnipeds is vital to understanding the hormonal control of fat regulation in these mammals that routinely undergo cycles of fasting and foraging. The anti-human leptin antibody in the Linco multispecies RIA kit used here has a wide cross-reactivity with leptin of other species. The kit has been used to measure leptin in carnivores [e.g., bear (Ursus arctos arctos) (19), mink (Mustela vison) (31), raccoon dog (Nyctereutes procyonoides) (29)], including some pinniped species [northern elephant seals (Mirounga angustirostris) (32, 33), sea lions (Eumetopias jubatus) (35), Antarctic fur seals (Arctocephalus gazella) (5)]. However, the present study calls into question the reliability of leptin measurements in gray seal serum obtained using this kit.

Previous reports have demonstrated that the Linco multispecies leptin RIA responds in the same way to a protein present in northern elephant seal serum as it does to human leptin (32). In this study, measured levels of leptin were very low and close to the detection limit of the kit. The %([B/B0]) values for gray

seal serum lay on the nonlinear section of the standard curve where incremental changes in leptin concentration do not produce a large change in assay response. The precision of the RIA in gray seal serum was very poor. Other studies on pinnipeds report much lower interassay and intra-assay variability using this kit (5, 32). However, it is not clear whether serum or kit QCs were used to calculate these values.

The modifications to the kit protocol may have introduced some of the variability seen here. Variation in the human leptin QC measurements was higher than the values reported by the manufacturers for variability in human samples (<15% compared with <9%). If modification of the kit protocol alone was the source of the high assay variability, interassay and intra-assay CV for QC and sample measurements would be similar. There is, however, a higher degree of variation in the gray seal serum results than in the QC results, which is probably due to poor detection of phocine leptin by the kit antibody and/or the presence of interfering substances in the serum.

The high % (B/Bo) values for gray seal serum in the RIA indicate very low levels of circulating immunoreactive leptin (<5 ng/ml), similar to those reported in other pinniped species (5, 32). Although these low values may be a consequence of an inability of the kit to detect seal leptin rather than a reflection of true concentrations, circulating leptin concentrations in other carnivores are low (20). In seals, high assay variability combined with low actual leptin concentrations may mask important patterns of change in leptin secretion. This could explain the reported absence in pinnipeds of a relationship between body fat and serum leptin levels (5, 32) that is normally found in terrestrial mammals (38). The polyclonal antiserum that we raised against an oligopeptide, conserved between the gray and harbor seals, cross-reacts with an epitope on seal leptin in serum samples and will be used in an assay to investigate the true nature of this relationship in phocid seals.

In summary, our findings reveal that the lung, blubber, and bone marrow in phocid seals express leptin. Inspection of the differences between seal and other mammalian leptins suggest it is very likely that seal leptin is a functional protein, similar in structure to the long-chain helical cytokine family. Further studies on the tissue expression of leptin, particularly in the lungs of other marine mammals, will be of interest, including comparative investigations with various cetacean and some otarid species, which dive on inspiration (21). Leptin mRNA expression patterns among various marine mammals, coupled with cell culture experiments, will enable further exploration of the control of pulmonary surfactant secretion by leptin in relation to the respiratory physiology of this diverse group of carnivores.

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GRANTS

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REFERENCES


15. Greenberg JA and Booser CN. The leptin-fat ratio is constant, and leptin may be part of two feedback mechanisms for maintaining the body fat set point in non-obese male Fischer 344 rats. Horm Metab Res 31: 525–532, 1999.


27. Masuzaki H, Ogawa Y, Sagawa N, Hosoda K, Matsumoto T, Mise H, Nishimura H, Yoshimasa Y, Tanaka I, Mori T, and Nakao K. Non-


36. Reilly JJ, Fedak MA, Thomas DH, Coward WAA, and Anderson SS. Water balance and the energetics of lactation in gray seals (Halichoerus grypus) as studied by isotopically labelled water methods. Water balance and the energetics of lactation in gray seals (Halichoerus grypus) as studied by isotopically labelled water methods.


