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Cardiovascular effects of Catestatin (CST) on Insulin Resistant, Hypertensive  
*db/db* mice.

A Thesis submitted in partial satisfaction of the  
requirements for the degree Master of Science

in

Biology

by

Arshi Jha

Committee in charge:

Professor Sushil K. Mahata, Chair  
Professor Yunde Zhao, Co- Chair  
Professor Milton Saier.

2011

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Co- Chair

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Chair

University of California, San Diego

2011

## DEDICATION

I dedicate this thesis to my parents and brother, for their unconditional love  
and support.

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ABSTRACT OF THE THESIS

Cardiovascular effects of Catestatin (CST) on Insulin Resistant, Hypertensive *db/db* mice.

by

Arshi Jha

Master of Science in Biology

University of California, San Diego, 2011

Professor Sushil K. Mahata, Chair  
Professor Yunde Zhao, Co-Chair

Catestatin (CST), an antihypertensive peptide, is known to decrease blood pressure (BP) in *Chga*<sup>-/-</sup> mouse models. This prompted us to examine whether CST was similarly able to decrease BP in a spontaneously generated

hypertensive and insulin resistant *db/db* mouse model. We used a non-invasive computerized tail-cuff method for measuring blood pressure, qPCR to determine gene expression, western blot for protein and high performance chromatography (HPLC) for determination of catecholamines after treatment with CST. Our results showed that CST was able to decrease BP in diabetic mice possibly by reducing systemic vascular resistance via the action of vasodilatory mediators, like Nitric Oxide (NO) and Atrial Natriuretic Peptide (ANP). These findings improve our understanding of CST as an antihypertensive peptide in general, as well as its role in diabetic type II mouse model.

## 1. INTRODUCTION

### **1.1. The Granins (Chromogranins/Secretogranins).**

First identified in the 1960s, while studying the physiological and pharmacological role of catecholamine in mammals, the granins are a group of acidic, heat stable, soluble secretory proteins (1, 2). The granins are approximately 180 to 700 amino acids in length and made of single polypeptide chain containing an N-terminus signal domain. This amino terminal signal guides the movement of the granin pro-proteins from ribosome to the endoplasmic reticulum and finally to the Golgi for further post-translational modifications. Studies have shown that the granins are not only co-localized within the secretory granules but also co-released with catecholamine from the chromaffin granules (3).

Granins are known to be precursors of several bioactive molecules. The three most important granins are, Chromogranin A (CHGA): first extracted from the adrenal medulla, Chromogranin B (CHGB): discovered from rat pheochromocytoma cell line (PC12) and Chromogranin C (also known as secretogranin II) (SCG-2) first found in the anterior pituitary (3, 4).

### **1.2. Importance of Chromogranin A and its properties.**

Chromogranin A (human CHGA<sub>1-439</sub>) is one of the most important and also the first identified granin of the Chromogranin/Secretogranin family. This acidic 48-kDa glycoprotein, located on chromosome 14q32.12 in humans, has been found to be ubiquitously present in secretory vesicles of endocrine,

neuroendocrine and neuronal cells (2-5). Further studies have shown that the presence of CHGA is most abundant in the adrenal medulla, followed by pituitary, brain, gastro intestinal tract and the pancreas. Granins, in particular CHGA and CHGB are known to have a regulatory function in formation of secretory vesicles (2). CHGA, like the other granins, is a pro-protein that gets cleaved by endopeptidases, namely prohormone convertase 1 and 2 (PC 1 and PC 2) to give rise to a plethora of bioactive proteins involved in numerous endocrine functions.

The major bioactive peptides include, pancreastatin, (hCHGA<sub>250-301</sub>) that regulates blood sugar levels by antagonizing the action of insulin, vasostatin 1 and 2 (hCHGA<sub>1-70</sub>), both preventing vasoconstriction and also regulating the attachment of fibroblast and coronary-artery smooth muscle, and lastly catestatin/CST (hCHGA<sub>344-364</sub>): which has a role in blocking the nicotine-induced release of catecholamines from the sympathoadrenal chromaffin cells (6-10).

### **1.3. Pathological relevance of Chromogranin A.**

CHGA, shortly after its discovery has played an important role as a general histochemical marker for neuroendocrine cells as well as carcinoid tumors (tumors arising from neuroendocrine cells) (3). CHGA is not only used as a biomarker for the tumors themselves but the circulating levels of CHGA in the plasma is used as a guideline in treatment of these tumors. Elevated levels of

CHGA has been correlated with diseases like neuroblastoma, and higher concentration of CHGA in blood is used as a measurement for the tumor mass (13, 14). CHGA has also proved to be an important marker in other cancers such as the endocrine tumors, gastrinoma, small cell lung cancers, prostate colon, and even breast cancers (2).

Along with the vast range of cancers mentioned above, CHGA is also being seen as a diagnostic tool not only in different cardiopathologies but also in many neurological diseases. The level of CHGA increases in preamyloid plaques of Alzheimer's disease, as well as in Lewy bodies in Parkinson's disease. Previous studies have shown that CHGA might play a role in activating the microglia and therefore contributing to the various neuronal pathologies (15-17).

The most important pathological role of CHGA, for the purposes of our study, is in hypertension. It is been shown that patients with hereditary hypertension have an increased level of plasma CHGA concentration. It is known that the catecholamine release takes place via exocytosis along with the release of CHGA (3, 4). CST acts by preventing the release of catecholamines and therefore CHGA itself; thus, showing a regulatory effect in hypertension (3). Interestingly CST levels are diminished in hypertensive individuals and in their normotensive offspring suggesting low CST level as an early predictor for developing hypertension (3-5).

#### **1.4. Identifying CST and its properties.**

CST was first identified in bovine CHGA, as the region that inhibited nicotine induced catecholamine secretion. Due to the inhibitory effect of this bioactive peptide on catecholamine release, it was named CST (10).

CHGA has about 8-10 basic sites that are the preferred supposed sites for endopeptidases and thus proteolytic cleavage (1). To identify the inhibitory domain 15 peptides were generated which covered about seventy-eight percent of functional CHGA. These peptides were then tested to see how well they were able to inhibit the secretion of nicotine induced catecholamine release. The only region that was able to successfully inhibit catecholamine secretion was found to be bCHGA<sub>344-364</sub> (4, 10 & 19).

#### **1.5. Pathological relevance and functions of CST.**

One of the many functions of CST is vasodilation (62). The two other important functions of CST for the purposes of our study are its effect on hypertension and cardiovascular system. It has been shown that CST, when injected into the rats intravenously is able to reduce blood pressure even after blockage of  $\alpha$  and  $\beta$  adrenergic receptors (20). This study further confirmed that CST was not only able to decrease blood pressure by decreasing catecholamine release, but it was also able to do so by decreasing peripheral resistance by increasing histamine release from the mast cells. In addition, to

its vasodilatory effects, CST has also been shown to regulate both positive and negative inotropic myocardial contraction (21).

### **1.6. Type II Diabetes and Hypertension.**

It is a known fact that a large patient population with diabetes type II also suffers from hypertension. The exact cause as to which disease strikes first and the interplay between the pathologies of the two diseases is still unclear. However, some data has suggested that diabetic patients, due to hyperinsulinemia, are likely to be predisposed to hypertension due to sympathetic hyperactivity (22).

Studies have shown that insulin resistance may be associated with sympathetic activation and arterial pressure. Sympathetic activation would then increase vasoconstriction via  $\alpha$  adrenergic stimulation and also via the activation of renin/angiotensin system causing an increase in blood pressure in obese subjects. Another theory relating hypertension and diabetes has regarded leptin (a satiety hormone that regulates energy intake and expenditure by regulating appetite and hunger) as the key hormone, which stimulates sympathetic response in mice, thus leading to hypertension in diabetic patients. The gene for leptin is located on chromosome 7q32.1 and it binds to the *ob* receptor in the brain to carry out the signaling pathways. Leptin's role in relating diabetes and hypertension is further strengthened by



the fact that hypertensive; non-diabetic patients are found to have increased levels of leptin(22-25).

### **1.7. Neuropeptide Y and Hypertension.**

Neuropeptide Y or NPY is a co-transmitter of the sympathetic nervous system. NPY is so called because of the tyrosine residues at the amino and carboxy ends (60). It has been shown to be co-released with nor-epinephrine and ATP, the two other transmitters of the sympathetic nervous system. In addition, NPY is said to positively regulate vasoconstriction and increases the action of noradrenaline (60).

### **1.8. Objective of the study.**

CST has successfully proven to decrease blood pressure in *Chga*<sup>-/-</sup> KO mice by decreasing the release of catecholamines from the secretory vesicles in the chromaffin cells. Therefore, we wanted to investigate whether CST was able to decrease the blood pressure in the insulin resistant, hypertensive, diabetic (*db/db*) mice and whether it played any role in easing the pathologies of the diabetic and hypertensive heart.

Therefore, we hypothesized that CST can decrease blood pressure in *db/db* mice. We suggested that CST does so mechanistically by rescuing systemic vascular resistance through decreasing plasma Neuropeptide Y levels. In addition, CST is able to increase the mRNA transcript levels of ANP (atrial

natriuretic peptide) and eNOS (enzyme nitric oxide synthase). ANP acts to reduce the water, sodium and adipose loads on the circulatory system. The enzyme eNOS catalyze the production of Nitric oxide (NO), a potent vasodilator.

We also examined the involvement of metabolic regulatory molecules like AMPK and ERK and their role in CST's action of lowering blood pressure in *db/db* mice.

Furthermore, CST is known for its inotropic effect in the wild type heart. We therefore hypothesized that CST might be able to rescue the myocardial restrains, namely diabetic cardiomyopathy a pathological condition in *db/db* mice.

## 2. MATERIALS AND METHODS

### **2.1. The *db/db* mouse model.**

Sixteen *db/db* male mice (8 weeks old), were bought from Jackson Laboratory (strain: B6.BKS (D)-LEPR DB/J). Three-four mice were housed in each cage situated in a temperature controlled room (at 72°F) with 12 hour: 12 hour light-dark cycle. The mice were allowed free access to standard chow and tap water for drinking.

At 18 weeks of age, the mice were sacrificed after 2 weeks of treatment with CST (5 µg/g daily, intraperitoneal injection) and tissues were harvested for further analyses.

### **2.2. Blood Pressure via the tail-cuff method.**

CODA High- Throughput system (Kent Scientific Corp., Torrington, Connecticut) was used to measure the Blood Pressure of the *db/db* saline and *db/db* CST treated mice. The heating tray was turned on about 30 minutes prior to the experiment and the temperature was set to about 35°C. 7 ear tagged mice were placed in medium sized (30-50kg body weight) holders and placed on the heating tray. The VPR (volume pressure recording) cuff and the occlusion (O) cuff were screwed on in their respective places, followed by the running the *BP* program called "CODA." The V and O cuffs were then tested for leaks, which if found could be fixed by changing the inflatable balloons on the cuff.

New experiment was started with the following conditions: acclimatization cycle was set to 10, and the total number of set was kept at 1; cycles per set were set at 60, along with time between cycles set to 20 seconds. Each mouse was assigned a number between 1-8 and placed on the heating plate according to the corresponding number. The tails of individual mouse was threaded through the V and O cuffs—the O cuff being closer to the body and the V cuff away from the body. Minimum venous return volume was set to 5 $\mu$ l, and the program was set to sensitivity override for mouse. Tail temperature was checked via a portable thermometer (Kent Scientific Corp.), and once around 35°C the program was run till completion. Data was saved and exported to Microsoft Excel for further analyses.

### **2.3. Tissue Harvest.**

At 18 weeks of age mice were sacrificed after 7-8 hrs of fasting between 12:30 pm to 3:30 pm. The mice were first deeply anesthetized by Isoflurane gas and blood was collected, centrifuged (2000 rpm at 4°C, for 10 minutes) and the plasma was stored in -80°C for the measurement of catecholamine in blood. Along with blood other harvested tissues included: heart, liver, adipose, muscle, carotid artery and adrenal glands. The liver was weighed and along with the rest of the tissues frozen in liquid nitrogen and then stored in -80°C for gene expression and protein analyses.

#### **2.4. Total RNA isolation.**

RNA was extracted using the Trizol kit from Invitrogen (Carlsbad, CA). Transverse section including left and right ventricle from the heart (30-50 mg) and blood vessels (5-7 mg) were homogenized in 1ml and 500  $\mu$ l respectively for about 60 seconds (s). The solution was mixed by vortexing and incubated at room temperature for 5 minutes (min). Chloroform was added (0.2 ml chloroform to 1 ml of Trizol) to each of the samples and mixed manually for 15 s. The samples were again incubated at room temperature for another five minutes, followed by centrifugation at 12000 g for 10 minutes at 4°C. The top, aqueous layer is transferred into a new tube. It is important to take caution and stay away from the interface to avoid contamination.

Isopropanol was added (1:2 trizol volume) to the samples and incubated at room temperature for 10 min. The samples were then spun down at maximum speed at 4°C and the supernatant was discarded. 70% ethanol, prepared in RNase free water was added to the RNA pellet for RNA wash. The samples were spun down at maximum speed for 10 min at 4°C and the supernatant was discarded. The pellet was left to air-dry for about 20-25 min. The RNA pellets were re-suspended in 50  $\mu$ l of RNase free water for the heart samples and 30  $\mu$ l for vessel samples. All the above steps were done under a fume hood.

## 2.5. cDNA preparation from RNA.

Bio-rad (Hercules, CA) iScript cDNA kit was used for generating cDNA from the isolated RNA. 30  $\mu$ l cDNA reaction was set up. 6  $\mu$ l of iScript reaction mix, 1.5  $\mu$ l of iscript reverse transcriptase and respective volumes of RNase free water and RNA was added to reach an overall concentration of 200  $\mu$ g. The prepared samples were then run through the thermal cycler with the following protocol: 5 min at 25°C, followed by 30 min at 42°C then 5 min at 85°C and hold at 10°C.

## 2.6. q (or RT) PCR reaction.

Gene expression levels were identified by quantitative RT-PCR. 25  $\mu$ l reaction was set up in each of the wells of a 96 well plate. 12.5  $\mu$ l of Syber green, 0.2  $\mu$ l of forward and reverse primers was added along with 10  $\mu$ l of water and 1  $\mu$ l of DNA template.

qPCR was done in an iCycler IQ Real Time PCR detection system (Bio-Rad Laboratories, Hercules, CA), using SYBR green chemistry. The icycler protocol was set according to the one defined by the company itself.

The oligonucleotide primers used for real-time quantitative PCR are listed in Table 1.  $\Delta C_T$  method was used to determine the relative expression of the qPCR products. This method uses the equation: fold induction =  $2^{-[\Delta C_T]}$ , where  $C_T$  = the threshold cycle, which represents the cycle number at which the sample's relative fluorescence rises above background fluorescence and  $\Delta C_T = C_T$  gene of

interest-  $C_T$  standard (GAPDH). Each sample was run in duplicate, and average  $C_T$  was determined. The data was then plotted in a bar graph.

**Table 1:** List of genes for qPCR

<i>Gene</i>	<i>Sequence (5'-3') F</i>	<i>Sequence (5'-3') R</i>
Natriuretic Peptide precursor A (ANP)	caacacagatctgatggttca	attgccgatctggacacc
Natriuretic Peptide precursor B (BNP)	cttctgcggcatggatct	cccagcggtgacagataaag
Phospholamban (PLN)	acgtcaccgaagccaag	tggtaagagaaagataaaaagtga
ATPase, Ca <sup>2+</sup> transporting, cardiac muscle, slow twitch 2 (SERCA 2a)	tcgaccagtcaattctttacagg	caggacagggcagtatgc
Procollagen, type1, $\alpha$ 1(Col1a1)	gtctcccggtcagagagg	accttgttgccaggtcac
Receptor for advanced glycation end products (RAGE)	cctgggaagccagaaattg	gacacacatgtccccacctt
Endothelin-1 (Edn1)	Tgctgttcgtgactttcaa	gggctctgactccattct
Nitric Oxide Synthase 3 (eNOS)	Ccagtgcctgcttcac	gcagggcaagttaggatcag
Myosin heavy polypeptide 6, cardiac muscle $\beta$ (MHC $\beta$ )	Acggatgccatacagaggac	aacacttggcgttgacagc
Myosin heavy polypeptide 7, cardiac muscle $\beta$ (MHC $\beta$ )	Ggcctcattgatgactctg	cgctgtcagcttgtaaag



## 2.8. HPLC Assay and ELISA

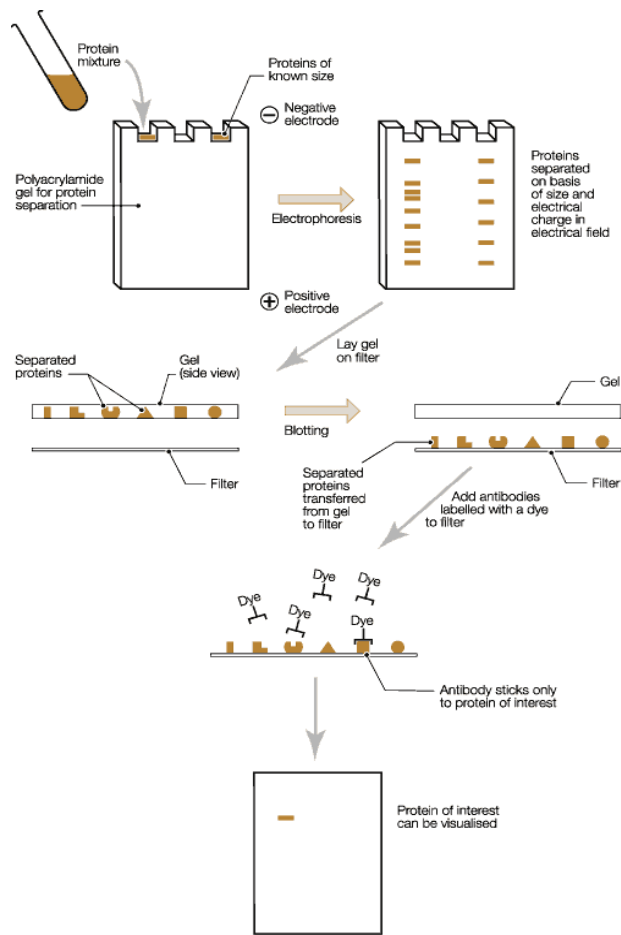
Fresh frozen adrenal glands were dropped into 0.5 mL cold PBS for homogenization for about 30 seconds. 250  $\mu$ l of the homogenized solution is extracted and 0.8 N perchloric acid is added in a 1:1 ratio. This mixture is then centrifuged at 8000 rpm for 15 min at 4°C to get rid of cellular debris. The supernatants are carefully transferred to a fresh eppendorf tube and 20 ng of DHBA is added. This mixed is then transferred to tubes containing 40-50 mg of activated alumina. The solution is then brought to pH 8.6 by adding Tris buffer. The catecholamines is then allowed to adsorb onto activated alumina in a shaker for about 30-45 mins. The alumina beads are washed and gently catecholamines are eluted using 400  $\mu$ l of 0.1N HCl. The sample is then loaded on to the injection rack (70-80  $\mu$ l) and the samples are run overnight to gather data.

*ELISA:* 50  $\mu$ l of sample was added and 25  $\mu$ l of Biotinylated protein was added to each of the respective wells. The protocol was followed as per provided in the company user manual (Phoenix Pharmaceuticals, Burlingame, CA).

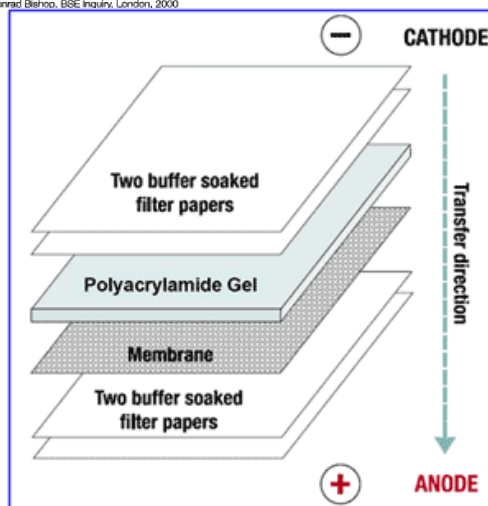
## 2.7. Western Blot analysis.

Adrenals and murine hearts of adult *db/db* mice were harvested and homogenized in 1 ml of ice cold lysis buffer containing 0.2 M sucrose, Tris maleate (pH 7.0) buffer, supplemented with 2 mM EDTA, pH 8.0, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and 10 µg/ml aprotinin. Cellular debris along with cytosolic fractions were isolated by centrifugation of the lysate at 12000 rpm for 10 mins at 4°C. The protein content was then determined by Bradford assay (BIO-RAD, Hercules, CA), using Bovine Serum Albumin (BSA) as the standard. Ten µg of protein from adrenals and murine hearts were used for Western immunoblot analysis of Phospho Erk (P-Erk), Phospho AMPK (P-AMPK), total Erk, total AMPK and CHGA proteins using specific antibodies (primary: Cell signaling, Beverly, MA and secondary: Sigma, St. Louis, MO).

Blots were probed with 1:1000 dilution of anti-P-Erk, P-AMPK, total AMPK, total Erk and CHGA. Membranes were then washed in phosphate buffer saline (PBS) supplemented with 0.05% Tween-20 and incubated with 1:5000 dilution of peroxidase conjugated anti-rabbit IgG (Sigma) followed by detection using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ).



Source: Konrad Blehoo, BSE Inquiry, London, 2000

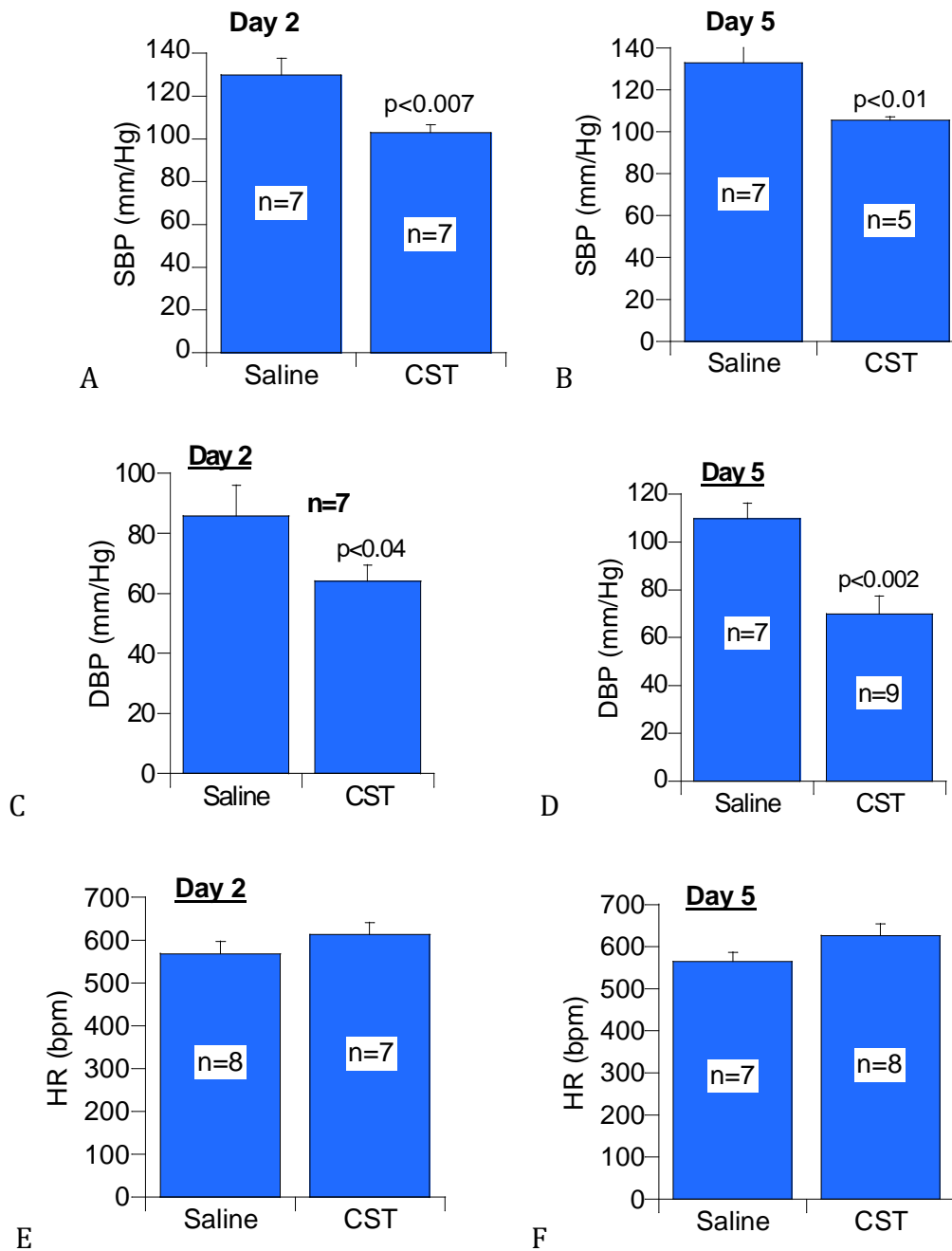


**Scheme 1:** Diagram of Western Blotting

### 3. RESULTS

### **3.1. Blood Pressure (BP) analysis in *db/db* mice treated with CST versus control (saline).**

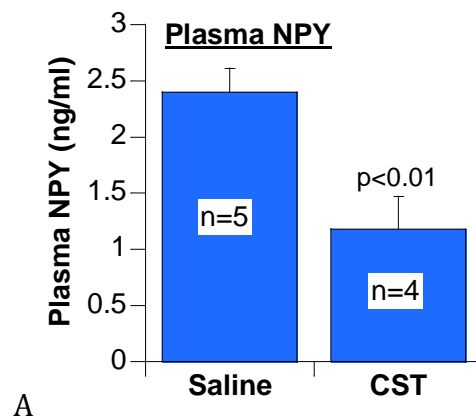
BP data revealed a steady decline in systolic pressure (SBP) in *db/db* CST treated mice versus the *db/db* saline (control). The first day of BP measurement was considered to be an acclimatization run for the mice, and therefore the data was dismissed as unreliable. Out of all the results of the tail cuff BP measurements performed during the following four days, SBP measurement of the second and the fifth day was presented (Fig. 1 A&B). SBP showed a significant decline in CST treated vs. the control. These measurements are taken during the second half of the experimental procedure. A significant decrease in diastolic blood pressure (DBP) in CST treated mice was also observed compared to the control (Fig. 1 C&D). However, there were no significant changes in heart rate (Fig. 1 E&F).



**Figure 1:** Graphs of Systolic blood pressure (SBP) in *db/db* CST treated mice vs. *db/db* Saline (control). Tail cuff method was used to measure the blood pressure of diabetic mice over a period of 5 days, one of which is disregarded as acclimatization day. A and B: Shows SBP measurements taken on day 2 and 5. C and D: Diastolic pressure measurement on day 2 and day 5. E and F: No change in HR is observed in CST treated vs. Saline mice.

### 3.2. Catestatin's effect on Neuropeptide Y levels in plasma.

ELISA assay of the neuropeptide Y plasma levels was performed. NPY is a known vasodilator, therefore we tested whether CST was able to alleviate vascular resistance by decreasing the plasma levels of NPY. Significant decreases in the levels of plasma NPY was seen in CST treated vs. the control, showing that CST was able to relieve vascular resistance via a decrease in NPY plasma levels (Fig. 2).

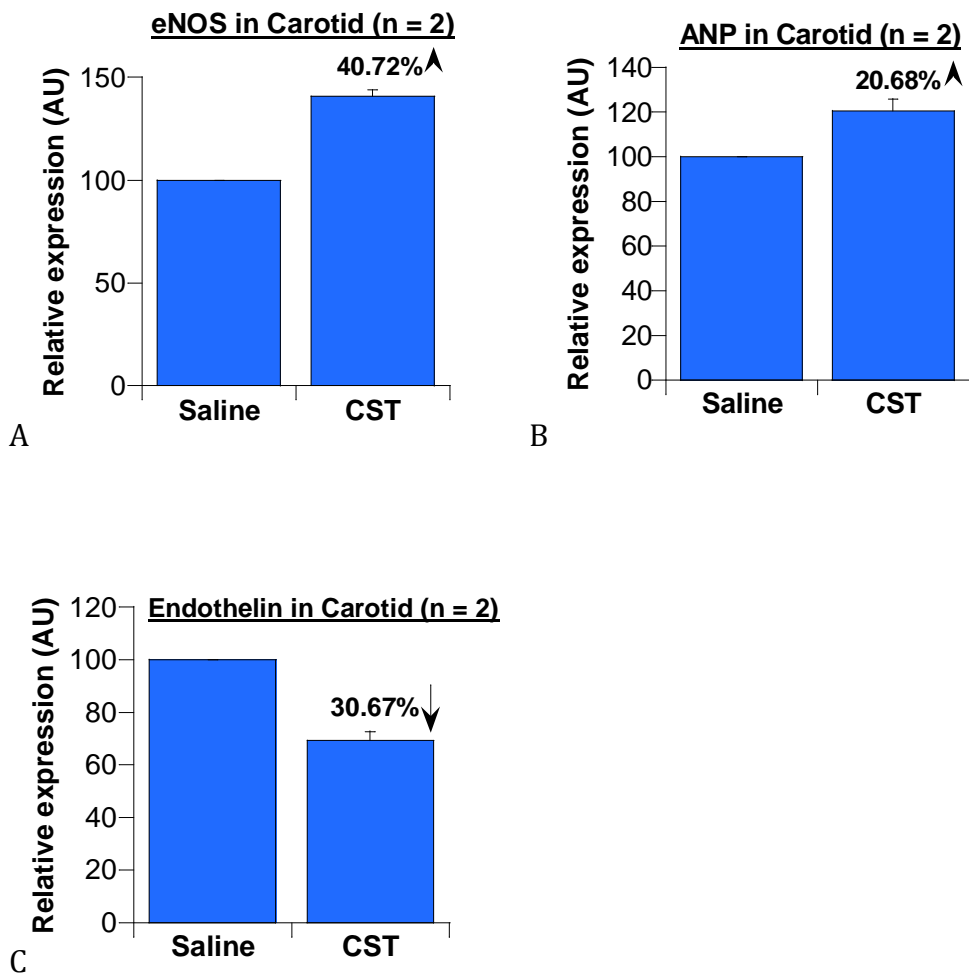


**Figure 2:** Graph of plasma NPY levels in CST treated vs. control.

### **3.3. Catestatin's effect on vasculature and systemic vascular resistance.**

Gene expression analysis of several vasodilatory proteins was done to show whether CST played a role in increasing the expression and thus regulating blood pressure via decreasing systemic vascular resistance. Data from the expression analysis of eNOS (enzyme involved in conversion of L-arginine to NO, a potent vasodilator) revealed that catestatin treated mice showed an increased expression in the blood vessel (carotid artery) versus the control (Fig. 3A). CST treatment also showed an increase in gene expression of Atrial Natriuretic Peptide (Fig. 3B), which is known to be a potent vasodilator in hypertensive mouse models (28). In addition, ET-1 (Endothelin-vasoconstrictor) showed a decrease in expression in experimental mice vs. the saline treated (Fig. 3C)

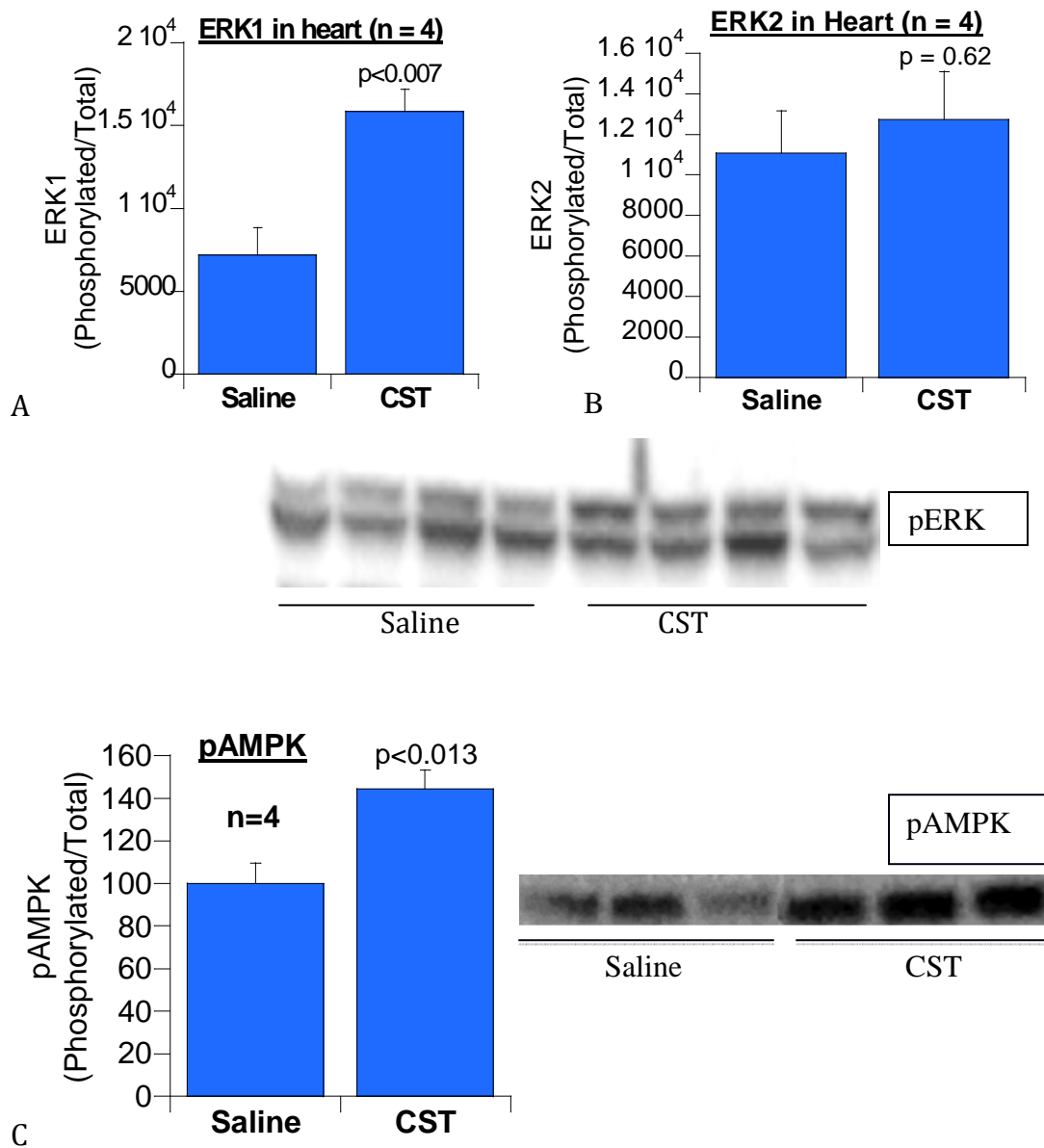




**Figure 3:** qPCR analysis of genes involved in blood pressure by vasoconstriction and vasodilation in *db/db* CST vs. saline murine carotid arteries. A: CST treated mice showed an increased level of the eNOS. B: Increase in another vasodilatory peptide, ANP, is observed in treated mice vs. the control. C: ET-1 showed significant decline CST treated mice vs. control.

### 3.4. Role of catestatin in activating important regulatory molecules.

Previous studies have shown the importance of regulatory metabolic molecule like AMPK and ERK1/2 in diabetes and hypertension. Phospho-Erk (pERK) is shown to increase the level of NO synthesis in CST treated *Chga*<sup>-/-</sup> mice and phosphorylated AMPK is involved in numerous pathways including contractile work of the heart, cardiac dysfunction as well as vasodilation (29, 30) in *db/db* and hypertensive mouse models. Therefore, protein level analysis of these regulators would confirm/negate CST's role on their activity. First, we looked at the protein levels of pERK1/2, corrected against total ERK, via western blot analysis in murine hearts. CST treated mice showed significant changes in the phosphorylated ERK1 level compared to the control (Fig. 4A), however, ERK2 phosphorylation showed no significant changes between the CST and the control (Fig. 4B). Next, pAMPK (phosphorylated or activated AMPK) protein levels were determined to show that CST treated mice did, in fact show elevated levels of phosphorylated AMPK vs. the saline treated (Fig. 4C).



**Figure 4:** Western blot gel pictures and quantification of metabolic regulator protein levels in heart of *db/db* CST vs. saline A and B: Expression level of pERK1 and 2, respectively, corrected with total ERK with the respective gel exposure, in CST treated vs. control mice. C: Expression of pAMPK corrected with total is graphed and presented with the respective gel exposure.

### 3.5. Catestatin's effect on cardiopathology.

Various effects of CST in regards to the cardiovascular systems are slowly emerging. Recent studies have shown CST to be a cardiosuppressive agent in hypertensive hearts (41) along with many others that shed light on the CST's importance on cardiac pathologies. Therefore, in order to understand the general effects of catestatin in ailing *db/db* mouse model gene expression analysis was used to quantify any structural and functional changes in the heart in CST vs. control mice.

Expression levels of eight different genes were looked at in treated vs. non treated murine hearts. RAGE, receptor for advanced glycation end products, was amongst the first genes to be analyzed. It is known to be a key player in diabetic cardiomyopathy due to its negative effects on cardiac contractility (54). Mouse heart treated with CST did not show a significant increase in RAGE expression compared to the control (Fig. 5A). Collagen 1a1 involved in fibrosis of the heart, was seen to show an increasing trend. However statistical analysis did not show the increase to be significant (Fig. 5B). Expression of Serca, Ca<sup>++</sup> ATPase, showed an increasing trend (not significant) in mice with CST treatment compared to the control (Fig. 5C). Phospholamban (PLN), a protein that regulates Serca 2a, seemed to show a decreasing non-significant trend (Fig. 5D). Two more genes (MHC $\alpha$  and MHC $\beta$ ) involved in contractility of the cardiac muscles were analyzed. MHC $\beta$  as well as

MHC $\alpha$ , showed an increase non-significant trend in mice treated with CST compared to the control Fig. 5E and 5F, respectively).

CST's effect on the gene expression level of potent vasodilator atrial natriuretic peptide (ANP), in murine heart, revealed a significant decline compared to the control (Fig. 5G). Brain natriuretic peptide, a biomarker for heart failure, showed an increasing trend in *db/db* CST treated murine hearts compared to the control, however, the data was not significant (Fig. 5H).

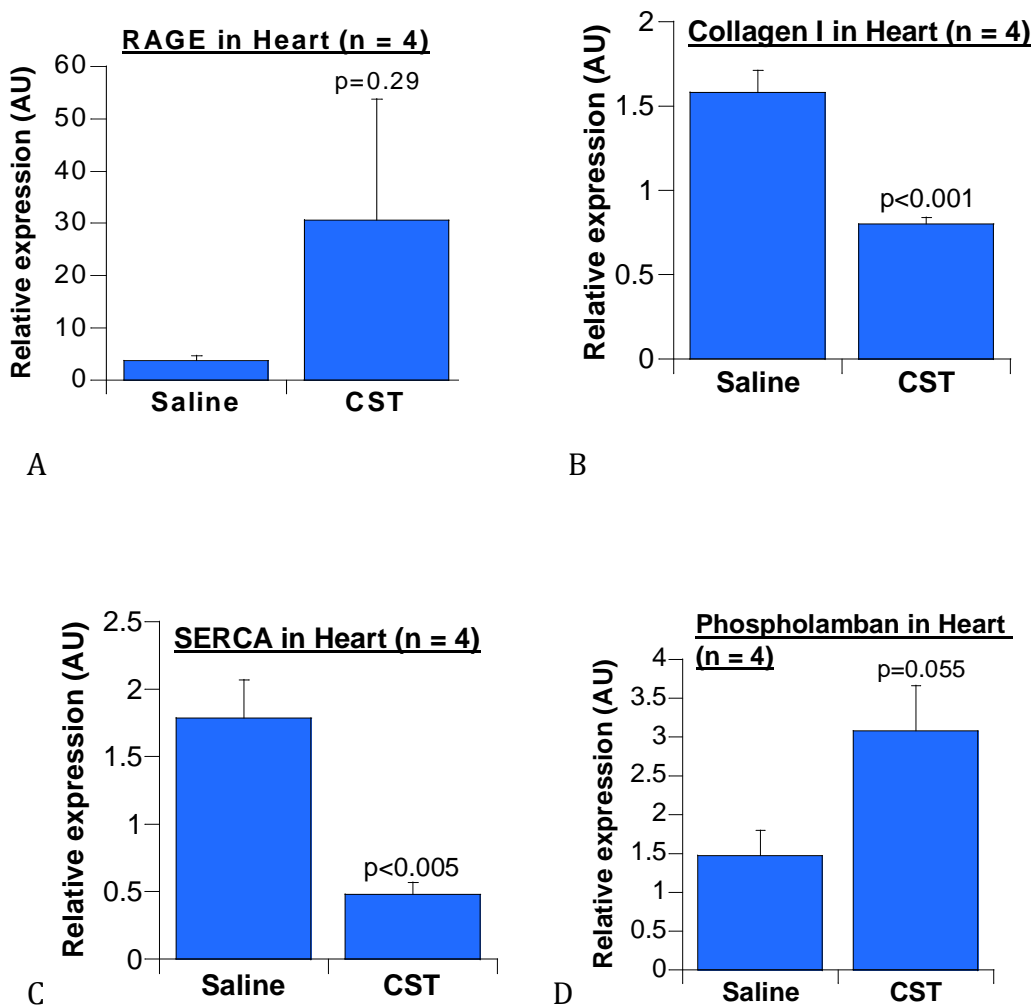
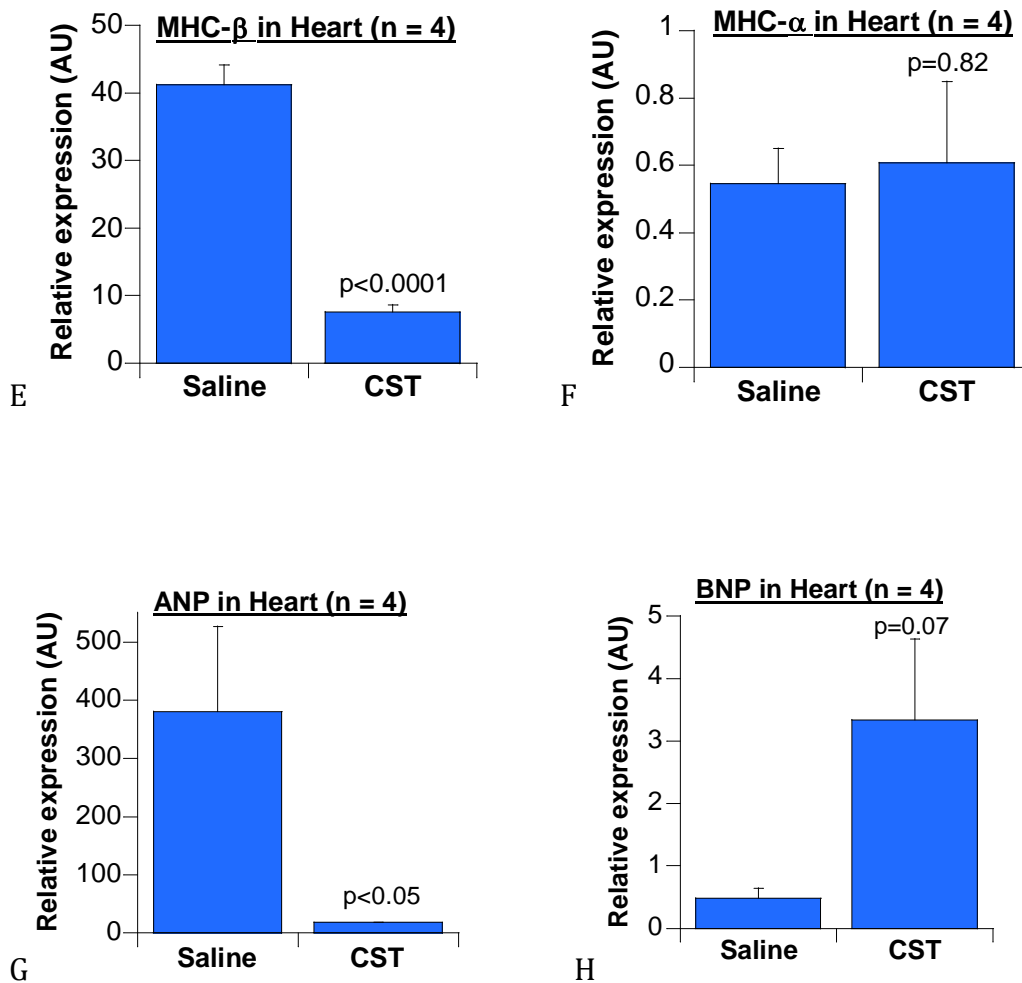


Figure 5 cont...



**Figure 5:** qPCR analysis of eight genes involved in cardiac myopathy in *db/db* CST treated vs. Saline murine hearts. A and B: is showing gene expression of RAGE and Collagen 1a1, which are involved in contractility in diabetic heart. C and D: expression of Serca and PLN, two other gene involved in contractility CST treated vs. the control. E and F: gene expression of MHC  $\beta$  and  $\alpha$  genes, respectively, affecting contractility in hypertensive heart, are shown. G and H: Expression levels of two hypertrophic markers, ANP and BNP, are observed in *db/db* CST treated vs. control.

#### 4. DISCUSSION

#### 4.1. CST and Blood Pressure.

CST is one of the major peptides arising from proteolytic processing of pro-protein CHGA of the Chromogranin/Secretogranin family. Studies have identified the key dibasic sites for action of endopeptidases, PC-1 or PC-2, (for the generation of CST) at the following location: hCHGA<sub>352</sub> and CHGA<sub>372</sub> (3 & 4). CST is a 21 amino acid long acidic, soluble polypeptide which inhibits catecholamine release from the chromaffin cells through a negative feedback mechanism (10). Several studies have shown that elevated circulating CHGA is associated with increased sympathetic tone in patients with idiopathic hypertension (31, 36). The level of CST in hypertensive patients and also normotensive people with history of familial hypertension is significantly diminished suggesting processing or packaging errors. Furthermore, high levels of catecholamine are observed in hypertensive individuals, therefore implying a strong correlation between CST, catecholamine and hypertension (63). In *Chga*<sup>-/-</sup> mice, lack of CST results in elevated circulating catecholamine levels which can be rescued by exogenous expression of CST (64).

Once Acetylcholine (Ach), a neurotransmitter in the parasympathetic nervous system, binds to the nicotinic cholinergic receptor, depolarization of the chromaffin cells takes place by a surge of Na<sup>+</sup> influx. This activates voltage gated Ca<sup>++</sup> channels to open, triggering the docking and release of chromaffin vesicles. Once released, CST binds to the nicotinic cholinergic receptor



noncompetitively (away from the active site of Ach) and prevents the release of catecholamine and granins in a negative feedback loop (10).

Due to the lack of availability of serum samples from the *db/db* mice, we were unable to establish the decrease in catecholamine levels in CST treated mice vs. the control. However, since HPLC analysis of catecholamine levels inside the adrenal showed no change in the two groups and CST was successfully able to reduce blood pressure in the diabetic mice, there was no reason for us to negate the present view of CST's mechanism in decreasing blood pressure (Supplementary Fig. 1). Knowing CST's role in decreasing hypertension in *Chga*<sup>-/-</sup>, we wanted to further our understanding of its involvement in blood pressure in *db/db* mice, which are predisposed to hypertension and other cardiac and vascular pathologies (64).

#### **4.2. Understanding the role of CST in easing vascular resistance in hypertensive, insulin resistant *db/db* mice.**

Our data clearly showed that chronic 2-week treatment with CST successfully decreased systolic and diastolic blood pressure in diabetic mice. As mentioned before, it has already been established that a decrease in catecholamine level by CST acts to decrease sympathetic activation followed by a decrease in blood pressure. In addition, it has also been shown that CST directly acts as a potent vasodilator in rat as well as in humans (62, 65). Knowing that CST has a direct role in cardiovascular system, we aimed to

understand the role of CST in the pathologically infested vasculature followed by the heart of *db/db* mice.

Blood pressure is dependent on several factors: cardiac output (Heart rate \* stroke volume) and systemic vascular resistance (38, 39). According to our data, CST showed no change in the heart rate of diabetic mice. Since CST is a known vasodilator, we investigated whether it was directly or indirectly involved in regulating the vascular tone in the diabetic model. We did this first, by analyzing the Neuropeptide Y (NPY) levels in the plasma. NPY is a known vasoconstrictor and is increased in patients with hypertension (61). CST was significantly able to decrease the levels of NPY leading us to believe that it was affecting vascular resistance through the action of NPY. Several transcript levels of vasoconstrictors (ET-1) and vasodilators (eNOS and ANP) were examined in the carotid of CST treated vs. untreated mice.

CST has been shown to act via eNOS in WT rat endothelial cells. This led us to test the expression levels of eNOS in carotid artery of *db/db* mice to see whether the response to CST treatment in these mice paralleled that in WT rats. Gene expression analysis showed an increase in eNOS transcript levels (40.72%) in CST treated mice vs. saline. Our data confirmed that CST is able to activate the phosphorylation eNOS. We therefore predict an increase of NO production in the endothelial cells which in turn decreases vascular resistance and eventuates in lowering of BP. To further test CST's role in decreasing vascular resistance, we looked at the expression of another known potent

vasodilator ANP (atrial natriuretic factor) secreted by the heart. Our data showed 20.68% of ANP transcript expression in mice with CST treatment vs. saline.

Thus CST's treatment may result in the activation of intermediates (eNOS and ANP) in vasodilation. We next tried to determine if CST also had an inhibitory effect on vasoconstrictors like Endothelin-1 (ET-1). The gene expression data showed that the level of ET-1 was greatly reduced in CST treated *db/db* mice vs. the control. Therefore we proposed that CST by both increasing the expression vasodilators, and decreasing vasoconstrictor expression level, decreases systemic resistance and thus reduces blood pressure in diabetic mouse model.

#### **4.3. CST and its influence over metabolic regulators in decreasing blood pressure.**

CST is said to activate phosphorylation of eNOS in endocardial endothelial cells of WT male Wistar rats by increasing the phosphorylation of ERK1/2 (39). Therefore, we wanted to investigate if ERK1/2 was involved in the signaling pathway through which CST acts in order to increase NO production in *db/db* mice. Our data showed a significant increase in phosphorylation of pERK1 but no change in protein expression level of p-ERK2 in CST treated vs. control was observed. Our data therefore showed that ERK1 was involved in CST's action of eNOS phosphorylation.

Another metabolic regulator known to play a role in NO mediated vasodilation in hypertensive Wistar rat aorta is, AMPK (40). AMPK plays a role in a plethora of metabolic pathways and has recently been shown to play a part in NO mediated vessel dilation. Since we know that AMPK levels are reduced in *db/db* mice we assayed protein expression level of AMPK after CST treatment. Interestingly, AMPK levels showed a significant increase in CST treated mice vs. control. Therefore, in totality, our data suggests that CST is able to activate phosphorylation of eNOS not only through pERK1 but also through pAMPK as well. This sheds further insight onto CST's mode of action in decreasing vascular resistance in insulin resistant hypertensive *db/db* mouse model.

#### **4.4. CST and Cardiac dysfunction in hypertensive *db/db* mice.**

The mouse model we decided to work with is predisposed to innumerable cardiomyopathies due to the simultaneous effect of two pathological conditions. Among the plethora of cardiac impairments plaguing the diabetic heart are hypertrophy, fibrosis, and contractile dysfunction (28, 29, 41, 54). Since CST is known to have regulatory effect in cardiac function in WT and *Chga* *-/-* mouse models and also frog heart, we wanted to see if CST was globally able to revert some of the cardiopathologies in *db/db* mice (1-4, 20, 39, 41). Multiple markers for cardiomyopathy in diabetes and hypertension were thus evaluated.

The first couple of genes we looked at were Serca-2a and PLN. Serca 2a and PLN are proteins involved in contractility of the heart. Serca 2a is a  $\text{Ca}^{++}$  ATPase, that transports  $\text{Ca}^{++}$  from the cytosol of the cell to the sarco(endo)plasmic reticulum at the expense of ATP. PLN is a protein that regulates Serca, thereby controlling cardiac contraction (29, 41). In hypertensive rats, mRNA levels of Serca 2a is diminished and levels of PLN are elevated (28, 41). According to our data, mRNA levels of Serca 2a in CST treated *db/db* murine hearts did not show a significant increase and PLN although showed a decreasing trend, was not significant. Therefore we proposed that Serca and PLN maintained a similar profile under CST treatment in hypertensive mice as well as the diabetic models. Furthermore,  $\text{MHC}\alpha/\beta$ , proteins also involved in regulation of contractility was analyzed. In WT mice more of  $\text{MHC}\alpha$  is expressed, compared to beta form, however, a diabetic heart is characterized by reactivation of  $\text{MHC}\beta$  from the  $\text{MHC}\alpha$  isoform (54). Therefore, keeping the importance of  $\text{MHC}\alpha/\beta$  in mind we decided to investigate whether CST was able to have an effect on reactivation of either forms of myosin heavy chain. Our data showed that both the isoforms of Myosin Heavy Chain ( $\alpha$  and  $\beta$ ) showed an insignificant increasing trend.

RAGE, receptor for advanced glycation end products, and Collagen1a1 (Col1a1) are two proteins that play a very important role in cardiac dysfunction. RAGE, produces a wide range of cardiac pathologies ranging from fibrosis to contractile dysfunction; Col1a1 also causes fibrosis in diabetic heart

(28, 29, 54). In the *db/db* model RAGE expression showed increased trend in CST treated as compared to control, however the data was not significant. Likewise, Col1a1 mRNA levels also showed an increasing trend in CST-treated mice. Some of the proteins, like RAGE, are known to undergo post-translational changes; therefore, a protein level analysis is necessary to truly determine the activity of the proteins (54).

mRNA levels of two hypertrophic markers, ANP and BNP (Brain Natriuretic Peptide) were also measured. As previously mentioned, the major role of ANP is vasodilation. Since CST is indirectly involved in vasodepression, through histamine release via mast cells (20), we tested to see if CST treatment had any effect on ANP gene expression levels. Unexpectedly, our data showed that ANP level was significantly lowered in CST treated vs. the control mice. Further analysis is needed to confirm any conjecture. In addition, BNP—an important maker for heart failure (42) was although increased in CST treated murine hearts vs. the control, the data was not statistically significant.

The mRNA expression data of these multiple genes involved in diabetic and hypertensive cardiomyopathy failed to reveal a clear picture of CST effect on the diseased *db/db* murine hearts. CST seems to show trends of both beneficial and detrimental effects in terms of its therapeutic potential in treatment of cardiomyopathy.

#### **4.5. Clinical relevance to working with hypertensive, insulin resistant *db/db*.**

Patients with Diabetes mellitus are more likely to develop hypertension as well as cardiac dysfunction (54). A few mechanisms have been proposed that are said to mediate the development of hypertension, including reduced baroreceptor response, endothelial dysfunction and vascular response (56). Therefore, having a peptide that is able to bring down BP as well as ease cardiopathologies could lead to a significant advancement in the treatment of diabetes as well as hypertension.

## 5. CONCLUSIONS



CST decreases BP in *db/db* mouse model. In this study, we report that CST has a direct effect on the vasculature. CST increases the production of NO, a vasodilator in blood vessels. It does so by activating an enzyme eNOS involved in the production of NO in endothelial cells. The activation of eNOS involves the signal transducer pAMPK while not pERK; pAMPK might be a key metabolic regulator in phosphorylating and activating eNOS in the *db/db* mouse models. However, this hypothesis needs to be validated further. CST also functions by blocking catecholamine release from chromaffin cells. This has already been established in other models and probably is a mode of action in the *db/db* mouse model as well.

Even though we were not able to elucidate the cardiopathologies corrected by CST in the *db/db* murine heart, enough data was gathered to guide future experiments in a systematic manner. For example, CST seems to have an effect on contractility as shown by the increasing trend of Serca 2a transcript levels in CST treated vs. the control. Therefore, more genes involved in contractility could be looked at to get a solid idea as to whether CST is truly able affect contractility in the *db/db* heart. In addition, even though there seemed to be a substantial, however not significant, increase in RAGE and Collagen (genes involved in fibrosis) mRNA expression, protein levels and activity should be analyzed.

The study at this juncture suggests that although CST in the diabetic mouse ameliorates hypertension, it has a heterogeneous effect on diabetic

cardiomyopathy pathways. Therefore, further research needs to be conducted to fully assess the mechanism of CST's action in the heart and vasculature.

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