Biology Honors Thesis

Neurotransmitter Metabolism by Monoamine Oxidase in Porcine Heart Differs by Location and is Increased with Obesity/ Metabolic Syndrome

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A thesis submitted to the Department of Biology, East Carolina University, in partial fulfillment of the requirements for Biology Honors Thesis

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April 26th, 2017

I hereby declare I am the sole author of this thesis. It is the result of my own work and is not the outcome of work done in collaboration, nor has it been submitted elsewhere as coursework for this or another degree.

Signed: Kendal Carter Date: 4/26/17

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ABSTRACT - Monoamine Oxidase (MAO), an outer mitochondrial enzyme, catalyzes the oxidative deamination of ncatecholamines and other biological amines, and in the process, produces a reactive aldehyde and H2O2. Our lab has recently shown that MAO activity (indirectly measured through H_2O_2 production) in right atrial myocardium of patients undergoing cardiac surgeries is associated with postoperative atrial fibrillation. Furthermore, our lab has shown that diabetic patients have higher MAO-A and -B content and rates of activity in this tissue, compared to nondiabetics. While recent studies have explored the connection between MAO and cardiac dysfunction, the chamber-specific content and activity of MAO within the heart is still unknown. Therefore, we characterized the regional enzymatic activity of MAO-A and -B based upon the hypothesis that the atria would have the highest MAO activity due to the greater density of local sympathetic nerve innervation in this region. For this study, porcine hearts were isolated from domestic pigs, as this model closely recapitulates the physiology of the human heart. One group of pigs were fed a high fat, high sucrose diet for 16 weeks to induce obesity/metabolic syndrome (n=4). Three MAO substrates (dopamine, norepinephrine, serotonin) and two isoform-selective inhibitors (clorgyline=MAO-A, deprenyl=MAO-B) were used to characterize MAO activities. Each enzyme content was measured using ELISA and confirmed with immunoblots. Kinetic

analysis showed that, across all substrates, when normalized to tissue protein, the left atrium had the highest maximal MAO-A and –B activity while the other three chambers were similar. Furthermore, maximal MAO activity in the left ventricle of pigs with metabolic syndrome was greater than left ventricle of control group. Our findings suggest that MAO activity differs by location in the heart (i.e., chamber heterogeneity). Upon confirmation in human heart, this information is useful to inform about clinical pharmacology applications where MAO inhibitors might be used.

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Introduction

Metabolic Syndrome

Metabolic syndrome is a collection of risk factors associated with metabolism that can increase one's risk of heart disease, stroke, and diabetes. There are five metabolic risk factors associated with this disease, and to be diagnosed with metabolic syndrome, a patient must display three or more. The risk factors include abdominal obesity, high triglyceride levels, hypertension, impaired fasting glucose, and low HDL cholesterol (1). Abdominal obesity is defined as a waistline greater than 35 inches for women and 40 inches for men. It often appears as the characteristic "apple shape" in which there is excess weight in the middle portion of the body rather than the extremities. High triglyceride levels are defined as levels greater than or equal to 150 mg/dL. Hypertension is a metabolic risk factor when blood pressure is greater than or equal to 130/85 mmHg. Impaired fasting glucose is defined as having a fasting glucose level greater than or equal to 100 mg/dL. This is also the same criteria to be considered pre-diabetic. Low HDL cholesterol is considered to be levels lower than 40 mg/dL in women and 50 mg/dL in men. In addition to presenting any of these risk factors, being on medication to treat any of these risk factors is also qualification for diagnosis (1).

In a study published by the Journal of the American Medical Association, from 2003-2012 the prevalence of metabolic syndrome in the U.S. was 33%, with an occurrence of 35.6% among women and 30.3% among men (2). Even more alarming is that of those over the age of 60, approximately 50% have metabolic syndrome. Metabolic syndrome increases risk of developing diabetes by 2-3.5 fold, as well as the risk of cardiovascular disease by 1.5-2 fold. In addition, 85% of people with type 2 diabetes have metabolic syndrome. The

National Cholesterol Education Program viewed cardiovascular disease as the primary clinical outcome of metabolic syndrome (3), and the American Heart Association estimates that metabolic syndrome will become the main risk factor for cardiovascular disease, above cigarette smoking (4).

Metabolic Syndrome Related Cardiomyopathy

The presence of each risk factor associated with metabolic syndrome negatively impacts of the cardiovascular system. Abdominal obesity has both direct and indirect impacts on the heart. The increase in stroke volume that is needed to keep up with the demand of the excess adipose tissue can lead to hypertrophy causing long term diastolic dysfunction (5). Cardiomyopathy can also be caused by the presence of adipocytes around the conduction cells of the heart. This can lead to conduction defects such as sinoatrial blocks or bundle branch blocks (5). In addition, obesity has been positively correlated with the occurrence of congestive heart failure and arrhythmias (5). Although the evidence is maybe not as strong as for high LDL-cholesterol, high triglyceride levels negatively impact the cardiovascular system by directly contributing to atherosclerosis which can lead to stroke or heart failure (6). Hypertension can lead to left ventricular hypertrophy, arrhythmias, and over time heart failure due to the high stress placed on the cardiac muscle (7). Impaired fasting glucose levels are also independently associated with the development of cardiovascular disease. Low HDL cholesterol levels lead to increased cardiovascular disease do to the absence of their protective effects in oxidizing lipoproteins and their low levels are correlated with an increase in atherosclerosis (8). Epidemiological studies show strong relationships between each of the metabolic syndrome risk factors and various cardiovascular complications. In addition to each risk factor having a negative impact on the cardiovascular system, the

different risk factors are all interconnected further intensifying the problems associated with metabolic syndrome. Obesity contributes to the development of hypertension, high LDL cholesterol, low HDL cholesterol, and hyperglycemia. Insulin resistance can lead to fatty liver disease which could directly cause high triglyceride levels in the blood (3).

Proposed Molecular Mechanism

Metabolic syndrome, diabetes, and cardiovascular complications are all closely interconnected and require further study to fully understand the mechanisms involved. Our lab has focused on a specific cardiovascular complication, metabolic syndrome associated cardiomyopathy. Cardiomyopathy is a disease of the heart muscle in which it becomes enlarged, thick, or rigid (9). It can lead to more extreme cardiac dysfunction such as arrhythmia or even heart failure. The cellular mechanisms that lead to metabolic syndrome associated cardiomyopathy are still unknown, but many avenues have been explored. Increased fibrosis has been investigated as a potential mechanism arising from the crossed-linked collagen and myocardial stiffness that occurs in people with metabolic syndrome. Another path that has been studied is increased fatty acid utilization and lipotoxicity that arise from the accumulation of triglycerides and increased serum fatty acid levels (10). The molecular mechanism of metabolic syndrome associated cardiomyopathy that my project is focusing on is related to mitochondrial dysfunction due to oxidative stress. Oxidative stress is a state of toxicity whereby production of reactive oxygen species, such as superoxide ($-O_2\cdot^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$), overwhelm the antioxidant/ detoxification pathways within the cell. Mitochondria have multiple antioxidant and detoxification systems to deal with the normal products of cellular metabolism and respiration. Some of the reactive oxygen species detoxifying pathways within the

mitochondria include enzymes that scavenge the free radicals such as α-tocopherol and glutathione reductase. Some of the other detoxifying pathways involve enzymes that reduce H_2O_2 to H_2O, such as phospholipid hydroperoxide glutathione peroxidase, catalase, and glutathione peroxidase (11). The overproduction of reactive oxygen species overwhelms these endogenous mechanisms, causing deleterious effects to the cell such as the formation and accumulation of lipid peroxidation products or the generation of reactive nitrogen species from nitric oxide (10, 12). Previous studies have shown that mitochondrial dysfunction leads to impaired respiratory capacity which was evident by the increased oxidative stress occurring within the mitochondria (13).

The Role of Monoamine Oxidase

Monoamine Oxidase is an outer mitochondrial enzyme that exists in two isoforms, MAO-A and –B. Its function is to metabolize biological amines such as serotonin (5-HT), norepinephrine (NE), and dopamine (DA), among others. MAO catalyzes their oxidative deamination and in the process produces two reactive products, H_2O_2 and a catechol aldehyde (figure 1). These products undergo detoxification to prevent oxidative stress on the cells. The catechol aldehyde produced is either DOPAL or DOPEGAL. DOPAL is further metabolized by alcohol dehydrogenase, and DOPEGAL is metabolized by aldehyde reductase or aldose reductase (14). The H_2O_2 is metabolized by the reactive oxygen species defense systems previously mentioned that scavenge and reduce it to H_2O.

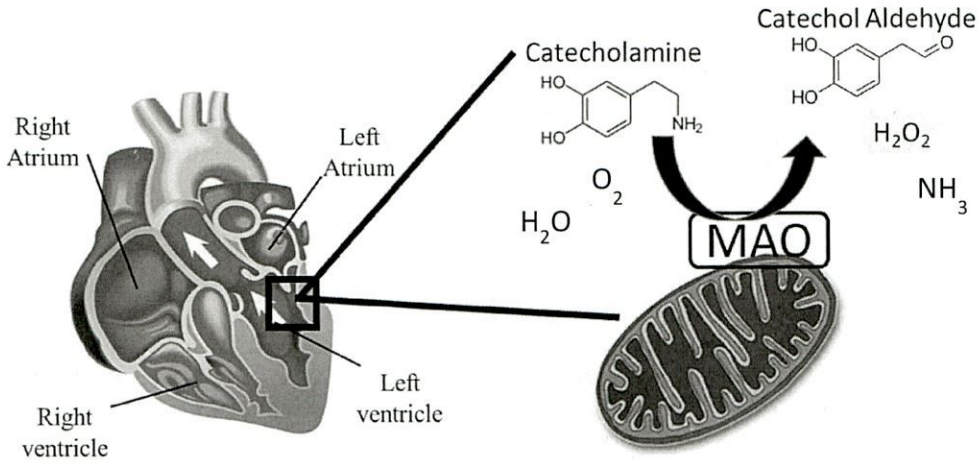

Figure 1. Oxidative Deamination of Monoamine Oxidase
MAO, located in the outer mitochondrial membrane, catalyzes the oxidative of catecholamines producing a catechol aldehyde, hydrogen peroxide, and a nitrogen species. The catechol aldehyde and hydrogen peroxide are toxic species that must be further metabolized in order to avoid their deleterious effects on the cell.

MAO has been extensively studied in the brain, as its generation of reactive oxygen species has been linked neurodegenerative disorders such as Parkinson's disease. Brain cells incubated in dopamine have shown inhibition of mitochondrial state three respiration up to 37% due to the oxidative products formed upon their deamination (15). MAO inhibitors have been developed and are currently used as pharmacological agents to combat the catecholamine cytotoxicity that is leading to the state of oxidative stress within the cells. MAO has also been studied in its link to catecholamine cytotoxicity in cardiomyocytes. It has been shown that rat cardiomyocytes incubated in serotonin resulted in apoptotic and necrotic cell death due to mitochondrial dysfunction (16).

Studies from our laboratory have established that both MAO-A and MAO-B proteins are expressed at higher levels in the right atrial appendages of diabetic patients (figure 2-A and B). Our studies have also shown that when norepinephrine is used as a substrate, MAO-A activity is higher in diabetic patients than in non-diabetic patients (Figure 2-C). While this data is useful in establishing the presence of increased MAO protein content in diabetes and

the role of MAO in oxidative stress on the cell, it only shows us what is occurring in the tissue of the right atrial appendage. Because diabetic cardiomyopathy starts at the level of the left ventricle, often spreads to the right ventricle, and sometimes affects the atria, a chamber specific comparison would allow a more complete view of how MAO is impacting the entire heart in the context of diabetes.

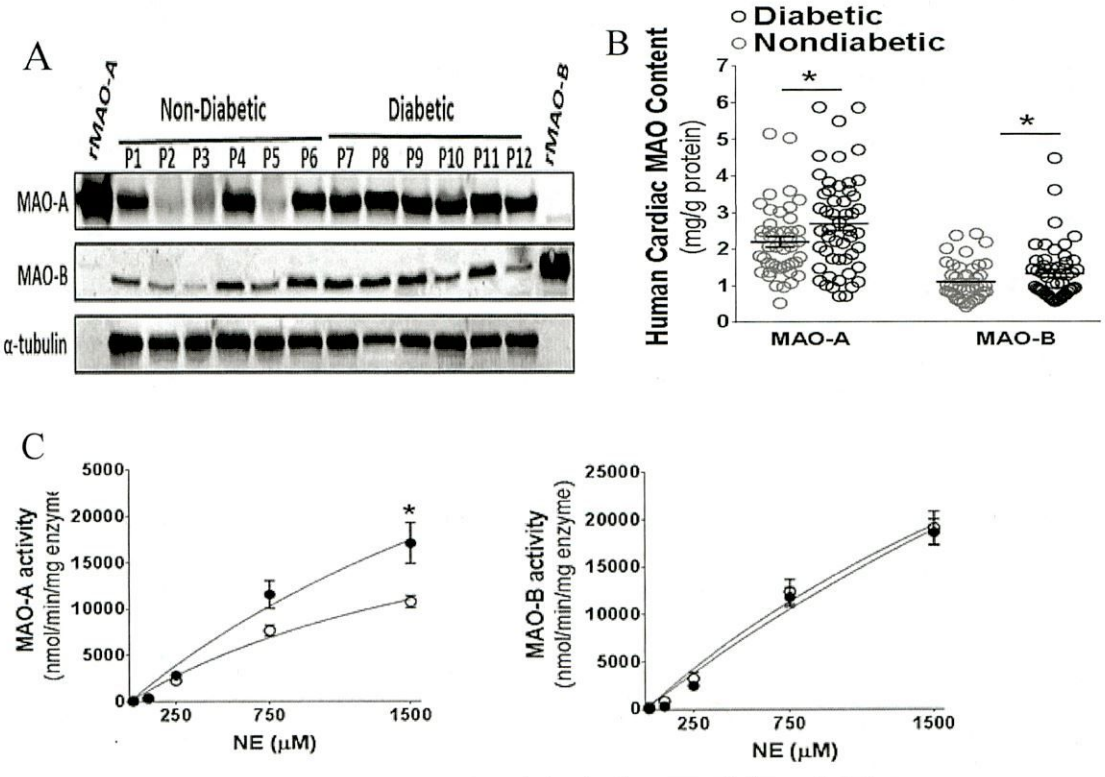

Figure 2. MAO-A and B content and activity in human right atrial tissue
Right atrial appendage tissue harvested during cardiac bypass surgery shows increased content in diabetics when compared to non-diabetics for both isoforms of MAO (A and B). This tissue also shows increased deamination of norepinephrine by MAO-A in diabetics when compared to non-diabetics.

A porcine model is being utilized for this study as the pig heart closely recapitulates the physiology of a human heart (17). The distribution of blood within the coronary artery system of a pig is almost identical to that of humans. The hearts of pigs and humans are also more similar in size than that of any other model that could be used. But most importantly,

like humans, pigs have a right-side dominant conduction system and have been successfully used in past studies to better understand the cardiac conduction system (17).

Hypothesis & Research Objective

The first research question I am seeking to address is to determine whether ventricular tissue and atrial tissue differ with regards to MAO content and activity. My hypothesis is that there will be higher content and activity in the atrial tissue compared to ventricle tissue due to the greater density of local sympathetic nerve innervation in this region of the heart. I will be exploring the differences among all four chamber-- the right atrium (RA), right ventricle (RV), left atrium (LA), and left ventricle (LV), as well as comparing the LV of a control and metabolic syndrome group. Of the chamber comparisons that will be made, the most interesting relationship I will be exploring is the activity level of the right atrium, the tissue in which we have performed human kinetic studies on, with the left ventricle, where diabetic cardiomyopathy occurs. My second research question is how does MAO content and activity differ among atria and ventricle in type 2 diabetes. Based on unpublished work in human diabetic patients in our lab and recently published work by other groups, I hypothesize that there will be higher levels of MAO activity in the metabolic syndrome group versus the control group.

Methods

Pig Model

Heart tissue from eight female domestic pigs was received from our collaborators at Mayo Clinic in Rochester, Minnesota for study. Four pigs, considered to be our model of metabolic syndrome, were fed a high-fat/high-fructose diet ad libitum (5B4L, protein 16.1%, ether extract fat 43.0%, and carbohydrates 40.8%, Purina Test Diet, Richmond, IN), with free access to water. Four control pigs were fed a standard chow diet. After seven months, the pigs were euthanized with pentobarbital-sodium (100 mg/kg IV, Sleepaway®, Fort Dodge Laboratories, Fort Dodge, Iowa) (18,19). Heart tissue was separated by chamber (RA, RV, LA, LV) and frozen in liquid N_2. Once received from the Mayo Clinic, the frozen tissue was stored at -80°C until assays were performed.

Enzyme Kinetics

Cardiac muscle samples frozen in liquid N_2 were homogenized in 10X (wt./vol) TEE buffer containing (in mmol/L) 10 Tris base, 1 EDTA, 1 EGTA, and 0.5% Tween-20, using a glass-on-glass tissue grinder (Kimber Chase). MAO activity assays were performed on the same day as the protein extraction because freezing samples or keeping them at 4°C overnight causes a dramatic loss in activity. H_2O_2 generation from MAO was determined by continuous monitoring of Amplex Red oxidation in the presence of horseradish peroxidase (1 U/mL) and superoxide dismutase (25 U/mL) using a spectrofluorometer (Horiba Jobin Yvon) equipped with a thermo-jacketed cuvette chamber maintained at 37°. MAO-A activity was determined by continuous monitoring of H_2O_2 production supported by titration of dopamine (25 uM, 75 uM, 150 uM, 300 uM), serotonin (5 uM, 25 uM, 75 uM, 150 uM, 500 uM), and norepinephrine (10 uM, 50 uM, 100 uM, 250 uM, 500 uM) in the presence of depryenyl, a

MAO-B inhibitor. MAO-B activity was determined by continuous monitoring of H_2O_2 production supported by the same titrations previously mentioned in the presence of clorgyline, a MAO-A inhibitor (20).

Protein Quantification

Bradford protein assay was performed using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) to determine the concentration of protein present in each sample. Standards and samples were loaded into a 96-well plate, incubated for 15 minutes, and absorbance read at 562 nm.

Enzyme Quantification

Enzyme-linked immunosorbent assays (ELISA) were performed using an EIR/RIA 96-well plate (Costar), human recombinant MAO-A and –B standards, and MAO-A and –B polyclonal antibodies (Santa Cruz Biotechnology). All sample solutions were prepared in 30 mM sodium carbonate/ 96 mM sodium bicarbonate, and all antibody solutions were prepared in 1X phosphate buffered saline. For MAO-A, the primary antibody was diluted 1:50 and the absorbance was read at 450 nm using TMBZ. For MAO-B, the primary antibody was diluted 1:50 and the fluorescence was read at excitation 567 nm and emission 590 nm using Amplex Ultra Red (Life Technologies). Western blot analysis was performed using MAO-A and –B monoclonal antibodies (Santa Cruz Biotechnology) on samples separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis transferred to a polyvinylidene fluoride membranes.

Statistical Analysis

MAO-A and –B activity and content between the four heart chambers was compared using a one way ANOVA test with the highest titration concentration as the representative

V_{max}. MAO-A and –B activity and content between the control and obese/insulin resistant LV was compared using a student's t-test. All data was analyzed using Prism-graph pad software. Statistical significance was determined by a p-value less than or equal to 0.05.

Results

MAO Content Analysis

Enzyme-linked immunosorbent assays (ELISA) revealed no statistical significance in MAO-B content between the four chambers of the pig heart (figure 3-A). There was also no statistical significance between the LV control and the LV obese/ insulin resistance in MAO-B content (figure 3-B). MAO-A content was not measured using ELISA method.

Western blot analysis showed a higher density of both MAO-A and –B in the atria than the ventricle regions of the heart (figure 3-C).

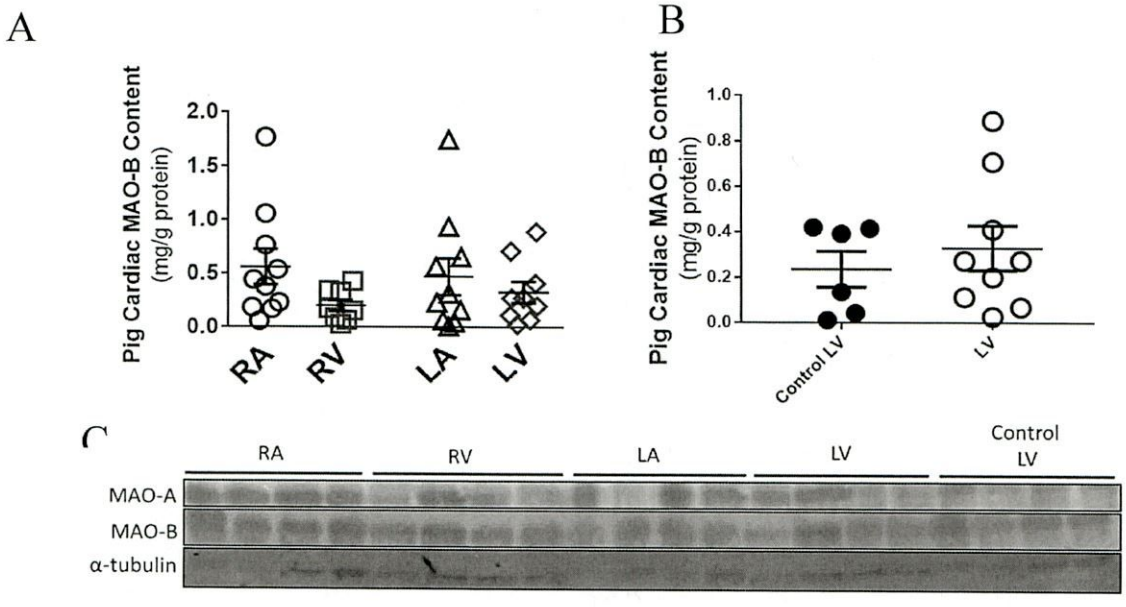

Figure 3. MAO-A and -B content of pig heart tissue
Enzyme linked immunosorbent assays were performed to measure MAO-B content. The four chambers of the pig heart were compared and revealed no statistical significance (A). The LV of control and obese/ insulin resistant pigs were compared and revealed no statistical significance (B). Western blot analysis was performed to compare MAO-A and –B content (C).

MOA Activity- Chamber Comparison

Maximal activity of MAO-A in the four chambers of the heart (RA, RV, LA, LV) for the substrates dopamine, 5-HT, and NE is depicted in figure 4-A. For the titration of dopamine, the kinetic curves of the RA, RV, and LA are very similar and almost

indistinguishable for every concentration. The activity of MAO-A in the LV is slightly lower. The activity of MAO-A on the substrate dopamine is statistically significant with a p-value of 0.05. For the titration of 5-HT, the curves for the RV and the LA very similar, with the activity in the RA falling below that and the activity in the LV even lower. The differences between the chambers for 5-HT is not statistically significant. For the titration of NE, there is no difference between the activities of MAO-A in the four chambers. The activity in the LA is slightly elevated but not to an extent that makes it significant.

Maximal activity of MAO-B in the four chambers of the heart for the substrates dopamine, 5-HT, and NE is depicted in figure 4-B. For all three substrates, the activity of MAO-B in the LA is higher than the other three chambers which do not show any differences. The differences seen are not statistically significant.

Figure 4-C depicts the maximal capacity of MAO-B in the four chambers of the heart for the three substrates normalized to mg of enzyme. For the titration of dopamine, the same trend that is seen in figure 4-B is followed but with an approximately 10-fold increase than when normalized to mg of protein. The differences seen here are statistically significant with a p-value of 0.02. For the titration of 5-HT, the same 10-fold increase is seen, but the activities between the four chambers appear to be more similar when normalized to enzyme content. For the titration of NE, and approximately 4-fold increase in activity is seen per mg enzyme than mg of protein. Also, the activity of MAO-B in the LA and LV is elevated compared to that of the RA and RV. The activity of MAO-B for the substrates 5-HT and NE do not show any statistical significance.

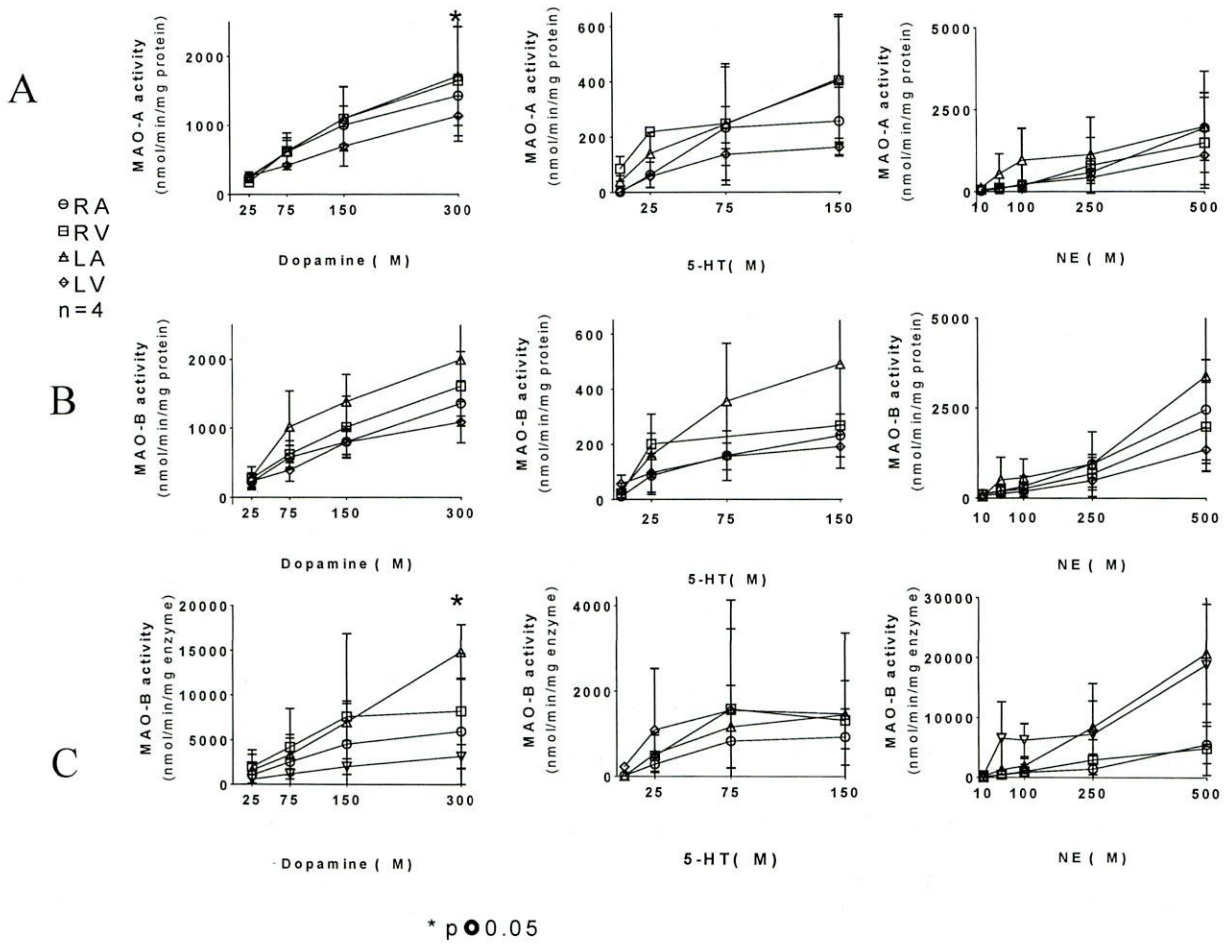

Figure 4. MAO-A and -B activity of pig heart tissue compared by chamber
Pig heart homogenate separated by chambers (RA, RV, LA, LV) was titrated with substrate (dopamine, 5-HT, NE) to measure MAO-A and MAO-B activity in nmol/min/mg protein (A and B). MAO-A activity for the substrate dopamine is statistically significant with a p-value of 0.05. MAO-B activity was further quantified to nmol/min/mg enzyme (C). MAO-B activity for the substrate dopamine is statistically significant with a p-value of 0.02.

MAO Activity- Control vs. Metabolic Syndrome

Maximal activity of MAO-A in control LV and obese/ insulin resistant LV for the substrates dopamine, 5-HT, and NE is depicted in figure 5-A. MAO-A showed higher activity in the control group for all three substrates, but this difference is not statistically significant.

Maximal activity of MAO-B in control LV and obese/ insulin resistant LV for the substrates dopamine, 5-HT, and NE is depicted in figure 5-B. MAO-B showed higher activity in the control group for all three substrates, but this difference is not statistically significant.

Figure 5-C depicts the maximal activity of MAO-B in control LV and obese/ insulin resistant LV for the substrates dopamine, 5-HT, and NE normalized to mg of enzyme. The control group showed higher MAO-B activity for the substrates dopamine and NE, but the obese/ insulin resistant group showed higher activity for 5-HT, but these differences are not statistically significant.

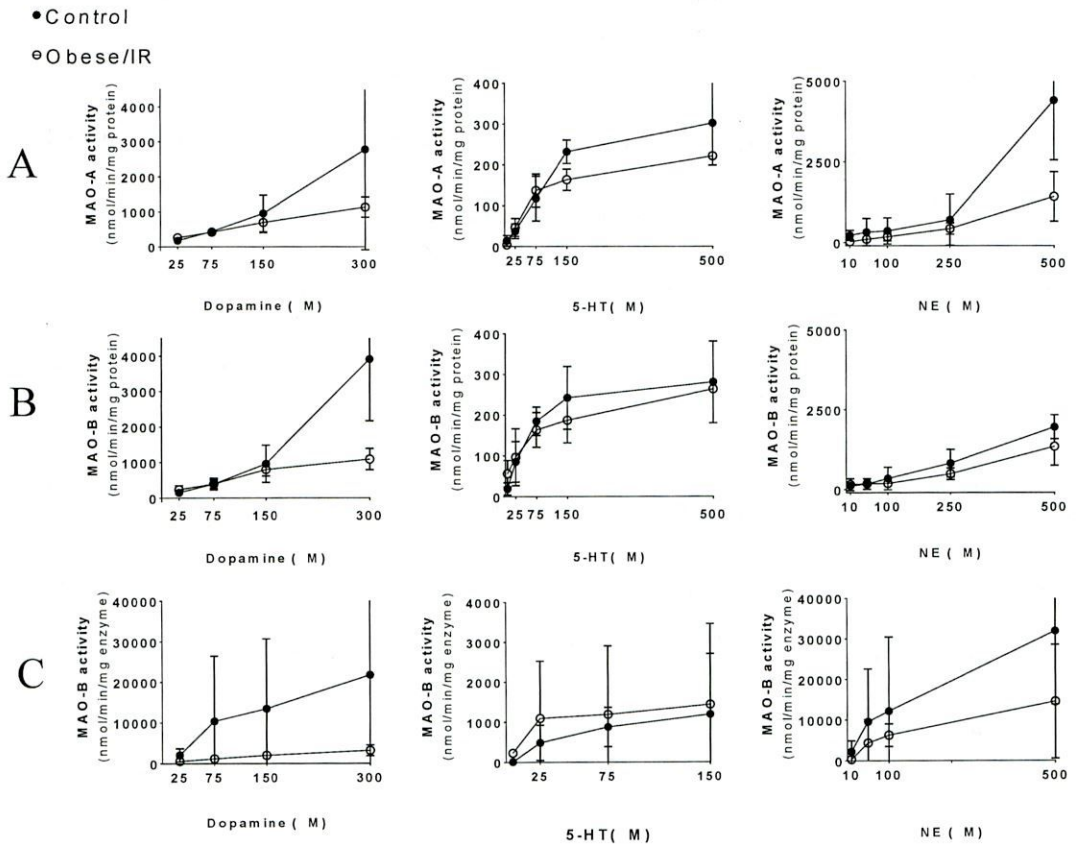

Figure 5. MAO-A and –B activity in control and obese/ IR pig heart LV tissue
Pig heart homogenate from control LV and obese/insulin resistant LV was titrated with substrate (dopamine, 5-HT, NE) to measure MAO-A and MAO-B activity in nmol/min/mg protein (A and B). None of the results were statistically significant. MAO-B activity was further quantified to nmol/min/mg enzyme (C). None of these results were statistically significant.

Discussion

MOA Characterization- Chambers

In order for the results obtained for the activity of MAO-A between the four chambers to be meaningful, it is important that they be reported per mg of enzyme rather than mg of protein. Without that step being completed, it is still possible to see trends in the data. For all three substrates, the atria have slightly higher activity than the ventricles, with the LA appearing to be the highest. I hypothesized that the RA would have the highest activity due to the sympathetic nerve innervation contained within it, but along that same thinking it makes sense that the LA would have high MAO activity as it is also highly innervated by both the sympathetic and parasympathetic nervous system (21).

For the activity of MAO-B between the four chambers of the pig heart, it makes sense that the results were statistically significant for dopamine and not 5-HT or NE because dopamine acts on both isoforms of MAO while 5-HT and NE act preferentially on isoform A.

An important factor to keep in mind when reviewing the results of this experiment is that although we know which chamber the heart tissue is coming from, we do not know from where in that chamber it was obtained. For ventricular tissue this doesn't make much of a difference as it is fairly uniform throughout consisting of strong muscle tissue to propel the blood through the body. For the atria, this detail is more significant because one sample could be from a portion that is dense in muscle fibers while another sample could be from a portion dense in nervous tissue (21).

An important trend to notice between the chambers is there is no significant difference between the activity in the RA and the LV. This leads us to believe that the previous data obtained from the human RA could be reflective of what is also occurring in

their LV, an area of the heart unobtainable for study. This study is a beginning step of the characterization of MAO within the heart to potentially target it for prevention of cardiovascular complications arising from metabolic syndrome or diabetes.

MAO Characterization- Metabolic Syndrome

The results obtained from the MAO-A and –B content and activity analysis of control LV versus obese/insulin resistant LV were not as expected. Based on the previous studies in our lab showing that, in humans, MAO content and activity is increased with the presence of diabetes, I expected the same relationship to be present in the pig model of metabolic syndrome. Because the opposite was true, further investigation into the metabolic state of the pig model used is warranted as well as the relevance of the pig model to the study of MAO in the context of metabolic syndrome.

A potential reason for the differences seen between the human right atrial data and that of the pig heart tissue could stem from the level of cardiac dysfunction experienced by the two groups. The human tissue used in both the diabetic and control group came from people who were experiencing enough cardiac dysfunction to warrant open-heart surgery. The pig model used for this study consisted of pigs that were obese, insulin resistant, and hypertensive, but not necessarily experiencing the same amount of cardiac dysfunction. This points to an underlying mechanisms that is causing the increased expression and activity of MAO-A and –B in the humans that is not seen in the pigs. The human tissue was also obtained from people with several co-morbidities not experienced by the pigs.

Another limiting factor of this study comes from the age of the pigs. For the study model, pigs were sacrificed at 7 months, and the mean age of the participants in the human tissue study was 65. There is no clear cut formula to compare pig years to human years, but

the age of the pigs can be put into better context. The growth of a pig's heart from birth to four months of age is similar to that of a human's in to their mid-teens (22). Also, domestic pigs become sexually mature between 4 and 6 months of age. Using this information, the age of the pig heart tissue studied is much lower than that of the human heart tissue. It has been shown that MAO expression correlates positively with age (23, 24). A research group in France showed that in a mouse model, there was a 7.5 fold increase in MAO-A mediated H_2O_2 production between 1 month of age and 24 months of age (25).

Of these possible limiting factors, the most plausible seems to be the differences that occur in the heart and even deeper in the mitochondria in the transition from metabolic syndrome to diabetes. While the two are closely linked, they are not the same disease and this could account for the trend differences between the pig samples and human samples.

Future Directions

In order to reach stronger conclusions about the data obtained in this study, the sample size needs to be increased to a power that will make it significant. With only four pigs in this study, there is a lot of variation and unidentified outliers. Also, the control of this experiment could be improved upon by studying the enzyme content and kinetics of control pigs for all four chambers rather than just the LV. If this were done, it would paint a clearer picture of what changes are happening in the heart and where they are occurring.

Another step to be made in this area of research is to quantify the amount of mitochondria within each sample studied. This would allow the data to be normalized to yet another standard beyond just the protein content as well as show what region of the heart is denser with mitochondria.

In conclusion, this study provided interesting information on the content and activity of MAO in the heart of pigs with metabolic syndrome. Further research on this topic will allow for a better characterization of the enzyme and its role in the etiology of the disease. This area should continue to be studied as it, along with previous research in our lab, has provided a promising starting point for the study of using MAO inhibitors to treat patients with metabolic syndrome and diabetes.

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