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Catestatin in Heart and Brain

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by

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DEDICATION

To my family, friends and fellow laboratory colleagues, who made this possible.

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ABSTRACT OF THE THESIS

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Chromogranin A (CgA) and its processed, bioactive peptides are known effectors numerous biological pathways. Initially, CgA was studied as an important prohormone peptide of the chromaffin cells of the adrenal gland. Previous studies have shown CgA's necessity in the exocytosis and the release of peptides stored within dense-core secretory granules. Catestatin is a product of CgA processing and serves as a major inhibitor of CgA by antagonizing CgA's effects. This thesis investigated the expression of CgA and catestatin in tissues outside the adrenal gland. We were particularly interested in the tissues outside the adrenal medulla that incur collateral damage as a result of increased sympathetic tone. Specifically, we investigated the processing and expression of chromogranin A into its inhibitory peptide, catestatin in the heart and brain. We

found not only a differential processing pattern between the adrenal, heart, and brain tissue, but also an age-dependent processing pattern.

1. INTRODUCTION:

1.1. General properties of Chromogranin A. Chromogranin A ($hCgA_{1-439}$) is a 48 kDa, acidic polypeptide and index member of the chromogranin/secretogranin protein family (1-3). Members of this family include chromogranin B ($hCgB_{1-657}$) (4) (1) and secretogranin II ($hScg\ II_{1-587}$) (5, 6); altogether, these peptides are packaged and exocytosed from dense-core secretory granules of the adrenomedullary sympathochromaffin cells and postganglionic sympathetic neurons (1, 7, 8). In addition, catecholamines, ATP, atrial naturetic peptide (ANP), and brain naturetic peptide (BNP) are also co-localized to these secretory granules and thus are released during granin exocytosis (7-12). While being co-stored with the aforementioned peptides, CgA is also responsible for vesicular biogenesis and co-release stimulation; CgA's expression in these secretory vesicles is not only necessary for exocytosis, but may also be sufficient (13-16).

1.2. Endocrine functions of Chromogranin A. In addition to serving as a key regulator of the secretory process, CgA also functions as a prohormone itself (1, 3, 17-19). Depending upon the species, CgA contains 8 to 10 well-conserved, dibasic residues (20-23); these are considered sites for potential cleavage by prohormone convertases (PCs) or cysteine proteases (24-27). For example, CgA's dibasic residues are prone to cleavage by PC2 and the lysosomal cysteine protease, Cathepsin L; both of which are co-localized with CgA and responsible for the endoprotealytic processing of the prohormone into its active

forms, either intra- or extra-cellularly (17-19, 26, 28-31). These biologically active peptides have an array of systemic functions; they include the dysglycemic peptide pancreastatin ($hCHGA_{250-351}$) (32-36), vasodilating vasostatin ($hCHGA_{1-76}$) (37), antimicrobial prochromacin ($bCHGA_{79-431}$), chromacin, (38) and multifunctional peptide catestatin (CST) ($hCHGA_{352-372}$; $bCHGA_{344-364}$) having inhibitory effects on catecholamine secretion, blood pressure, cardiac contractility and relaxation (39-46). CgA functions not only to derive these peptides, but it, along with other granin-family proteins, are also responsible sorting the cleaved hormones and concentrating them within their secretory granules (47).

1.3. Physiological and pathological consequences of Chromogranin A.

When CgA and CgA-derived peptides are released from their secretory granules, they circulate through the plasma as soluble peptides (1, 2); upon reaching the heart, CgA-derived peptides, such as vasostatin and catestatin, have the potential to exert negative inotropic and lusitropic effects on Langendorff-perfused rat heart (45, 48, 49). Originally, elevated CgA levels were used as a biomarker and prognostic tool for neuroendocrine tumors such as pheochromocytomas, carcinoid tumors, and neuroblastomas (2, 50-52). It was not until recently that researchers associated elevated CgA plasma levels with those who suffered from chronic heart diseases, including arterial hypertension (43), chronic heart failure (53), complicated myocardial infarctions (54, 55), dilated and hypertrophic cardiomyopathy (12), and acute coronary syndrome (55); furthermore, each study concluded that CgA serves some unknown

pathophysiological role in the aforementioned conditions and can be used as an effective prognostic tool. Thus CgA and its bioactive derivatives serve as important regulators in maintaining cardiovascular homeostasis (12, 45, 46, 54, 56, 57).

In the brain, CgA's role has not drawn as many associations. Whereas CgA is markedly elevated in patients with acute coronary syndromes, it is not elevated in patients who have suffered from cerebrovascular accidents (stroke). (55)

1.4. General properties of Catestatin. The CgA-derived catecholamine inhibitory fragment, catestatin was of particular interest to us. In humans, the CST peptide is a result of cleavage roughly between the 352nd and 372nd amino acid of CgA (hCHGA$_{352-372}$). Amongst functioning as an inducer of histamine release, and an antimicrobial and antimalarial agent (58, 59), CST also serves as a counter regulatory peptide for CgA and thus an inhibitor of catecholamine release (39, 40, 46). CST has 3 common variants: Pro370Leu, Arg374Gln, and Gly364Ser (41, 42). The variant, Pro370Leu, is the most potent inhibitor, however one of the more rare, whereas the variant Gly364Ser is more abundant (41, 42).. Moreover, the variant Pro370Leu is unable to be cleaved by Cathepsin L and thus could not generate an active CST peptide (19). While CgA promotes the formation of secretory vesicles and the release of catecholamines into the blood (2, 10, 13, 47), it is further elicited by activation of nicotinic acetylcholine receptors (nAChR) (39, 40, 46). As CgA undergoes intracellular protealytic processing (18, 19, 28), processed and circulating forms of its

bioactive peptide, CST, noncompetitively antagonizes these nAChR (39, 40, 46). On a molecular level, CST accomplishes this feat by antagonizing the Na^+ and Ca^{2+} signal transduction pathway found when nAChR is stimulated (39, 40, 46). As CST noncompetitively antagonizes nAChR, it hinders the release of the dense-core secretory granules, thereby inhibiting the effects of CgA. CST has a low IC_{50} of 200 nM (39, 40, 46), and is therefore potent and can immediately act as a autocrine/paracrine (39, 40, 46) inhibitor on the secretion of the dense-core secretory granules that contain CgA, catecholamines, ANP, etc. Interestingly, the more abundant isoforms of CST, Gly364Ser has also been linked to a reduced risk in the development of hypertension in humans (60).

1.5. Physiological and pathological consequences of Catestatin.

Furthermore, CST has been shown to promote vasodilation (61, 62), regulate myocardial contraction (negative inotropic effect), and relaxation (negative lusitropic effect) (45). This inhibitory function is dose-dependent on the amount of CST in circulation. From a pathophysiological standpoint, a decreased level of CST has been found in the plasma of patients with essential hypertension and also in normotensive mice with a genetic predisposition for hypertension (43, 63). This data suggests that the counter regulatory relationship between CgA and CST has important bearing on maintaining cardiovascular homeostasis. Indeed, by genetic ablation of the *Chga* gene (consequently preventing the expression of endogenous CST), this leads to an increase in blood pressure. This increase, however can be “rescued” by treatment with exogenous CST (43). Hence, this inhibitory dose dependency and pathological relevance lead us to

investigate the potential processing of CgA into its biologically active peptide, CST in tissues other than the adrenal medulla.

1.6. Aim of study. Previous findings have elucidated a 10-fold discrepancy in the concentration of CgA in the endocytic secretory vesicle and its immediate release into the extracellular matrix (ECM) (46). Since CgA is a soluble peptide that travels through the circulatory system and exerts endocrine/paracrine affects, this would further dilute the active peptides derived from it. However, if other tissues do in fact express CgA and CST, there would be a much more concentrated level of CgA and CST, whence they could act in a concentrated, paracrine/autocrine manner; much like CST's action in the sympathochromaffin cells. Previous studies have only investigated the role of CgA as an endocrine/paracrine effector. In this study, we sought to elucidate the expression and processing of CgA and CST in other tissues, including the heart and the brain. The heart is of particular interest due to the collateral damage it incurs during the development of metabolic and cardiac diseases. Previous researchers established CgA as an independent predictor of mortality due to its elevation in populations with a genetic predisposition and those with chronic and acute cardiac conditions. To determine whether other tissues independently express CgA and process CST, we homogenized heart, brain, and adrenal tissue from mice to investigate the differential expression patterns of CgA and CST amongst the aforementioned tissues. Overall, we determined that mice do in fact express and process CgA into CST in tissues such as the heart and the brain. Although processing occurs in both the heart and the brain, they have different

patterns of expression throughout development. Consequently, those who possess diminished CST levels in the heart, may be put at a further disadvantage. Since CgA overproduced in patients, likely for compensatory reasons, those with heart disease cannot produce enough CST to counter the development of disease. Once the disease has commenced, the heart also faces collateral damage from the diseases. Our results demonstrated that CgA's expression pattern in the brain varies from that in the heart and the adrenal gland. This may also lend further insight into the prognostic irrelevance of CgA in patients who suffered from a stroke. However, since we did not specifically isolate portions of the brain that are known effectors of neuroendocrine functions (i.e. the hypothalamus and the pituitary gland); it is difficult to reach a conclusion about true expression pattern of CgA in the brain. These differential expression patterns give further insight into the development of essential hypertension and other cardiac diseases.

2. MATERIALS AND METHODS:

2.1. SDS-PAGE and Western blot analysis. We raised a colony of healthy mice to the age of 5-6 months and subsequently sacrificed them using isoflurane gas. Surgery was performed immediately thereafter where brain, heart, and adrenal tissues were extracted and homogenized. Homogenized tissue samples were prepared with 4X loading dye and reducing agent. They were separated in a 10% SDS-PAGE (Novex precast gel; Invitrogen, San Diego, CA) and transferred onto a nitrocellulose membrane using electroporation. The membrane was blocked with PBS containing 0.1% Tween 20 (TBS-T) with 5% nonfat dry milk for 1 hour. Rabbit polyclonal antihuman catestatin [1:5000 in tris-buffered saline with 0.1% Tween 20 (TBS-T) and 2% BSA] and goat polyclonal anti-actin (1:500 in TBS-T and 2% BSA) were used to probe for our protein of interest with an overnight incubation period at 4°C. The membrane was incubated with secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit (1:3000 in TBS-T and 2% BSA), and donkey anti-goat (1:2000 in TBS-T and 2% BSA) for 1 hour at room temperature. The probed membrane was then developed by the Supersignal west pico chemiluminescent substrate (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol.

2.2. Immunohistochemistry. 12-well plates were fixed with flame-sterilized cover slips and mice cardiomyocyte cultures, derived from 4-day-old pups were incubated for a period of 96 hours. Mice cardiomyocyte cells were washed with PBS and fixed with 2.5% paraformaldehyde for 20 minutes at room

temperature. Fixed cells were permeabilized for 10 min with 0.5% Triton X-100 in PBS, incubated for 5 min with 150 mM glycine in PBS, and exposed for 30 min to 5% BSA in PBS. Cells were then incubated for 30 min at room temperature with rabbit polyclonal anti-human CgA antibody (1:1000 dilution), goat polyclonal anti-human CgB antibody (1:100 dilution, Santa Cruz), rabbit polyclonal anti-human ScgII antibody (1:2000 dilution), goat polyclonal anti-human β-Myosin Heavy (MYH) Chain antibody, or goat polyclonal anti-ANP antibody in PBS containing 2% BSA. Cells were subsequently washed and incubated for 30 min with a Alexa Fluor 594-conjugated donkey anti-goat IgG (1:350; Invitrogen) and Alexa Fluor 488-conjugated donkey anti-rabbit at (1:250; Invitrogen) together with 1 μg/ml Hoechst 33342 in PBS containing 1% BSA. Cover slips were washed with PBS, mounted in SlowFade antifade kit (Invitrogen). Three-dimensional images were captured on a DeltaVision deconvolution microscopy system operated by SoftWoRx software (Applied Precision, Issaquah, WA), using oil immersion objectives (60x magnification, for single cell viewing; 20x, for multiple cell viewing)

2.3. High performance liquid chromatography (HPLC). Mice were sacrificed and whole heart tissue were extracted and homogenized with Tris-maleate buffer. The supernatant was lyophilized and dissolved in water. A Symmetry C18 column (5 μm, 4.6x, 250 mm) was used to separate out the peptides of the heart extract. Equilibrium occurred in 0.1% trifluoroacetic acid (TFA) and the peptides were eluted with a 10%-100% acetonitrile gradient. Fractions were subjected to a slot blot using a nitrocellulose membrane and

probed with rabbit polyclonal catestatin antibody (1:5000) and secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:3000). The blots were then incubated with Supersignal west pico chemiluminescent substrate (Thermo Scientific, Rockford, IL) for 5 minutes and placed on the developer to visualize the presence of CgA or CgA-derived peptides.

2.4. Electrospray. Mass spectroscopy was performed through the University of California, San Diego Chemistry Department. Figures were composed using Data Explorer software (Applied Biosystems) where we typically see a mass error less than 0.1% (*i.e*. within ± 50 ppm, parts per million). The resulting CgA-derived fragments' masses were obtained from the MALDI analyses and were analyzed in the program Protein Prospector (http://prospector.ucsf.edu).

2.5. Immunoprecipitation. Heart tissue from 30 day to 1-month-old mice were homogenized in tris-maleate buffer. Samples were then subjected to a 10kDa-cut eppendorf and pre-cleared with nonspecific rabbit serum. They were subsequently incubated with a catestatin specific antibody overnight. The resulting immunocomplex was then subjected to protein G plus protein A agarose and eluted with acetonitrile/water/TFA. The eluted sample was concentrated via lyophilization and sent for mass spec analysis. Quality assessment of the immunoprecipitation was done by western blot analysis

2.6. Matrix assisted laser desorption (MALDI)/ ionization time-of-flight (TOF) mass spectroscopy of IP Sample. For MALDI-TOF, the immunoprecipitated fractions of CgA-derived peptides were acidified with 0.1%

trifluoroacetic acid, and purified using a Zip Tip (Millipore, Billerica, MA). One μl of each eluted fraction was mixed with 4 μl cyano-4-hydroxycinnamic acid (Agilent Technologies, Inc, Santa Clara, CA). One μl of this mixture was spotted onto a MALDI plate and subjected to air-drying. We used a PE Biosystems Voyager DeSTR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) with a nitrogen laser, and delayed extraction and reflectron mode to obtain the mass spectra. Calibration for all MALDI spectra was performed using two common peaks previously identified by MS/MS analysis. Figures were composed using Data Explorer software (Applied Biosystems) where we typically see a mass error less than 0.1% (*i.e*. within ± 50 ppm, parts per million). The resulting CgA-derived fragments' masses were obtained from the MALDI analyses and were analyzed in the program Protein Prospector (http://prospector.ucsf.edu).

3. RESULTS:

3.1. CgA and other granin proteins are expressed in murine heart.

First, we wanted to establish the presence of CgA, the index member of the chromogranin/secretogranin protein family, and its co-stored vesicular proteins CgB and Scg II in the heart. We sacrificed healthy mice between 5 to 6 months old with isoflurane and surgically excised the heart. We then homogenized each sample, combined the cell lysates and concentrated the sample to approximately 20 μg and 40 μg of protein. We subjected the 20 μg and 40 μg concentrated cell lysates to PAGE and electroblotted onto a nitrocellulose membrane. With three membranes, one membrane intended for each granin protein, we probed for CgA, CgB, and Scg II with anti-CST* (see note[1]), anti-CgB, and anti-ScgII, respectively. Upon probing using chemiluminescence, we saw the appropriate bands for each granin protein. It is important to consider the high number of acidic amino acid residues contained within all the granin protein's structure, thus they run anomalously on PAGE and their apparent molecular weight (MW) differs from the weight deduced by primary amino acid sequencing (1). The CST-probed Western Blot illustrated that murine heart contained some faint immunoreactivity at 75 kDa, the apparent molecular weight of full length CgA;

[1] It is important to note that we used CST as our primary antibody when conducting our Western blots. This is due to the ready availability of the antibody and the similarity in sensitivity of the antibody to both CgA and CST due conservation occurring in the sequence of both.

however there are more pronounced bands at the weight of 37 kDa and 27 kDa (Figure 1A). Thus suggesting that the heart has a greater expression of smaller, post-translationally modified CgA forms. The immunoblot for CgB appropriately displays a band at its apparent PAGE MW, 100 kDa (1) (Figure 1B). Furthermore, the immunoblot for Scg II also displays an appropriate immunoreactivity band at 87 kDa (5) (Figure 1C). The packaging of prohormone convertases, such as Cathepsin L, within granin-containing secretory vesicles allows for the intracellular processing of CgA, CgB and Scg II, and thus the presence of smaller, processed fragments on each granin protein's membranes. To further elucidate this point, we used immunohistochemical methods with cultured mice cardiomyocytes. We kept an inventory on the content of these dense-core secretory granules by tagging our granin proteins with immunofluorescent antibodies (Figure 2). As our Western Blots confirmed, there are numerous vesicles within the cardiomyocyte expressing each granin proteins in addition to ANP, which is an important peptide responsible decreasing blood pressure during hypertensive episodes. Previous research has highlighted the co-storing of these molecules and thus confocal microscopy was not performed. Thus, this data elucidates the expression of granin proteins outside the adrenal medulla and suggests the wide spread expression of the respective granin proteins in other tissues. With the presence of CgA established throughout the body, we investigated the processing of CgA into its smaller bioactive forms. Specifically,

we further researched the relative expression patterns of full length CgA and processed CgA in the adrenal gland, heart, and brain.

3.2. CgA and CST have different levels of expression in murine heart and adrenal tissue. After establishing the presence of the granin proteins in the heart, we wanted to observe if such tissues outside the adrenal medulla had protealytic processing capabilities. Both prohormone convertase 2 (PC2) and Cathepsin L (CTSL), a cysteine protease are known to be ubiquitously expressed in sympathochromaffin cells, cardiomyocytes, etc. (19, 64) thus we suspected that these tissues had their own potential to process CgA into its active hormone peptides and thus regulate themselves in a more potent, autocrine manner. Once again, we used SDS-PAGE and Western Blotting to observe the differential protein expression patterns between the adrenal and heart tissue. We used adrenal tissue from healthy mice and ran concentrated, homogenized and combined samples out on SDS-PAGE. When we probed for CgA with anti-CST, we noted that only two very heavy, distinct bands surfaced at 75 kDa and 55 kDa. Whereas in the heart extract, we noted several smaller peptides that ran on the PAGE at 37 kDa, 27 kDa, and 15 kDa; although there was a faint band at around 50 kDa, it is notably diminished from the adrenal extract. From these results, we suspected that within the adrenal medulla there is less endoprotealytic processing of CgA. On the other hand, there is an extensive amount of processing occurring in the heart from the original full length CgA peptide. Such processing could have implications on the heart where smaller forms of CgA imply that there

was endoprotealysis of CgA that resulted in the production of its bioactive peptides. In particular, production of the antihypertensive fragment, CST results in a negative inotropic and lusitropic effect in rodent heart. In fact, previous research has drawn an association between CTSL deficiency and the progression of dilated cardiomyopathy in mice. Moreover, this data may lend further insight into the development of essential hypertension and dilated cardiomyopathy in patient's with diminished CST expression.

The full length CgA fragment appeared to be highly prevalent in the adrenal extract, however we wanted to further investigate if smaller processed forms were present. Since full length CgA is abundant on the membrane, it limits the exposure time of the blot, and thus hides any other less abundant proteins from being developed. To overcome this, we cut the membrane and developed the lower portion of the membrane for a longer period of time. After exposing the lower membrane for a longer period of time, we noted a similar pattern to the heart extract with the presence of smaller, processed forms of CgA at 37 kDa, 27 kDa, and 15 kDa (Figure 4). Although both tissues express the processed forms, the ratio of full length CgA to processed CgA in the heart is much lower in the heart versus the adrenal gland.

3.3. Age-dependent processing of CgA in the adrenal medulla.

CgA is known to have a higher level of expression in hypertensive humans; whereas CST is known to be diminished in humans who are hypertensive and also in normotensive individuals who are the offspring of a hypertensive parent.

CST serves as a negative regulator of CgA and thus inhibits the release of catecholamines and other stress-induced peptides; a disruption in this delicate feedback system, as seen in middle-aged individuals, is tied to the pathophysiological role of CgA in the development of hypertension and cardiovascular failure. Since the adrenomedullary production of CgA is intimately interwoven into maintaining sympathetic tone, we first wanted to investigate the expression of CST in regards to specific age groups in the adrenal medulla. Thus, we wanted to investigate if there was an age-dependent processing mechanism of CgA in healthy mice. To investigate this further, we had four subsets of healthy mice that we sacrificed at 10d, 30d, 60d, and 90d, respectively. We used SDS-PAGE and Western Blots to elucidate the level of CgA and its processed products in homogenized adrenal tissue from each age subset. We faced the same overdeveloping dilemma in this experiment, where we wanted to observe the processing of CgA into its smaller processed forms. As in Figure 4, we cut the membrane at around 55 kDa so we could expose the film for a longer period of time and thus see if there were smaller CgA immunoreactive peptides present in the adrenal tissue. Indeed, in every age group of mice, there were smaller 50 kDa-processed forms that was previously unresolved from the other Western blots (Figure 3); the smaller forms were similar to those seen in the heart at 50 kDa, 37 kDa, 27 kDa, and 15 kDa. Although, we are constrained in our knowledge of the exact peptide that is running on the blot, we can infer that these smaller processed forms of immunoreactive CgA have are decreasing size due to

endoprotealysis with prohormone convertases. Even more intriguing was the emergence of even smaller peptides on the blot with increasing age of the murine subsets. Upon normalizing the intensity of CgA to actin, we noted that there was the greatest expression of CgA in the subset of mice at age 60d and 90d (Figure 5A). We hypothesize from this data that the healthy age-dependent processing occurs later in life may serve a protective role against pathogenesis. Moreover, if in fact the processing mechanism between CgA and CST is disrupted, this would result in the less endoprotealysis of CgA into CST, and a diminished amount of CST in the plasma. A lack of CST removes the autocrine negative feedback loop put on the secretion of CgA and catecholamine, thus resulting in a higher concentration of CgA and catecholamines in the plasma. High levels of CgA, and catecholamines, such as epinephrine have been noted in hypertensive patients. Since we previously demonstrated that cardiomyocytes have their own localized expression of CgA and CST, we wanted to investigate if other tissues, such as the heart, have a similar age-dependent processing mechanism.

3.4. Age-dependent processing of CgA in murine heart and brain.

We surgically excised and homogenized 10d, 30d, 60d, and 90d heart and brain and used SDS-PAGE and Western blots to investigate total postranslationally modified CgA fragments in the heart and brain in 10d, 30d, 60d, and 90d old mice. Interestingly, the heart and the brain both, had their own unique age-dependent processing mechanism. Like in the adrenal tissue, there is an emergence of smaller processed peptides of CgA as the mice aged in murine

heart; hence there is also an age-dependent mechanism (Figure 6A). This age-dependent mechanism could have major implications in the heart because the production of CST is essential for counter regulating CgA, exerting vasodialating, negative inotropic, and negative lusitropic effects; all of which serve a cardioprotective role. Furthermore, the local processing of CgA into CST (and its other biologically active peptides) allows CST to regulate in an autocrine/paracrine manner; by acting locally, these peptides are not diluted in the circulation and thus have a greater concentration and ability to act on the heart. Thus, in healthy heart tissue, there are multiple safeguards established by CST in maintaining cardiovascular homeostasis and the development of other cardiac diseases. Unlike the heart, the brain has a very different age-dependent processing mechanism. In healthy mice, the brain has the greatest amount of processing occurring at a very young age (around 10d) and tapers its processing as the population gets older (Figure 7). Thus from these findings, we cannot draw a similar parallel as we did in the heart, where an emergence of processing CgA into CST at a later age may provide a safeguard against the development of hypertension. Further research is needed to determine whether certain specific regions of the brain , particularly those responsible for neuroendocrine activation, like the hypothalamus, have an age-dependent processing pattern as seen in the adrenal medulla and the heart.

3.5. Identifying the processed CgA peptides in cardiomyocytes. The Western Blots we constructed had their constraints and only allowed us to see if

there was in fact an age- and tissue-dependent processing mechanism that yielded smaller, biologically active peptides that were immunoreactive with the CST antibody. Therefore, these blots only highlighted the presence of postranslationally modified CgA peptides and not the true identity of the smaller protealytic fragments. These findings lead us to continue probing for the identity of the smaller, protealytic fragments on the Westerns. To accomplish this feat, we used Reverse Phase HPLC (RP HPLC) to purify and elute CgA and CST from heart extract of healthy mice. We were particularly interested in fractions containing peptides within a 59.00 to 64.00 retention time (RT) and a 72.00 to 77.00 RT (Figure 8A). When we performed a slot blot of the following retention times to confirm the presence of CST-immunoreactive peptides with rabbit anti-catestatin antibody, we indeed found that these two ranges of RT were had some reactivity with the antibody; particularly the second set of elutants with RTs of 72.00 to 77.00 were particularly abundant in comparison to those with RTs between 59.00 to 64.00 minutes (Figure 8B). This lends further support to our Western Blot, which demonstrated that heart tissue had a greater concentration of smaller processed CgA-derived fragments versus the larger and full length, prohormone form of CgA. Now that we had confirmed the presence of smaller, processed peptides in the heart extract, we wanted to identify the products, thus we used mass spectroscopy and electrospray to identify the peptide fragments eluted from the RP HPLC. With assistance from the University of California, San Diego Department of Chemistry, the MS analysis elucidated the identification of several

peptides that nearly matched the length of CST and also had basic amino acid residues on their N-term and C-term. These dibasic amino acid cleavage sites are a hallmark of CST and other bioactive peptides derived from CgA. (Table 1) From these findings, we can reasonably infer that the smaller protealytic products that we visualized on both the Western Blot and HPLC were CST. Thus, in the heart, there is a local production of CST that has the capability of feeding back on the heart in an autocrine fashion. The brain, as a whole, lacks the progressive protealytic cleavage of CgA into its smaller bioactive forms and thus the development of its associated pathologies may not be related to a production mishap and regulation of CgA.

3.6. Confirming the presence of CST in cardiomyocytes. To lend further support to our argument that CST is in fact the locally processed peptide from CgA in cardiomyocytes, we took one more approach at isolating the peptide in a more specific manner. We sacrificed our 30d to 1-month-old murine pups and surgically excised the heart and pre-cleared with nonspecific rabbit antiserum; the homogenized tissue was subjected to CST-antibody specific immunoprecipitation (IP) to pull down any CST if in fact it was present in the tissue. The peptides were eluted and subjected to MALDI-TOF mass spectroscopy. From the samples we submitted, we found similar results as the last MS study. Smaller peptides, similar to the size of synthetic CST (~7.1 kDa) were also detected by the MS (Figure 9); these peptides also contained the hallmark dibasic cleavage sites flanking both ends of the peptides, suggesting that they were

cleaved from CgA in their local tissues. The remaining aliquots lefts from the IP were ran out on an SDS-PAGE gel and subjected to a Western Blot with anti-CST antibody; these samples were ran out next to a synthetic CST, as means of comparing the identities of the two small peptides. We noted that both the heart sample subjected to IP and the synthetic CST ran identically on the gel by materializing bands at 20.6 and 7.1 kDa, thus giving reasonable proof that the smaller, processed peptide that we had visualized throughout our study was in fact CST. Hence, from this study we conclude that the heart acts as its own autocrine regulator by locally producing CST in its tissue. Furthermore, the heart produces this inhibitory peptide, shown to have a strong association with the development of hypertension when diminished, and thus has the ability to maintain homeostasis locally.

4. DISCUSSION:

4.1. Chromogranin A and its pathophysiological role in heart failure.

Chromogranin A is an established biomarker of hypertension and cardiac failure (2, 12, 43, 54, 55). This association between plasma CgA levels and the development of cardiovascular disease has been contributed to CgA's involvement in adrenosympathogenic activity. First, plasma CgA levels are closely associated with setting the sympathetic tone (7, 8), as demonstrated by its catecholamine and Ca^{++}-dependent aggregation properties (10, 65). Secondly, Kim T, et al (13) demonstrated CgA's necessity and probable sufficiency in stimulating the biogenesis of secretory granules as they noted a decrease in the number of secretory granules upon siRNA targeted against CgA and an increase upon treatment with exogenous CgA. Lastly, Mahapatra NR, et al (43) noted that the genetic ablation of CgA consequently lead to an increase in plasma catecholamine level and increased blood pressure (43, 66). And thus, a mishap in the endoprotealytic processing of CgA into its bioactive peptides carries pathological consequences.

4.2. Catestatin role in maintaining cardiovascular homeostasis.

The bioactive peptide we were most concerned with was catestatin. CST is the result of endoprotealytic processing within the secretory granule at hCgA's dibasic site around CgA_{344} and CgA_{364} is exposed to either PC2 or CTSL (17, 19,

29). This 7.1 kDa fragment is cleaved from CgA and serves as a potent, noncompetitive inhibitor of catecholamine secretion in vivo. CST provides multiple cardioprotective mechanisms; it negatively feeding back on CgA and also exerts a negative inotropic and lusitropic effect on the heart (45). Diminished levels of CST are noted in both hypertensive mice and normotensive mice with a genetic predisposition (63). Furthermore, *Chga*-KO mice, which consequently knock out CST, also display the hallmarks of adrenosympathogenic over activity. This phenotype was reversible upon treatment with exogenous CST (43). Researchers reason that CgA attempts to provide an immediate compensatory mechanism when the heart is put under excessive volume and pressure (e.g. conditions caused by cardiac disease). Although CgA is initially attempting to repair the damage by releasing itself, its processed forms, and ANP, prolonged exposure to these peptides in the plasma carries detrimental effects and is inversely related to survival. Hence, CST and CgA's intimate, counter regulatory relationship serves a function in maintaining cardiovascular homeostasis.

Expression of CgA and the production of its bioactive peptides was originally discovered in the adrenal medulla and was subsequently found to be expressed ubiquitously throughout mammalian tissue (1-3, 67). Metz-Boutigue MH, et al (28) postulated that processing could also occur extracellularly by proconvertases such as PC2; the processed peptide could then act systemically on organs other than the adrenal gland and postganglionic neurons. In this process, the secretory granins concentration drops 10-fold alone from its concentration

intracellularly to immediately outside the cell and considerably more once diluted by the entire plasma volume. Pieroni M, et al (12) demonstrated that CgA is expressed in human myocardium. Prior studies have only alluded to CgA and its bioactive peptides exerting an endocrine or paracrine effect (39-41). Local expression CgA and subsequent processing into CST would allow the peptide and its derivatives to act in an autocrine/paracrine manner; since CST has such a low IC_{50} such local processing would allow for a quicker and more potent inhibition. In addition, the local expression of CgA would diminish the diluting effects due to the proximity of the site of action.

4.3. Differential processing of CgA in murine tissues. The sympathochromaffin cells of the adrenal medulla are known to highly express full length CgA. Catecholamines are both far more concentrated in the adrenal gland and also have inhibitory activity on endoprotealytic processing (68). Our present investigation demonstrated that the amount of full length CgA in the adrenal extract was far more prominent than in the heart and thus the adrenal has far less extensive endoprotealysis than the heart. (Figure 3B) In contrast, upon viewing the heart extract, we visualized very little full length CgA on the blot, whereas we visualized a substantial amount of processed CgA at 37 kDa, 27 kDa, and 15 kDa (Figure 3A). Although our polyclonal antibody could not uniquely identify the protein products on the membrane, the peptides visualized on the blot still provide evidence of the CgA fragment remaining and the CgA fragment cleaved.

4.4. Age-dependent processing of CgA and its probable role in the cardiovascular pathogenesis. Essential hypertension is typically surfaces in an adult human's midlife. Essential hypertension has also been proven to carry a dominant, genetic component. Previous studies have elucidated the genetics behind CgA and its association with essential hypertension, and with chronic heart failure. Such studies have shown a decrease in CST and an elevation of CgA plasma level in the abovementioned diseases (2, 63). This led us to investigate the processing of CgA into its bioactive peptides. Specifically, its cardioprotective peptide, CST in the development of hypertension. CST acts analogously as a "break" on the heart by a few methods: (1) inhibits catecholamine release by acting as a noncompetitive inhibitor, antagonizing the nAChR signal transduction pathway in myocytes and thereby inhibiting the adrenosympathogenic over activity. (2) Acts locally on the heart by exerting a negative inotropic and lusitropic effect. (3) Stimulates mast cells to release histamine and thereby induce vasodilation and decreasing the blood pressure and the stress on the heart. A local expression of CST would enhance these effects as the concentration of CST would be much larger than that expected of adrenomedullary-processed CST diluted throughout the plasma. Because hypertension is typically a disease that fully manifests itself later in life, we investigated an age-dependent processing mechanism in the chromaffin cells, the heart and the brain. Each respective organ had their own unique age-dependent processing mechanism. In the adrenal tissue, the greatest amount of processing occurred in mice at 60 days

old. The heart extract has a similar age-dependent processing mechanism, where we see a significant amount occurring at a later age in healthy mice. We suspect that this age-dependency processing may have gone awry in mice that have developed essential hypertension or are the offspring of a parent with hypertension. As O'Connor, DT et al (63) demonstrated, a diminished level of CST in the plasma is seen in both sets of mice. Dysfunctional processing of such a cardioprotective peptide in the heart could potentially introduce hypertensive risk factors unseen before (69).

4.5. Age-dependent processing of CgA and its ambiguity in neurovascular pathogenesis. The brain extract, in contrast, had a very unique age-dependent processing mechanism where we saw, for whole brain, the greatest amount of processing occurring in the first 10 days of life and tapering off from there. Because the brain is a very complex system and each component has its own unique physiological purpose, it is difficult establish if CgA's processing throughout the whole brain is an accurate representation of the physiological processes occurring. To better understand this, CgA's age-dependent processing pattern ought to be investigated in hormones pertinent to adrenosympathogenic activity such as the members of hypothalamic-hypophyseal axis.

Although processing in the brain remains to be elucidated, we established that there is an age-dependent processing mechanism of CgA into CST and its other bioactive peptides in healthy mammalian tissues. Such an age-dependent

processing mechanism in healthy mice illustrates that the development of hypertension and other cardiovascular diseases could be a result of a botched processing mechanism.

5. APPENDIX:

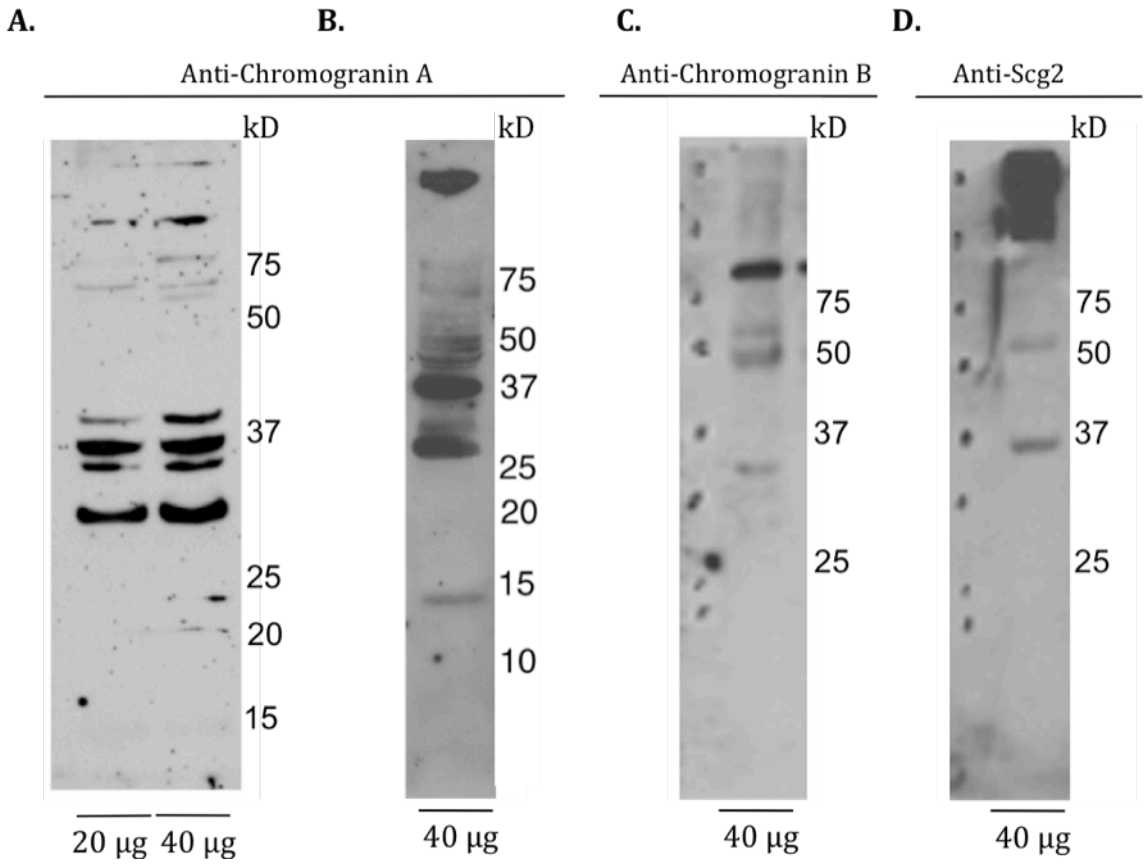


Figure 1: Western blot of mice cardiomyocyte lysates for CgA, CgB, and Scg II. Invitrogen 10% SDS-PAGE gels were loaded with (A) 20 µg and 40 µg of homogenized cardiomyocyte lysates or (B-D) 40 µg of heart tissue extract from mice. Protein content was transferred to a nitrocellulose membrane and probed with (A, B) rabbit polyclonal anti-CST antibody (1:5000) and HRP conjugated goat anti-rabbit secondary antibody. (1:3000)(C) goat polyclonal anti-CgB (1:100)and HRP conjugated donkey anti-goat secondary (1:350) (D) rabbit polyclonal anti-ScgII (1:2000)and HRP conjugated goat anti-rabbit secondary antibody (1:3000)

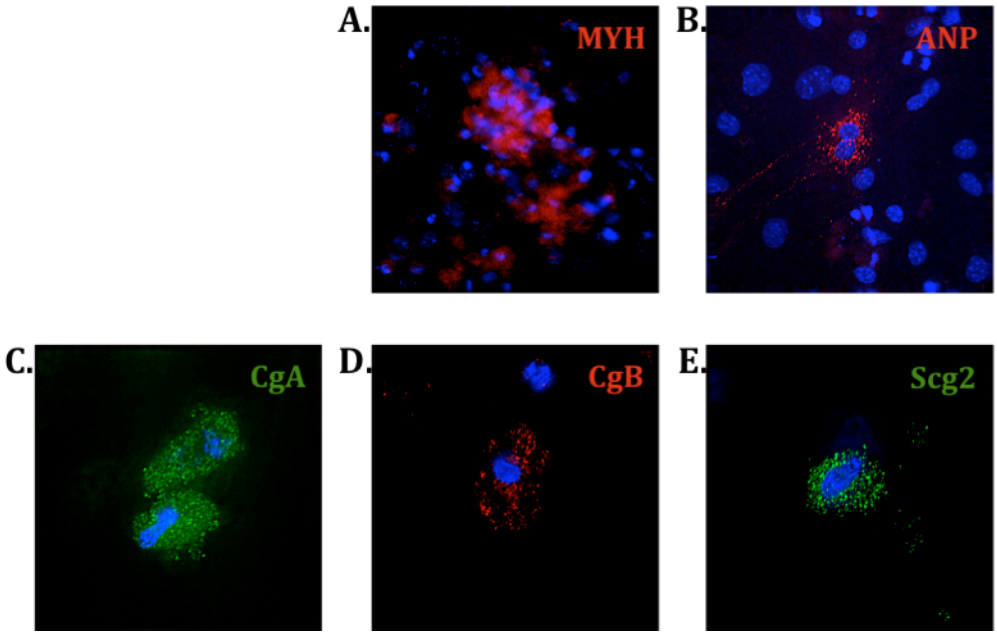


Figure 2: Immunomicroscopy of murine cardiomyocytes. Mice cardiomyocyte cells were washed and fixed with 2.5% paraformaldehyde. Fixed cells were permeabilized and then incubated with (A) goat polyclonal anti MyH, (B) goat polyclonal anti-ANP, (C) rabbit polyclonal anti-CgA (1:1000), (D) goat polyclonal anti-CgB (1:100), and (E) rabbit polyclonal anti-ScgII (1:2000), and washed and incubated for 30 min with a Alexa Fluor 594-conjugated donkey anti-goat IgG (1:350; Invitrogen) and Alexa Fluor 488-conjugated donkey antirabbit at 1:250; (Invitrogen) together with 1 μg/ml Hoechst 33342. Coverslips were washed and mounted in SlowFade antifade kit (Invitrogen). Three-dimensional images were taken using SoftWoRx software (Applied Precision, Issaquah, WA) on a DeltaVision deconvolution microscopy system, using oil immersion objectives (x60 (granin) x20 (upper)).

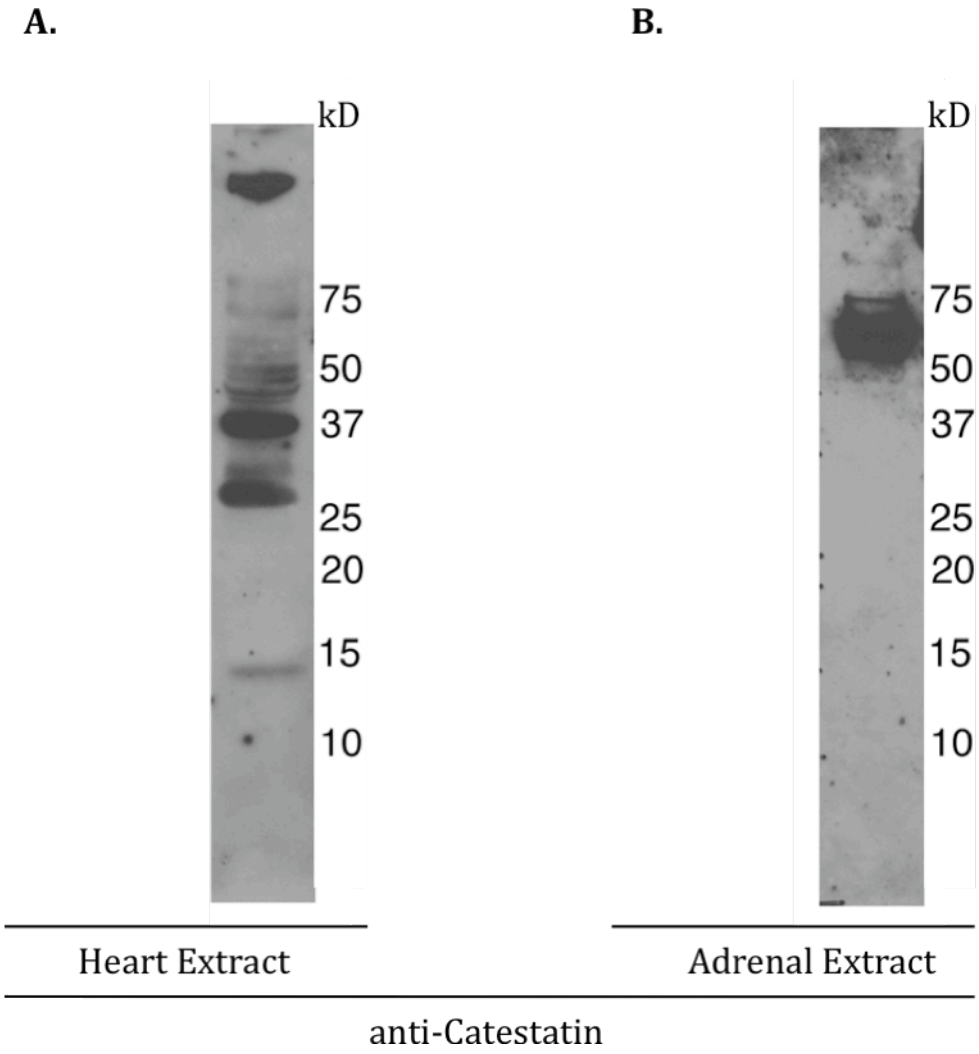


Figure 3: Differential expression of CST in murine heart and adrenal tissues. (A) Invitrogen 10% SDS-PAGE gels were loaded with 75 µg of homogenized cardiac and (B) 25 µg adrenal tissue and subjected to Western blot probed with rabbit anti-human CST primary antibody (1:5000) and HRP conjugated goat anti-rabbit secondary antibody (1:3000).

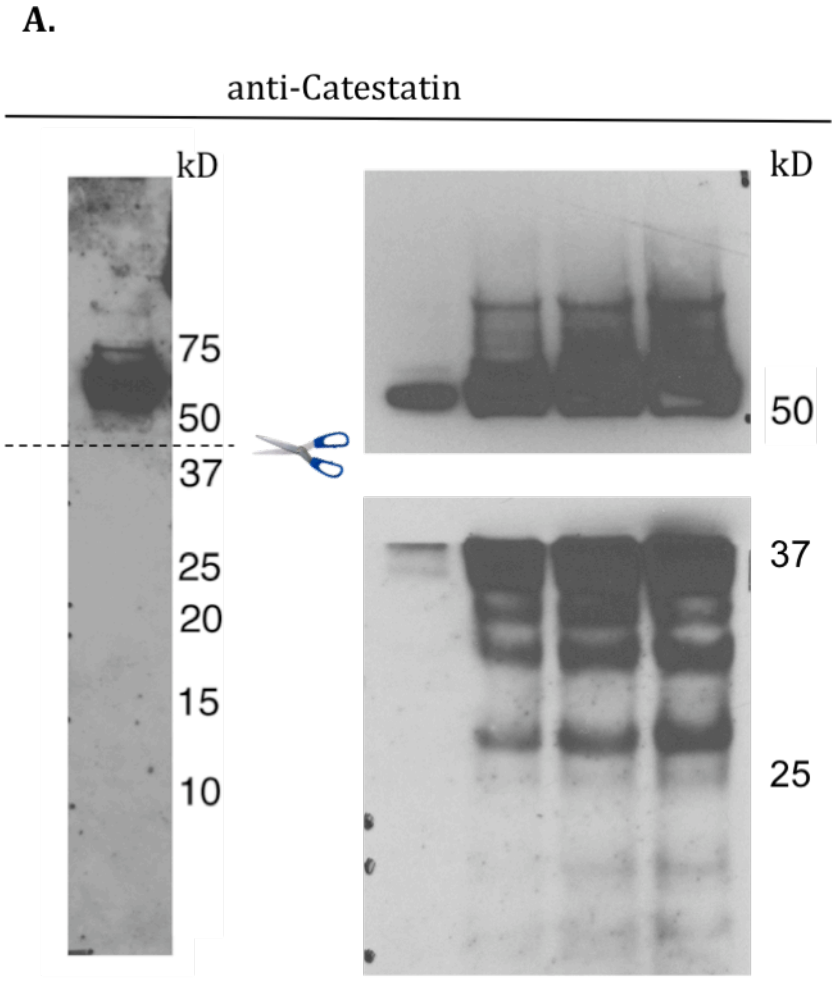
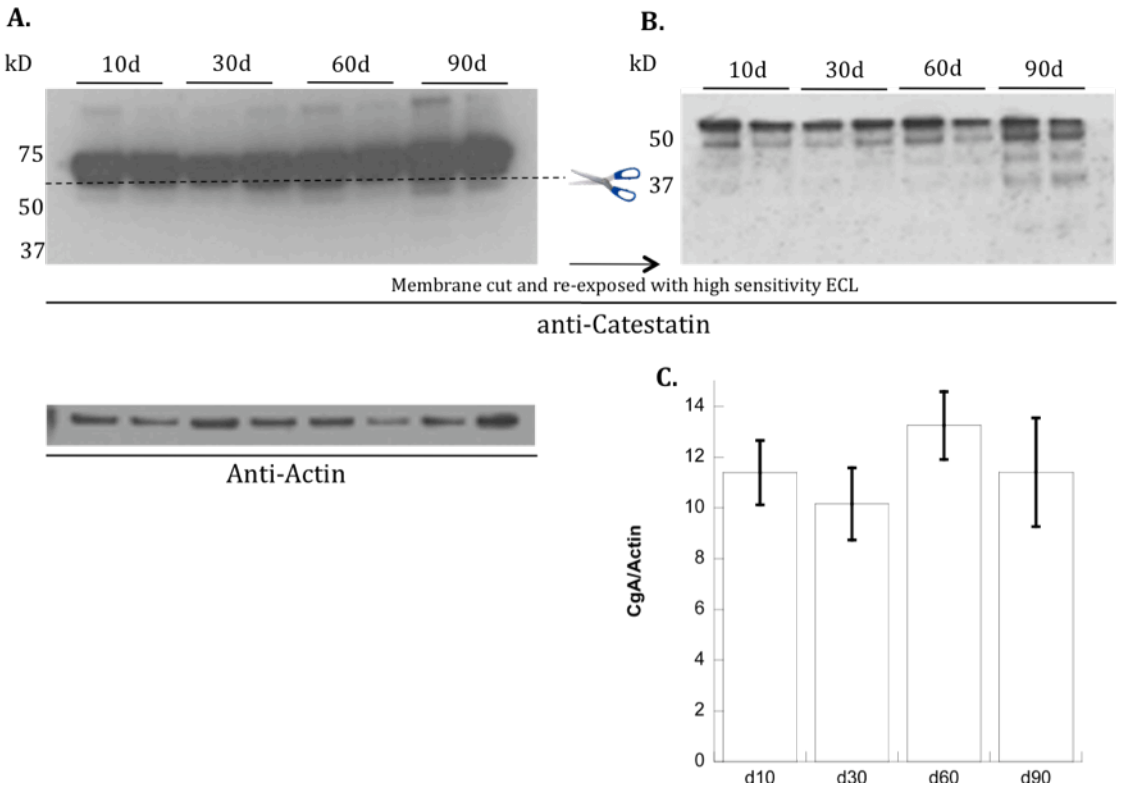
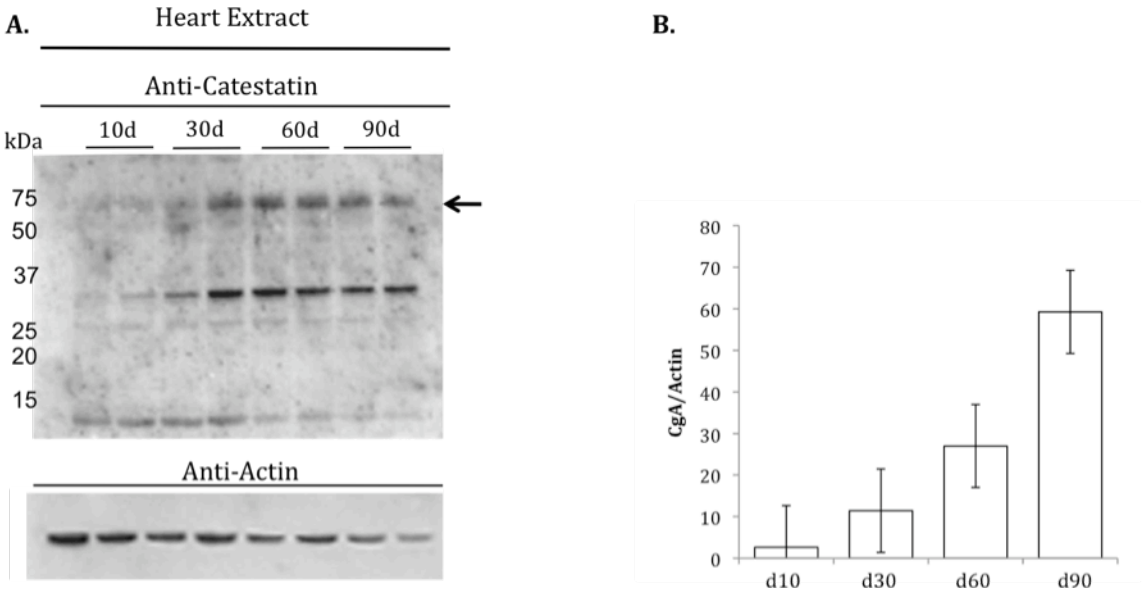


Figure 4: Expression of Processed CgA in the Adrenal Gland. Cut and reexposed the upper and lower membranes with Supersignal west pico chemiluminescent substrate



<u>Figure 5:</u> Western blot of CST expression in mice at age 10d, 30d, 60d and 90d in adrenal tissue. (A) 20 µg aliquots of murine adrenal tissue taken at age 10d, 30d, 60d, and 90d, respectively were loaded onto InVitrogen 10% SDS-PAGE gels and transferred onto a nitrocellulose membrane. The membrane was probed with rabbit anti-human CST antibody (1:5000) and HRP conjugated goat anti-rabbit secondary antibody (1:3000). The membrane was subsequently stripped and reprobed with diluted 1:500 goat polyclonal anti-actin antibody and HRP conjugated donkey anti-goat secondary antibody (1:2000). (B) The membrane in (A), probed with CST was cut at approximately 55 kDa and re-exposed with Supersignal west pico chemiluminescent substrate (C) Densitometry (BioRad) normalized the amount of CgA full length to actin in each of the samples



<u>Figure 6:</u> Age-dependent processing of CgA in murine heart (A) 75 μg samples of murine heart tissue taken at age 10d, 30d, 60d, and 90d, respectively were loaded onto InVitrogen 10% SDS-PAGE gels and transferred onto a nitrocellulose membrane. The membrane was probed with rabbit anti-human CST antibody and goat anti-rabbit secondary antibody. The membrane was subsequently stripped and reprobed with actin antibody and secondary. (B) Densitometry (BioRad) normalized the amount of CgA full length to actin in each of the samples.

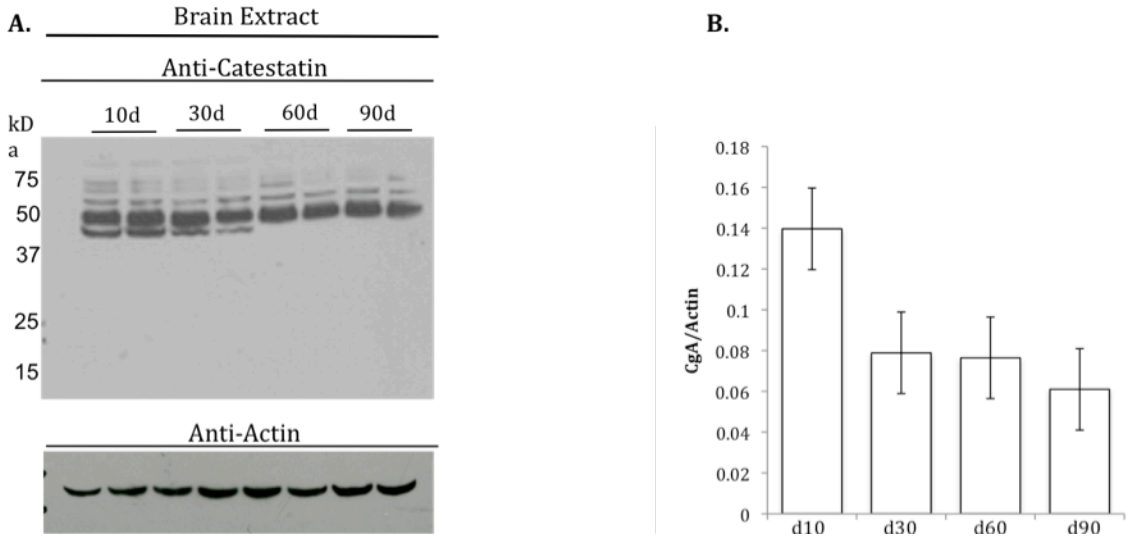
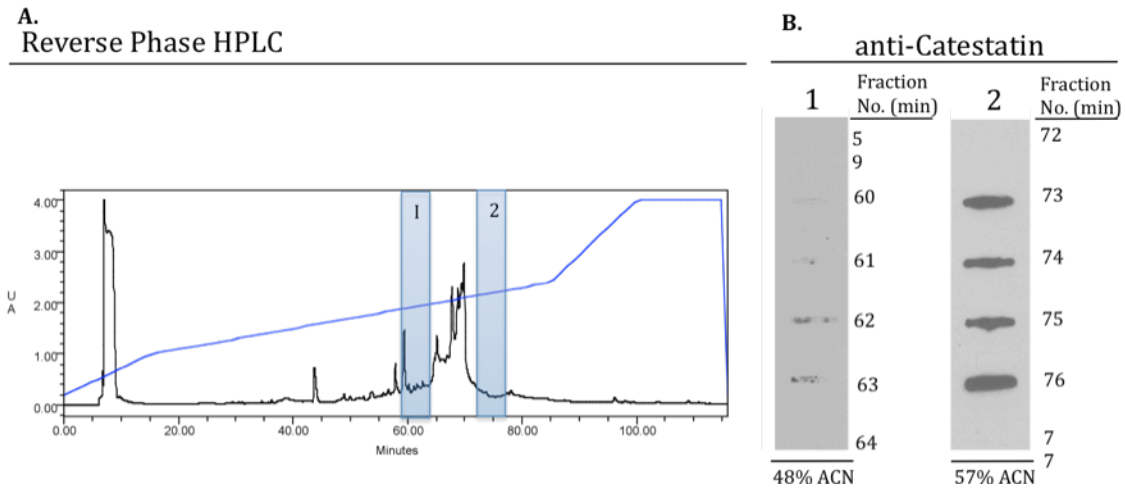


Figure 7: Age-dependent processing of CgA in brain. (A) 75 µg samples of murine brain tissue taken at age 10d, 30d, 60d, and 90d, respectively were loaded onto InVitrogen 10% SDS-PAGE gels and transferred onto a nitrocellulose membrane. The membrane was probed with rabbit anti-human CST antibody (1:5000) and goat anti-rabbit secondary antibody (1:3000). The membrane was subsequently stripped and reprobed with mouse anti-chicken actin antibody (1:500) and goat anti –mouse secondary antibody (1:2000). (B) Densitometry (BioRad) normalized the amount of full-length CgA to actin for each of the samples.



<u>Figure 8</u>: Eluting CgA and CST with Reverse phase HPLC and identifying with slot blot: (A) Homogenized and lyophilized whole heart tissue were subjected to a Symmetry C18 column (5 µm, 4.6x, 250 mm). Equilibrium occurred in 0.1% trifluoroacetic acid and the peptides were eluted with a 10%-100% acetonitrile gradient. (B) Slot Blot of fractions taken from RT 59-64 min. and 72-77 minutes. Probed with rabbit anti-CST antibody (1:5000) and goat anti-rabbit antibody (1:3000)

Table 1: Electrospray and Mass Spectroscopy Analysis of HPLC fraction. **Electrospray** Performed through the Department of Chemistry, Resulting peptide masses obtained from the MALDI analyses were analyzed in the program Protein Prospector (http://prospector.ucsf.edu) to identify the possible fragments of the respective proteins.

Mass (Monoisotopic) submitted	Mass (Monoisotopic) matched	Sequence	Cleavage sites	
4989.67	4989.37 4989.54	343-382 367-408	E/E K/E	R/S P/S
5148.74	5148.52	346-387	E/E	S/F
5057.51	5057.52	357-398	K/R	P/G
3061.55	3061.50	360-385	S/R	K/L
4341.31	4341.17	362-398	M/D	P/G
3034.66	3034.56	364-389	Q/L	R/T
4989.78	4989.54	367-408	K/E	P/S
5051.6	5051.47	374-416	R/L	E/A
5644.11	5643.69	375-422/376-423	L/E or E/G	E/E or E/G
3294.72	3294.67	378-405	E/D	G/W
4757.5	4757.41	382-421	D/R	F/E
5244.85	5244.63	383-426	R/S	E/E
2478.34	2478.36	385-405	M/K	G/W
1754.94	1754.87	387-401	L/S	Q/L
2676.45	2676.41	387-408/388-409	S/F or L/S	S/S or P/S
5187.64	5187.51	387-430	L/S	S/A
2369.22	2369.2	394-413	Y/G	D/S
3828.98	3828.88	396-427	F/R	E/E
2008.99	2009.00	397-413	R/D	D/S

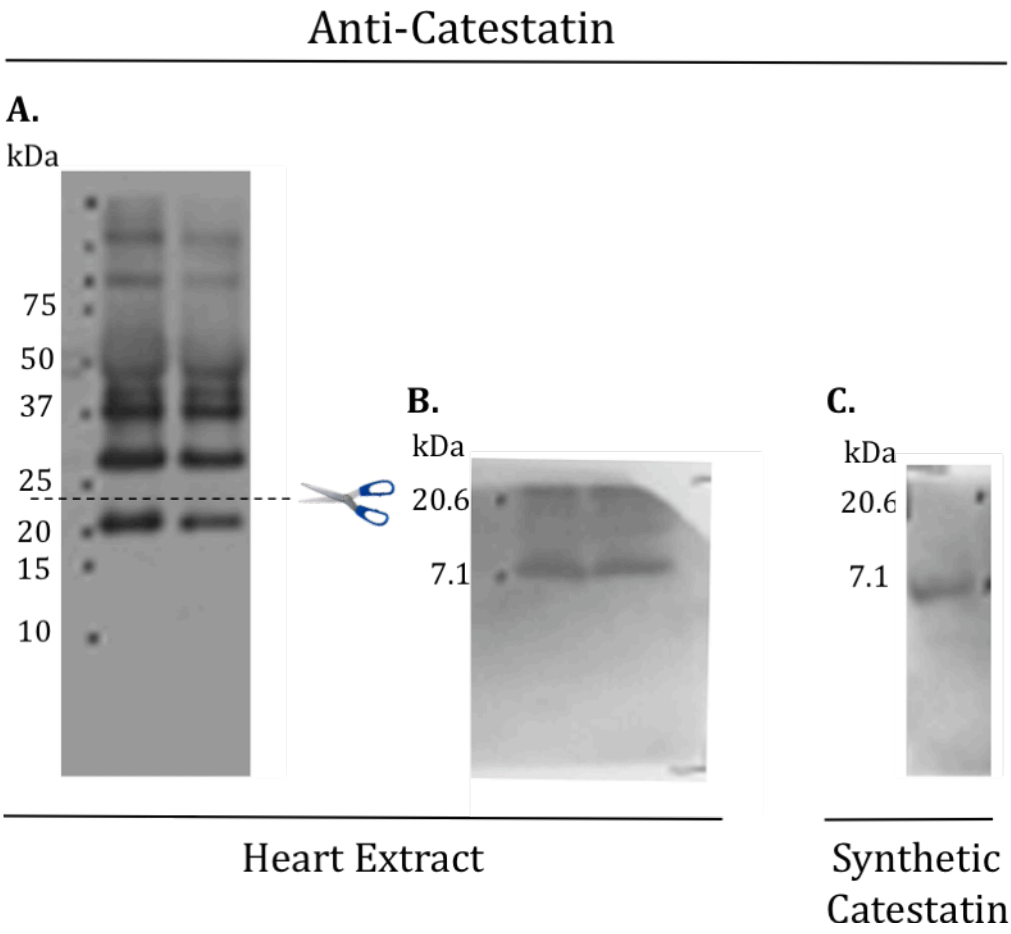


Figure 9: Immunoprecipitation of CST in murine cardiomyocytes Whole heart tissue was extracted and homogenized in tris-maleate buffer and homogenized. Samples were then subjected to a 10kDa cut-off eppendorf and incubated with rabbit anti-CST antibody. The immunocomplex was pulled with G Plus/protein A-agarose beads and eluted with acetonitrile/water/TFA for SDS-PAGE and Western Blotting analysis. 2 samples of elutant were electroblotted and probed with rabbit anti-CST and goat anti-rabbit secondary. (B) The membrane was cut and re-exposed for a longer period of time (C) Synthetic CST was electroblotted and probed with rabbit anti0CST and goat anti-rabbit secondary.

Table 2: MALDI-TOF analysis of CST immunoprecipitated heart extract. Immunprecipitated fractions of CgA-derived peptides were acidified in 0.1% trifluoroacetic acid, and purified using a Zip Tip (Millipore, Billerica, MA). 1 µL of each fraction was mixed with 4 µl -cyano-4-hydroxycinnamic acid (Agilent Technologies, Inc, Santa Clara, CA). 1 µl of said solution was spotted onto a MALDI plate and air-dried. Mass spectra were acquired on a PE Biosystems Voyager DeSTR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) with a nitrogen laser, operating in delayed extraction and reflectron mode. Calibration of MALDI spectra was done by 2 common peaks. The mass error of this method is typically less than 0.1% (*i.e.* within ± 50 ppm, parts per million). Resulting peptide masses obtained from the MALDI analyses were analyzed in the program Protein Prospector (http://prospector.ucsf.edu) to identify the possible fragments of the respective proteins

Mass (average) submitted	Mass (average) matched	Sequence	Cleavage sites	
4097	4097.6503 4098.6272	357-390/358-391 383-417	K/R R/W R/S	T/R R/A A/R
4228	4225.8254	356-390	D/K	T/R
5733	349-395 360-407	349-395 360-407	R/L S/R	F/R R/P

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