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Plasma Angiotensin II, Arginine Vasopressin and Atrial Natriuretic Peptide in Free Ranging and Captive Seals and Sea Lions

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ABSTRACT. We used radioimmunoassay methods to quantify arginine vasopressin (AVP), atrial natriuretic peptide (ANP), and angiotensin II (Ang 11) in plasma samples from harbor seals *(Phoca vitulina richardsii),* Weddell seals *(Lepconychotes wedellii),* northern elephant seals *(Mirounga angustirostris),* ringed seals *(Phoca hispida),* California sea lions *(Zalophus californianus),* and Steller sea lions *(Eumetopius jubatus).* Plasma concentrations of AVP, ANP, and Ang II in these pinniped species were within the ranges reported for other vertebrates under resting conditions. However, there were species, geographic and developmental variations in these hormones: Levels of AVP in plasma samples from adult Steller sea lions and harbor seals were higher than in pups of the same species; higher levels of plasma ANP were found in wild captured Alaskan Steller sea lions and in hunted ringed seals; differences in plasma levels of all three hormones were found throughout the geographic distribution of harbor seals and Steller sea lions in Alaska. This is the first report on circulating concentrations of vasoactive hormones in pinnipeds, and demonstrates that further studies are needed to ascertain the natural variability in these levels with the impact of molting, fasting, diving and environmental factors in seals and sea lions. COMP BIOCHEM PHYSIOL 119C;l:l-6, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Seals, sea lions, marine mammals, vasoactive hormones

INTRODUCTION

As vasoactive hormones, the role of both angiotensin II (Ang II) and arginine vasopressin (AVP) is to produce constriction of vascular smooth muscles (10,33), while that of atrial natriuretic peptide (ANP) is to antagonize such constrictor effect (3). Deviations from baseline plasma concentrations of ANP, AVP, and Ang II during exercise and water-immersion have been reported for a variety of vertebrate species. In humans and other mammals, plasma concentrations of ANP, AVP, and aldosterone, and plasma renin activity increase in response to exercise in an intensity-related fashion (17,22). During head-out immersion the plasma level of ANP increases in humans (14) and dogs (31) while AVP (9,19,26) and the renin-angiotensin-aldosterone systems (RAAS) (9) are inhibited. Freshwater turtles responded to water immersion and diving with changes in circulating vasoactive hormones (2) similar to those observed in humans and dogs. It is possible that Ang II, AVP, and ANP participate in the control of the changes in heart rate, blood pressure, and redistribution of blood flow observed in marine mammals during natural diving- and sleep-associated apnea (5,6,13). However, in order to explore this hypothesis it is first necessary to obtain information on the circulating levels of these hormones in marine mammals at rest. Of these hormones, only AVP has been studied in marine mammals, and mostly in its antidiuretic role (28,29, 32). The purposes of the present study were to determine plasma concentrations of ANP, AVP, and Ang II in a variety of diving mammals and to establish potential species and geographic differences.

MATERIALS AND METHODS

Blood samples were collected from Steller sea lions *(Eumetopias jubatus)* and harbor seals *(Phoca uitulina)* at haul-out sites and rookeries at various locations in the Aleutian Islands and the Gulf of Alaska. Weddell seals *(Lepeonychotes weddellii)* were studied in McMurdo Sound, Antarctica, while northern elephant seals *(Mirounga angustirostris)* were studied at Año Nuevo State Reserve, California. Additional samples were obtained from Steller sea lions that had been captured as pups in the wild and raised at the Vancouver Aquarium, Vancouver, British Columbia, Canada, and from harbor seals maintained at SeaWorld-Hubbs Research Insti-

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tute, San Diego, CA. Samples were also obtained from California sea lions *(Zalophus californianus)* from SeaWorld-Hubbs Research Institute and from ringed seals *(Phoca hispida)* from native hunters in Barrow, AK. We analyzed a total of 181 plasma samples from Steller sea lions, 161 harbor seals, 6 California sea lions, 33 Weddell seals, 10 elephant seals, and 5 ringed seals. Age was estimated based on morphometric data as follows: newly-born to 16-week-old animals were classified as pups, yearlings were considered animals from the weaning period to about 1 year old, subadults were estimated to be 2 to 3 years old, and adults were estimated to be at least 4 years old. During sampling, pups and yearlings were manually restrained. Steller sea lion adults and subadults, as well as some pups, were darted with Telazol® and, if necessary, further anesthetized with either Halothane® or Telazol(21); some Steller sea lion pups were anesthetized with Halothane, and adult harbor seals (Prince William Sound) were anesthetized with ketamine/diazepam intramuscularly (i.m.) at standard doses (18).

Blood samples (5 mL) were taken by venipuncture from either a hind flipper vein, the extradural vein (phocid seals}, or the dorsal pelvic vein (sea lions), immediately transferred into a chilled test tube containing 0.125 M ethylenediaminetetraacetic acid (EDTA) (Vacutainer 6450, Becton-Dickinson Ltd., Rurherford, NJ) and centrifuged at 4000 X g for 10 min. An angiotensin-converting enzyme (ACE) inhibitor, o-phenanthroline (0.025 M, 100 uL/mL plasma, P-9375; Sigma Chemicals, St Louis, MO) (11) was added to the recovered plasma. Samples were placed in a liquid nitrogen-cooled CryoPac shipper (-196°C) and transported to the University of Alaska Fairbanks, where they were archived at -70°C until extraction for radioimmunoassay (RIA). Plasma osmolality (Osm_{pl}) was not measured; osmometers were not available at the remote field locations. In our hands, freezing/thawing of plasma samples significantly compromises the accuracy of the Osm_{pl} determinations.

Immunoreactive (ir) material was extracted from plasma by using prepacked octadecasilyl-silica cartridges (Sep-Co1 C_{18}, Phoenix Pharmaceuticals, Mountain View, CA) according to a method adapted from Hartter (20). Each cartridge was used for only one sample. The cartridge was sequentially washed with 100% methanol (glass distilled, HPLC grade, Sigma Chemicals), 90% methanol in 0.5% trifluoroacetic acid (TFA, HPLC/Spectrograde, Sigma Chemicals), and distilled water. Thawed plasma (1 to 2 mL) was slowly passed through the cartridge, which was then washed with 8 mL distilled water, and the immunoreacrive material was eluted with a 90% methanol-0.5% TFA solution. The eluate was dried in a vacuum sample evaporator and concentrator (Labconco, Kansas City, MO) and stored at -20°C for RIA on the next day. Prior to analysis, samples were reconstituted to their original volume with RIA buffer (Phoenix Pharmaceuticals). The percent recovery from the extraction procedure was determined by adding known amounts of synthetic peptide (5 to 100 pg/mL, Phoenix Pharmaceuticals) to pooled plasma (quality control). Measurements of extracted plasma samples were not corrected for extraction efficiency, which ranged from 90 to 110%. Biochemical identity of the ir material obtained from pinniped plasma samples has not yet been assessed; thus, in this paper will be referred to as ANP-, AVP-, and Ang II-like ir material, accordingly.

The concentrations of AVP-, ANP-, and Ang II-like ir material in plasma samples were analyzed using commercially available RIA kits, which include antibodies raised in rabbits against the human peptides (Phoenix Pharmaceuticals). Using a second antibody, goat anti-rabbit immunoglobulin G serum, the antibody-bound material was removed. After centrifugation at 3000 X g for 30 min at 4°C, the supernatant was aspirated, and the radioactivity in the bound fraction was counted using an automatic gamma counter (Micromedic 200+, Micromedic Systems Inc., Horsham, PA). During all RIA procedures, reagents and samples were kept on ice. All samples were run in duplicate, and replicates were run within the same assay. Each assay included a standard curve generated with serial dilutions of the synthetic peptide provided by the manufacturer, and the quality control for the species for which unknown samples were being analyzed in that assay. Only values that fell within 20 to 80% of the dose-response curve (the linear portion of the curve) were considered. All dilutions were made using RIA buffer. Cross-reactivity between antibodies and the material extracted from pinniped plasma samples was determined by assaying pooled plasma serially diluted in RIA buffer and then comparing the resulting curve with that given by the standard peptide dilutions. The amount of the pooled plasma which yielded 50% inhibition of binding of the labelled hormone to the antibody was compared with the amount of standard peptide giving the same inhibition, and expressed as percentage of that of the standard (7). This test was performed in all pinniped species for each hormone analyzed.

Data were analyzed using ANOVA followed by multiple comparison Student-Neuman-Keuls tests, and non-paired t-tests with Bonferroni adjustment for multiple comparisons (41), running the statistical software, SYSTAT® (SPSS, Chicago, IL). Significance was assumed when $P < 0.05$. Final results are presented as mean ± standard error of the mean. Information on gender and age was not available for all samples; results were analyzed and are presented for the subset where this information was procured.

RESULTS

Concentrations of AVP-, ANP-, and Ang II-like ir material extracted from pinniped plasma samples are presented in Table 1. Results are expressed as picograms of ir material

TABLE 1. Plasma concentrations of arginine vasopressin (AVP)-, atria1 natriuretic peptide (ANP)-, and angiotensin II (ANG II)-like immunoreactive material in several pinniped species

Species	*n*	AVP	ANP	ANG II
Steller sea lions				
Adults	20	14.2 ± 1.5	139.3 ± 7.8[b]	55.8 ± 11.9
Subadults	1	6.5	6.5	20.5
Yearlings	5	6.2 ± 1.7[d]	32.0 ± 13.6[d]	24.6 ± 4.0
Pups	155	7.2 ± 0.4[d]	88.3 ± 6.4[d]	46.9 ± 3.3
Total/average	181	7.9 ± 0.4[b]	92.0 ± 5.7	47.0 ± 3.1
California sea lions				
Adults	1	10.2	26.9	8.4
Pups	5	4.7 ± 1.0	31.7 ± 5.4	7.6 ± 0.8
Total/average	6	5.6 ± 1.2	30.9 ± 4.5	7.7 ± 0.6
Harbor seals				
Adults	68	15.9 ± 2.5	30.4 ± 4.4[a,c]	29.5 ± 3.7
Subadults	41	11.4 ± 1.3	23.7 ± 4.5	34.2 ± 6.6
Yearlings	15	16.2 ± 4.0	46.8 ± 13.9	29.0 ± 9.6
Pups	17	8.4 ± 2.0[d]	25.3 ± 9.1[a]	24.0 ± 5.5
Unknown	20	10.2 ± 1.3	20.3 ± 2.8	20.8 ± 2.9
To&/average	161	13.3 ± 1.2"	28.5 ± 2.7[a,c]	29.0 ± 2.6[a]
Weddell seals				
Adults	2	12.0 ± 0.1	20.3 ± 0.2	9.0 ± 0.2
Yearlings	10	7.5 ± 2.2	52.0 ± 3.8	13.7 ± 2.6
Pups	21	2.6 ± 0.5	18.2 ± 2.3[a]	17.0 ± 2.5[a]
Total/average	33	4.4 ± 0.8[b]	24.5 ± 3.1[a,c]	15.6 ± 1.8[a]
Elephant seals				
Pups	10	7.9 ± 4.9	23.5 ± 2.8	33.2 ± 3.4
Total/average	10	7.9 ± 4.9	23.5 ± 2.8[a,c]	33.2 ± 3.4
Ringed seals				
Adults	5	9.3 ± 1.8	126.8 ± 38.4[b]	14.0 ± 6.0
Total/average	5	9.3 ± 1.8	126.8 ± 38.4	14.0 ± 6.0

Data are expressed as pg/mL extracted plasma and presented as mean ± SEM. *n* = Number of samples (one sample per animal) assayed.
[a]P < 0.05 compared to Steller sea lions.
[b]P < 0.05 compared to harbor seals.
[c]P < 0.05 compared to ringed seals.
[d]P < 0.05 compared to adults of the same species.

per milliliter (pg/mL) of extracted plasma. The sensitivity of the RIA systems (50% depression of tracer binding) for each hormone was as follows: AVP, 1.8 ± 0.2 pg/tube; ANP, 6.4 ± 0.6 pg/tube; Ang II, 4.1 ± 0.3 pg/tube (n = 15). Intra-assay errors (coefficient of variation percent) were: AVP, 3.7 ± 0.1%; ANP, 3.9 ± 0.3%; Ang II, 3.1 ± 0.2% (n = 10). Inter-assay coefficients of variation were: AVP, 5.3 ± 0.2%; ANP, 4.2 ± 0.4%; Ang II, 5.6 ± 0.3% (*n* = 15). For all three hormones, the least detectable concentration was 0.1 pg/mL. Dilutions of all seal and sea lion species showed parallelism with the synthetic peptides provided by the manufacturer, yielding 75 to 85% cross-reactivity (Fig. 1). No diurnal, seasonal or annual cyclic patterns in hormone concentrations were detected because insufficient information precluded testing for effects of time, of day, or year. There was no statistically significant effect of gender or anesthesia on AVP-, ANP-, or Ang II-like ir material concentration in any of the pinniped species analyzed.

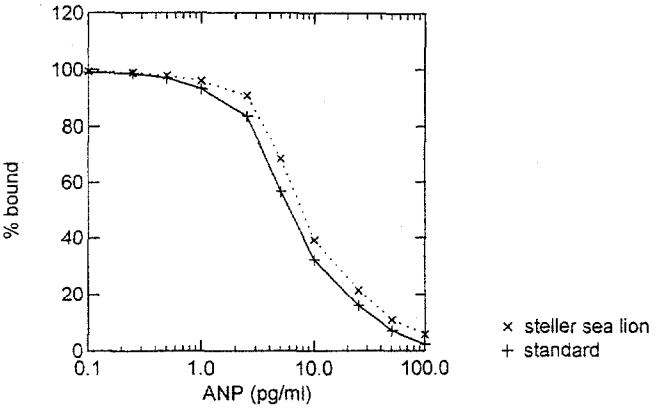

FIG. 1. Representative estimation of the percentage cross-reaction from the amount of material required to produce 50% inhibition in the radioimmunoassay. Results shown are for estimating cross-reactivity between the atria1 natriuretic peptide (ANP) standard provided by the manufacturer (Phoenix Pharmaceuticals) and Steller sea lion plasma. Solid line, standard; dashed line, Steller sea lion pooled plasma.

Thus, data was combined for further statistical analyses and the results presented include data for both males and females, as well as anesthetized and manually restrained animals.

Vasopressin

The use of antibodies raised against human AVP yielded 80% cross-reactivity with pinniped plasma samples. The concentration of AVP-like ir material in plasma samples from all seal and sea lion species analyzed ranged from 1 pg/mL to over 16 pg/mL and demonstrated significant developmental and regional differences. Steller sea lion pups and yearlings and harbor seal pups had lower levels of AVP-like ir material compared to adults of the same species (Table 1). Additional analyses revealed that AVP-like ir material was significantly lower (P < 0.05) in Steller sea lion pups from Southeast Alaska (4.7 ± 0.4 pg/mL, n = 41) (where the population is stable) than in pups from Aleutian Islands (7.8 ± 0.6 pg/mL, n = 46) and Gulf of Alaska (8.8 ± 0.9 pg/mL, n = 53), both locations at which the populations are declining. Harbor seal pups sampled in Southeast Alaska (stable population) appeared to have higher concentrations of AVP-like ir material (26.1 ± 9.1 pg/mL, n = 2) than did captive harbor seals in California (6.2 ± 1.4 pg/mL, n = 11) and wild harbor seals in Prince William Sound (5.6 ± 0.5 pg/mL, n = 4) (population declining). However, this pattern changed in yearlings, such that harbor seal yearlings from Kodiak (population increasing) appeared to have lower levels of AVP-like ir material (8.4 ± 2.6 pg/mL, n = 2) than yearlings from Prince William Sound (20.3 t 7.2 pg/mL, n = 8) (population decreasing).

Atrial Natriuretic Peptide

Anti-human ANP serum displayed a 75% cross-reactivity with pinniped plasma samples and also showed patterns by region, age and species. The average levels of ANP-like ir material in plasma samples from Steller sea lions and ringed seals were significantly higher than in samples from harbor seals, Weddell seals, and elephant seals ($P < 0.05$) (Table 1). Adult Steller sea lions had significantly higher ($P < 0.05$) levels of ANP-like ir material than did younger conspecifics. Among the Steller sea lion pups, those sampled in the Aleutian Islands (115.2 ± 13.1 pg/mL, n = 46) and the Gulf of Alaska (118.9 ± 10.0 pg/mL, n = 55) (declining populations) had significantly higher ($P < 0.05$) levels of circulating ANP-like ir material than those from Southeast Alaska (31.6 ± 3.5 pg/mL, n = 41) (population stable) and those kept at the Vancouver Aquarium (2 1.7 ± 0.6 pg/mL, n = 9). Levels of ANP-like ir material in hunted ringed seals (126.8 ± 38.4 pg/mL, n = 5) were as high as those in Steller sea lions (all age classes) from Aleutian Islands (113.4 ± 12.9 pg/mL, n = 47) and Gulf of Alaska (124.3 ± 7.7 pg/mL, n = 75) (declining populations). This pattern was age-dependent and subadult harbor seals in Southeast Alaska had higher levels of ANP-like ir material (59.1 ± 20.2 pg/mL, n = 3) than subadult harbor seals in Kodiak (16.2 ± 5.1 pg/mL, n = 6), and Prince William Sound (21.6 ± 5.0 pg/mL, n = 28) ($P < 0.05$).

Angiotensin II

Plasma samples from pinnipeds exhibited an average 85% cross-reactivity with the human Ang II antiserum used. Samples from Steller sea lions had a higher average concentration of Ang II-like ir material than harbor seals and Weddell seals ($P < 0.05$), unlike the results for AVP- and ANP-like ir material. However, there was no statistically significant effect of age on circulating levels of Ang II-like ir material in any of the seal and sea lion species analyzed. The concentration of Ang II-like ir material in plasma samples of Steller sea lion pups from the Gulf of Alaska (69.2 ± 6.4 pg/mL, n = 49) was significantly higher ($P < 0.05$) than that in samples of pups from the Aleutian Islands (32.4 ± 4.8 pg/mL, n = 43), Southeast Alaska (40.7 ± 5.4 pg/mL, n = 41), and the Vancouver Aquarium (23.8 ± 2.0 pg/mL, n = 9). Plasma samples of harbor seals of all ages from Prince William Sound had significantly ($P < 0.05$) higher Ang II-like ir material (36.2 ± 4.7 pg/mL, n = 79) than did samples of harbor seals from Southeast Alaska (20.3 ± 1.7 pg/mL, n = 45).

DISCUSSION

Sufficient cross-reactivity was obtained between each of the antibodies against the human peptides and plasma samples from pinnipeds to justify the use of commercial kits. Successful determination of AVP-, ANP-, and Ang II-like ir material in plasma samples from Weddell seals, harbor seals, northern elephant seals, ringed seals, California sea lions, and Steller sea lions was achieved by the use of commercially available antibodies. This suggests that these hormones are present in the plasma of pinnipeds. The amino acid sequences for these peptide hormones are highly conserved among vertebrates (1,24,39). Detailed biochemical analyses are needed in order to verify the biochemical identity of the ir material found in seal and sea lion plasma samples.

Vasopressin

The levels of AVP-like ir material in pinnipeds (Table 1) were higher than those reported for conscious humans, in which the average circulating AVP concentration is 4 pg/mL (34). However, plasma AVP concentrations in conscious rabbits [9.4 ± 3.2 pg/mL (25)] and rats [10.4 ± 2.5 pg/mL (40)] are similar to values found in the pinnipeds sampled in this study. Similarly, the levels of plasma AVP-like ir material found in all pinniped species were comparable to those found in a previous study on fasting, post-weaned northern elephant seal pups 134.8 ± 18.2 pg/mL (early fasting) to 4.8 ± 1.3 pg/mL (late fasting) (32)]. The northern elephant seal pups in the present study were in the mid to late fasting stage. In general, pups had lower levels of AVP-like ir material than adults, but this was statistically significant ($P < 0.05$) only for Steller sea lions and harbor seals. Johnson et al. (23) reported higher plasma AVP concentration in healthy elderly than in younger humans; however, Clark et al. (8) did not find differences between healthy young and old subjects. In addition, some of the yearling, subadult, and adult harbor seals were molting at the time of sampling. Evidence suggests that hypophysial peptides may regulate molting in vertebrates and invertebrates (12,30,35).

Atrial Natriuretic Peptide

The ANP-like ir material detected in plasma samples from California sea lions, Weddell seals, harbor seals, and northern elephant seals were similar to values reported for conscious humans [54.0 ± 5.0 pg/mL (16)] and fresh water turtles [47.0 ± 3.5 pg/mL (2)]. However, the concentrations of ANP-like ir material were relatively high in samples from adult Steller sea lions and ringed seals (Table I).

Angiotensin II

The concentration of Ang II-like ir material in samples from northern elephant seals, ringed seals, harbor seals, Weddell seals, and California sea lions (Table 1) were similar to values reported for conscious humans [12.0 ± 2.1 pg/mL (38)] and other terrestrial mammals [rabbit, 18.5 ± 4.0

pg/mL (37); rat, 29.3 ± 3.5 pg/mL (40); calf 57.2 ± 1.0 pg/mL (4)].

Unlike samples collected from all other species, samples from ringed seals were taken from animals that had been shot, which may explain the elevated ANP-like ir material concentrations in this species. However, their levels of AVP- and Ang II-like ir material were not distinct from other species. All other seals and sea lions were captured when they were on land, and presumably had been on land for some time. It is unlikely that the high levels of ANP-like ir material found in Steller sea lions were brought about by differences in sample manipulation or the diving history of the animals just prior to blood sampling. Molting may not by itself be responsible for these higher values since, unlike harbor seals, Steller sea lions were not molting at the time of sampling. Factors that may contribute to the variability in the concentrations of these hormones include variations in fluid and electrolyte balance, and anesthesia.

Most interestingly, the circulating levels of ANP- and Ang II-like ir material in Steller sea lion samples collected in the Aleutian Islands and the Gulf of Alaska were higher than those from Steller sea lions in Southeast Alaska and the Vancouver Aquarium. Similarly, the concentration of AVP- and Ang II-like ir material in harbor seal samples from Prince William Sound was higher than that in harbor seal samples from Kodiak, Southeast Alaska, and California. Furthermore, concentrations of the vasoactive peptides in samples of Steller sea lions and harbor seals from Southeast Alaska, the Vancouver Aquarium and California were closer to those in California sea lions, northern elephant seals, and Weddell seals, as well as to those in terrestrial mammals.

The populations of Steller sea lions and harbor seals at the Aleutian Islands, Gulf of Alaska, and Prince William Sound have been declining for the past 20 years (27,36) to the point that a portion of the Steller sea lion population has recently been proposed as “Endangered” (15). The cause of the decline is not obvious, but we are addressing the possibility that the hormonal differences we found may reflect nutritional, physiological, and/or genetic distinctions among the populations.

In summary, the plasma concentrations of AVP-, ANP-, and Ang II-like ir material in samples from northern elephant seals, harbor seals, Weddell seals, and California sea lions are similar to those reported for many terrestrial mammals, but showed interesting natural variation. Studies in out laboratory are being conducted to address the possibility that the differences in vasoactive hormone concentrations found in Alaskan pinnipeds may be inherent to genetic stocks or may be due to physiological and/or pathological states. Clearly, further research is needed to evaluate interactions among vasoactive hormones and physiological events pertinent to the natural histories of seals and sea lions, such as molting, fasting, and diving.

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