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Neuroendocrine evaluation of cardiac disease

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Heart failure is a complex clinical syndrome wherein reduced systolic or diastolic performance of the heart results in increased activity of the adrenergic nervous system, overexpression of atrial (ANP) and brain (BNP) natriuretic peptides, activation of the renin-angiotensin-aldosterone system (RAAS), increased synthesis and release of endothelin and arginine vasopressin (AVP), and amplified expression of proinflammatory cytokines, such as tumor necrosis factor-α, interleukin-1, and interleukin-6 [1,2]. Neuroendocrine responses to developing heart failure have been well documented in human patients, and recently conducted studies support the assertion that qualitatively similar responses operate in dogs and cats with heart disease. Understanding these complex systems is vital to understanding the modern treatment of heart failure, which is largely based on the concept of blunting or otherwise modifying the excessive operation of certain maladaptive neuroendocrine responses, such as the adrenergic system and the RAAS. It is becoming increasingly evident that measurement of particular neuroendocrine markers offers diagnostic, prognostic, and therapeutic information not easily obtained by routine clinical evaluation, sophisticated imaging, or hemodynamic assessments.

Plasma catecholamines

When cardiac output is depressed and blood pressure falls, the adrenergic nervous system is activated. This results in an elevated heart rate, augmented myocardial contractility, and the selective redirecting of blood flow to vital centers. The systemic effects of generalized sympathetic stimulation include arteriolar constriction, which helps to maintain tissue perfusion pressures.

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Myocardial performance, already compromised by underlying heart disease, is negatively affected by the resulting mismatch of afterload to contractility. This consequence is exaggerated in patients with chronic heart failure, wherein downregulation of cardiac β_1-receptors further diminishes the contractile response. Adrenergic venous constriction results in increased venous return (preload) augmenting cardiac output, but the resulting increases in venous and capillary pressures aid in the development of symptomatic congestion. Chronic exposure to high norepinephrine levels contributes to pathologic vascular and cardiac remodeling, promotes arrhythmogenesis, and induces premature death of myocytes [1–3]. Moreover, increased sympathetic discharge is a potent stimulus of the RAAS and contributes to elevated circulating concentrations of AVP and endothelin [1,2]. The interactions of these systems are sufficiently complex that unintended and unpredicted consequences may be observed in individual patients in varying circumstances. For example, administration of a β-receptor–blocking drug removes the adrenergic stimulus for renin release, but renin levels may paradoxically increase if the negative chronotropic and inotropic effects of beta-blockade serve to diminish effective renal perfusion.

Norepinephrine and epinephrine are small-molecular-weight hormones synthesized by sequential modification of the amino acid, L-tyrosine. The adrenal medulla synthesizes and stores norepinephrine and epinephrine and releases them into the circulation in response to acute stress. Lacking the enzyme phenylethanolamine *N*-methyltransferase, peripheral nerves do not synthesize or release epinephrine (Fig. 1). Norepinephrine (but not epinephrine) plays a central role as a neurotransmitter and is constantly released from terminal sympathetic nerve endings. Despite reuptake and inactivation of most of the norepinephrine released in this fashion, a small portion leaks into the circulating blood so that plasma levels of norepinephrine, measured at rest can serve as a useful index of sympathetic nervous system activity. Plasma norepinephrine concentrations in human congestive heart failure (CHF) patients correlate with the severity of heart failure and are inversely related to survival [4]. In addition, rising concentrations of norepinephrine in human patients treated for CHF correlate with a decline in clinical status [5]. Importantly, catecholamine plasma concentrations rise in many circumstances other than heart failure, including emotional stress and physical exertion, emphasizing the rather poor specificity of such measures. For these reasons, interpretation of plasma catecholamine levels in individual animals is always likely to be problematic. Obtaining suitable resting samples from dogs and cats in a clinical setting is challenging, and there is large variation in such measurements even in patients appropriately categorized according to the severity of underlying heart disease.

For reasons already mentioned, blood sampling for measurement of plasma catecholamine levels is ideally accomplished via preplaced indwelling catheters from rested and relaxed subjects. Mean plasma epinephrine and norepinephrine levels obtained and measured in this fashion in healthy

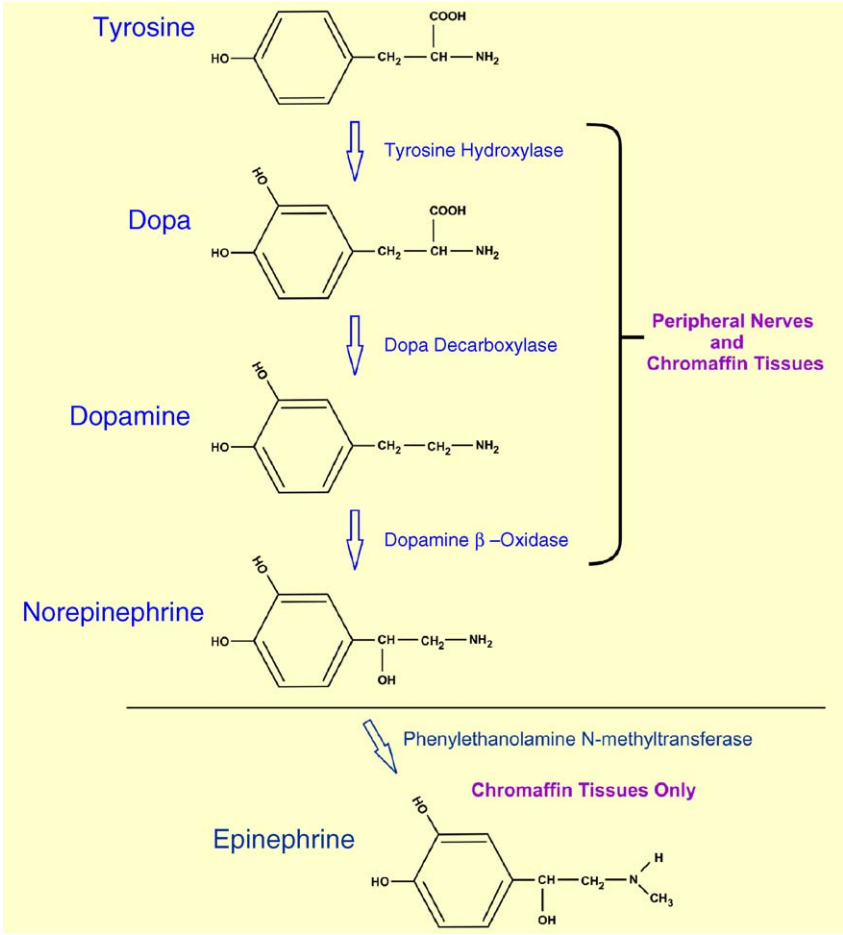

Fig. 1. Norepinephrine and epinephrine are synthesized by sequential modification of the amino acid, L-tyrosine. The enzyme phenylethanolamine N-methyltransferase is present in the adrenal medulla but not in the peripheral nerves, which neither synthesize nor release epinephrine. For this reason, plasma norepinephrine levels are a better indicator of basal sympathetic nervous system activity than epinephrine, which tends to rise more in response to acute stressors.

research cats are 221 and 424 pg/mL, respectively (D.F. Hogan, DVM, D.D. Sisson, DVM, unpublished observations). When blood is obtained via jugular venipuncture from unsedated client-owned cats, mean plasma epinephrine and norepinephrine levels are greater than 250 and 1000 pg/mL, respectively [6]. There can be little doubt that cats experience blood sampling at a veterinary teaching hospital as a stressful event even when attempts are made to minimize excitement and taxing distractions. The requirements for sample handling are also rigorous, because plasma catecholamines are subject to oxidation, necessitating the use of antioxidants

to preserve the samples before analysis, cold centrifugation, and storage at temperatures less than 0°. Plasma epinephrine and norepinephrine concentrations are best determined by high-pressure liquid chromatography (HPLC), which is cumbersome and expensive to perform, generally limiting such measures to a research environment.

Limited studies of circulating catecholamine concentrations in dogs and cats with spontaneously occurring heart disease have been conducted, but the value of plasma norepinephrine as an independent predictor of mortality in dogs or cats has not been determined. In cats with CHF or systemic thromboembolism caused by hypertrophic cardiomyopathy (HCM) and restrictive cardiomyopathy (RCM), we found that plasma epinephrine and norepinephrine concentrations are greater than 2000 and 2500 pg/mL, respectively [6]. In cats with HCM or RCM that are not in heart failure, plasma epinephrine and norepinephrine concentrations are above 1500 and 1700 pg/mL, respectively [6]. In 1990, Ware et al [7] reported significantly elevated plasma norepinephrine levels in dogs with heart failure caused by dilated cardiomyopathy (DCM) and degenerative valve disease (DVD) compared with normal dogs. In this study, plasma concentrations of norepinephrine correlated directly with the severity of heart failure; they tended to be higher in dogs with DCM compared with dogs with DVD. Plasma epinephrine levels in dogs with heart failure were also slightly higher than those measured in control dogs, but the difference was not statistically significant. Observations from a much larger population of dogs (D.D. Sisson, DVM, unpublished data) indicate that plasma norepinephrine and epinephrine concentrations are significantly elevated in dogs with CHF (New York Heart Association [NYHA] class III and IV) caused by DCM and DVD. More modest elevations are found in dogs with more modest disease (NYHA class I and II). These results provide convincing evidence of increased sympathetic nervous system activity in dogs and cats with naturally occurring heart disease not unlike that observed in human patients with chronic heart failure [4,5]. Given the established adverse consequences of chronic exposure to adrenergic stimulation and the proven efficacy of beta-blockers in human trials, there is overwhelming evidence supporting the need for carefully designed clinical trials evaluating the efficacy of β-receptor–blocking drugs in dogs and cats.

Natriuretic peptides

ANP and BNP (B-type) are initially elaborated from cardiac mRNA as long peptide sequences, termed *pre-proANP* and *pre-proBNP*, respectively [8]. Removal of a signal peptide from each yields shorter peptides, termed *proANP* and *proBNP*, which, in healthy animals, are stored in membrane-bound granules in the atria for later release. The mature active ANP and BNP hormones are cleaved from the carboxy- or C-terminal ends of the

proANP and proBNP molecules and released into the circulation together with their respective amino- or N-terminal fragments, usually termed *NT-proANP* and *NT-proBNP*. The structures of mature ANP and BNP are similar in that both contain a 17–amino acid ring closed by a disulfide bond between two cysteine residues. The sequence and number of amino acids comprising ANP and BNP are dissimilar, however, because they are encoded by different genes [9]. In healthy human beings, cats, and dogs, circulating forms of BNP and ANP are probably derived mainly from the atria [10–12]. A third natriuretic peptide, C-type or CNP, is found primarily in the brain and vascular endothelium. Circulating levels of CNP are much lower than those of ANP and BNP in healthy animals and human beings, suggesting that it acts in a paracrine fashion, inducing local relaxation of vascular smooth muscle and inhibiting vascular remodeling.

Sudden rises in plasma ANP and BNP levels are accomplished by their release from atrial storage granules mainly by the stimulus of atrial stretch. Sustained increases in circulating ANP and BNP, as seen in patients with heart disease, are accomplished by increased mRNA expression in different regions of the heart [8]. In some species, plasma BNP concentrations rise dramatically and often surpass ANP levels as the major site of BNP production switches from the atria to the ventricles [13–15]. We found that cats with HCM demonstrate marked increases in the expression of BNP in the atria and ventricles [16]. Others studying dogs with experimental pacing-induced heart failure reported that ventricular BNP expression remains rather modest and that the atria remain the predominant source of most circulating BNP [17]. Some caution is warranted in the interpretation of such studies, because gene expression is difficult to quantify and tissue levels do not necessarily reflect the amount of peptide synthesized and released. Nonetheless, a growing body of experimental evidence indicates that the patterns of ANP and BNP gene expression vary in different species and in relation to the type of underlying heart disease [18,19].

The physiologic actions of ANP and BNP generally oppose those exerted by the RAAS [15,20]. ANP and BNP act via the A-type natriuretic peptide receptor (NPR-A) to induce natriuresis and diuresis by inhibiting tubular sodium transport in the inner medullary collecting duct of the kidney. This same receptor type mediates vasorelaxation of systemic and pulmonary arterioles, thereby decreasing systemic and pulmonary vascular resistance. Additional actions of ANP and BNP mediated by NPR-A include direct inhibition of the release of renin by the kidney and the release of aldosterone from the adrenal cortex. A second receptor, the B-type natriuretic peptide receptor (NPR-B), responds to ANP and BNP but preferentially mediates vasodilation from locally produced CNP. Mature ANP and BNP are removed from the circulation by the clearance receptor, C-type natriuretic peptide receptor (NPR-C), which internalizes ANP and delivers it to lysosomes for degradation, and by a membrane-bound ectoenzyme, neutral endopeptidase, which cleaves ANP into inactive peptide fragments. Neutral

endopeptidase shows greater activity for ANP than for BNP, and NPR-C exhibits greater selectivity for ANP than for BNP, offering an explanation for the longer plasma half-life of BNP [15,20,21]. N-terminal fragments of proANP (NT-proANP) and proBNP (NT-proBNP) are removed more slowly from the circulation than their C-terminal counterparts, because clearance of these peptides is more dependent on renal excretion. As a result, NT-proANP and NT-proBNP plasma levels are higher than and not as labile as their C-terminal counterparts. In general, the N-terminal peptides are more sensitive markers of heart disease, and their levels tend to correlate more closely with the severity of underlying heart disease [22,23]. Importantly, assays measuring N-terminal fragments are also more likely to be affected by altered renal function; thus, this variable must be taken into consideration when interpreting plasma levels of an individual patient.

The amino acid sequence of active C-terminal ANP is remarkably similar in different species (Fig. 2). Human, canine, feline, bovine, porcine, and ovine ANP share the same 28–amino acid sequence [11]. Although more variable, there is also sufficient homology between the N-terminal amino acid sequence of ANP such that some of the assays developed to measure NT-proANP in human beings can be used for the same purpose in dogs and cats [11,24]. For example, NT-proANP radioimmunoassay (RIA) kits designed for use in human beings (Biotop OY, Turku, Finland) can be used in dogs and cats, because the antibody employed in this kit is directed to amino acid residues 80 to 96 of human ANP, a sequence identical to that observed in canine and feline NT-proANP over this region. This assay is a coated-tube RIA and does not require chemical extraction, simplifying the assay procedure. In contrast to the homology demonstrated by ANP in different species, the structure of BNP is quite variable in different mammals [16]. The amino acid sequences of mature BNP in dogs and cats are markedly different from the human BNP sequence (Fig. 3). For this reason, assays designed to measure BNP in human

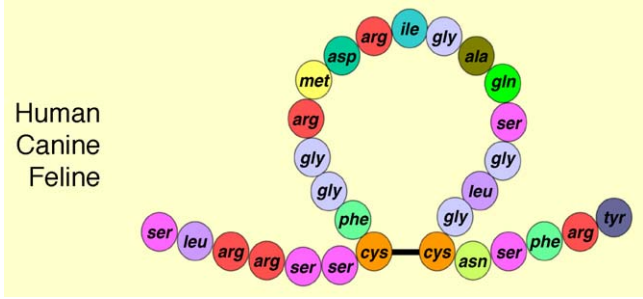

Fig. 2. The amino acid sequence of mature atrial natriuretic peptide (ANP) is highly conserved in mammals. The amino acid sequence of mature ANP is identical in human beings, dogs and cats, and there is considerable homology in the N-terminal fragments of proANP peptide as well. Thus, many ANP assays developed for use in human patients can be used to measure ANP in canine and feline plasma samples.

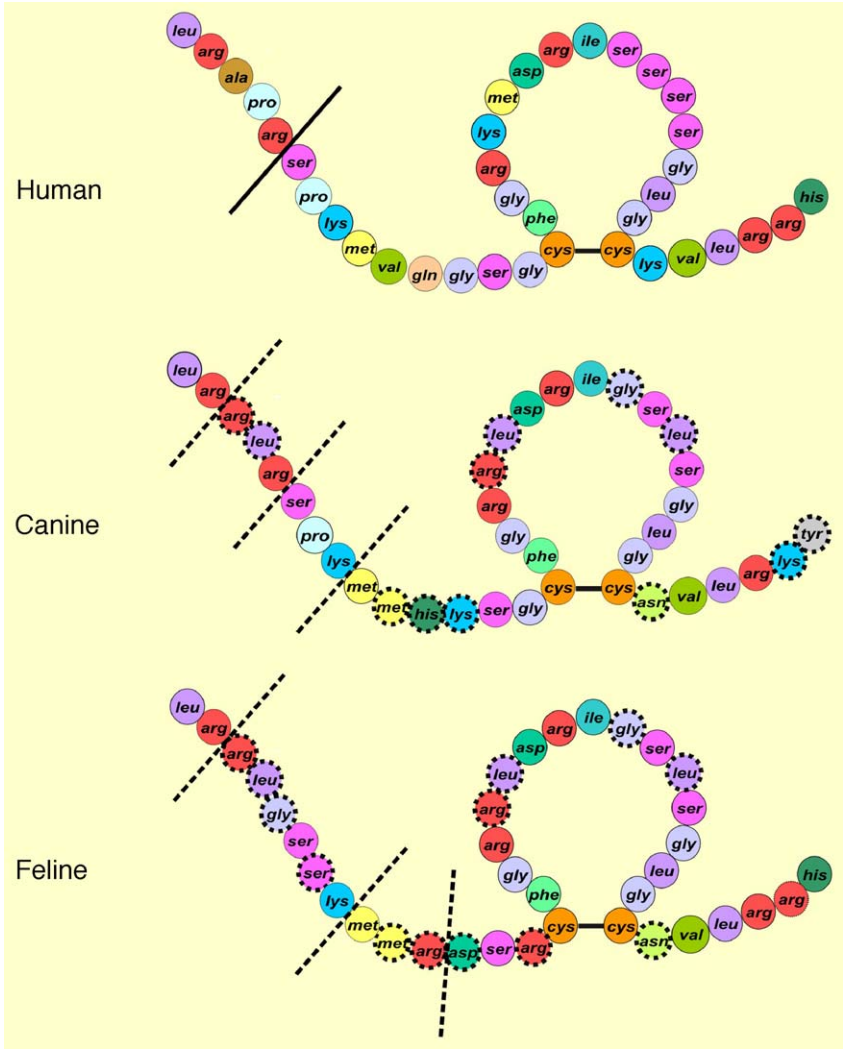

Fig. 3. The amino acid sequence of mature brain (B-type) natriuretic peptide (BNP) is not as highly conserved in mammals as that of atrial natriuretic peptide. The amino acid sequence of mature BNP in dogs and cats varies considerably from that in human beings. Those amino acids that are different from their counterparts in the human BNP molecule are identified by an interrupted envelope. The sequences of canine and feline BNP are similar enough to allow measurement by a radioimmunoassay using antibodies directed against canine BNP.

beings tend not to work well in dogs and cats. We have used a commercially available RIA kit (Phoenix Pharmaceuticals, Mountain View, CA), that employs antibodies specific for the 32–amino acid carboxy-terminus of canine BNP to measure mature peptide concentrations in dogs and cats. The structure of mature feline BNP is sufficiently homologous with canine BNP to

permit the use of the canine antibody assay, but feline-specific BNP antibodies should improve the sensitivity and specificity of the assay.

The prospect of screening veterinary patients suspect for possible heart disease or heart failure is an exciting possibility, but much technical work and clinical verification remain to be accomplished before natriuretic peptide assays can be incorporated into routine practice. The only method currently available to measure canine or feline BNP is an RIA, and this method is tedious to perform. Blood is ideally collected in chilled tubes, and aprotinin is added to inhibit proteolysis. The plasma is separated by cold centrifugation, and the sample is stored at −70°C until the assay is performed. The sample must then be subjected to a time- and labor-intensive extraction process before the RIA can be performed. Clearly, such laborious procedures do not lend themselves to routine clinical testing. Nonetheless, if measurement of BNP levels is shown to have sufficient clinical merit, it is quite feasible to develop a user-friendly sandwich BNP enzyme-linked immunosorbent assay (ELISA) that could be performed in any veterinary practice setting.

Heart failure in human patients is characterized by substantial elevations of ANP and BNP, and a large number of studies of human subjects have shown that measurement of plasma natriuretic peptide concentrations, especially BNP, are helpful for discriminating patients with dyspnea caused by heart failure from those with pulmonary disease or other disorders [23,25–29]. Two assays, one measuring BNP and the other NT-proBNP, have been approved by the US Food and Drug Administration (FDA) for identifying human patients with heart failure. The BNP assay is a bedside test, whereas the NT-proBNP is an automated assay suitable for simultaneous processing of large numbers of samples [30]. Which assay is superior is a matter for debate, but the BNP assay has been evaluated in greater numbers of patients in more studies. In the Breathing Not Properly Multinational Study, a BNP level lower than 50 pg/mL had a negative predictive value of 96%, whereas a BNP level greater than 100 pg/mL was 90% sensitive for detecting heart failure in human patients [30]. The mean BNP concentrations of patients with NYHA class III and IV heart failure were 8- to 10-fold fold higher than the cutoff value for subjects without heart failure [31]. In a recently reported study of cats with myocardial disease, measures of plasma BNP levels seem to have similar diagnostic potential [6]. Plasma BNP levels elevated more than 10-fold distinguished cats with heart failure from control cats better than plasma ANP levels, which were increased 4- to 5-fold. The diagnostic potential of plasma BNP levels does not seem as promising in dogs. Plasma BNP concentrations do not increase markedly until the later stages of heart failure (NYHA class III and IV) in dogs with DVD, and the magnitude of the change is less dramatic than that observed in cats and human beings [32–34]. We have observed similar patterns in dogs with DVD and DCM, suggesting that there may be a species difference in the magnitude of BNP expression. In contrast to cats and human beings, plasma NT-proANP levels are currently more useful than BNP levels as a marker of heart disease and heart failure in

dogs [24,32–35]. It is not clear at the present time whether this finding reflects a species-related difference in natriuretic peptide physiology or whether it is simply an artifact of a primitive BNP assay. This observation was first noted in Cavalier King Charles spaniels with DVD [32], and our laboratory subsequently confirmed it to be true in other breeds with DVD or DCM. In a recently completed study of dogs presented for dyspnea, we found that plasma NT-proANP was better than plasma BNP or endothelin (ET)-1 for identifying dogs with CHF and that all three peptide assays outperformed serum troponin as markers of CHF in dogs [36]. We are currently testing the hypothesis that BNP is the superior marker of CHF in cats in a prospective study of similar design.

In humans, circulating BNP concentrations are elevated in asymptomatic patients with systolic left ventricular (LV) dysfunction, in patients with LV diastolic dysfunction, in patients with ventricular hypertrophy caused by systemic or pulmonary hypertension, and in patients with HCM [18,37–39] Some have even advocated using circulating BNP levels to screen patients for early LV dysfunction. Based on our preliminary data, it is likely that the measurement of plasma BNP will aid in the early identification of cats with HCM. Such testing also might clarify the status of cats with equivocal results when evaluated by other diagnostic modalities, including echocardiography. The ultimate clinical utility and prognostic value of natriuretic peptide assays remain uncertain in dogs and cats. Studies of human patients with CHF have proven that plasma BNP levels are particularly useful in formulating an accurate prognosis, particularly when measured before and after therapy [30,39–42] In one study, treatment guided by BNP monitoring was superior to that based solely on other clinical methods of evaluation [43].

Renin-angiotensin-aldosterone system

The major circulating form of renin is prorenin, which is formed in juxtaglomerular cells in the kidney from preprorenin by removal of a signal peptide and by glycosylation during transport through the rough endoplasmic reticulum [44]. Prorenin is an inactive prohormone that is converted to the active renin enzyme by removal of a 43–amino acid segment within intracellular storage granules or after release into the circulation. The half-life of activated plasma renin is on the order of 10 to 20 minutes [45]. Major stimuli for the release of the renin from the juxtaglomerular apparatus include β_1-adrenergic stimulation, decreased effective renal perfusion, and reduced sodium reabsorption by the renal tubules [44,46]. Angiotensin II inhibits renin, exemplifying the phenomenon of classic feedback inhibition. The main action of renin is to accelerate the conversion of the large prohormone, angiotensinogen, to the decapeptide, angiotensin I, which is subsequently converted to the octapeptide, angiotensin II, via angiotensin-converting enzyme (ACE; Fig. 4). The precursor of angiotensin II and III,

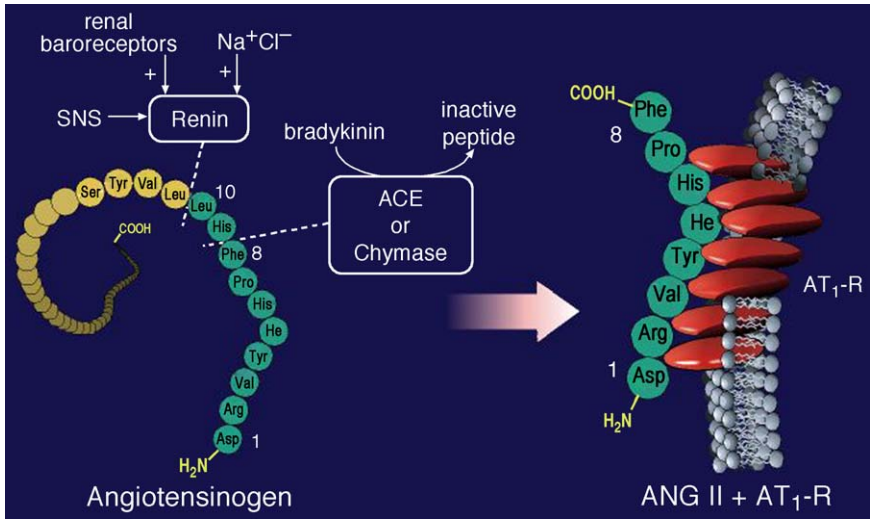

Fig. 4. The conversion of the large prohormone, angiotensinogen, to the decapeptide, angiotensin I (ANG I), is catalyzed by the enzyme renin. ANG I is subsequently converted to the octapeptide, angiotensin II (ANG II), mainly via angiotensin-converting enzyme. Other tissue enzymes, including cathepsin G, elastase, tissue plasminogen activator, chymase, and chymostatin-sensitive AII-generating enzyme, may also mediate the conversion of ANG I to ANG II.

angiotensinogen, is a globular glycoprotein produced in the liver and released into the circulating plasma, which serves as the primary storage reservoir [44]. In human beings, and presumably in domestic animals, the production of angiotensinogen is upregulated and plasma levels are increased by infection, thyroid administration, and hyperadrenocorticism [44]. Conversely, production is downregulated in hypothyroidism and Addison's disease.

ACE is a membrane-bound zinc metallopeptidase that is present in virtually all tissues and body fluids. It is a long single-chain protein with more than a thousand peptide residues complexed with large amounts of sugar in the form of fucose, mannose, galactose, and *N*-acetyl-glucosamine as well as with sialic acid [44]. The sugar content varies depending on the source of isolation. ACE acts by cleaving terminal dipeptides from the C-terminus of the substrate peptide; hence, it is a dipeptidyl carboxypeptidase. The selectivity of ACE is such that it cleaves any substrate peptide, R1-R2-R3-OH, where R1 is a protected L-amino acid, R2 is any L-amino acid except proline, and R3 is any L-amino acid with a free carboxy-terminal. Thus, ACE converts angiotensin I to active angiotensin II and also inactivates the potent vasodilator, bradykinin. Although the conversion of angiotensin I to angiotensin II occurs mainly via the action of ACE, it can also be accomplished by the actions of cathepsin G, elastase, tissue plasminogen activator, chymase, and chymostatin-sensitive AII-generating enzyme (CAGE) [46,47]. The importance of these alternate pathways is species

dependent [47]. Sequential actions of aminopeptidase and ACE acting on angiotensin I also produce angiotensin III, a seven–amino acid peptide (heptapeptide) that has actions similar to but less potent than angiotensin II [46,48]. In addition to its recognized prominence in the kidney, local tissue renin-angiotensin systems have been demonstrated in a number of different organs, including the brain, heart, blood vessels, and adrenal glands [1,2,46,49,50] These local renin-angiotensin systems play an important role in the development of the constellation of changes constituting pathologic remodeling, including vascular and myocardial hypertrophy, inflammation, and fibrosis.

The physiologic actions of angiotensin II have been thoroughly explored and reported [44,47,48]. All the important physiologic effects of angiotensin II are mediated by angiotensin-1 receptors (ARBs), which are abundantly located in the blood vessels, kidneys, liver, heart, and pituitary and adrenal glands [44,48]. In addition to its role as a potent vasoconstrictor, angiotensin II promotes sodium and water retention via direct effects on the renal tubules and contributes to this effect by stimulating aldosterone production and release from the adrenal glands. The half-life of circulating angiotensin II is on the order of 1 or 2 minutes because it is rapidly hydrolyzed by circulating and tissue angiotensinases to inactive peptide fragments. From an evolutionary perspective, angiotensin II and aldosterone are seen to play essential roles in regulating sodium and water balance and maintaining vascular pressure when the circulating blood volume is compromised by hemorrhage or salt and water deprivation. When inappropriately elevated for excessive periods, as in chronic heart failure, angiotensin II and aldosterone induce detrimental vascular and ventricular remodeling processes [46,49,50]. The end result of these processes is further damage to an already compromised heart, accelerating the clinical deterioration and premature demise of patients with heart disease. Advances in the treatment of heart failure and systemic hypertension realized in the last decade have resulted largely from the use of compounds that prevent the formation of angiotensin II via inhibition of ACE and block the interaction of angiotensin II with ARBs or antagonize the actions of aldosterone. The benefits of these treatment strategies include longer survival times and an improved quality of life. Of these agents, only ACE inhibitors are approved for the treatment of heart failure in dogs and people, although aldosterone antagonists and ARBs certainly have demonstrated efficacy in improving outcome in human patients with CHF.

Major stimuli for aldosterone production and release include angiotensin II, elevated plasma potassium levels, and corticotropin (ACTH) as well as the dominant physiologic effect of aldosterone related to its effects on the kidney. Aldosterone acts on epithelial cells of the distal collecting ducts, where it diffuses into the cytoplasm and binds to cytoplasmic mineralocorticoid receptors (MRs) [44,46,49]. After its entry into the nucleus, activated MR induces a cascade of events that ultimately increases absorption of sodium

ions and excretion of potassium. Aldosterone mediates similar sodium-conserving processes in the sweat and salivary glands and in the colon. This sodium-conserving effect constitutes the classic view of aldosterone as an integral component of the RAAS. Based on extensive studies conducted over the last decade, the role of aldosterone, like angiotensin II, is now better understood [1,2,46]. Other messengers, including plasma catecholamines, ET-1, and AVP, promote the production and release of aldosterone into the tissues and blood [46,49]. In this context, the failure of ACE inhibitors to restore and maintain normal plasma aldosterone concentrations uniformly in patients with CHF is understandable [1,2,46]. Moreover, aldosterone production is not confined to the adrenal gland. MRs are more widely distributed than previously realized, and aldosterone exerts important physiologic effects in addition to those related to sodium, water, and potassium homeostasis. In patients with heart failure, aldosterone contributes to generalized vasoconstriction via direct MR-mediated stimulation of sympathetic nervous system activity, via inhibition of norepinephrine uptake and degradation in the periphery, and via other complex actions promoting endothelial cell dysfunction [44,46,49,50]. Aldosterone also contributes to baroreceptor dysfunction in heart failure, enhancing the activity of the sympathetic nervous system and diminishing the activity of the parasympathetic limb. Aldosterone produced locally in tissues like the brain, vasculature, and myocardium mediates important biologic processes that are still only partly understood. Of particular importance is the emerging role of aldosterone as a mediator of inflammation and fibrosis in the processes of pathologic remodeling in the vasculature, kidney, and heart [46,49,50].

Commercial assays of all the various components of the RAAS are routinely performed at only a few specialized diagnostic laboratories. Plasma renin activity (PRA) rather than renin concentration is typically used to evaluate the initiating action in the of renin-angiotensin-aldosterone cascade [51]. PRA assays typically measure the rate of formation of angiotensin I generated by the action of the enzyme renin acting on angiotensinogen. In our laboratory, the rate of angiotensin I formation is determined via a competitive binding RIA. Like renin, ACE assays determine enzyme activity rather than the quantity of circulating ACE. The techniques used and the utility of measurements of plasma ACE activity are controversial. A variety of substrates, including furylacryloyl-Phe-Gly-Gly, Hip-His-Leu, Phe-His-Leu, Hip-Gly-Gly, and angiotensin I, are mixed with a plasma sample, and the rate of the reaction is determined via spectrophotometric, fluorometric, or radiochemical detection hardware [52–54]. The results obtained from different assays are not comparable. If repeated sampling is anticipated or when groups or individuals are to be compared, it is advisable to use a single validated assay from a reliable laboratory. Hamlin and Nakayama [55] used an ultraviolet kinetic assay to measure ACE activity in dogs but did not specify the substrate. In their evaluation of the pharmacokinetics and pharmacodynamics of benazepril in dogs and cats, King et al

[56,57] validated a commercial radioassay kit based on the substrate H-Hip-Gly-Gly. When assessing the degree of plasma ACE inhibition, it is advisable to measure other components of the RAAS [58]. Plasma angiotensin II levels should fall, whereas renin levels and the ratio of angiotensin I/angiotensin II should rise with effective ACE inhibition [54,59,60]. Most importantly, all these assays are underused in veterinary clinical studies and in clinical practice given their value for assessing owner compliance and dose response to an administered ACE inhibitor.

Measurement of angiotensin levels and various metabolites in plasma is probably best accomplished by HPLC-RIA [61]. With this method, peptides are first separated by liquid chromatography, and RIA techniques then are used to quantify the peptides of interest. Because this technique is difficult and expensive, measurement of angiotensin I and angiotensin II levels is often accomplished using competitive RIA or enzyme immunoassay kits [62]. Although these kits show little cross-reactivity between angiotensin I and angiotensin II, peptides like angiotensin III do cross-react. Depending on the information desired, this may or may not be problematic. By comparison, serum or plasma aldosterone concentrations are relatively easily and accurately determined using commercially available competitive binding RIA kits [63].

Interpretation of the results of all the various RAAS assays can be problematic in individual human beings or animals. CHF is but one of many patient circumstances causing increased release of renin from the kidney. Low-salt diets, dehydration, blood loss, and vigorous exercise stimulate renin release from the juxtaglomerular apparatus as a consequence of diminished renal blood flow. More interestingly, PRA and aldosterone concentrations are not always elevated in patients with overt CHF [64,65]. As already mentioned, the physiologic effects of the RAAS include volume expansion and vasoconstriction, both of which serve to diminish renin production. As a result, operation of the RAAS tends to be phasic and to conceal its activation. Accordingly, it is not surprising that there is uncertainty regarding the initiation of RAAS overexpression in dogs and cats with heart disease. There is fairly uniform agreement that the RAAS is activated in dogs and cats with cardiomyopathy and signs of CHF, particularly if diuretics have been administered [6,35,66,67]. There is disagreement regarding the presence of RAAS activation before the onset of overt CHF. Häggström et al [68] reported low plasma concentrations of angiotensin II and aldosterone in Cavalier King Charles Spaniels with valvular heart disease 1 year before, 1 to 6 months before, and at the time of onset of overt CHF. These investigators concluded that fluid retention in the early stages of developing heart failure may not be caused by activation of the plasma RAAS and that other mechanisms may be responsible for early sodium and water retention in dogs with mitral regurgitation (MR). They further hypothesized that increased circulating concentrations of ANP effectively suppress the plasma RAAS in dogs with early compensated DVD. In apparent contradiction, Pedersen and

his colleagues [69,70] reported increased PRA and elevated concentrations of aldosterone in asymptomatic and mildly symptomatic Cavalier King Charles Spaniels with DVD, even when diet was taken into account. Studies conducted in our laboratory show substantial elevations of PRA and serum aldosterone levels in dogs with overt CHF caused by MR and DCM as well as in cats with HCM or RCM. Activation of the RAAS is particularly marked in dogs and cats with acquired heart disease when furosemide is used to alleviate congestive signs. In most dogs and cats with less severe heart disease (NYHA class I and II), PRA and aldosterone concentrations are within the normal range or only slightly elevated.

Endothelin

Vascular tone is modulated by the endothelium-derived vasodilators, nitric oxide (NO) and prostacyclin, and by the complex actions of the potent endothelium-derived vasopeptide, endothelin. [71,72] Three related peptides, ET-1, ET-2 and ET-3, comprise the endothelin family [73]. Circulating endothelins are derived from larger peptides produced by vascular endothelial cells (myocytes and a variety of other cells) in a sequence of steps analogous to that described for natriuretic peptides [73–75]. Thus, preproendothelin gives rise to biologically inactive proendothelin, also termed *big endothelin*, which, in turn, is cleaved at the N-terminus to yield the mature peptide. The active mature peptide, ET-1, is derived from inactive big ET-1 by the action of a membrane-bound metallopeptidase, endothelial-converting enzyme (ECE), and is the predominant circulating form of endothelin produced by endothelial cells. The mature peptide has two intramolecular disulfide bridges linking cysteine residues, producing a double-ring structure (Fig. 5). ET-1 mRNA expression and ET-1 production are stimulated by hypoxia and mechanical factors, including stretch and low shear stress; by vasoactive substances, such as angiotensin II, AVP, norepinephrine, and bradykinin; and by growth factors and cytokines, including transforming growth factor-β, tumor necrosis factor-α, and interleukin-1 [74–76]. Other vasoactive endothelin derivatives are produced by the action of tissue chymases, but the biologic importance of this and other alternate pathways is not yet clear [77].

ET-1 acts via two receptors, ET_A and ET_B, to exert complex biologic effects serving to maintain normal vascular tone [74–78]. Vasoconstriction of smooth muscle, increases in myocardial contractility, and aldosterone secretion are among the more prominent effects mediated by ET_A receptor stimulation. Chronic stimulation of ET_A receptors and persistently elevated ET-1 levels cause proliferation and hypertrophy of vascular smooth muscle and myocardial hypertrophy. Thus, ET-1 is one of several mitogenic substances incriminated in the pathologic remodeling of the vasculature and heart in response to chronic hypertension and heart failure. Vasodila-

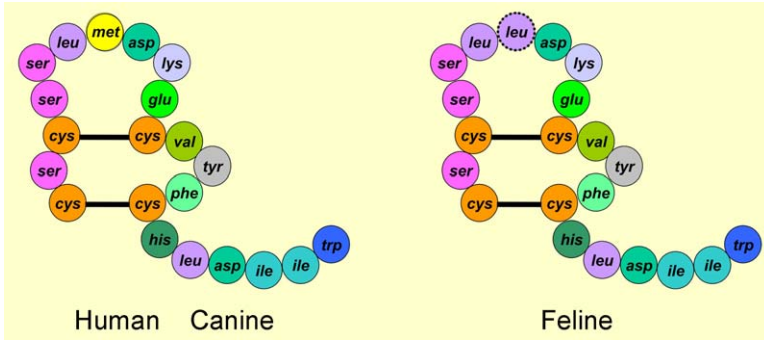

Fig. 5. The amino acid sequence of mature endothelin-1 (ET-1) is highly conserved in mammals. The amino acid sequence of mature ET-1 is identical in human beings and dogs. Interestingly, feline ET-1 differs by only one amino acid switch at position 7, where leucine is substituted for methionine. Despite this difference, human assays for ET-1 work well on plasma from dogs and cats.

tion, mediated by increased NO production, and aldosterone secretion result from stimulation of endothelial cell ET_B-receptors. Increased NO levels, in turn, inhibit ET-1 synthesis, exemplifying a negative-feedback mechanism. After intravenous injection of ET-1, blood pressure first declines transiently and then increases, reflecting the action of these two receptor subtypes. The interactions of endothelin and the RAAS are complex, but the net effect is suppression of renin production and stimulation of aldosterone secretion [77].

In healthy people and animals, most circulating ET-1 is derived from the vasculature and ET-1 levels are low, reflecting its paracrine role in the maintenance of normal vascular tone. Increased plasma concentrations of ET-1 and big ET-1 have been documented in human patients with heart failure, and their levels seem to correlate with disease severity [78,79]. Moreover, plasma endothelin levels have been shown to correlate inversely with survival [80]. Myocardial ET-1 production is thought to substantively contribute to the approximately twofold elevation of circulating endothelin levels observed in patients with CHF [77–79]. ET-1 concentrations are also consistently elevated in patients with pulmonary hypertension and some forms of renal disease but, interestingly, not in patients with systemic hypertension [74,80].

The structure of the 21–amino acid sequence of ET-1 is highly conserved in mammals such that canine ET-1 is identical to human ET-1 and feline ET-1 differs by only 1 amino acid switch at position 7, where leucine is substituted for methionine (see Fig. 5) [73,81]. The biologic significance of this switch is uncertain. We recently identified and validated a sandwich ELISA assay designed for use in human subjects that uses antibodies directed at amino acids 8 through 21 of human ET-1, which are identical to amino acids 8 through 21 of dogs and cats [82,83]. Using this assay, we

demonstrated that plasma ET-1 levels more than double in dogs with CHF caused by DVD or DCM and increase more than threefold in cats with cardiomyopathy and CHF or systemic thromboembolism [82,83]. Significant but more modest elevations are observed in dogs and cats with less severe disease. In a recently reported study of dogs presented for dyspnea, we confirmed that plasma ET-1 levels were elevated in dogs with CHF, although they were less accurate than plasma NT-proANP for differentiating dogs with CHF from those with dyspnea from other causes [36]. Therapeutic strategies based on blocking ET receptors and inhibition of ECE have not produced convincing clinical benefits as yet, but several studies are still in progress [84,85]. Interestingly, in a small number of dogs with overt CHF, we observed that plasma endothelin levels declined substantially after treatment with conventional therapy (digoxin, furosemide, and an ACE inhibitor).

Vasopressin and adrenomedullin

AVP, often referred to as antidiuretic hormone (ADH), is a nonapeptide with the amino acid arginine at position 8 [86,87]. The amino acid sequence of the mature peptide is highly conserved in most mammals and is identical in human beings, dogs, and cats [88]. Interestingly, the amino acid structure of vasopressin differs from that of oxytocin (OT) at two amino acid positions, suggesting that the encoding genes of these two peptides arose from a common precursor [86]. Provasopressin is produced by neurons whose cell bodies are located in the hypothalamus. This propeptide is derived from preprovasopressin and consists of the mature vasopressin peptide linked via a short processing signal to neurophysin II and its associated copeptin. Provasopressin is processed into the mature peptide, vasopressin, in vesicles that are transported along the length of axon to the posterior pituitary, where they become secretory granules containing the active peptide within the nerve endings [86]. Release of vasopressin from the neurohypophysis into the circulation is stimulated by increased plasma osmolality or hypovolemia. When plasma volume is reduced, stretch receptors in the atria and large veins decrease their firing rate, stimulating release of AVP [86]. Sympathetic stimulation and angiotensin II also stimulate AVP release [86,87]. After its release, vasopressin reacts with V_{1A} receptors in the vasculature and heart, mediating weak vasoconstrictive and inotropic actions, and with V_2 receptors in the kidney, stimulating water reabsorption [89,90]. This latter effect is accomplished via regulation of the number of aquaporin-2 water channels inserted into the luminal membrane of cells in the renal collecting ducts [89,90]. Baroreceptor V_2 receptors respond to elevated plasma AVP levels by augmenting baroreceptor reflexes, which lower the heart rate to maintain arterial blood pressure in the normal range.

Elevated plasma AVP levels are detectable in some human patients with CHF, particularly those with severe heart failure and dilutional hypona-

tremia [86,91]. The paradox of increased AVP release in the face of reduced plasma osmolality and high filling pressures may be a result of baroreceptor signaling caused by low arterial blood pressure [86]. Whatever the mechanism, selective V_2 or combined V_{1A}/V_2 receptor antagonists have been shown to normalize plasma sodium concentrations and to alleviate congestive signs in affected patients [92–94]. Conivaptan, a combined V_{1A}/V_2 blocker, has shown efficacy in dogs with experimentally induced heart failure and in human patients with severe symptomatic CHF [94,95]. Few data are available regarding circulating AVP levels in dogs or cats with spontaneously occurring heart disease, but this knowledge deficit will likely be remedied in the near future.

Adrenomedullin (ADM) is a potent natriuretic and vasodilating 52–amino acid peptide detected in a variety of tissues, including the adrenal medulla, heart, lung, and kidney [96,97]. ADM production is stimulated by a variety of chemical and mechanical stimuli, including a number of inflammatory stimuli, suggesting an important role for ADM in inflammation and patients with endotoxic shock [97]. Circulating levels of ADM are elevated in human patients with CHF, and ADM immunoreactivity is increased in failing human and canine ventricles [98,99]. Current consensus favors an autocrine/paracrine function for ADM [96(97)]. Interestingly, ADM exerts an inotropic effect on myocardial cells and attenuates myocardial hypertrophy and collagen production.

Summary

Current evidence favors the view that regardless of etiology, there is a predictable sequence of neuroendocrine activation that operates in most dogs and cats with progressive heart disease and that it is largely but not entirely independent of etiology. The natriuretic peptides and sympathetic nervous system seem to be early responders to developing cardiac and hemodynamic perturbations in both species. BNP plays a particularly prominent role in cats, possibly as a reflection of disease etiology. Shortly thereafter, plasma endothelin concentrations rise, reflecting the impact of the hemodynamic alterations on the vasculature. Endothelin and the natriuretic peptides directly suppress plasma renin release but have divergent effects on aldosterone. Activation of the tissue RAAS may operate early on to further the progression of heart failure, but evidence of plasma RAAS activation occurs comparatively late and near the time of development of overt CHF. Finally, in animals with severe CHF that are prone to hypotension, vasopressin levels may also rise, contributing to the retention of free water and congestion that is refractory to diuretics. Measurement of vasopressin levels in dogs and cats with heart disease must be accomplished to confirm this hypothesis. Although oversimplified, this scenario seems to be consistent with data obtained in human, canine, and feline patients. These observations

provide some impetus for evaluating ACE inhibitors in cats and β-receptor–blocking drugs in dogs and cats. Perhaps we are also a little closer to identifying useful biochemical markers that can aid in the diagnosis of heart disease, guide therapy, and improve our understanding of the biologic processes occurring in our patients.

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