INACTIVATION OF AT1a RECEPTORS ATTENUATE LACTATE ACCUMULATION AND IMPROVE CARDIAC PERFORMANCE AND ACID-BASE HOMEOSTASIS DURING ENDURANCE EXERCISE

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

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ABSTRACT

Ahmad Alhajoj, M.S., Department of Pharmacology and Toxicology, Wright State University, 2014. Inactivation of AT1a receptors Attenuate Lactate Accumulation and Improve Cardiac Performance and Acid-Base Homeostasis during Endurance Exercise.

Angiotensin Type 1 (AT1) receptors are involved in cardiovascular pathology. Losartan, the AT1 receptor blocker, in combination with exercise has been shown to be effective in improving cardiac performance. The goal of the study was to investigate the role of AT1a receptors on cardiac function, and exercise tolerance in response to aerobic exercise using AT1a receptor knockout (AT1aKO) mice. An exercise wheel system was used for the exercise paradigm. Male (C57BL/6) wild type (WT) and AT1aKO mice were randomly assigned to four groups: WT control (n=6), WT exercise (WTEX, n=8), AT1a KO (KO) control (n=5), and AT1a KO exercise (KOEX, n=8). Mice were forced to run at a velocity of 8 m/min for 1 hour, 3d/wk, for 7 wks. Echocardiography was conducted at baseline and 7 wks. Lactate was measured during several exercise sessions. Electrolytes and cardiac histology were assessed post-sacrifice. Results showed a significant increase in ejection fraction (EF %) in KOEX (72.5±1.5%) vs. WTEX and KO control (63.4±1.2% and 63.2±2.2%, respectively). Mitral valve assessment revealed a marked decrease in E-wave velocity in WTEX compared to WT control at baseline (74±1.9 vs.90±3.5 cm/s, p<0.05), while E/A wave ratio remained unchanged. Cardiomyocyte diameter was larger in WTEX compared to KOEX (29.5±0.7 vs. 25.8±0.5 µm, p<0.05). Heart to body weight ratio was significantly higher in WTEX vs. KOEX (5.5±0.2 vs. 4.3±0.1 mg/g, p<0.001). Masson’s Trichrome staining revealed higher collagen levels in
WTEX myocardium compared to WT control and KOEX (16% vs. 5%). Blood lactate accumulation values were greater at 5 and 60 min of wheel running in WTEX (4.2±0.4 and 3.9±0.5 mmol/l) vs. KOEX (3.0±0.2 and 2.9±0.2 mmol/l). A basic metabolic panel revealed higher [HCO₃⁻] in KOEX vs. WTEX (21.7±0.6 vs. 13.7±1.8 mmol/l). Cl⁻ was lower in KOEX compared to WTEX (102.5±2.5 vs. 113.5±2.7mmol/l, p<0.05). In conclusion, AT1aKO mice exhibited improved cardiac performance without myocardium hypertrophy, greater exercise endurance, and enhanced metabolic activity in response to chronic exercise. These results suggest that the AT1a receptor is an important mediator of exercise induced cardiac dysfunction and acid-base imbalance during exercise training.
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1. INTRODUCTION and LITERATURE REVIEW

Benefits of Exercise

Two hundred and fifty thousand death cases occurring annually in the United States are associated with the lack of physical activity and daily body exercise (Myers et al., 2003). The relationship between regular physical activity and its positive impact on human health has been well discussed in the past few decades. Accumulating evidence suggests that daily exercise training exhibits several cardiac benefits to human health by minimizing coronary artery disease risk factors (e.g. reduction in resting systolic/diastolic blood pressure, serum triglyceride, total body fat, intra-abdominal fat, increased serum high density lipoprotein cholesterol, and improved glucose tolerance). Evidence also shows that exercise lowers the mortality rate associated with coronary artery disease and extends life span by up to several years (ACSM, 2010). Regular physical activity, in combination with diet regime and medications, promotes blood sugar control and weight loss in patients with type 2 diabetes mellitus and is considered an essential element in diabetes therapy (Sigal et al., 2006).

In general, oxygen demand increases during exercise work and the heart is responsible for pumping enough oxygenated blood to the muscle. Thus, heart pumping capacity incrementally increases during exercise as a result of elevation in hemodynamic parameters, including stroke volume and cardiac output, leading to heart structural change or what has been called the “athletes heart” (Baggish et al., 2011; Andrew et al.,
1966; Sullivan et al., 1991). Regular exercise is shown to be cost-effective in minimizing cardiac death rate following myocardial infarction (MI) and is highly recommended as an essential part of cardiac rehabilitation program for patients with coronary heart disease (CHD) (Jolliffe et al., 2001; Achttien et al., 2013). The combination of angiotensin converting enzyme (ACE) inhibitor, angiotensin II (Ang II) receptor blocker, and exercise has a positive effect in the treatment of congestive heart failure (Gašanin et al., 2013). Although the recommended dose for the exercise paradigm (frequency, duration, intensity) needs further studies, the American Heart Association (AHA) suggests at least 30 minutes of exercise per day for five days a week as a regular physical activity for healthy life style (American Heart Association (AHA) recommendation for physical activity, 2013).

Renin Angiotensin System (RAS)

Among the top ten global fatality risk factors hypertension is attributed to 7.1 million deaths worldwide annually, which represents thirteen percent of the total fatality risk factors (World Health Organization Report, 2002). Moreover, the World Health Organization has reported that hypertension is the major leading cause for cerebrovascular disease and is responsible for approximately 49% of all heart attacks every year (World Health Organization Report, 2002). The renin angiotensin system plays a prominent role in regulating blood pressure and heart function. It has been observed that higher levels of circulating renin are associated with a higher risk of developing cardiovascular and cerebrovascular disease (Schrader et al., 2007; Unger et al., 2002). Angiotensinogen is an α-2-globulin polypeptide produced by the liver and represents the precursor protein for the peptide hormones angiotensin I (Ang I) and
angiotensin II (Ang II). Renin, also known as an angiotensinogenase, is a kidney synthesized proteolytic enzyme that is specific in catalyzing the cascade of Ang II formation, while angiotensin converting enzyme (ACE) is a non-specific converter of inactive Ang I to the active pressor Ang II. Angiotensin I is also formed by the action of renin on angiotensinogen. Renin cleaves the polypeptide bond between specific amino acid residues on angiotensinogen, creating the ten-amino acid peptide angiotensin I.

**Angiotensin II and AT1 receptor**

Angiotensin II (Ang II) or Ang (1-8) is the key element of the renin angiotensin system and the one with the highest cardiovascular potency among the angiotensin oligo-peptide family. It has a pivotal effect on many body organs and modulates various body function including blood pressure, water and electrolyte homeostasis, neuronal function, myocardium ischemia, and other cardiovascular system functions (Figure 1). However, research has been conducted on the drawback effect of Ang (II) overproduction and its role in cardiovascular disease development. Chronic Ang (II) infusion has been used in research to induce high hypertension in animal models and consequence pathological complications for other organs (Alghamei et al., 2012; Ruiz-Ortega et al., 2001). In addition, long-term infusion of Ang II may lead to myocardium and aortic remodeling as well as enhancement of the inflammatory response in vasculature (Alghamri et al., 2012).
Figure 1: Effects of angiotensin II in several body organs.
AT1 receptor

There are two main receptors through which Ang II produces its effects, AT1 and AT2. Both receptor subtypes have strong affinity to the Ang II octapeptide. However, the majority of Ang II impact on various body systems is mediated by the subtype angiotensin II type I (AT1) receptor. Binding of Ang II to the AT1 receptors, which are dominant in vital organs, including heart, kidney, blood vessels, lung, skeletal muscle, adipose tissue, and brain causes dissociation of G proteins (guanosine nucleotide-binding proteins) and consequently induces several intracellular changes. Activation of AT1 receptors has been associated with production of reactive oxygen species (ROS) particularly in the cardiovascular system, which initiates vasoconstriction and stimulates cellular growth (Gasparo et al., 2000). In addition Ang II has a direct action in stimulating cardiac hypertrophy and heart failure; the effect mediated through the activation of AT1 receptor (Gasparo et al., 2000). Nevertheless, AT1 receptor blocker has been shown to counteract the deleterious effects on cardiac myocytes resulting from elevation in cardiac Ang II levels (Ferreira et al., 2007).

AT1a and AT1b Receptors

The two main subtypes for the AT1 receptor are ninety five percent similar in protein structure and both pose almost the same ligand binding and transduction characteristics, but differ in their location throughout the body organs and transcripational regulation. In mice, AT1a receptors are widely distributed in several body tissues, while AT1b receptors are known to be expressed only in brain, adrenal gland, and testicular tissues (Burson et al., 1994). The presence of both subtype receptors has not been reported in humans (Bergsma et al., 1992).
RAS and exercise performance

The role of the renin angiotensin system in physical performance has been a point of debate for many years. The relation between RAS and exercise performance in human athletes was noted after evaluating the dominant subtype gen (I/D allele) of angiotensin converting enzyme (ACE) that cleaves Ang I to the active form Ang II (Gayagay et al., 1998; Saul et al., 1999). When the human polymorphic angiotensin I converting enzyme (ACE) gene is present (I allele), ACE activity is reduced, while its absence (D allele) is associated with higher ACE activity in the circulation. Several studies has been conducted on human ACE gene background suggesting that exercise endurance improvement is associated with I allele (low ACE activity) rather than D allele (high ACE activity) (Gayagay et al., 1998; Saul et al., 1999; Montgomery et al., 1999, 1998). A study on Russian athletes proposed that the D allele gene is abundant in athletes who perform short duration (less than one minute) activity while the I allele gene is in excess in athletes who perform outstanding medium distance exercise (between one to twenty minutes) (Nazarov et al., 2001). However, the relation between ACE genotype and long distance activity was not detected in the Russian study. Moreover, skeletal muscle metabolic efficiency (Montgomery et al., 2000) and mechanical efficiency (Williams et al., 2000) have been attributed to lower ACE activity in the circulation. On the other hand, Bahi et al (2003), examined the previous findings regarding the relationship between ACE activity and physical performance in an animal model using ACE inhibitor, perindopril, and reported no involvement of ACE activity in endurance time and/or muscle metabolic capacity.
In 2001, the relation between RAS and exercise tolerance was studied using AT1a receptor inactivation. Even though the exercise performance was not the area of interest, Mistslberger et al. 2001, found that mice lacking the AT1a receptor exhibited greater levels of wheel running compared to a wild type (WT) group. It was not until 2012 when the bond between low RAS activity and exercise performance was verified using AT1a KO mice (Murphy et al., 2012). This author reported that mice lacking the AT1a receptor showed greater distance covered, movement speed, latency to fall, and improved body strength in a rotarod exercise test. However, lactic acid measurement as well as blood pH and electrolytes assessments were not a part of this study. A recent study conducted in our lab revealed significantly greater distance and velocity in AT1a deficit mice during swimming exercise. Interestingly, Gašanin et al. (2013), reported improvement in exercise time in human patients with congestive heart failure (CHF) following Candesartan (AT1 receptor blocker) treatment as an additional treatment with ACE inhibitor. These findings suggest that physical performance could be improved by lowering or inactivating RAS in the circulation. However, none of the previously mentioned investigations studied the underlying mechanism through which inactivating of RAS promotes enhanced physical performance.

**Lactic acid (brief history)**

Lactic acid was first detected in sour milk and given the name “lactic” by Carl Wilhelm Scheele in 1780 (Robergs et al., 2004). By 1810, the scientist was able to confirm the natural presence of lactic acid in other organic items such as blood, meat, fresh milk, and food products. Lactic acid has been used as a preservative in some food products such as
cucumbers, and as an ingredient to make cheese and bread, and in brewing and flavoring beer. The chemical name is 2-hydroxypropanoic acid (Figure 2).

Chemical structure:

![Chemical structure of lactic acid](image)

Figure 2: The addition of hydrogen in the conversion of pyruvate to lactic acid.

**Lactic acidosis**

Lactic acidosis is considered the major cause of exercise-induced metabolic acidosis (Böning et al., 2005). There are two main types of lactic acidosis; type A and B. Type A lactic acidosis results from severe tissue hypoxia which is the most abundant type in exercise activity. Type B lactic acidosis is occurring due to underlying systemic disease such as liver problems or due to exposure to toxic substances.

**Lactic acid and exercise**

It is well documented that there is a direct relation between lactate production and exercise intensity. During severe muscular work (high intensity), insufficient oxygen in the muscle accelerates anaerobic glycolysis and becomes the predominant source of ATP needed causing the release of lactic acid into the muscle through a series of reactions. This increase in lactate production occurs during the first few minutes of almost all
exercise forms because it takes time for the aerobic pathway to predominate. When the aerobic pathway is unable to maintain the ATP required for the activity, lactic acid accumulates and physical work is stopped due to severe muscular fatigue resulting from sharp decreased in muscle pH (Bang et al., 1936) (Figures 3 & 4). In addition, the continuous elevation of muscle lactic acid production leads to increase lactate diffusion into the blood stream causing a drop in blood pH too. At this point, the performer reaches the metabolic capacity limit and will be forced to stop exercising due to high levels of accumulated acid (mainly lactic acid) in muscle and blood. Lactate measurement is widely used in human athletes as well as in horses as a marker for exercise endurance and considered the best indicator for the type of energy used during physical activity (Billat et al., 1996). Ferreira et al (2007) measured the maximal lactate steady state concentration (MLSS) in trained mice. The MLSS is the point where the maximal exercise capacity achieved without continual blood lactate accumulation or the point where the lactic acid production is in equilibrium with its clearance from the blood. The lactic acid production may fluctuate in untrained subjects and in some forms of exercise, with both anaerobic and aerobic systems participating in energy production during physical activity.
Figure 3: Rest to exercise transition and predominant metabolic pathway. (Modified from Powers & Howley, 2001)

Figure 4: Lactate accumulation during long distance running shows that two athletes were forced to stop exercising due to blood lactate accumulation (from www.lactate.com)
**Bicarbonate Buffer System**

There are three main buffer systems in the body through which the blood resists the change in pH: the bicarbonate, the phosphate, and the plasma protein buffer systems.

Among these three buffer systems, bicarbonate (HCO$_3^-$) buffer works as the main controller for elevated blood lactic acid during exercise (Beaver et al., 1986). Bicarbonate ions (HCO$_3^-$) react with the extra hydrogen ions (H$^+$) present in the blood to form carbonic acid (H$_2$CO$_3$) which dissociates into water and carbon dioxide (CO$_2$) (as shown in the equation). Then, the lung through hyperventilation will excrete CO$_2$.

\[
\text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^- 
\]

Therefore, bicarbonate buffer plays a critical role in maintaining blood pH during physical activity and hence, improves exercise performance. Accumulating evidence suggests that exercising under alkaline condition or ingestion of sodium bicarbonate (NaHCO$_3$) enhances exercise endurance and/or capacity (Price et al., 2003; Wilkes et al., 1983; Zajac et al., 2009). Moreover, Carr et al., 2011, and Zajac et al., 2009, recommended using oral NaHCO$_3$ to increase training intensity and improve exercise performance in young athletes.

**Echocardiography**

Echocardiography is widely utilized as a non-invasive and reliable method for assessment of cardiac structure and function in humans. In the past few years, murine members (mice and rodents) have become the ideal model to study the mechanism of cardiac dysfunction resulting from genetic manipulation and the demand for the adapted technique has increased. The ability to translate the imaging techniques as well as the conceptual framework from human to mice models is one of the compelling benefits of
echocardiography despite some limitations. Relatively rapid heart rate (600 beat per minutes) and small size of the heart makes it necessary to select an echo transducer with a frequency of more than 10 MHz in order to acquire an enhanced temporal and spatial resolution in mice heart (Shumin et al., 2011). Tanaka et al (1996), reported on the significance of using echocardiography in morphological and functional evaluation of the mouse heart. Because of its non-invasive and cost-effective advantages and its accuracy in assessment of the murine heart, echocardiography becomes one of the most effective scanning exams for cardiac function in genetically and functionally manipulating mice (Syed et al., 2002). Inge Elder was able to record the first M-mode echocardiography (Picard et al., 2008) opining the door for early clinical application of the M-mode echocardiography to estimate the hemodynamic parameters and cardiac structure in many clinical cases (Popp et al., 1975).

Parasternal long axis and short axis views in 2-Dimension (2-D) and M-mode parameters derived through transthoracic echocardiography (TTE) are commonly used in providing accurate left ventricular measurements (Litwin et al., 1994; Morgan et al., 2004). Kiatchoosakun et al., 2002, recommended the use of 2-D area length in determination of left ventricular mass as an alternative for in vivo measurement. Short axis 2-D and guided M-mode views are highly reliable in detecting systolic dysfunction (Tanaka et al., 1996). Ejection fraction (EF) and fractional shortening (FS) both are localized among most important parameters derived from long and/or short axis end diastolic (EDA) and end systolic area (ESA), as well as diameters (EDD and ESD). Ejection fraction is a critical factor in prognosis of myocardial infarction and heart failure (Otterstad et al., 2002; Aaronson et al., 1997) while fractional shortening is another important tool in left
ventricular systolic function estimation (Gardin et al., 1995). Other valuable
echocardiography parameters include the peak velocity of early rapid filling (E-wave)
and the filling during systole (A-wave) that is useful in measuring LV inflow through the
mitral valve (Arnlov et al., 2004). Myocardial performance index (MPI) along with E/A
ratio provides important prognosis for the risk of future congestive heart failure (CHF)
(Arnlov et al., 2004).
2. HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

We hypothesized that the absence of AT1a receptor positively affects cardiac function and structure and improve exercise tolerance during chronic physical activity.

Specific aims

Two specific aims were tested in the current study

1. To test the hypothesis that inactivation of AT1a receptor enhances cardiac performance and prevents cardiac hypertrophy in response to exercise training.
2. To test the hypothesis that absence of AT1a receptor attenuates lactate accumulation and improves exercise endurance and metabolic activity.
3. METHODS

Experimental protocol

Adult, 20-22 week old mice were randomly assigned to four groups: WT control (WTCON) (n=6), AT1a KO control (AT1aKOCON) (n=5), WT exercise WTEX (n=8), and AT1a KOEX (n=8). An exercise wheel system was used for the exercise groups to force mice to run in speed rate of 8m/min for 1 hour and 3d/wk (Monday through Friday) for 7 weeks during light cycle. Initially, mice were adapted to exercise wheel system for five days with starting speed of 4 m/min for one hour and gradually increased in the speed by 1 m/min every day. In the fifth day, the required speed (8 m/min) was achieved and maintained for the rest of the experiment. Echocardiography measurements were performed at baseline and 7 weeks. Body weight and fat composition (fat mass, lean tissue mass, free water, and total body water) were measured at baseline and after 7 weeks. Blood lactate level measurements were analyzed at multiple exercise sessions (baseline, 5, 10, 15 and 60 minutes) of wheel running over one week using Lactate pro meter (Lactate Pro LT–1710, Akray (KDK) Japan). Plasma corticosterone were also assessed in both control and exercise groups by radioimmunoassay method. A cage stress test was performed by collecting urine at baseline and 2 hours after exercise. Electrolyte assessment was done for all groups using Piccolo® xpress chemical analyzer (Abaxis North America, Union City, CA) as well as blood pH using a glass electrode. At experimental termination, mice were sacrificed by decapitation and body organs.
including heart, aorta, kidney and quadriceps were immediately collected and frozen in liquid nitrogen or fixed in 4% paraformaldehyde. All animal experimental protocols were approved by the Wright State University (WSU) Animal Care and Use Committee. Fixed tissues were embedded with paraffin and sent to AML laboratory (AML Laboratories, Inc., Baltimore, MD) for sectioning and staining.

**Animals**

Adult (20-22 weeks old) Male (C57BL/6) wild type (WT) and AT1aKO mice were housed at 22 °C with a 12:12 hour dark-light cycle and had free access to standard mouse chow and tap water. The AT1a KO mice breeding strain was obtained from Drs. Coffman and Gurly from Duke University, Durham, NC, USA. Mice were genotyped for AT1a gene expression and sent to the WSU lab.

**Exercise wheel system**

The exercise wheel system (model 20 (80800A) Lafayette Instrument, Lafayette, IN) is designed to provide maximum flexibility in performing forced exercise work with speed range of 0.9m/min to 11.4m/min (Figure 5). The capacity of the system is up to 20 exercise wheels with one mouse per wheel. The wheels are electronically controlled and driven by speed motor that force the mice to perform walking or running exercise job. The system also has a small control panel that process several functions such as: exercise time, resting time, number of cycles, and exercise wheel speed. Each wheel is surrounding by elastic safety mesh netting that protect the mice tails from getting cut accidently while exercising. For exercise groups, mice ran on the wheel system 3d each week (Monday through Friday) during the light cycle. Each session lasted for 60 min of wheel running (8 meters/min) between 9 and 10am.
Assessment of heart function was done using a Siemens ACUSON Sequoia 512 echocardiography machine, with fifteen megahertz transducer that allows enhanced images in small animals (model 15L8, Siemens Medical Solutions Inc., Malvern, PA). Mice were isolated in a small chamber and anesthesia was induced using 3% isoflurane in 1L/min of oxygen (Surgivet Anesco® Isotec 4™). Mice were moved to a warm pad in order to maintain normal body temperature and isoflurane was adjusted to 1.5 % in 0.5 L/min of oxygen for anesthesia maintenance during the experiment using a nose cone. A hair remover cream (Nair®, Church & Dwight Co., Inc., Township, NJ) was applied to
the chest area followed by alcohol swab. An ultrasound transmission gel was wormed and applied to the chest area and the transducer was positioned and manipulated until the required image is obtained.

A three second 2-dimensional (2D) video clip was recorded for the left ventricle through a parasternal long axis view and end diastolic area (EDA) as well as end systolic (ESA) area measurements were obtained. The transducer was then manipulated to get the 2-D short axis view video clip and 2-D guided M-mode (2-D M-mode) for interior LV wall parameters including the left ventricular end diastolic diameter (LVEDD), end systolic diameter (LVESD), ventricular septal wall thickness (SWT), and posterior wall thickness (PWT). The relative wall thickness was calculated using M-mode parameters with the formula $RWT = [(SWT+PWT)/LVEDd]$. A 2-D M-mode image was analyzed according to the American Society of Echocardiography standards (Sahn et al., 1978). Pulsed Wave Doppler echocardiography was recorded to assess mitral valve function. Mice were tilted backward and the prop was directed upward such that the prop was orthogonal with the apex of the heart (Respress & Wehrens, 2010). The peak velocity of early rapid filing (E-wave), and the filing during systole (A-wave) were measured and the E/A ratio was calculated (Figure 6). Isovolumetric contraction time (IVCT) and isovolumetric relaxation time (IVRT) were also obtained through pulsed wave assessment. EF% was calculated automatically by the Echo following end diastolic and end systolic volume determination. FS% derived from M-mode elements and calculated using the formula $FS\% = [(LVEDd+LVESd)/LVEDd]$. 
Figure 6: Representative several forms of Echocardiography Images and Video clips.

Lactate levels measurements

Lactate was measured over multiple exercise sessions using lactate pro test meter (Lactate Pro LT–1710, Akray (KDK) Japan) and lactate pro test strips. The lactate meter was calibrated using calibration and check strips to ensure that the test meter was operating correctly. Each sample requires 5 µl blood. Results from the sample are available within 60 s. Mice were individually removed from the wheels and blood was sampled from the tail at 0, 5, 10, 15, and 60 minutes of wheel running on several days through one week. Lactate measurement was performed on the second week of the
experiment at the same time each day (between 9-10am). After blood sampling, mice were returned to wheels to continue the exercise period.

**Body fat composition measurements**

Body fat composition assessment was performed using EchoMRI-100™ that measures total body fat, free water and lean tissue mass. Each mouse was weighed and inserted individually into a long-narrow cylinder with manual input for the mice ID and weight into a computer. The scanning process lasted for 2-3 minutes for each individual animal and the results were downloaded from the computer data base.

**Heamatoxyline and eosin staining (H&E) for heart cells**

Paraffin was removed from paraffined slides containing 5 µm of fixed heart tissue by dipping the sections in xylene, ethanol with several concentration (100%, 95%, 80%, 70%), and water. The slides were then submerged with heamatoxyline and eosin stain for 10 minutes. H &E stains the cardiomyocyte nuclei dark blue and other parts of the cell are stained in pink. After 10 minutes, the slides were washed with indirect tap water and allowed to dry on a slide warmer. Finally, they were dehydrated with xylene and covered slipped with Permount™ adhesive (Fisher Scientific, Pittsburgh, PA).

**Cardiomyocyte size assessment**

Pictures of heart sections were taken at 20X magnification using a Leica Microsystem® DMR microscope with Optronics® PictureFrame™ imaging software to acquire the images. The white balance was modified using the software’s ROI white balancing tool, and the images were adjusted using exposure, gain and gamma tools. Three different images in multiple locations were taken randomly for cardiomyocytes in the LV wall.
with the same camera setting and light intensity. Cardiac cell diameters were analyzed using Metamorph® image analysis software (Ver. 7.6.3). Cardiomyocyte diameters were traced manually using a trace selection tool and three measurements were taken for each picture and the average was calculated in µm.

**Picro-Sirius red staining for aorta**

Picrosirius red staining used to stain type I, and III collagen in the aortic wall. Five-micron paraformaldehyde-fixed aorta sections were de-paraffinized using xylene and ethanol at several concentrations (write out concentrations), and water. Then, the sections were stained with Weigert’s iron haematoxylin staining kit (ENG Scientific, Inc., Clifton, N.J.) for eight minutes. The slides were washed with tap water and stained in a picrosirius red solution containing five grams of Direct Red 80 (Sigma-Aldrich, St. Louis, MO) and 500 ml of picric acid solution (Sigma-Aldrich, St. Louis, MO). After one hour incubation with picro-sirius red staining, the slides were washed with acidified water and dehydrated with ethanol. Finally, they were washed with xylene and covered with a Permount™ adhesive.

**Quantification of Picro-Sirius red**

Images were taken at 5x magnification for aorta sections under polarizing light using a light microscope. Polarizing light allows a clear and selective appearance for the collagen fibers in the aortic wall with a dark black background for the rest of the section, so that it prevented any unwanted fibers from being reflected. An external light source transmitter was used and connected to the microscope via external port. Images were calibrated using a section of the control group aorta to adjust light intensity and polarization at 90 degrees as well as microscope and camera settings. Once selected, settings remain
unchanged for the rest of the images. Metamorph®7.6.3 was used to quantify the collagen using the selection tool. Threshold area of collagen was traced and normalized to the total area of the aorta.

**Masson’s trichrome staining for collagen**

Masson’s trichrome staining colors the collagen fiber blue and the nuclei black and the rest of the tissue red. The slide sections were de-paraffinized by 100%, 95%, and 70% alcohol followed by washing with distilled water. The slides then were re-fixed in Bouin's solution, which was composed of picric acid, formaldehyde, and glacial acetic acid, for 1 hour at 56 °C and washed again with tap water until the yellow color disappeared. Weigert’s iron haematoxylin solution was applied for 10 minutes followed by tap and distilled water washing. After that, the sections were stained with Biebrich scarlet-acid fuchsin solution (AR173 // Artisan™ Masson’s Trichrome Stain Kit) for 10-15 minutes followed by phosphomolybdic-phosphotungstic acid solution until the red color of collagen was removed. Then, the sections were stained with aniline blue for approximately 15 minutes followed by water rinsing and 1% of acetic acid solution. Finally, the sections were dehydrated with ethanol and mounted with xylene-based mounting media and slide cover slips.

**Quantification of collagen in cardiomyocytes**

Pictures with 20x magnification were taken for collagen stained heart tissue using a Leica Microsystem® DMR microscope. Metamorph® (Ver. 7.6.3) was used to analyze images. Software was calibrated according to image resolution using the calibration distance and calibrated by region tools. The load region tool was applied to select the target area of the image in three locations of the LV image and the region size was fixed for all other
images. After measuring the target region, the threshold color of the collagen was adjusted by manipulating the saturation and the intensity of the image to the required level (Figure 7). The proper level of saturation could be adjusted by direct clicking on the image so that the collagen fiber is traced. The measured threshold was normalized to the selected area using integrated morphometry analysis. The result was then recorded on an Excel spreadsheet.

Figure 7: Metamorphic quantification of collagen content in heart section stained with Masson’s Trichrome. A, selected area of analysis; B, adjust color threshold; C select the blue color of collagen, D; take the measurement and export to Excel spreadsheet.
Corticosterone radioimmunoassay

Mice urine was collected for all groups (control and exercise) and aliquoted to several Eppendorf tubes and stored immediately at -80 °C. For the cage stress test, urine was collected at baseline and two hours after exercise. Urinary corticosterone was measured using the ImmuChem™ double antibody corticosterone 125I RIA kit (MP Biomedical, Orangeburg, NY, USA). Urine was diluted to 1:100 dilutions and the assay was set up as in table 1.

Table 1: Corticosterone radioimmunoassay procedure (samples and reagents set up).

<table>
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<th>Tube #</th>
<th>Description</th>
<th>Ster.Dil.</th>
<th>Std or ctrl</th>
<th>Sample</th>
<th>^125^I Cort</th>
<th>Cort Ab</th>
<th>Prec. Rgt.</th>
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<td>(NSB)</td>
<td>100</td>
<td>---</td>
<td>---</td>
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<td>3,4</td>
<td>(Zeroes)</td>
<td>50</td>
<td>---</td>
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<td>50 ul</td>
<td>50 ul</td>
<td>200 ul</td>
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<tr>
<td>5,6</td>
<td>25 ng</td>
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<td>21end</td>
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</tbody>
</table>
All assay tubes were mixed via a vortex mixer and incubated at room temperature (22-25 °C) for two hours. Precipitant solution (red reagent) (200µl) was added to the tubes followed by vortexing. Tubes were then centrifuged at 2300-2500 rpm for 15 min and the supernatant decanted. Finally, the precipitant was counted using a gamma counter system (Micromedic 4/200 plus systems, Inc. Huntsville, Alabama, USA).

**Blood electrolyte assessment**

Blood electrolytes were analyzed for all experimental groups after sacrificing using the Piccolo Xpress™ Chemistry Analyzer (Piccolo Xpress® Chemistry Analyzer, Abaxis, Inc., Union City, CA). This “point of care” analyzer has been shown to be precise in providing multiple blood analytes using 100 µl of blood, serum, or plasma sample (Park, Ko, Kim, and Song, 2009). Sample blood was pipetted into a basic metabolic panel disc and results printed within 12 minutes. Bicarbonate, sodium, potassium, blood urine nitrogen, and chloride were simultaneously measured in each mice blood sample.

**Blood pH measurement**

Blood pH was measured immediately after sacrificing using a pH meter and micro pH glass electrode (S220 SevenCompact™ pH/Ion). The pH meter was calibrated using three buffers (pH 4, pH 7, and pH 10). The electrode was rinsed between calibrations. The electrode was inserted into the blood tube and a result recorded. Because pH is affected by temperature, blood temperature was recorded during pH measurements and was normalized to the change in pH using the following formula (Ashwood, Kost, and Kenny, 1983)

\[ pH = pHm + \left[-0.0147 + 0.0065 (7.4 - pHm)\right] (37 - t) \]
Statistical methods

Experimental data were analyzed using prism 5® (GraphPAD Software, Inc; version 5.01). In general, two-way ANOVA was used with Bonferroni post-tests to compare means. Unpaired t-test was used in aorta to compare the collagen content between exercise groups only. All data are expressed as means ± SEM. Results are considered significant when p is <0.05. One way ANOVA with post-hoc test was used in muscle collagen analysis to compare the control to exercise groups.
5. RESULTS

Results of echocardiography analysis

Baseline 2-D echocardiography measurements showed no changes between groups. Ejection fraction (EF %) was similar at baseline between WT and AT1aKO mice as well as LVESa and LVEDa. 2-D M-mode analysis showed no differences in fractional shortening (FS %) as well as in LVEDD and LVESD between control groups. Pulsed wave Doppler imaging analysis was also similar in all groups at the baseline. These results indicate normal heart function for the AT1a deficit mice.

Effect of chronic exercise on cardiac function

Seven weeks of running wheel exhibited several changes on cardiac function and geometrical structure. Long axis view revealed a significant increase in EF% in AT1aKOEX (72.5%) compared to WTEX and AT1aKO at baseline (64.3% and 63.2±2.2%, respectively). However, WTEX showed a slight increase (not significant) in EF% (Figure 8). LVEDa was significantly greater in AT1aKOEX compared to WTEX (0.186 vs. 0.165 cm2), while the LVESa were almost similar between groups. Short axis analysis for fractional shortening (FS %) showed a slight (non-significant) increase in both exercise groups compared to control (Figure 9). Assessment of pulsed wave Doppler revealed a lower E-wave in WTEX (74 cm/s) as compared to WT at baseline (90 cm/s) (Figure 10). However, the E/A ratio remain unchanged between groups. IVCT was
higher in WTEX compared to baseline while there was no significant different was seen in IVRT between groups. Table 2 showed several echocardiography parameters.

**Cardiomyocyte size quantification**

Cardiac cell diameter was measured on H&E stained section using computerized image analysis (Figure 13). Significant enlargement in cardiomyocyte diameter (width) was observed in WTEX as compared to AT1aKOEX group (29.5µm vs. 25.8µm, \( p<0.05 \)) (Figure 14). This indicates cardiac cell hypertrophy resulting from long term wheel running exercise. Finding was consistent with the cardiac hypertrophy seen in WTEX determined by heart to body weight (H/BW) ratio (Figure15). However, while there was no significant change between WTEX and WT control in cardiomyocyte diameter, analysis showed higher cardiac cell diameter in AT1aKO control group as compared to AT1aKOEX. Finding suggests the role of AT1a receptor in inducing cardiac hypertrophy in response to chronic exercise.

**Masson’s trichrome staining for collagen in heart tissue**

A section of LV wall was analyzed for trichrome collagen staining (Figure 11). Long term exercise showed greater collagen accumulating in WTEX myocardium as compared to AT1aKOEX and WT control (16% vs. 6% and 5%, respectively). However, AT1aKO control showed also higher collagen percentage when compared to corresponding exercise group (Figure 12). Accumulating collagen is a good indicator for impaired cardiac relaxation and filling as well as LV myocardium remodeling and stiffness.
**Picro-serius red staining analysis for aorta**

One-way ANOVA showed no significant difference in the ascending thoracic aorta collagen content between groups (Figure 16 & 17). However, T-test revealed a marked increase of collagen percentage in WTEX aorta when compared to AT1aKOEX (Figure 18). High level of collagen deposition on the aortic wall indicates vessel stiffening, and impaired aortic relaxation and elasticity. These finding suggest a trend toward an aortic remodeling in WTEX that increase aortic resistance and might affect systolic and diastolic function of the heart negatively.

**Lactate measurements during wheel running**

Blood lactate analysis was performed to exercise groups in order to examine the exercise endurance on wheel running system. Results showed similar baseline lactate level between groups. However, after five minutes of wheel running with a velocity of 8m/min, lactate was markedly increase to 4.2 mmol/L in WTEX compared to AT1aKOEX (2.9 mmol/L) and to WTEX at baseline (2.4 mmol/L). Lactate concentration was also higher at 60 minutes of wheel running in WTEX compared to AT1aKOEX and to baseline (Figure 19). In general, there was a trend toward more lactate level over the whole running sessions in WTEX than AT1aKOEX. These finding was consistent with the over activity of the AT1aKO mice discussed in the introduction of this thesis. Since angiotensin II is the most important protein in RAS, this result is also support the concepts that links the exercise performance to lower RAS activity in the circulation.

**Assessment of bicarbonate buffer (HCO3-) in the blood**

Bicarbonate is the most important buffer during exercise work. In order to verify the last finding regarding lactate level in blood, [HCO3-] concentration was tested for all groups.
post sacrifice. While there was no significant change observed between control groups, results revealed greater [HCO3-] in AT1aKOEX compared to WTEX (21.2 vs. 13.7 mmol/L, respectively) (Figure 21). These results reflect higher levels of bicarbonate buffer in AT1aKOEX during exercise which works against elevated lactic acid concentration in blood and delay or terminate muscle fatigue onset. Finding was consistent with previous lactate level measurement result.

**Blood pH assessment**

Blood pH or hydrogen ion [H+] concentration was analyzed to assess the effect of chronic exercise on acid-base balance and to further confirm the previous finding of lactate and bicarbonate buffer concentration. At baseline, blood pH was similar between groups. Interestingly, blood pH was significantly higher in AT1aKOEX compared to WTEX (7.41 vs. 7.28) (Figure 23). Blood pH was also lower in WTEX compared to WT control. These results suggest acidic pH resulting from chronic exercise in WTEX and the lacking of enough bicarbonate buffer to resist change in pH.

**Electrolyte measurement results**

In addition to bicarbonate buffer, chloride (Cl-) and sodium (Na+) were also analyzed in blood using chemical analyzer. Post-hoc analysis revealed greater CL- level in WTEX compared to AT1aKOEX while there was no significant change between control groups (Figure 22). CL- is in continuous change with HCO3- through the cell membrane through what is called “chloride shift”. Thus, low level of CL- in AT1aKOEX resulted from exchange with HCO3- in the erythrocyte which enhanced the resistance to acidic pH during exercise. Na+ analysis showed no change between groups by two-way ANOVA
However, there was a trend toward lower Na+ in AT1aKOEX when compared to WTEX alone (142.5 vs. 153.8 $p<0.05$ by t-test). Body Fat composition was also measured for all groups (Table 5)

**Corticosterone assessment**

**Plasma corticosterone measurement**

Plasma corticosterone showed a significant change in response to chronic exercise stimulation. Results revealed lower corticosterone levels in both exercise groups as compared to control (Figure 25). However, there was no significant change seen in plasma corticosterone levels within the treatment groups.

**Urinary corticosterone results**

Urinary corticosterone analysis was done for exercise groups only at baseline and after 2 hours of exercise at week 7. Results showed greater corticosterone levels in both exercise groups as compared to the baseline. There was no significant change was observed between groups after exercise (figure not shown).

**Cage stress analysis (urine sample)**

Mice urine samples were analyzed for cage stress stimulation. Even though there was increase in cortecosterone level following 30 min of cage change, Tow-way ANOVA revealed no significant effect of cage change between groups (Figure 26).

**Inflammatory response**

Inflammatory analysis was done for plasma samples in all experimental groups (Figure 29). Results showed higher interleukin one alpha (mIL-1α) in WTEX as compared to other groups (Figure 27). Keratinocyte chemoattractant (mKC) chemokine was in greater
levels in AT1aKO control compared to AT1aKOEX (Figure 28). However, t-test revealed higher levels in (mKC) in WTEX as compared to WT control (Table 4).

**Quadriceps skeletal muscle collagen**

Quantification of quadriceps skeletal muscle collagen content revealed greater collagen percentage in WTEX as compared to WT control (Figure 30 & 31). One way ANOVA with Tuky post-hoc test used to differentiate means in this analysis. The high collagen content shown by Masson’s Trichrome staining in WTEX group indicated skeletal muscle fibrosis that might result from low muscle pH during exercise and the consequence fatigue.
Figure 8: Percent ejection fraction (EF%) for control and after 7 weeks of wheel running exercise in WT and AT1aKO mice. Results showed increase in EF% in AT1a KOEX compared to WTEX and AT1a KO control. Data were analyzed using repeated measure two-way ANOVA. Bonferroni post-tests was used to compare means; *$p<0.05$ vs. WTEX; †$p<0.05$ vs. AT1aKO control. Data are mean ± SEM.
Figure 9: Percent fractional shortening (FS %) for control and after 7 weeks of wheel running exercise in WT and AT1aKO mice. Two-way ANOVA showed non-significant change between groups. Data are means ± SEM.
Figure 10: Peak velocity of early rapid filling (E-wave) for control and after 7 week of wheel running exercise. Result showed significant decrease in E-wave in WTEX as compared to WT control. Post hoc test was used to compare means. Data are mean ± SEM.*p<0.05 vs. WT control.
Table 2: Effects of 7 weeks of wheel running exercise on cardiac function in WT and AT1aKO mice, echocardiography parameters.

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<tr>
<th>Parameter</th>
<th>WTCon (n=5)</th>
<th>AT1a KOCon (n=5)</th>
<th>WTEX(n=8)</th>
<th>AT1a KOEX(n=8)</th>
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<td>0.177 ± 0.006</td>
<td>0.165 ± 0.006</td>
<td>0.186 ± 0.004†</td>
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<tr>
<td>LVEDd</td>
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<td>0.389 ± 0.015</td>
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<td>9.86 ± 1.47</td>
<td>13.29 ± 1.16†</td>
<td>9.38 ± 0.56</td>
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</table>

Data were analyzed using two-way ANOVA and post hoc test. Data are means ± SEM. †p<0.05 vs WTEX; * p<0.05 vs. control. LVEDa, left ventricular end diastolic area; LVEDd, left ventricular end diastolic diameter; LVESa, left ventricular end systolic area; LVESd, left ventricular end systolic diameter; FS%, fractional shortening; SWT, septal wall thickness; PWT, posterior wall thickness; RWT, relative wall thickness; EF%, ejection fraction; E-wave, peak velocity of early rapid filling; A-wave, filling during systole.
Figure 11: Masson’s Tricrome staining for collagen in heart section in AT1aKO and WT mice. WT control (A), WTEX(C), AT1aKO control (B), AT1aKOEX (D). Image were taken at (20X) magnification. Blue color represents collagen content in myocardium which is a good indicator for myocardium remodeling.
Figure 12: Quantification of collagen content in cardiac cell in AT1aKO and WT mice at baseline and after 7 weeks of wheel running exercise. Result showed greater collagen percentage in (collagen %) in WTEX and AT1aKO control as compared WT control and AT1aKOEX. Data are means ± SEM. *P<0.01.
Figure 13: Effect of wheel running exercise on cardiomyocyte diameter (µm).
Representative histological sections at (20X) magnification of LV myocytes diameter in WT control (A), AT1aKO control (B), WTEX(C), and AT1aKOEX (D). The arrows indicate the line showing the width of each cardiac myocyte that was traced across the middle of the nuclei using image software. Greater cardiomyocyte diameters were observed in WTEX and AT1aKO control.
Figure 14: Quantification of cardiomyocyte diameter in WT and AT1aKO mice at baseline and following 7 weeks of wheel running exercise. Results showed lower cardiac cell diameter in AT1aKOEX as compared to AT1aKO control and to WTEX. Data are means ± SEM. *p<0.05 vs. WTEX; †p<0.05 vs. AT1aKO control.
Figure 15: Heart weight/Body weight ratio was calculated for control and exercise WT and AT1aKO. Result showed higher heart to body weight ratio in WTEX group compared to AT1aKOEX. Data were analyzed using Two-way ANOVA and Bonferroni post-tests. Data are means ± SEM.*p <0.05 vs. AT1aKOEX.
Figure 16: Picro-serius red staining of aorta sections in WT control (A), AT1aKO control (B), WTEX(C), and AT1aKOEX(D). Image were taken at (5X) magnification.
Figure 17: Quantification of collagen percentage in aorta sections in control and exercise WT and AT1aKO mice. Results showed no significant change between groups. Data are means ± SEM.
Figure 18: Collagen content in aorta sections in exercising groups. Unpaired T-test revealed higher collagen in WTEX as compared to AT1aKOEX. Data are means ± SEM. *P<0.05 vs. AT1aKOEX.
Figure 19: Blood lactate levels in WT and AT1aKO during wheel running exercise. The velocity was fixed at 8m/min and lactate was sampled at baseline and over several exercise times. Results showed higher lactate level in WTEX at 5 and 60 min of wheel running compared to baseline and to AT1aKOEX. Data are mean ± SEM. *p<0.05 vs. baseline; †p<0.05 vs. AT1aKOEX at the same exercise time. Note that lactate was assessed for exercise groups only.
Figure 20: Blood glucose levels vs. time of wheel running exercise in WT and AT1aKO mice. The velocity was fixed at 8m/min. Two-way ANOVA with Bonferroni post-tests showed greater blood glucose levels in WTEX at 60 min as compared to baseline and to 5 min of wheel running. Result also revealed higher blood glucose at 60 and 10 min of wheel running in WTEX vs. AT1aKOEX. *p<0.05 vs. baseline; †p<0.05 vs. 5 min running; # p<0.05 vs. AT1aKOEX at the same exercise time. Data are means ± SEM. Note that data for blood glucose at 15 min wheel running is not shown.
Figure 21: Blood bicarbonate [HCO3-] buffer for control and exercise WT and AT1aKO. The analysis was done post-sacrifice. Bonferroni post-test revealed significant higher HCO3- in AT1aKOEX as compared to WTEX. Data are means ± SEM. *p<0.001 vs. WTEX.
Figure 22: Chloride levels in blood for control and exercise WT and AT1aKO. Bonferroni post-tests showed higher chloride in WTEX as compared to AT1aKOEX. Data are means ± SEM. *p<0.05 vs. AT1aKOEX
Figure 23: Effect of 7 weeks exercise of wheel running on blood pH in WT and AT1aKO mice. Blood pH was measured post sacrifice. Two-way ANOVA and Bonferroni post-tests were used in data analysis. Results revealed lower blood pH in WTEX as compared to baseline and to AT1aKOEX. Data are means ± SEM.
*p<0.05 vs. baseline; †p<0.001 vs. AT1aKOEX.
Figure 24: Levels of Sodium (Na+) in the blood for control and exercise WT and AT1aKO. Two-way ANOVA showed no significant changes between groups in response to chronic exercise training. Data are means ± SEM.
Figure 25: Effect of chronic exercise of wheel running on plasma corticosterone in WT and AT1aKO mice. Two way ANOVA showed significant decrease in plasma corticosterone in both exercise groups after 7 week of wheel running as compared to control. Bonferroni post-test analysis used to differentiate means. Data are means ± SEM. *p<0.05 vs. baseline.
Figure 26: Effect of change of cage on urinary corticosterone levels in WT and AT1aKO mice at baseline and after 30 min of cage change. Result showed no significant differences in urinary corticosterone between groups in response to cage changes. However, a slight increase was observed in both groups following cage changes. Data are means ± SEM.
Figure 27: Effect of long term exercise on inflammatory cytokines response in WT and AT1aKO. Results revealed greater interleukin 1-alpha (IL-1a) proteins in WTEX as compared to AT1aKOEX and to corresponding baseline. Data are means ± SEM. 
*p<0.05 vs. baseline; †p<0.05 vs. AT1aKOEX.
Figure 28: keratinocyte chemoattractant (mKC) chemokine was expressed in greater amount in AT1aKO control compared to WT. However, there was a trend toward higher (mKC) protein in WTEX vs. WT control as shown by unpaired t-test.*p<0.05

Table 3: Unpaired t-test showing the significance between exercise groups (mKC).

<table>
<thead>
<tr>
<th>Column A</th>
<th>WTCONT.</th>
<th>vs</th>
<th>Column B</th>
<th>WTEX.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unpaired t test</td>
<td></td>
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</tr>
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<tr>
<td>P value summary</td>
<td></td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Are means signif. different? (P &lt; 0.05)</td>
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</tr>
<tr>
<td>One- or two-tailed P value?</td>
<td></td>
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<td>Two-tailed</td>
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<tr>
<td>t, df</td>
<td></td>
<td></td>
<td>t=2.322 df=14</td>
<td></td>
</tr>
</tbody>
</table>
Figure 29: Representative inflammatory response to chronic wheel running exercise in control and exercise WT and AT1aKO. mIL-2, interleukins-2; mIL-5, interleukin-5; mIL-4, interleukin-4; mIL-6, interleukin-6; mTNFa, tumor necrosis factor a; mIL-12, interleukin-12; mIL-10, interleukin-10; mIFNγ, interferon γ; mIL-1b, interleukin-1 beta; mMIP-2, macrophage inflammatory protein-2. Data are means ± SEM. Result showed no significant different between groups.
Table 4: Representative body fat composition, heart weight, and heart/body weight ratio for control and exercise WT and AT1aKO mice

<table>
<thead>
<tr>
<th></th>
<th>WT-control</th>
<th>AT1aKO-control</th>
<th>WTEX</th>
<th>AT1aKOEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>26.86 ± 0.35</td>
<td>35.11 ± 1.68*</td>
<td>26.97 ± 0.37</td>
<td>34.04 ± 1.45†</td>
</tr>
<tr>
<td>Body fat (g)</td>
<td>1.978 ± 0.16</td>
<td>5.632 ± 1.08*</td>
<td>2.338 ± 0.30</td>
<td>4.215 ± 0.48†</td>
</tr>
<tr>
<td>Body lean (g)</td>
<td>21.21 ± 0.24</td>
<td>24.90 ± 1.04*</td>
<td>21.05 ± 0.29</td>
<td>23.92 ± 0.80†</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.132 ± 0.009</td>
<td>0.146 ± 0.014</td>
<td>0.148 ± 0.007</td>
<td>0.144 ± 0.006</td>
</tr>
<tr>
<td>Heart/body weight (mg/g)</td>
<td>5.019±0.24</td>
<td>4.763±0.49</td>
<td>5.490±0.23#</td>
<td>4.250±0.11</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM. Results showed higher body fat composition in AT1aKO mice at baseline and post exercise as compared to WT without significant effect of exercise between groups. Heart/body weight ratio was significantly higher in WTEX vs. AT1aKOEX. *p <0.05 vs. WT control; †p <0.05 vs. WTEX; #p<0.05 vs. AT1aKOEX.
Figure 30: Sections of quadriceps muscle stained with Masson’s trichrome at (5X) magnification. A, WT control; B, WTEX; C, AT1aKO control; D, AT1aKOEX. Collagen content (blue) were dominant in WTEX as compared to WT control.
Figure 31: Muscle collagen content in control and exercise WT and AT1aKO mice. One way ANOVA with Tukey post-hoc test was used in data analysis. Result revealed greater collagen fraction percentage in WTEX as compared to WT control. Data are means ± SEM. *p<0.05 vs WT control.
6. DISCUSSION

Heart function
Exercise training has been shown to be effective in improving cardiac dysfunction and is an essential part in cardiac rehabilitation program in patients with coronary heart disease (Medeiros et al., 2007; Achttien et al., 2013; Schober and Knollmann et al. 2007). Angiotensin II participates in cardiac remodeling and hypertrophy (Ichihara et al 2001; Alghamri et al 2012). While several studies reported the role of AT1 receptor in exercise-induced cardiac hypertrophy and remodeling in patient subjects (Thu´rmann et al 1998; Xu et al., 2008; Jain., et al 2000) few information are available on normal animals. In this study we investigated the role of AT1a receptor in cardiac function and structure in response to moderate exercise stimulus in non-infarct (normal) model using AT1a receptor deficit mice. Our finding suggested that exercise training increase EF% in AT1aKO mice as compared to WTEX and to the corresponding control group. FS% was slightly increase (non-significant) in both exercise groups. These results are consistent with a previous study performed in our laboratory on swimming mice with the same genetic background, age, gender and exercise frequency (data not shown). Higher EF% in mice lacking AT1a receptor might be a consequence of enhanced or higher left ventricular end diastolic area (LVEDa) in response to chronic exercise. In the other hand, WTEX showed lower in the peak early filling velocity (E-wave), which is considered an indicator for early stage of diastolic dysfunction (Nagueh et al., 2009).
Histological evaluation suggested myocardium hypertrophy in WTEX as shown by higher cardiomyocyte diameter. This result was supported by greater heart to body weight ratio in WTEX as compared to AT1aKOEX mice. These changes were associated with greater collagen content in myocardium WTEX group. Collectively, findings suggested an essential role for AT1a receptor in cardiac remodeling and hypertrophy during exercise training. Our result is consistent with a recent study reported on the effect of angiotensin II receptor blocker (losartan) in preventing cardiac remodeling in normotensive rate during chronic exercise training (Libonati et al., 2012). Our finding is also consistent with the result from Barauna et al 2008. This author reported the role of losartan in preventing resistance training-induced cardiac hypertrophy in animal model. Echocardiography analysis was not a part of the previous two mentioned studies. It’s worth to note that, however, higher cardiacmyocyte diameter and greater collagen content were also observed in sedentary AT1aKO group. Those findings point toward the importance of combine the inactivation of AT1a receptor with exercise training in order to prevent myocardium remodeling and hypertrophy and improve cardiac response. In the treatment of heart failure, Kemi et al (2007), reported that exercise and angiotensin II receptor blocker (losartan) mediated their effects by different mechanisms in the improvement of aerobic capacity and heart function in myocardial-infarct rats. Another study suggested that exercise training in conjunction with angiotensin II receptor blockade is required to achieve the maximum benefits in post-MI ventricular remodeling treatment plan (Jain et al 2000). In contrast, treatment with angiotensin II receptor blocker (Irbesartan) alone has been reported to be non-effective in minimizing heart
failure outcome (death rates or hospitalization) and/or in improvement of LV ejection fraction in human patients (Massie et al 2008).

In addition, there was an indication of aortic remodeling in WTEX group shown by a trend toward higher collagen fraction level in aortic wall (by t-test), which could affect the left ventricular ejection fraction negatively.

**Exercise tolerance**

The relationship between exercise performance and low RAS activity in the circulation was discussed in the introduction of this thesis. Another important objective of the current study was to identify the role of angiotensin II receptor in exercise tolerance. Results suggested lower lactate levels in AT1a deficit mice as compared to WT during wheel running exercise. This observation could justify the over activity of AT1aKO mice reported in Mistlberger et al., 2001 during wheel running exercise, and in Murphy et al., 2012 in response to rotarod exercise test. Lactate accumulates in lower levels in AT1a deficit mice which blunt acceleration in metabolic acidosis and delays muscle fatigue onset leading to improvement in the physical performance. Moreover, since angiotensin II is the main peptide in RAS system cascade, our finding is consistent with the concepts that relate the exercise performance improvement to lower ACE activity in the circulation (Gayagay et al., 1998; Saul et al., 1999; Montgomery et al., 1999; Montgomery et al., 1998). In order to confirm the role of AT1a receptor in minimizing metabolic acidosis resulting from exercise-induced lactic acidosis, blood pH was assessed post-sacrifice. Interestingly, finding reported lower blood pH in WTEX as compared to WT control and to AT1aKOEX groups. The exact mechanism through which AT1a receptor inactivation reduces lactic acidosis and resists changes in blood pH during exercise needs further
investigation. However, lactic acid generated during physical activity buffered mainly by bicarbonate buffer (HCO3-) system (as mentioned in the introduction). Therefore, electrolyte analysis (including bicarbonate) was performed after sacrifice. Higher bicarbonate buffer was observed in exercise AT1aKO blood mice. This observation was associated with low level of blood chloride ion (CL-) in exercise AT1aKO. Bicarbonate penetrates the erythrocyte cell membrane through exchange with intracellular chloride ion, a process called Cl–/HCO3- shift. Consequently, Bicarbonate buffer and chloride ion results could be explanation for the low levels of lactic acid and higher blood pH in mice lacking AT1a receptors during exercise training. Ingestion of sodium bicarbonate (NaHCO3) or exercising under alkaline condition improves exercise performance and capacity (Price et al., 2003). To the author knowledge, however, this is the first data evaluate lactate levels, blood pH, and electrolytes in exercise AT1a deficit mice. Glucose measurements were also taken during exercise and the results suggested greater blood glucose in WTEX at 60 and 10 min of wheel running as compared to AT1aKOEX at the same time. In contrast, blood glucose levels were not significantly affected during exercise in AT1aKO group. The role of AT1a receptor in maintaining almost stable blood glucose noted in this experiment need to be identified. Nevertheless, since the absence of AT1a receptor associated with enhanced physical performance, skeletal muscle required to utilize more glucose to keep continues energy supply. This clarification is consistent with a study suggested that RAS-inhibition could increase skeletal muscle-glucose uptake and glucose transport system during exercise training (Henriksen and Jacob et al 1995) which may lead to maintain stable blood glucose levels.
Quadriceps Skeletal muscle collagen assessments results indicated greater collagen levels in WTEX as compared to WT control group. Elevated collagen levels could be an indication for skeletal muscle tissue damage or fibrosis (Lorts et al 2012). This observation could be a consequence of metabolic acidosis and the impairment in metabolic efficiency reported in the above findings. Absence of AT1a receptor is associated with increase in skeletal muscle strength up to 25% and enhancement in the exercise performance and the whole body function (Murphy et al 2012). Collectively, improvement in exercise tolerance reported in this study is attributed to enhancement in metabolic efficiency and skeletal muscle mechanical efficiency in mice lacking AT1a receptor.

The way that the reduction of RAS is associated with improvement in exercise capacity and performance has not been fully understood. One explanation, depending on our finding, is that inhibition of AT1a receptor can increase the level of bicarbonate buffer (the first line buffer for lactic acid) during exercise training which minimize the level of blood lactate accumulation leading to reduction in metabolic acidosis and delay or prevent muscle fatigue onset. Bicarbonate buffer, in the form of sodium bicarbonate, has been shown to be effective in increasing exercise endurance in human and recommended to be used in youth athletes to improve performance for high intensity competitions (Zajac et al 2009). Another possible clarification is that inhibition of AT1a receptor would decrease vascular resistance (Gašanin et al 2013), and hence, increase cardiac output and blood flow to the skeletal muscle. Our echocardiography investigation showed higher left ventricular ejection fraction in AT1aKOEX animal which indicated greater blood supply to the target organs and that would enhance the exercise performance. In
heart failure patients, muscle fatigue considered one of the most common symptoms that need to be resolved as a part of heart failure therapy. However, some investigators reported that skeletal muscle fatigue in heart failure patients is not related mainly to reduction in the cardiac output, but it might attributed to skeletal muscle metabolic inefficiency and the consequence muscle mechanical abnormality (Montgomery and Brull et al 2000; Harridge et al 1996). Regular physical training improves the symptoms of heart failure by enhancement of skeletal muscle performance and metabolic efficiency (Montgomery and Brull et al 2000). Evidence suggested that skeletal muscle metabolic efficiency could be impaired by of Ang II administration in animal model (Brink et al.1996). Moreover, Ang II could also interfere with mitochondrial respiration and impaired mitochondrial efficiency. RAS inhibition, by ACE inhibitor (ramipril) has been reported to be effective in minimizing lactate production and increasing levels of ATP and creatine phosphate (an important source of ATP) in ischemic heart animal (Linz et al 1986).

Inflammation and exercise

Several studies reported that physical activity may reduce inflammation in human subjects (Abramson et al 2002; Ford et al 2002). In the current study, however, two inflammatory markers out of twelve cytokines were expressed in higher levels in WTEX plasma: interleukin-1-a (mIL-1a) and the chemokine murine growth-regulated alpha protein (mKC). Ang II is an important mediator for inflammation and plays a crucial role in inflammatory response (Suzuki et al 2003). Therefore, a possible explanation for expression of (mIL-1a) and (mKC) inflammatory markers could be related to elevated Ang II in the circulation during exercise in WTEX. In addition, these inflammatory
markers could be also related to the impairment in metabolic process and to the cardiac remodeling and hypertrophy observed in WTEX group.

**Effect of exercise on stress response**

Conflict data are available regarding the relationship between exercise training and stress management. While several studies suggested that regular physical activity may play a role in stress reduction (Starzec et al 1983; Hare et al 2013), other investigators reported an opposite role of exercise on stress levels (Girard and Garland et al 2001; Hu et al 1998; Härkönen et al 1990). A study suggested that involuntary and/or forced exercise is associated with elevation in corticosterone response while there was no effect of voluntary exercise in corticosteron levels (Ke et al., 2011). Ang II has been reported as an important stress mediator and has a role in stimulation of corticosterone synthesis (Rainey et al 1991; Saavedra et al 2007). The current study was reported lower plasma corticosterone in both exercise groups as compared to control. However, no effect was noticed for AT1a receptors in plasma corticosterone response to exercise. These results support the concept that regular physical activity may improve stress management. In contrast, urinary corticosterone was greater in both exercise groups following 2 hours of exercise compared to the baseline at the week 7 of experiment. It is important to note that plasma cortecosterone in this study was compared to control groups (different animal), while urine corticosterone was taken at rest and after two hours of wheel running exercise for the same animals. In addition, corticosteron measurement in urine sample before and after the cage change reported no differences between groups.
7. Conclusion

Angiotensin Type 1 (AT1) receptors are involved in cardiovascular pathology. Lozartan, the AT1 receptor blocker, in combination with exercise has been shown to be effective in improving cardiac performance. Accumulated evidence reported the relationship between exercise tolerance improvement and lower RAS in the circulation. In the current study AT1aKO mice exhibited improved cardiac performance without myocardium hypertrophy, greater exercise endurance, and enhanced metabolic activity and skeletal muscle mechanical efficiency in response to chronic exercise. These results suggest that the AT1a receptor is an important mediator of exercise induced cardiac dysfunction and acid-base imbalance during exercise training. To the author knowledge, this study was the first in estimating exercise endurance (lactate assessment) blood pH, and electrolytes analysis in normotensive exercise AT1a receptors deficit mice. AT1a receptors may also prevent expression of inflammatory proteins resulting from chronic exercise. Regular physical activity could provide a positive effect in stress reduction as shown by lower plasma coticosterone in exercise animal.
APPENDIX A

Role of AT1a Receptors in Cardiac Function and Acid Base Homeostasis during Exercise Endurance. Ahmad M Alhajoj, Mahmoud S Alghaniri, Roberta L Pohlman, Nadja Grobe, Debora Nakamoto, Mariana Morris, Wright State University, Dayton, OH

Angiotensin Type 1 (AT1) receptors are involved in cardiovascular pathology. Lozartan, the AT1 receptor blocker, in combination with exercise has been shown to be effective in improving cardiac performance. The goal of the study was to investigate the role of AT1a receptors on cardiac function, and exercise tolerance in response to aerobic exercise using AT1a receptor knockout (AT1aKO) mice. An exercise wheel system was used for the exercise paradigm. Male (C57BL/6) wild type (WT) and AT1aKO mice were randomly assigned to four groups: WT control (n=6), WT exercise (WTEX, n=8), AT1a KO (KO) control (n=5), and AT1a KO exercise (KOEX, n=8). Mice were forced to run at a velocity of 8 m/min for 1 hour, 3d/wk, for 7 wks. Echocardiography was conducted at baseline and 7 wks. Lactate was measured during several exercise sessions. Electrolytes and cardiac histology were assessed post-sacrifice. Results showed a significant increase in ejection fraction (EF %) in KOEX (72.5±1.5%) vs. WTEX and KO control (64.4±1.2% and 63.2±2.2%, respectively). Mitral valve assessment revealed a marked decrease in E-wave velocity in WTEX compared to WT control at baseline (74±1.9 vs.90±3.5 cm/s, p<0.05), while E/A wave ratio remained unchanged. Cardiomyocyte diameter was larger in WTEX compared to KOEX (29.5±0.7 vs. 25.8±0.5 µm, p<0.05). Heart to body weight ratio was significantly higher in WTEX vs. KOEX (5.5±0.2 vs. 4.3±0.1 mg/g, p<0.001). Masson’s Trichrome staining revealed higher collagen levels in WTEX myocardium compared to WT control and KOEX (16% vs. 5%). Blood lactate accumulation values were greater at 5 and 60 min of wheel running in WTEX (4.2±0.4 and 3.9±0.5 mmol/l) vs. KOEX (3.0±0.2 and 2.9±0.2 mmol/l). A basic metabolic panel revealed higher [HCO₃⁻] in KOEX vs. WTEX (21.7±0.6 vs. 13.7±1.8 mmol/l). Cl⁻ was lower in KOEX compared to WTEX (102.5±2.5 vs. 113.5±2.7mmol/l, p<0.05). In conclusion, AT1aKO mice exhibited improved cardiac performance without myocardium hypertrophy, greater exercise endurance, and enhanced metabolic activity in response to chronic exercise. These results suggest that the AT1a receptor is an important mediator of exercise induced cardiac dysfunction and acid-base imbalance during exercise training.

Abstract was published in Hypertension (Hypertension. 2013) Abstract was presented in HBPR 2013, New Orleans, LA
Aortic Remodeling in Angiotensin II Treated ACE2 Knockout Mice
Ahmad M Alhajoj, Mahmoud Alghamri, David R Cool, William C Grunwald Jr, Khalid M Elased, Nathan Weir, Mariana Morris, Wright State University, Dayton, OH

Angiotensin converting enzyme 2 (ACE2) is a protective regulatory protein that catalyzes conversion of Ang II to Ang-(1-7). There is much information on ACE2’s role in cardiac and renal pathologies, but limited data on ACE2 in vasculature. The objective was to study the role of ACE2 in the Ang II response as related to aortic remodeling and inflammation using ACE2 knockout (KO) mice. Eight week (C57Bl/6) male ACE2 KO and wild type (WT) mice (n= 7-8) were infused with Ang II (1000 ng/kg/min, 4 wks). Aortic structure (collagen, dimension), oxidative stress, and inflammatory cytokines were measured. Results showed a significant decrease (p<0.05) in aortic luminal area in Ang II ACE2 KO over WT (74.51µm²/pixel vs. 91.50 µm²/pixel). Picrosirius red staining showed higher collagen in the outer aortic wall in Ang II infused ACE2 KO mice compared to Ang II WT (55% vs. 19%). Dihydroethidium (DHE) staining indicated marked elevation in superoxide generation following Ang II infusion in ACE2 KO (18%) compared to WT (7%). A BioPlex system was used to quantify a panel of 23 cytokines in aorta. It revealed higher macrophage inflammatory protein (MIP-1α) in Ang II ACE2 KO vs. Ang II WT mice (13.5 vs. 3.3 pg/ml). Two-way ANOVA showed a significant effect of treatment (p< 0.02) for interleukin 1-α (IL-1α). Findings indicate that the aorta is a target site for Ang II’s pathological effects, an action which involves ACE2.

Abstract was published in Hypertension (Hypertension. 2012) Abstract was presented in HBPR 2012, Washington, DC
APPENDIX C

Inactivation of AT1a Receptor Attenuates Lactate Accumulation and Enhances Cardiac Performance during Exercise Endurance. Ahmad M Alhajoj, Mahmoud S Alghamri, Roberta L Pohlman, Nadja Grobe, Debra Nakamoto, Mariana Morris, Wright State University, Dayton, OH

Renin Angiotensin System (RAS) is involved in cardiovascular pathology. Losartan, the AT1 receptor blocker, in combination with regular physical activity has been shown to be effective in improving cardiac function. **Objective**: The purpose was to study the role of AT1a receptors on heart function, and exercise tolerance in response to chronic exercise using AT1a receptor deletion (AT1aKO) mice. **Method**: An exercise wheel system was used for the exercise paradigm. Male (C57BL/6) wild type (WT) and AT1aKO mice were randomly assigned to four groups: WT control (n=6), WT exercise (WTEX, n=8), AT1a KO (KO) control (n=5), and AT1a KO exercise (KOEX, n=8). Mice were forced to run at a velocity of 8 m/min for 1 hour, 3d/wk, for 7 wks. Echocardiography was conducted at baseline and 7 wks. Lactate was measured during several exercise sessions. Electrolytes, blood pH, and cardiac histology were assessed post-sacrifice. **Results** showed a significant increase in ejection fraction (EF %) in KOEX (72.5±1.5%) vs. WTEX and KO control (64.3±1.2% and 63.2±2.2%, respectively). Mitral valve assessment revealed a marked decrease in E-wave velocity in WTEX compared to WT control at baseline (74±1.9 vs. 90±3.5 cm/s, p<0.05), while E/A wave ratio remained unchanged. Cardiomyocyte diameter was larger in WTEX compared to KOEX (29.5±0.7 vs. 25.8±0.5 µm, p<0.05). Heart to body weight ratio was significantly higher in WTEX vs. KOEX (5.5±0.2 vs. 4.3±0.1 mg/g, p<0.001). Masson’s Trichrome staining revealed higher collagen levels in WTEX myocardium compared to WT control and KOEX (16% vs. 5%). Blood lactate accumulation values were greater at 5 and 60 min of wheel running in WTEX (4.2±0.4 and 3.9±0.5 mmol/l) vs. KOEX (3.0±0.2 and 2.9±0.2 mmol/l). A basic metabolic panel revealed higher [HCO$_3^-$] in KOEX vs. WTEX (21.7±0.6 vs. 13.7±1.8 mmol/l). Blood pH was significantly lower in WTEX as compared to KOEX (7.28 vs. 7.41, p<0.05). **In conclusion**, mice lacking AT1a receptor exhibited improved cardiac function without myocardium hypertrophy, higher exercise endurance, and enhanced metabolic response under chronic exercise stimulation. Findings indicate that the AT1a receptor is an important mediator of exercise induced cardiac dysfunction and acid-base imbalance during exercise training.

*Abstract was accepted in the American College of Sport Medicine annual meeting (ACSM. 2014) Abstract to be presented at ACSM meeting in Orlando, Florida*
APPENDIX D

Role of AT1a Receptor in Cardiac Hypertrophy in Response to Aerobic Exercise Training. Nakamoto, D.N.¹, Alhajoj, A.², Silva, J.G.F.¹, Alghamri, M.², Pohlman R.², Moriss, M.²

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² WRIGHT STATE UNIVERSITY- BOONSHOFT SCHOOL OF MEDICINE, DAYTON, OH, EUA

Accumulating evidence suggests that the angiotensin type1 (AT1) receptor plays a role in the cardiac hypertrophy produced by chronic exercise. The goal was to study the role of AT1a receptors in cardiomyocyte structure in response to aerobic exercise training using AT1a receptor deletion (AT1a KO) mice. Male (C57BL/6) wild type (WT) and AT1a KO mice were randomly assigned to four groups: WT control (WTCONT, n=6), WT exercise (WTEX, n=8), AT1a KO control (KOCONT, n=5), AT1a KO exercise (AT1a KOEX, n=8). An exercise wheel system was used for the chronic exercise paradigm. Mice were forced to run at a velocity of 8m/min for 1 hour, 3 days/week, for 7 weeks. At the experiment termination the mice were sacrificed with collection of heart for histological evaluation. The results show change in heart body weight ratio as well as cardiomyocyte structure. Cardiomyocyte diameter was higher in WT exercise as compared to AT1a KO exercise (29.5µm ± 0.68 vs. 25.8µm ± 0.52, p<0.05). Heart to body weight ratio was significantly higher in WT exercise vs. AT1a KO exercise (5.5mg/g ± 0.23 vs. 4.3mg/g ± 0.11, P<0.001). Masson’s Trichrome staining revealed higher collagen levels in WT exercise myocardium compared to WT control and AT1a KOEX (16% vs. 7%). Findings indicate that AT1a receptors are involved in preventing cardiac hypertrophy in response to aerobic exercise training. The results suggest a pathological role for AT1a receptor in regulation of heart function under exercise stimulation.

Abstract was presented in experimental biology meeting (2013), Brazil
APPENDIX E

Effects of Chronic Swim Stress on Endocrine and Cardiac Function in Angiotensin AT1a Receptor Knockout Mice. Najat Almahrouq, Ahmad Alhajoj, Mahmoud Alghamri, Mariana Morris, Roberta L Pohlman. Wright State University BoonshoftSchool of Medicine, Dayton, OH

Stress is an event or stimulus that causes an often abrupt but always large change in autonomic activity and hormone secretion. Angiotensin II (ANGII) is one of the main neurohormonal mediators that is stimulated by stress. The goal of this study was to assess the influence of chronic swim stress on adrenal and cardiac function in AT1 receptor knockout mice. Adult male AT1 receptor knockout (KO) and wild type (WT) mice were challenged to moderate exercise and were compared to sedentary groups (n= 6/group). Exercise paradigm was swimming, 1 hour 3d/ wk for 7 weeks. Urine and plasma corticosterone (CORT) were measured by radioimmunoassay (RIA). Urinary catecholamines were measured using high pressure liquid chromatography (HPLC) with electrochemical detection. Cardiac function was assessed by echocardiography (Echo).

Results showed that plasma CORT was lower in both KOEX and WTEX as compared to sedentary groups (3 fold decrease). Urinary CORT was used to show the dynamic response to exercise stress. The stimulatory change was 10 fold higher in WTEX as compared to 5 fold in KOEX. Urinary catecholamine’s results indicate that there are no significant differences in Norepinephrine (NE) and Epinephrine (EP) responses between the KOEX and WTEX. The swimming distance (cm) as well as the swimming velocity (cm/s) were significantly higher in KOEX as compared to WTEX, {F (1, 32) =9.35, p < 0.01}. In addition, Echo results showed a higher ejection fraction (EF %) in KOEX versus WTEX (75 % versus 55 %). Assessment of mitral valve function showed a higher A-wave velocity in KOEX as compared to WTEX. We concluded that ANG II AT1 receptor deficient mice showed improved cardiac response and reduced adrenal stress response to chronic exercise.

Poster was presented in Central Research Forum (2012), Wright State University, Dayton, OH.
Aortic Remodeling in Angiotensin II Treated ACE2 Knockout Mice

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Angiotensin converting enzyme 2 (ACE2) is a protective regulatory protein that catalyzes conversion of Ang II to Ang-(1-7). There is much information on ACE2’s role in cardiac and renal pathologies, but limited data on ACE2 in vasculature. The objective was to study the role of ACE2 in the Ang II response as related to aortic remodeling and inflammation using ACE2 knockout (KO) mice. Eight week (C57Bl/6) male ACE2 KO and wild type (WT) mice (n= 7-8) were infused with Ang II (1000 ng/kg/min, 4 wks). Aortic structure (collagen, dimension), oxidative stress, and inflammatory cytokines were measured. Results showed a significant decrease (p<0.05) in aortic luminal area in Ang II ACE2 KO over WT (74.51µm²/pixel vs. 91.50 µm²/pixel). Picrosirius red staining showed higher collagen in the outer aortic wall in Ang II infused ACE2 KO mice compared to Ang II WT (55% vs. 19%). Dihydroethidium (DHE) staining indicated marked elevation in superoxide generation following Ang II infusion in ACE2 KO (18%) compared to WT (7%). A BioPlex system was used to quantify a panel of 23 cytokines in aorta. It revealed higher macrophage inflammatory protein (MIP-1α) in Ang II ACE2 KO vs. Ang II WT mice (13.5 vs. 3.3 pg/ml). Two-way ANOVA showed a significant effect of treatment (p< 0.02) for interleukin 1-α (IL-1α). Findings indicate that the aorta is a target site for Ang II’s pathological effects, an action which involves ACE2.

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Chronic Exercise in Mice as a Mediator for Immunological Diseases

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**Objectives:** To study the effect of several forms of exercise, running and swimming, on cardiac function and inflammatory markers. To evaluate a mouse model of exercise for possible use in study of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and gulf war illness (GWI).

**Methods:** Adult male (C57BL/6) mice were assigned to three groups: wheel running exercise (RE) (n=10), swimming exercise (SE) (n=5), and sedentary control group (n=6). Mice were subject to exercise for 1 hour, 3 days/week, for 7 weeks. RE were forced to run at a velocity of 8 m/min in a wheel running system, while SE group performed voluntary swimming in warm water. Echocardiography was conducted at baseline and 7 weeks. At experiment termination, mice were sacrificed and plasma was collected for inflammatory cytokines analysis.

**Results:** Cardiac measurements showed a significant decrease ($p<0.05$) in ejection fraction (EF %) in SE compared to RE and control ($54.23\pm1.71\%$ vs. $64.31\pm2.27\%$ & $63.61\pm2.20$ respectively). Fractional shortening (FS %) was lower in SE compared to RE and sedentary ($0.28\pm0.02\%$ vs. $0.37\pm0.01\%$ & $0.35\pm0.01\%$ respectively, $p<0.05$). End systolic area (ESA) was markedly increased in SE group ($0.116\pm0.002CM^{2}$) compared to RE and control ($0.092\pm0.005$ & $0.095\pm0.002CM^{2}$). Inflammatory cytokines assays revealed greater murine tumor necrosis factor (mTNFa) in SE ($31.34\pm9.3$ pg/ml) vs. control group ($1.894 \pm 1.0$ pg/ml), $p<0.05$. mIL-1α was elevated ($p<0.03$ in the RE group compared to controls. There was a significant effect of treatment ($p<0.05$) between groups for the chemokine murine growth-regulated alpha protein (mKC) as determined by one-way ANOVA. During inflammation, mKC contributes to neutrophil activation.

**Figure 1**

- Mice
- Plasma mTNFa (pg/ml)

**Legend**
- Swimming mice
- Control mice

**Figure 2**

- Mice
- Plasma mIL-1α (pg/ml)

**Legend**
- Running mice
- Control mice
Conclusion: The findings indicate that chronic exercise paradigms may result in different pathologies specifically as related to cardiac and immune effects. Our research group has reported differences in exercise effects in both ME/CFS and GWI, compared to each other and to controls. The results presented indicate that this murine model may be useful in both ME/CFS and GWI.

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9. REFERENCES


