

Adrenomedullin Binding Protein in the Plasma of Multiple Species: Characterization by Radioligand Blotting

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Abstract Frequently, peptide hormones circulate in plasma associated with specific binding proteins that modify the clearance and biochemical activities of the peptide. Our experimental approach was to use ^{125}I -ligand blotting procedures to probe for the presence of specific adrenomedullin (AM) binding proteins (AMBP). Plasma proteins from chick, calf, dog, goat, guinea pig, human, mouse, pig, rabbit and sheep blood were separated electrophoretically in 10% nonreducing SDS-polyacrylamide gels and transferred to nitrocellulose. Nonspecific binding of tracer was blocked on the nitrocellulose with a hydrolyzed casein matrix. Blots were probed with synthetic human ^{125}I -AM. Autoradiogram scanning of blots revealed a mixture of 140- and/or 120- kD protein complexes that bound ^{125}I -AM in all species tested. Binding of the ligand was specific as judged by a linear competitive displacement of the tracer binding from human, bovine and pig plasma AMBP bands with increasing concentrations of nonlabelled AM in the binding buffer. A series of peptide fragments of AM representing amino- and carboxyterminal regions of the hormone, or amylin, calcitonin gene-related peptide (CGRP), or insulin failed to displace intact ^{125}I -AM from ligand blot bands. Bovine plasma proteins from healthy and parasitized calves with an infection-related stunting syndrome were separated electrophoretically, transferred to nitrocellulose and probed with ^{125}I -AM; phosphoimage densitometry analysis revealed a 67% decrease in AMBP band intensity in the 120 and 140 kD proteins from infected calves. We conclude that a specific binding protein(s) for AM exists in mammalian and avian blood that might impact on the bioactivity and function of AM in health and disease.

Introduction

Adrenomedullin (AM) is a 50 (rat) to 52 (most other species studied) amino acid hydrophobic peptide hormone present in a variety of mammalian species having diverse physiological effects on blood pressure, metabolism, reproduction, and development (1,2). Different actions of several peptide hormones are mediated not only through changes in circulating plasma concentrations but also through accessibility to specific receptors as facilitated or retarded by binding proteins present in extracellular fluids. For example, one of the most widely studied classes of hormone binding proteins is the insulin-like growth factor (IGF) binding proteins (IGFBPs). These proteins function to specify, enhance or block IGF actions on cells by regulating the hormone's ability to bind to cell surface receptors (3). With the development of the ligand blotting technique following SDS-PAGE (4) Hossenlopp et al. demonstrated that there were multiple IGFBPs in various body fluids that could be individually resolved on nitrocellulose with autoradiography.

With similarity to the interference that IGFBPs have on the IGF-I RIA (5), our RIA validation for measurement of AM in plasma suggested that an AM-binding protein (AMBP) may exist. Empirical observations and measurements with the RIA for AM demonstrated (a) high molecular weight elution of ^{125}I -AM in mixture with plasma from Sephadex G-50, (b) a displacement of ^{125}I -AM from primary assay antibody by

increasing volumes of serum not parallel to the displacement slope of synthetic human AM in the assay standard curve, and (c) greater than 100 percent binding (B_0) of ^{125}I -AM in the plasma assay matrix precipitated with polyethylene glycol-treated precipitating antibody. The use of short-column C-18 reverse phase chromatography (6) resulted in the elimination of the interference in the RIA for AM.

Investigations of AM actions are relatively infant in comparison with IGF-I. However, like IGF-I, the multiple diverse physiological actions of AM and the presence of a class of proteins that can bind with AM are consistent with the potential for AM to be distributed selectively to cell receptors when bound to a specific class(es) of plasma proteins. We employed a radioligand blotting technique to establish that AMBP(s) were present in the plasma of several animal species and further determined the specificity of the binding between AM and this plasma protein on nitrocellulose.

Materials and Methods

Serum or plasma was harvested from calf (4-5 mo old), pig (6-7 mo old), goat (2 yrs old), dog (6-8 yr old), mouse (4-6 mo old), chicken (6-8 wk old), guinea pig (6 mo old), sheep (1-2 yr old), rabbit (1 yr old) and adult male human volunteer blood samples, pooled by species, and stored at -20°C until needed. An initial determination of high molecular weight plasma proteins capable of binding synthetic human ^{125}I -AM (Phoenix Pharmaceuticals, Mountain View, CA) was obtained by gel filtration chromatography using Sephadex G-50

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(Pharmacia, Uppsala, Sweden) equilibrated with 50 mM phosphate, 50 mM disodium EDTA, 135 mM NaCl, 0.1% tween 20 and 0.125 % alkaline-hydrolyzed casein (AHC, 7), pH 7.2. For solution binding studies of ^{125}I -AM to plasma proteins, bovine plasma (0.1 or 0.3 ml) was mixed with 50,000 cpm ^{125}I -AM, incubated at 4 °C overnight and applied to the molecular sieve column with phosphate buffered saline in a final volume of 0.5 ml. One ml fractions were collected using gravity flow and the radioactivity quantified.

Further characterization of AMBP(s) was performed using radioligand blotting procedures similar to those described for IGF binding proteins in plasma (4). Briefly, plasma or serum from the respective animal species was diluted 1:5 with water and further mixed 1:2 with SDS TRIS-glycine-glycerol loading buffer and incubated for 10 min at 70 °C. Volume equivalents of 1.5 μl of the original plasma or serum were loaded onto 10% acrylamide gels in nonreducing conditions. Molecular weight markers (43 kD, 78 kD and 116 kD) were added to complementary lanes on each gel to assess the relative size of resolved proteins. Proteins were separated at 115 v for 1.5 h and transferred to 0.2 μm nitrocellulose using a semi-moist transfer (Bio-Rad, Hercules, CA) at 18 v for 42 min. Nitrocellulose blots were incubated for 15 min with 1.5% NP-40[®] (Sigma, St. Louis, MO). Nonspecific binding was blocked with a 4 hour incubation of the nitrocellulose in 0.01M TRIS-buffered saline containing either 1% BSA (RIA grade, Sigma, St. Louis, MO) or 0.1% AHC. Incubations with ^{125}I -AM were performed overnight, at 4 °C with agitation, with 80,000 CPM ^{125}I -AM per 5 ml buffer. The next morning, the nitrocellulose was washed 10 min with 0.2% Nonidet-P-40, and further washed 4 times for 15 minutes per wash with TRIS- buffered saline. The nitrocellulose was loaded into a phosphoimaging cassette (Molecular Dynamics, Sunnyvale, CA) overnight for analysis of resolved band images. Additional characterization of banding patterns was accomplished by autoradiography of the same blots on Kodak AR-5 film with a 4-day exposure at -80 °C.

Removal of apparent binding proteins from plasma was accomplished using a C-18 reverse-phase Sep-Pak[®] RIA preparatory technique as validated in our laboratory (6). Briefly, C-18 Sep-Pak[®] (short body) cartridges (Waters Inc., Milford, MA) were activated with sequential 5 ml washes with chloroform and methanol, followed by 5 ml 1.8% NaCl. For extraction, 1 ml plasma was mixed with 1 ml 0.01M TRIS-saline containing 0.1% AHC and 0.05% Triton X-100. This mixture was applied to the column and washed with 5 ml 1.8% NaCl. The material adsorbed onto the column usually assayed by RIA for AM was eluted with 80% isopropanol-water. The eluate was frozen, lyophilized, and reconstituted in 200 μl TRIS-saline with 0.01% Triton X-100. Samples of this material were subjected to ^{125}I -AM blotting.

Competitive displacement of ^{125}I -AM from electrophoresed human, bovine, and pig proteins on blot strips was

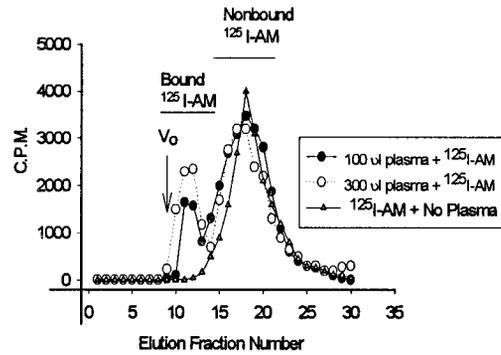


Fig. 1 Elution pattern of ^{125}I human AM from Sephadex G-50 when the tracer is preincubated with plasma for 12 hours at 4 °C.

performed to assess the specificity of the AM binding. Blot strips were incubated in buffer containing 0.1% AHC, ^{125}I -AM, and concentrations of synthetic human AM (Phoenix Pharma., Mountain View, CA) ranging from 10^{-10} to 10^{-7} M. Residual band intensity with 10^{-6} M AM established nonspecific binding and was subtracted from the bands at other AM concentrations. Band intensities were resolved by phosphoimaging densitometry. Specificity of AM for the binding protein(s) on the blots was assessed by coincubating ^{125}I -AM overnight with nonlabeled synthetic AM_{1-52} (10^{-6} or 10^{-8} M) or AM_{1-12} , AM_{13-52} , AM_{26-52} fragments and insulin, amylin and CGRP (Phoenix Pharm., Mountain View, CA), at 10^{-9} M.

Since plasma AM concentrations are known to change in some disease states (8), comparisons of band patterns and intensities on nitrocellulose blots of bovine plasmas from healthy and parasitized calves were used to determine whether AMBP content was affected by health status. Nitrocellulose transfer blots of plasma proteins from 4-mo old healthy male calves ($n=3$) and calves infected with a vascular endothelial-resident parasite (*Sarcocystis cruzi*; $n=4$) were probed with ^{125}I -AM. Plasma from infected calves was obtained on day 30 post oral inoculation (250,000 oocysts) at the peak of expression of clinical signs of the acute phase response associated with the eruption of schizonts from the vascular endothelium (9).

Data on the effects of parasitic infection on the amount of AMBP in calf plasma were statistically analyzed using an analysis of variance approach based on the general linear models procedure of SAS (10).

Results

Sephadex G-50 chromatography of plasma revealed that the elution pattern of ^{125}I -AM shifted from a single peak low molecular weight entity of approximately 6 kD to a dual peak pattern with a high molecular entity eluting at the column void volume, V_0 (>40 kD) when the ^{125}I -AM was preincubated

with plasma or serum (Fig. 1). Increasing the plasma volume of incubation from 100 to 300 µl resulted in an increase in the radioactivity eluting in the column V_0 .

When the method of AMBP detection by ^{125}I -ligand blotting on nitrocellulose was being refined, variability in background nonspecific binding of ^{125}I -AM to nitrocellulose blocked with albumin was bothersome and the intensity of the background increased with ageing tracer. We found AHC in TRIS buffer superior to other commonly used proteins, including purified bovine albumin, to block ^{125}I -AM nonspecific background. As seen in Fig. 2a, blocking with bovine albumin resulted in a general background approximately 4 to 5 times higher (by densitometry) than that observed when hydrolyzed casein was used to block the blot. Furthermore, specific, distinct bands for AMBP were observed only in the presence of the AHC, mostly as a function of the signal to noise ratio. Finally, ligand blotting analysis of C-18-chromatographed plasma confirmed that the reverse phase separation technique used to prepare plasma for RIA analysis effectively eliminated AMBP from the eluate matrix (Fig. 2b).

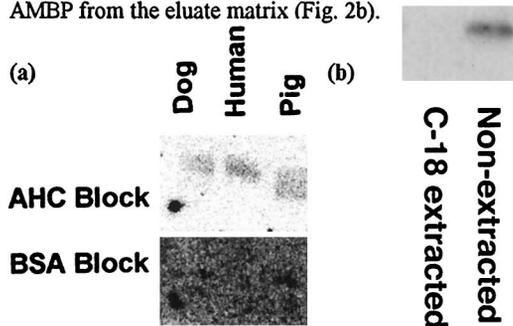


Fig. 2. (a) Background nonspecific binding is increased and band resolution decreased by preblocking the nitrocellulose with bovine serum albumin in contrast to the lower nonspecific binding with 0.1% AHC where bands were clearly resolved with a more favorable signal to noise ratio. (b) After extraction of plasma through C-18 reverse phase column, protein species that specifically bound ^{125}I - human AM on nitrocellulose could not be detected by ligand blotting suggesting that the procedure is efficient in removing AMBP from plasma.

Autoradiograms of proteins transferred to nitrocell-

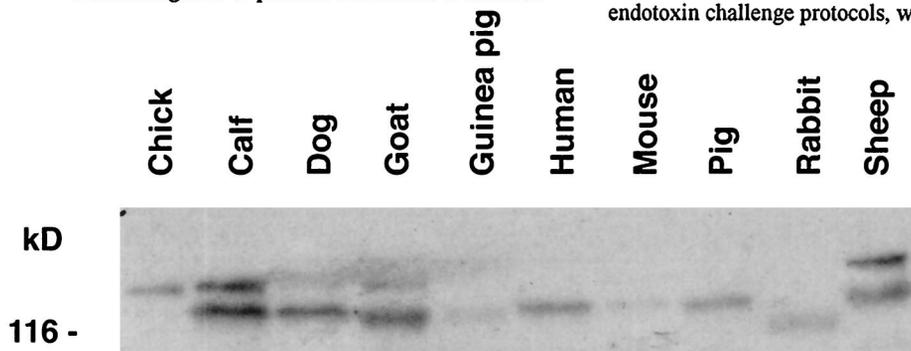


Fig. 3. AM binding protein(s) are detectable by ^{125}I - human AM ligand blot procedures and are characterized as 120 or 140 kD moieties in the plasma or serum of the 10 species tested.

ulose and probed with radiolabelled ^{125}I -AM demonstrated that plasma from all species tested contained a 120 to 125 kD protein (s) that bound ^{125}I -AM (Fig. 3). In plasma from the calf, goat, sheep, and to a lesser extent the dog, an additional band was prominent at 140 kD.

The specificity of the binding of ^{125}I -AM to the human, bovine, and pig plasma AMBPs was demonstrated by a linear competitive displacement of ^{125}I -AM from the bands with increasing concentrations of nonlabeled synthetic AM₁₋₅₂ (Fig. 4-a). Displacement of the ^{125}I -AM was largely complete with intact AM₁₋₅₂ (10^6); slight displacement was apparent with AM fragment 13-52 but not with shorter C- or N-terminal fragments or related peptides (not shown) even when used at concentrations ten-fold greater than the highest concentration of intact AM₁₋₅₂ tested (Fig. 4-b).

Analysis of AMBP bands revealed a 67 percent decrease in AMBP in plasma of calves undergoing an acute phase response to a parasitic infection ($P < 0.03$, Fig. 5) compared to healthy calves.

Discussion

The present data demonstrates for the first time the existence of 120-140 kD protein(s) in the plasma of several species capable of binding ^{125}I -AM in a specific and reversible competitive fashion on nitrocellulose and forming circulating high molecular weight binding protein-AM complexes. This is consistent with unpublished observations in our laboratory where ^{125}I -AM migrated in a 1% agarose gel with different relative mobilities depending on whether the ^{125}I -AM was in a protein-free, BSA, or plasma protein solution matrix. It was particularly interesting that plasma from ruminant species (calf, goat, and sheep) displayed a prominent additional binding protein band at 140 kD. A trace of a protein at this 140 kD size was also detectable in plasma from the dog. Whether these 120 and 140 kD proteins represent different glycosylated versions of the same protein remains undetermined at this time. In the present study, the 67 percent difference in measured AMBP in the plasma of healthy or parasitized calves suggests that the abundance of these binding proteins varies between states of health and disease. Using similar animal infection models or endotoxin challenge protocols, we have demonstrated

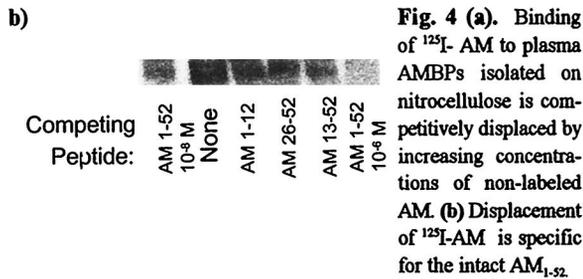
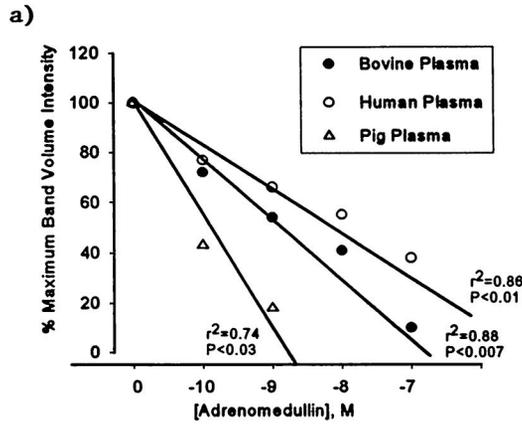


Fig. 4 (a). Binding of ¹²⁵I-AM to plasma AMBPs isolated on nitrocellulose is competitively displaced by increasing concentrations of non-labeled AM. (b) Displacement of ¹²⁵I-AM is specific for the intact AM₁₋₅₂.

significant decreases in the amounts of IGF binding proteins, BP-2 specifically, in bovine plasma (9,11). Because healthy and parasitized calves had similar plasma concentrations of total proteins, albumin and globulins, the measured decrease in AMBPs in parasitized calves reflects a specific response in this class of protein (s) to the onset of a disease stress. Thus, the availability and presentation of AM to tissues in states of health and disease may vary by virtue of the patterns of binding proteins present at those times.

Only speculation on the role of AMBP can be made at this time. By analogy with the better characterized IGFs, the role could encompass such functions as storage reservoirs for otherwise short-lived peptides, protease/ clearance protecting proteins, and agents capable of regulating the partitioning of AM to tissues such that ultimately the duration of function in the body and site-specificity of action are fine-tuned. Thus, AMBP may chaperon the ligand providing a molecular basis for discriminating between actions of AM on different tissues or at different times in states of nutrition, health and disease. An ability to specify AM binding to particular tissues mediated through a class of transport binding proteins may be particularly relevant to explaining the separation of some discrete biological actions of AM and CGRP in spite of the reported crossbinding between AM and CGRP on AM and CGRP receptor subtypes (12). In addition, localized production of AMBPs might participate in compartmentalizing the many actions of AM on

metabolism, reproduction, angiogenesis, apoptosis (1), and the separation of paracrine and endocrine actions of AM on cells.

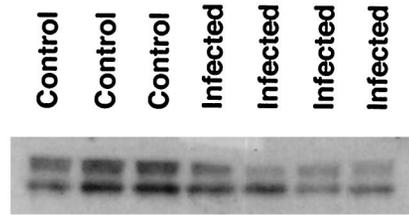


Fig. 5. The mean intensity of plasma AMBP ligand blot bands (and therefore implied plasma AMBP content) was 67 percent lower in parasitized calves compared to healthy calves (P<0.03).

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