Renin-angiotensin system enzymes in post-mortem myocardial and renal tissue: a pilot study

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Introduction

Myxomatous mitral valve disease (MMVD) and dilated cardiomyopathy (DCM) are the most common causes of cardiac morbidity and mortality in dogs [1-3]. Activation of the circulating renin-angiotensin-aldosterone system (RAAS) attends to the progression of MMVD [4, 5] and DCM [6, 7]. The RAAS is an important family of enzymes and hormones that regulates fluid and blood pressure homeostasis. In heart disease, the RAAS becomes deranged and promotes disease progression. RAAS blockade with angiotensin-converting enzyme inhibitors (ACEi) alone and with spironolactone has been shown to improve survival in dogs with heart failure due to MMVD (ACVIM Stage C) and DCM [1, 8-15]. However, survival is still relatively short once heart failure develops.

One reason for the ACEi shortcomings may be increased local (tissular) generation of angiotensin II (Ang II) despite ACE suppression in circulation [16-18]. The local RAAS in dogs with heart disease remains a mystery relative to the circulating RAAS, in part due to the difficulty of obtaining tissue samples from patients.

To study local RAAS activation, myocardial and renal tissue samples must be collected post-mortem and analyzed for angiotensin peptide formation [16, 17]. This poses a technical challenge, since tissue collection is often delayed until several hours post-mortem and minimal data exists on how long the RAAS enzymes remain stable in post-mortem myocardial and renal tissue samples [19].

Objectives

This summer, determine:

1. If the activity of ACE, chymase, ACE2, and prolyl carboxypeptidase (PCP) (Fig 1) can be measured in myocardial and renal tissue samples that are retrieved within 36 hours post-mortem.
2. Whether ACE or chymase contributes more to Ang II production in myocardial and renal tissue samples.

Beyond this summer, determine:

1. The stability of five key RAS enzymes (ACE, chymase, ACE2, PCP, NEP) (Fig 1) in myocardial and renal tissue samples over time post-mortem.
2. The activity of the five major RAS enzymes in the heart and kidneys of pet dogs with naturally-occurring MMVD and DCM.
3. How ACE inhibitors affect tissue RAS expression in the heart and kidneys of pet dogs with naturally-occurring MMVD and DCM.

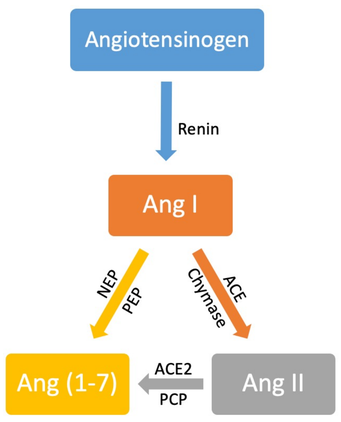


Fig 1. Simplified scheme of angiotensin (Ang) I, Ang II, and Ang (1-7) forming RAS enzymes. Adapted from [16].

Hypotheses

1. ACE, chymase, ACE2, and PCP activity can be measured in samples that are retrieved within 36 hours post-mortem, a realistic timeframe to collect samples from client-owned animals.
2. Chymase, not ACE, will be the predominant Ang II-forming enzyme in myocardial and renal tissue samples.

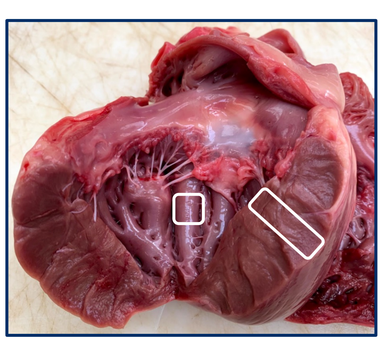
Methods

At necropsy, 2 g of left ventricular posterior wall (LVPW), 2 g of interventricular septum (IVS) at the same level, and 2 g of kidney parenchyma were collected. 1 g of tissue per site (LVPW, IVS, kidney) was immediately frozen at -80 °C and the remaining 1 g of tissue per site was returned to the necropsy cooler (4 °C). Seven hours later, the remaining 1 g of tissue per site was transferred from the necropsy cooler (4 °C) to freezer (-80 °C).

We have sampled 20 dogs so far, 9 of which were sampled according to this protocol and 5 of which are included in this preliminary analysis (Table 1).

Table 1. Tissue collection times (in hours post-mortem). Data from Dogs 1-5 (bolded) are included in this preliminary analysis.

	Dog 5	Dog 1	Dog 6	Dog 7	Dog 8	Dog 9	Dog 2	Dog 4	Dog 3
Sample 1	**4.5**	**6.5**	6.75	8.5	17.5	22	**22.5**	**23.5**	**28**
Sample 2	**11.5**	**13.5**	13.75	15.5	24.5	29	**29.5**	**30.5**	**35**



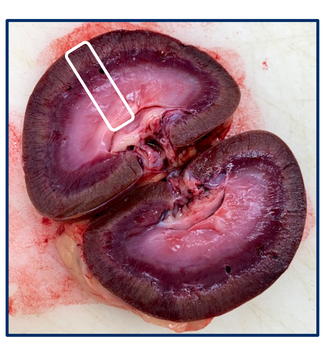


Fig 2. Photographs of a heart (L) and kidney (R) analyzed for RAS enzyme activity according to the technique described in Figure 3. White boxes delineate locations of tissue harvesting.

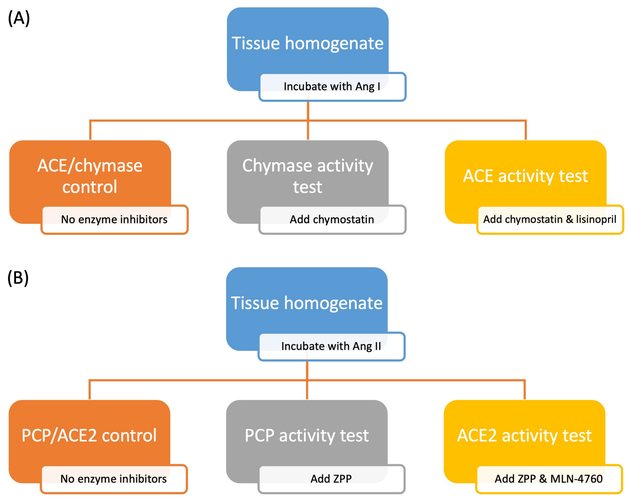


Fig 3. Overview of enzyme activity analysis performed at Attoquant Diagnostics in Vienna, Austria as described in [17]. Angiotensin peptide formation rate, our surrogate for enzyme activity, was measured via liquid chromatography with tandem mass spectrometry (LC-MS/MS) and recorded in (pg/µg tissue)/hr for all trials. Chymostatin = chymase inhibitor, lisinopril = ACE inhibitor, ZPP = PCP inhibitor, MLN-4760 = ACE2 inhibitor. Attoquant Diagnostics proved in a concurrent experiment that chymostatin does not inhibit ACE and ZPP does not inhibit ACE2. (A) Chymase and ACE activity assay overview. Quantified Ang II formation rate. (B) PCP and ACE2 activity assay overview. Quantified Ang (1-7) formation rate.

Results

Objective 1: detect RAS enzyme activity

There was measurable activity of ACE, chymase, ACE2, and PCP in myocardial and renal tissue samples retrieved within 36 hours post-mortem. The graphs in Figure 4 display Ang II formation rate over time in myocardial and renal tissue samples from Dogs 1-5. The percentages next to each colored line denote the change in Ang II formation rate between the two time points sampled for each dog. The bar graphs in Figure 5 depict the ratio of Ang (1-7):Ang II formation rate in the myocardium at both sample collection points for each dog.

Objective 2: determine predominant Ang II-forming enzyme

The scatter dot plots in Figure 6 display the median and range of Ang II formation rates measured in the control, chymostatin, and chymostatin + lisinopril tests for each myocardial and renal tissue sample from Dogs 1-5. A non-parametric ANOVA was used to evaluate the data. The asterisk indicates $p < 0.05$.

All myocardial and kidney samples incubated with chymostatin and lisinopril blocked Ang II formation entirely. All myocardial and kidney samples incubated with ZPP and MLN-4760 blocked Ang (1-7) formation entirely.

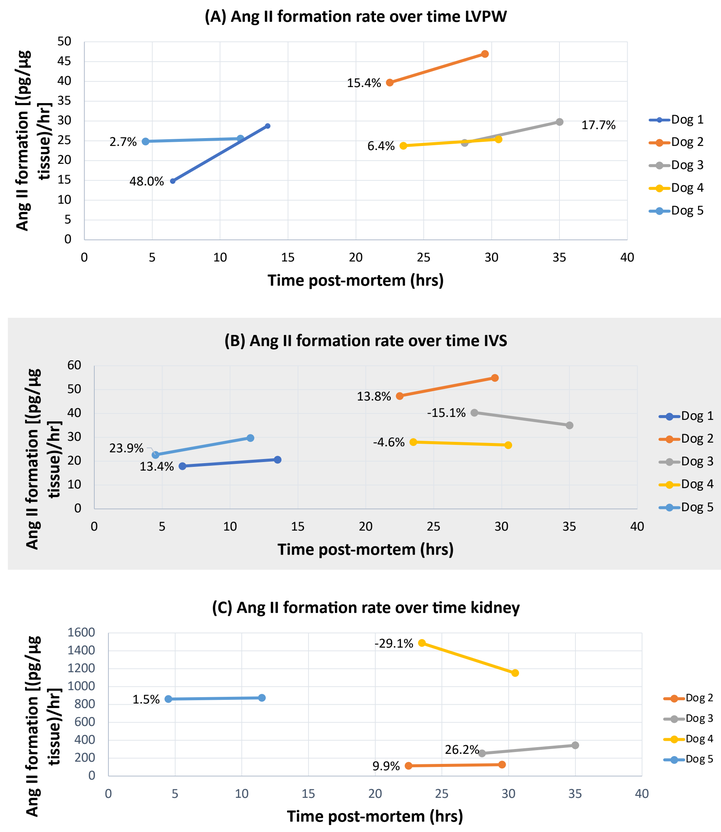


Figure 4. Ang II formation rate over time in (A) LVPW, (B) IVS, and (C) renal tissue samples from Dogs 1-5.

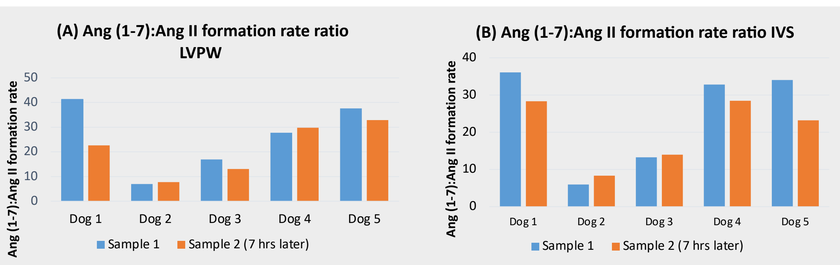


Figure 5. Ratios of Ang (1-7):Ang II formation rates in the (A) LVPW and (B) IVS of each dog at each sample point.

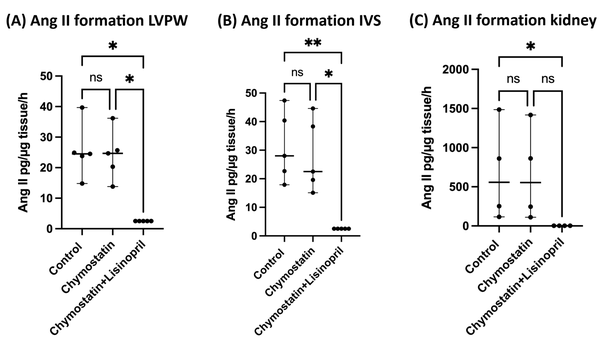


Figure 6. Ang II formation rates measured in the control, chymostatin, and chymostatin + lisinopril tests for each (A) LVPW, (B) IVS, and (C) renal tissue sample from Dogs 1-5.

Discussion

Objective 1: detect RAS enzyme activity

ACE, chymase, ACE2, and PCP activity were successfully estimated in myocardial and renal tissue samples retrieved within 36 hours post-mortem. The amount of variation in angiotensin formation rates between samples collected from the same dog seven hours apart differed between individuals. We cannot distinguish if changes in angiotensin peptide formation are due to the time difference between sample collection or inter-organ/dog variation. The intra-assay coefficient of variation is <10%. An analysis of enzyme stability over time is forthcoming.

The apparent stability of the Ang (1-7):Ang II formation rate ratio over time in each dog suggests that the relative contributions of each enzyme to angiotensin peptide synthesis are reasonably stable over time. The similarity of this ratio between two disparate sites (LVPW and IVS) suggests that either site is appropriate for RAS activity analysis. The Ang(1-7):Ang II formation ratio is useful to determine the overall balance (maladaptive to adaptive) of the RAS and may prove to be a helpful biomarker.

Further data analysis will allow us to determine an appropriate sample collection protocol for future studies. Likely, we will aim to collect tissues in a uniform and early (i.e., 4-6 hours) post-mortem window.

Objective 2: determine predominant Ang II-forming enzyme

Surprisingly, ACE, not chymase, appeared to be the predominant Ang II-forming enzyme in the myocardial and renal tissue samples. Ang II formed at very similar rates in the control and chymostatin trials, suggesting that chymase contributes relatively little to Ang II production in the tissues. In contrast, Ang II formation dropped significantly when lisinopril was added to the samples. This implicates ACE as the major Ang II-forming enzyme in the tissues. It also underscores ACE rather than chymase as a target for chronic diseases of these organs.

In contrast, other studies have cited chymase, not ACE, as the major Ang II-forming enzyme in canine heart and kidneys [20, 21]. These earlier studies used detergents in the tissue homogenization process, which has been shown to alter enzyme activity [20] and should be avoided. Current methods freeze and powderize the tissue and re-suspend it in a phosphate buffered saline.

Other influences on enzyme activity may have affected our results. Although we avoided harvesting major vessels in the tissues, our samples still included small coronary arteries and veins. Angiotensin converting enzyme is bound to cell membranes of endothelial cells, therefore the inclusion of vasculature in our tissue samples could skew our results. Additionally, whereas studies that found chymase dominance in the tissues harvested samples in a matter of minutes post-mortem, we collected our samples 4+ hours after death. Perhaps the post-mortem microenvironment rapidly changes the relative activity and balance of ACE and chymase in the tissues. We will need to retrieve fresh samples to investigate the validity of this hypothesis.

Conclusions

- ACE, chymase, ACE2, and PCP activity can be estimated in samples that are retrieved within 36 hours post-mortem, a realistic timeframe to collect samples from client-owned animals.
- The Ang (1-7):Ang II formation rate ratio remained stable between variable 7-hour time intervals post-mortem.
- ACE, not chymase, is the predominant Ang II-forming enzyme in myocardial and renal tissue samples.

Future Directions

We will harvest tissues from pet dogs in heart failure due to naturally-occurring heart disease. Our goal is to collect tissues from dogs in a relatively early and uniform time period post-mortem. We intend to study one group of dogs in heart failure on ACE inhibitors and a second group of dogs in heart failure not on ACE inhibitors. With myocardial and renal tissue samples from these cases, we will characterize the activity of ACE, chymase, ACE2, PCP, and NEP and describe how ACE inhibitors affect enzyme activity.

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