ALTERED SUBSTRATE METABOLISM AND CONTRACTILE FUNCTION IN THE AGING MYOCARDIUM

By

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ABSTRACT

Aging is associated with declining cardiac contractile function as well as changes in mitochondrial energetics and substrate metabolism. Neither the relationship between age-related changes in mitochondrial energetics and declining cardiac contractile function nor, the molecular mechanisms involved with these changes have been determined. We measured unloaded shortening properties and NADH fluorescence simultaneously during continuous stimulation at 4 Hz in isolated intact cardiac myocytes from Young (6 month) and Old (33 month) male Fischer 344 x Brown Norway F_1 hybrid rats (FBN) as a way to measure mitochondrial function. Secondly, we determined the impact stress has on cardiac myocyte contractile function during continuous stimulation at 2 Hz. We utilized rotenone to induce a mitochondrial stress and isoproterenol to induce a workload stress on the myocytes. We found significant correlations between changes in NADH and contractile properties in old myocytes but not in young. We also found that when old myocytes are stressed either metabolically with rotenone or increasing workload with isoproterenol they demonstrate greater decrements in contractile function compared to young myocytes. We explored the role of alterations in substrate metabolism on age-related changes in contractile function of isolated left ventricular myocytes from Young and Old FBN rats. Contractile properties were measured in single, intact myocytes that were continuously stimulated in Ringers solution containing either Glucose (11mM) or Oleate (0.4mM) as the fuel substrate. Myocytes from young hearts had better contractile function when glucose was the substrate, with a smaller workload-dependent decline in function, compared to when oleate was the substrate. In aged myocytes, the opposite was true, with old myocytes showing less workload-dependent decline in contractile function when oleate was the substratem compared to glucose.We determined gene expression differences in hearts of Young (6 mo), Old

(33 mo), and old exercise trained (Old + EXE) (34 mo) FBN rats, using Qiagen PCR arrays for Glucose, Fatty acid, and Mitochondrial metabolism. Old rats demonstrated decreases ($p < 0.05$) for key genes in fatty acid oxidation, mitochondrial function, and AMP-activated protein kinase (AMPK) signaling. There were no differences in the expression of genes involving glucose metabolism with age. These gene expression changes appear to occur prior to altered protein translation as we found no differences in the protein content of peroxisome proliferator activated receptor gamma, coactivators 1 alpha (PGC-1α), peroxisome proliferator activated receptor alpha (PPARα), and AMPKα_2 between young and old hearts. Four months of exercise training was unable to attenuate the decline in the gene expression changes in aged hearts. Despite this lack of change in gene expression, exercise-trained rats demonstrated increased exercise capacity compared to their sedentary counterparts. Taken together, these data suggest when old cardiac myocytes are stressed they demonstrate contractile dysfunction which is related to altered metabolism or mitochondrial function, while the molecular mechanisms involved with these changes are unequivocal and are likely due to changes in protein activity.

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INTRODUCTION

In 2015, there are over 40 million Americans that are 65 years and older, which represents ~ 13% of the total U.S. population. By the middle of the century, the number of people aged 65 or older is expected to rise to nearly 88 million, which is estimated to be about one quarter of the total U.S. population[1–4]. The incidence of heart failure is directly related to age [5]. Heart disease is the leading cause of death for both men and women and is a major cause of disability for the aged population[6]. For example, approximately 610,000 Americans die from heart disease each year[6]. In 2015, heart disease was projected to cost $320.1 billion, including health care services and lost productivity[7]. Therefore, an understanding of the impact aging has on cardiac function is critical to the prevention of cardiovascular disease in the fastest growing segment of our population.

The mammalian heart is characterized by its plasticity, in that cardiac structure and function change in response to aging and pathology. Progressive remodeling of the myocardium involves complex and interrelated processes in myocytes at the cellular and subcellular level[8,9]. With aging, the number of myocytes decreases and the area where cell death occurred is replaced by collagen, a process known as fibrosis[9–11]. The remaining, viable myocytes increase in size[8,9,12–14]. There are notable functional changes to the remaining myocytes, that compromise contractile function[11,14–20]. The interrelationship between myocyte loss and the remaining myocytes may be due to metabolic changes in the cell. Apoptosis involves mitochondrial dysfunction with age[21,22] and metabolic changes involves altered mitochondrial function[23–26]. The relationship between mitochondrial function and substrate metabolism is demonstrated by the finding that nearly all (>95%) of the ATP for contractile work is derived from oxidative phosphorylation[27]. This review summarizes the results of studies that have investigated: 1) the effect of age on cardiac function,

2) cardiac metabolism, how metabolism is altered with age and the effects of substrate on contractile function, and 3) the possible molecular mechanisms involved with altered cardiac metabolism in aging and how exercise might mitigate these changes.

Cardiac Aging

Aging is associated with a decline in cardiac function in humans as well as rodents[28]. The aging rat heart shows several structural and functional changes in both systolic and diastolic function[11,12,14,20]. Age-related changes in the myocardium may have two separate but related mechanisms a) changes in myocardial structure due to myocyte death/loss or b) changes in the functional capacity of the remaining myocytes[9,29].

Cell loss

Remodeling of the aging heart involves a significant loss of cardiac myocytes (~30% fewer than young hearts), reactive hypertrophy of the remaining cells, and increased connective tissue[8,12,14,30]. Altered function may, in part, be the result of increased ventricular stiffness related to fibrosis and increased collagen content[11,31]. Indeed, the loss of ventricular compliance has been demonstrated by an increase in the end-diastolic pressure-volume relationship (EDPVR) slope with age[20]. Apoptosis (i.e., programmed cell death), is one of the mechanisms for the age-related loss of myocytes[32]. The mitochondria play a key role in regulating apoptosis. There are numerous death-inducing signals, such as reactive oxygen species (ROS), which can trigger mitochondria to release apoptogenic proteins such as cytochrome c and apoptosis inducing factor (AIF) into the cytosol[22,33]. A recent study has demonstrated that the specific compartmentalization of apoptogenic proteins implies that mitochondrial mediated cell death signaling is highly active in the aged myocardium, which may contribute to progressive cardiac pathophysiology[21]. The roles of autophagy and necrosis have also been demonstrated to be

important mechanisms in cardiomyocyte loss with age[10,34,35]. The reduction in the number of cardiomyocytes undoubtedly contributes to the decline in cardiac functional capacity in aged animals. The loss of cardiomyocytes with age also increases the mechanical burden on the remaining myocytes.

Functional changes in the remaining myocytes

A significant contribution to decreased ventricular function is due to age-related changes in myocardial contractile function. Pacher et al[20] demonstrated decreases in the end-systolic pressure-volume relationship slope (ESPVR), stroke work and the dP/dt_{max} (rate of pressure development). The rate of relaxation or $-dP/dt_{max}$ has also been observed to decrease with age [11,14]. In addition, action potential duration, Ca^{2+} transient duration and contraction time are prolonged with age[15,16]. The changes in action potential duration, Ca^{2+} transient, and contraction time are in part due to reduced inactivation of the L-type Ca^{2+} current, I_{Ca} and reduced peak transient outward K channel current, I_{TO}[36].

The molecular mechanisms for the contractile changes may in part involve changes in Ca^{2+} handling by the sarcoplasmic reticulum (SR). Reuptake of Ca^{2+} into the SR by the SR Ca^{2+} ATPase (SERCA) is primarily responsible for the decline of the intracellular Ca^{2+} transient during the activation of the myocardium. Aging is associated with a decrease in SERCA content[17] of rat myocardium, decreased SERCA activity[37], and a depressed rate of Ca^{2+} sequestration into the SR[38].

Isolated cardiac myocytes have been used to study the cellular basis for age-related alterations in ventricular function. Results from shortening measurements in intact , electrically stimulated ventricular myocytes indicate a prolonged time to peak shortening[39] and decreases in percent shortening and rate of shortening (dL/dt)[40]. Maximal unloaded shortening velocity and the rate

of tension development were reported to decrease with aging in skinned myocytes[18]. These latter results are likely due to the progressive increase in β-MHC isoform expression and reduced α-MHC isoform expression that occurs with aging in rodents[18]. Since maximal unloaded shortening velocity is determined by the MHC isoform and its ATPase activity, loaded shortening velocity and power output measurements in a cellular setting are important determinants of the ability of the heart to perform external work. With age there is a reduction in peak power output, which is defined by the velocity at peak power output[19].

Mitochondrial changes with aging

In addition to the above age-related changes in Ca^{2+}-handling and contractile proteins, there is also evidence of age-related changes in mitochondrial function in the myocardium. Since the myocardium derives nearly all of its energetic needs from the oxidation of pyruvate and fatty acids within the mitochondria, mitochondrial changes have great potential to contribute to cardiac dysfunction with age. Progressive impairment of mitochondrial function is a hallmark of aging[41,42]. Mitochondrial energetics deficiency has been widely documented in heart failure in both human patients and mouse models[43]. The mechanisms may include biogenesis that is inadequate to match the increasing demand[44], and increased mitochondrial uncoupling and decreased substrate availability[45]. Besides mitochondrial energetic deficiency playing a role in altered myocardial function, severe mitochondrial dysfunction plays a critical role in apoptosis[32,43].

The myocardium is a vital organ with high metabolic demand and rich in mitochondria, it is especially vulnerable to mitochondrial oxidative damage. The mitochondrial free radical theory of aging proposes that mitochondrial ROS attack mitochondrial components, causing mitochondrial DNA (mtDNA) damage and mitochondrial dysfunction. Mitochondrial

dysfunction is followed by a vicious cycle between increased mitochondrial damage and further ROS production, which causes functional decline of cells and organ systems that eventually lead to death[46]. Indeed, mitochondrial ROS production in the heart has been reported to increase with age[47,48]. Membrane poly-unsaturated fatty acids (PUFAs) undergo lipid peroxidation by ROS, producing various lipid peroxide products[49]. Some of these lipid peroxides (i.e. 4-hydroxy-2-nonenal (HNE)) attack protein sulfhydrl groups to induce altered protein conformation[49]. HNE modification of mitochondrial protein occurred in hearts isolated from senescent rats, but not in young hearts[50]. Furthermore, exposure of intact cardiac mitochondria to HNE resulted in a decline in NADH-linked respiration[51], due, in part, to HNE inactivation of α-ketoglutarate dehydrogenase and pyruvate dehydrogenase[51,52]. Several studies have documented age-dependent impairment of mitochondrial function, primarily a decline in mitochondrial respiratory capacity (state 3) due to diminished activity of complexes I and IV, with complexes II, III, and V relatively unaffected[53,54].

Alterations to the mtDNA have also been observed during the aging process. There are increases in mtDNA deletion mutations and the number of cardiomyocytes that express electron transport system (ETS) abnormalities with age[14]. A number of deletions can occur, the most commonly studied in humans is a deletion mutation that removes all or parts of the genes for complexes I, IV, and V of the electron transport chain[41]. Since the myocardium derives almost all of its ATP for contractile work from oxidative phosphorylation of glucose and fatty acids, and there is significant evidence that the mitochondria within the remaining myocytes have impaired mitochondrial enzyme activity, it is plausible that altered mitochondrial function contributes to contractile dysfunction in the aged myocardium. While it seems clear that age is associated with

significant changes in myocardial mitochondria, the effect of these changes on cardiac function is not well understood.

Cardiac metabolism

One possible effect of age-related decreases in mitochondrial function is a fundamental change in cardiac energy metabolism. Under non-ischemic conditions almost all of ATP formation in the heart is generated from oxidative phosphorylation in the mitochondria, with the remainder derived from glycolysis and guanosine 5'-triphosphate (GTP) formation in the tricarboxylic acid (TCA) cycle. The heart has a low ATP content and high rate of ATP hydrolysis; hence, there is complete turnover of the myocardial ATP pool approximately every 10s under normal conditions[27]. Approximately 60-70% of ATP hydrolysis fuels contractile work, and the remaining 30-40% fuels the sarcoplasmic reticulum Ca^{2+}-ATPase and other ion pumps, such as Na/K-ATPase[55–57]. Mitochondrial oxidative phosphorylation is fueled by electrons transferred from carbon moieties by oxidation/reduction reactions that generate NADH and $FADH_2$ produced primarily from the β-oxidation of fatty acids, the TCA cycle, and less extensively the pyruvate dehydrogenase (PDH) reaction and glycolysis. The rate of oxidation of carbon fuels, NADH and $FADH_2$ reduction, flux through the electron transport chain, oxygen consumption, oxidative phosphorylation, ATP hydrolysis, actin-myosin interaction, and external contractile power are exquisitely united (Fig 1). Thus an increase in contractile work results in an increase in all of the constituents of the system[27].

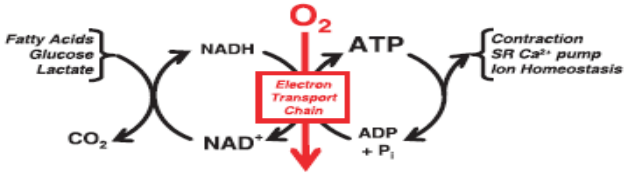

Figure 1: Linkage between cardiac power, ATP hydrolysis, oxidative phosphorylation, and NADH generation by dehydrogenases in metabolism. Adapted from[27].

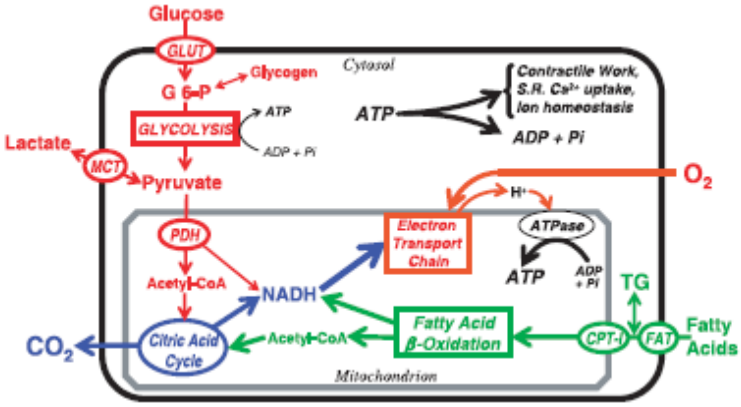

Figure 2: The pathways and regulatory points of myocardial substrate metabolism. Adapted from[27].

The regulation of myocardial metabolism is linked to hormone concentrations, inotropic state, coronary flow, arterial substrate concentration, and nutritional status of the myocardial tissue[58–61]. In healthy hearts, the rates of flux through metabolic pathways resulting in ATP formation are set by the external power generated by the myocardium and the rate of ATP hydrolysis (Fig 2). The rates of flux through metabolic pathways are controlled at the level of gene expression and translation of key metabolic proteins as well as complex regulation exerted by phosphorylation, allosteric compounds and substrate/product relationships.

Carbohydrate metabolism

The glycolytic pathway converts glucose-6-phosphate and NAD^+ to pyruvate and NADH and nets two ATP for each molecule of glucose. Pyruvate and NADH can be shuttled into the mitochondria to generate CO_2 and NAD^+ and complete the process of full oxidation via the TCA cycle and the ETS; or converted to lactate and NAD^+ in the cytosol[62].

The fate of pyruvate

Pyruvate from glycolysis can have several fates: 1) conversion to lactate, 2) oxidative decarboxylation to acetyl CoA, 3) carboxylation to oxaloacetate or malate. Pyruvate decarboxylation is the key irreversible step in carbohydrate oxidation and is catalyzed by pyruvate dehydrogenase (PDH). PDH is highly regulated, primarily by phosphorylation/dephosphorylation by pyruvate dehydrogenase kinase (PDK) and PDH

phosphatase [63,64]. In addition to lactate formation and oxidation by PDH, pyruvate enters the TCA cycle via carboxylation to malate or oxaloacetate[65]. These anaplerotic reactions maintain the pool size of TCA cycle intermediates[66].

Fatty Acid Metabolism

The rate of fatty acid uptake into cardiomyocytes is primarily determined by the concentration of non-esterified fatty acids (NEFAs) in the plasma[67], and secondarily by fatty acids released from chylomicrons and very-low density lipoproteins hydrolyzed by lipoprotein lipase in capillary endothelial cells[68]. Once in the cytosol the fatty acid is activated to long-chain acyl-CoA which can either be esterified and packaged as a triglyceride or converted to long-chain acylcarnitine by carnitine palmitoyltransferase 1 (CPT-1). CPT-1 is highly regulated by malonyl-CoA, which binds to CPT-1 on the cytosolic side of the enzyme[69]. CPT-1 exists on the outer mitochondrial membrane, and once its product acylcarnitine is made, the acylcarnitine is translocated into the mitochondrial matrix by carnitine acyltranslocase (CAT) in exchange for free carnitine. Once the long-chain acyl CoA is in the matrix, a four-step process known as β-oxidation occurs that produces NADH, $FADH_2$, and acetyl-CoA during each cycle.

Substrate competition in the myocardium

At rest, fatty acids are the primary substrate utilized for ATP supply in the myocardium and glucose uptake and oxidation is inversely related to arterial fatty acid concentration[70]. FAs have been seen to be the predominate fuel in isolated myocytes as well[71,72]. The reciprocal regulation of fatty acids and glucose is known as the glucose/fatty acid cycle or the Randle cycle, which has been describe in classical works[62,63,73–75]. The primary regulator of fatty acid inhibition of glucose oxidation comes at the level of PDH. High rates of fatty acid oxidation increase acetyl-CoA/CoA, NADH/NAD, which activates PDH kinase leading to concomitant phosphorylation

and inhibition of PDH. Secondly, excess citrate formation in the TCA cycle during high rates of fatty acid oxidation is inhibitory to phosphofructokinase-1 (PFK-1), which is a regulated step of glycolysis[75]. Conversely, inhibition of fatty acid oxidation increases glucose and lactate uptake and oxidation by decreasing levels of citrate in the cytosol and decreased inhibition of PFK-1. Also, lowering the levels of acetyl-CoA and NADH in the mitochondrial matrix will relieve inhibition on PDH[76]. The control of glucose-inhibition of fatty acid oxidation in heart muscle appears to require the aid of insulin. When glucose infusion occurred prior to exercise this produced hyperinsulinemia, which inhibited palmitate oxidation at the level of CPT-1[77]. Lactate uptake and oxidation is also enhanced when its plasma concentration increases, and this reduces the oxidation of other substrates[78,79].

Creatine Kinase and its impact on cardiac contractile function

ATP-requiring reactions are inhibited by the accumulation of the products of ATP hydrolysis, namely ADP and P_i. Furthermore, the heart uses energy reserve systems to maintain a high phophorylation potential ([ATP]/[ADP][P_i]), which promotes a favorable free energy change (ΔG_{ATP}) to drive ATPase reactions during variations in work output. The primary energy reserve compound in the heart is phosphocreatine (PCr)[80]. Creatine Kinase (CK) has several isoforms that are found within both cytosolic and mitochondrial compartments, and catalyze the reaction: PCr + ADP $\leftrightarrow$ Cr + ATP[81]. A study using cardiomyopathic hamsters demonstrated decreases in ATP, PCr, Cr, and creatine kinase (CK) enzyme activity which was accompanied by decreased contractile performance. Treatment of cardiomyopathic hamsters with enalapril (angiotensin converting enzyme inhibitor used to treat hypertension), increased phophoryl transfer through CK and improved contractile performance[82]. These results suggest that decreased CK flux leads to decreased capacity for ATP synthesis and may lead to attenuated contractile performance.

Another study demonstrated that hamsters with heart failure have an impaired contractile reserve (defined as the ability to increase the rate-pressure product -RPP), and that this is linearly related to the product of total CK activity (V_{max} (maximum velocity of an enzymatic reaction) and [Cr]). Thus, failing hearts had lower energy reserve and lower contractile reserve[83]. One of the functions of CK is to maintain a low [ADP]. The possibility that high cytosolic [ADP] is sufficient to slow dissociation of the cross-bridges enough to slow relaxation in the intact heart has been investigated[84,85]. In the normal heart, this was accomplished by chemically inhibiting CK to varying degrees[84]. In the hypertrophied heart, contractile dysfunction was the result of perturbations in the CK-PCr that occur during hypertrophy and failure[85]. In both settings, a direct relationship between increased LV end diastolic pressure and increased [ADP] was found. Taken together, these data demonstrate that increases in the average cytosolic [ADP] is sufficient to slow cross-bridge cycling and contribute to overt diastolic dysfunction. One of the aspects not explored in these aforementioned studies, is the consequence of slowed cross-bridge cycling on the rate of myocardial pressure development or the rate of shortening. Secondly, much of what we know about the consequences of altered metabolism have been studied in the context of altered CK activity and overt cardiac disease, but what is still unknown is what other aspects of cardiac energy metabolism dysfunction may ultimately contribute to contractile dysfunction in the setting of aging.

Effects of aging on cardiac metabolism

There is some evidence that cardiac metabolism changes with age[24–26,86], but there is very little work describing the metabolic effects of aging alone, without the presence of other pathologies. Changes to cardiac metabolism have also been seen in cardiac hypertrophy and models of heart failure and these changes appear to be somewhat similar to age-related changes.

Therefore, changes in cardiac metabolism observed in these disease models are often used to provide some insight into what may be occurring with age and make up for the paucity of information on aging *per se*.

A re-expression of some of the fetal metabolic genes has been demonstrated in heart failure[87–89]. During the fetal stages of the developing mammalian heart, glucose is the primary substrate for energy production, while rates of fatty acid oxidation are low[90]. In the fetal and immediate newborn heart, there is a reliance on glycolysis due to higher activities of enzymes in the glycolytic pathway[91]. In the hypertrophied heart there is an increase in glycolysis that is uncoupled from glucose oxidation[92,93]. One other important finding in heart failure/hypertrophy is that, along with an increased reliance on glycolysis uncoupled to glucose oxidation there is the upregulation of genes involved in anaplerosis (enzymes that produce TCA cycle intermediates from other substrates) to maintain TCA cycle flux[94,95]. In the hypertrophied setting, this leads to decreased dP/dt_{max} and the rate-pressure product (RPP)[94].

Some of these similar metabolic phenotypes have been observed in aging. In a recent study comparing young and old (29 v 69 yrs of age) subjects, Positron Emission Tomography (PET) was used to show that older hearts reduce fatty acid uptake and oxidation without an increase in glucose utilization compared to young[86]. These results indicate that older humans have an attenuated ability to utilize fatty acids, and rely proportionally more heavily on glucose, yet glucose utilization itself did not increase. Studies in the aged Fischer 344 rats have indicated similar effects to these studies of human aging[24,25]. A study done on the isolated, perfused rat heart demonstrated that with age there was a decrease in cardiac efficiency (cardiac power/MV_{O2}) along with reduced fatty acid oxidation when normalized to heart tissue mass[25]. McMillin et al[24] observed a decrease in palmitoylcarnitine oxidation, CPT-1 activity, and

carnitine exchange in isolated cardiac mitochondria from aged Fischer 344 rat hearts. Another important finding from this study was that in adult hearts the normal Randle cycle was observed (fatty acid inhibition of glucose uptake), however, in the aged hearts there was no decrease in glucose utilization seen when oleate was present.

It appears that age is associated with decreases in FA oxidation with an increased reliance on glucose. However, one study on aged Wistar rats, demonstrated an increase in fatty acid oxidation with no change to glucose oxidation. Lactate oxidation was significantly reduced in this study[26]. These data confound the widely assumed notion of reduced fatty acid oxidation with age, and demonstrates the need for further exploration of substrate utilization and substrate effects on contractile function in aged hearts. It has been postulated that the altered substrate metabolism is considered adaptive or necessary to maintain function in the aged or pathological environment. Specifically, in terms of oxygen cost, glucose oxidation is more efficient (~3.1 ATP/O) than that of FAs (~2.8 ATP/O)[96]. Also, cardiac overexpression of Glut1 in the myocardium slowed the development of heart failure in a model of pressure overload hypertrophy[97]. These results suggest that increasing glucose utilization may be a beneficial adaptation. However, it has been demonstrated that reductions in fatty acid oxidation in the hypertrophied setting preceded mitochondrial and contractile dysfunction[23]. The finding from Doenst et al[23] suggests that the attenuation of FA oxidation and increased reliance on glucose oxidation may be maladaptive. Thus the question of whether changes in substrate metabolism are adaptive or maladaptive is currently unclear in the aged setting.

Effects of Substrate on Cardiac Contractile Function

As summarized above, it appears that with age there is altered cardiac metabolism, but whether or not changes in substrate utilization may affect contractile function with age has not

been clearly delineated. However, there is evidence to suggest substrate-specific effects on contractile performance of the myocardium in young animals and in models of disease. A few studies demonstrated that at a given MVo_2, cardiac power is greater when the heart is oxidizing more glucose and lactate, and less fatty acids[98–101]. A study in isolated rat hearts demonstrated that the ESPVR and RPP at a given MVo_2 was decreased when fatty acids rather than glucose was the sole substrate[101]. A classical study by Mjos demonstrated that, in dogs, increasing the rate of fatty acid uptake by the heart with infusion of heparin (lipoprotein lipase activator) and triglyceride emulsion enhanced oxygen consumption in the heart by 26% without changing the dP/dt[98]. These data suggest that fatty acids reduce the cardiac efficiency. The proposed mechanisms behind fatty acid-induced decreases in cardiac efficiency are thought to be, in part a lower ATP/O_2 ratio, meaning fatty acids require more oxygen per molecule of ATP production compared to glucose[101]. Secondly, fatty acids may waste ATP and hence O_2, through the extrusion of long-chain fatty acids out of the mitochondria via uncoupling protein 3 (UCP3)[102]. However, the extrusion of fatty acids from the mitochondria via UCP3 may only be essential when fatty acid uptake and utilization is high, such as diabetes and fasting.

There is conflicting evidence that fatty acids reduce contractile performance[58,103]. Willebrands and Van der Veen[103] showed that fatty acids were the only substrate to maintain contraction amplitude and frequency throughout the duration of a 60 minute perfusion protocol. Neely et al[58] demonstrated that when glucose was the sole substrate, increased cardiac work was associated with a reduced phosphate potential and a reduction in the ability to supply carbon substrates to the TCA cycle. Secondly, when palmitate was added to the perfusate with glucose, energy production was not substrate limited, palmitate oxidation increased linearly with oxygen consumption, and peak pressure developed by the heart could be maintained much longer. This

finding suggests that fatty acid oxidation is necessary for the maintenance of energy supply meeting energetic demands of the myocardium.

The functional effects of increased fatty acid oxidation were explored using a cardiac-specific Acetyl CoA Carboxylase knockout ($ACC2^{-/-}$) in a model of pressure overload hypertrophy. In this study, not only was fatty acid oxidation increased and glucose oxidation decreased, the hearts demonstrated remarkable differences in cardiac structure and function compared to control hypertrophied hearts. In particular, fractional shortening, (RPP) and (PCr/ATP) were all improved, while cardiac fibrosis, myocyte size, and contribution of substrates to anaplerosis were all decreased in the $ACC2^{-/-}$ hypertrophied hearts compared to control hypertrophied hearts[104]. These results suggest that enhanced fatty acid oxidation rates throughout the life of the transition from healthy to hypertrophy results in attenuation of the pathological state by improving several aspects of cardiac structure and function. In another hypertrophy model, there was an increase in malic enzyme which ultimately, decreased flux through PDH. However, when dichloroacetate (a known activator of PDH) was added to the perfusate, flux through malic enzyme decreased and pyruvate oxidation increased, normalizing the RPP and dP/dt_{max}[94]. This study provides evidence that alteration in cardiac metabolism has a profound impact on contractile function of the heart.

Doenst et al[23] in an elegant study illustrated that substrate oxidation, mitochondrial function and contractile function are inextricably linked. This study assessed in vivo and ex vivo cardiac function during the progression of pressure-overload hypertrophy. Cardiac contractile function, substrate oxidation, and mitochondrial function were all assessed after 2, 6, 10, and 20 weeks of aortic banding. Following aortic banding, impaired fatty acid oxidation preceded contractile and mitochondrial dysfunction, however, mitochondrial dysfunction occurred in parallel to reduced

ejection fraction. These findings suggest that changes in substrate utilization may occur during the compensated stage of hypertrophy, and mitochondrial and contractile dysfunction is a process that occurs over time. Lastly, substrate oxidation changes without contractile dysfunction may be a marker where therapeutic intervention may be of benefit to the myocardium.

There is some cellular evidence for substrate-specific effects on cardiomyocyte shortening properties and Ca^{2+} handling[105,106]. In both of the aforementioned studies, an acute switch (five minutes of incubation in palmitate) to palmitate perfusion of the cardiomyocytes produced a decrease in percent shortening as well as a reduction in the Ca^{2+} amplitude [105,106]. In Haim et al[105], palmitate-induced decreases in percent cell shortening were accompanied by an increase in repolarizing Kv (voltage-gate potassium) currents. The increase in Kv currents reduced action potential duration and Ca^{2+} amplitude. Fauconnier et al[106] demonstrated that the effect of palmitate-induced negative inotropy was precipitated by ROS, since antioxidants abolished the palmitate-induced decreases in cell shortening and Ca^{2+} amplitude.

Therefore, there is data to suggest that there are substrate-specific effects on cardiac contractile properties. However, most of the data comes from in vivo or isolated perfused whole hearts, and there is very little information on substrate effects on cellular contractile properties. Importantly, we do not know from the previous literature whether contractile effects observed with substrates are due to changes in Ca^{2+} handling alterations, cross-bridge cycling or due to changes in energy availability (i.e., metabolism). Also, in whole heart studies there are multiple substrates in the perfusate and therefore, it is difficult to surmise how each substrate impacts contractile function. There have only been two studies to date that have approached substrate-

specific effects at the cellular level, and none of the cellular studies have been performed on aged cardiomyocytes.

Molecular mechanisms involved in altered metabolism with age

The molecular mechanisms associated with altered cardiac metabolism have been explored in a number of studies[40,107–109]. Importantly, the reduction in fatty acid uptake and oxidation seen in various studies of aging coincides with a finding of decreased mRNA and protein content of PPARα in old rat hearts[107]. PPARα is a nuclear transcription factor which upregulates genes involved in fatty acid oxidation[110]. Along with a reduction to PPARα expression with age, two gene targets for PPARα; β-hydroxyacyl dehydrogenase and CPT-1 mRNA were reduced with age[107]. Furthermore, it has been shown that peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is reduced in the aged left ventricle[40]. PGC-1α is a transcriptional coactivator of PPARα and also is known to stimulate mitochondrial biogenesis[111]. One mechanism for age-related changes in substrate metabolism may be related to altered AMPK activity. There is evidence that AMPK activity declines with age[40,108,109]. AMPK activity is known to increase glucose and fatty acid catabolic processes, thus improving energy liberation in the setting of metabolic stress[112–118]. Reduced AMPK activity was associated with reduced GLUT4 and PGC-1α expression in the heart[40], and reduced mitochondrial biogenesis in skeletal muscle[109].

Effects of exercise training on cardiac metabolism and mitochondrial biogenesis

The majority of studies probing the effects of exercise on changes in cardiac metabolism have been performed in young rodents[119–122]. The general consensus from these studies is that exercise training increases or does not change the gene expression and protein content of PGC-1α, GLUT4, PPARα and some of its downstream genes involved in fatty acid oxidation (CPT-1b,

CD36, FATP1, MCAD)[119–122]. In a few of these studies, investigators examined the effects of pathological pressure-overload hypertrophy in young rodents and saw no changes in the expression of PGC-1α and PPARα along with associated genes involved in fatty acid oxidation[119,121]. However, one study demonstrated that although there were no changes in the expression of PGC-1α and PPARα, GLUT4 and PDK4 expression were decreased, suggesting an impairment in glucose metabolism[121]. One other study that assayed gene expression changes in pathological hypertrophy demonstrated that genes involving fatty acid oxidation and the glycolytic pathway were increased[122].

One study that looked at the impact that exercise training had on aged Wistar rats demonstrated increases in PPARα gene expression, protein content and DNA binding activity with regular swimming exercise[107]. These findings were associated with increases in downstream PPARα targets, such as CPT-1 and 3-hydroxyacyl CoA dehydrogenase (HAD), along with increases in citrate synthase and cytochrome c oxidase (COX) activity.

There are other proteins that impact cardiac metabolism that can be affected by exercise training, such as phosphorylated AMPK (p-AMPK) and phosphorylated Acetyl CoA Carboxylase (p-ACC). It is well known that p-AMPK increases fatty acid oxidation by phosphorylation of ACC, which normally acts to convert acetyl CoA to malonyl CoA (known inhibitor of CPT-1), but with phosphorylation of ACC, malonyl CoA levels are reduced and this activates CPT-1. A study examining exercise training effects in the heart demonstrated increases in the ratio of p-AMPK/AMPK and p-ACC/ACC, suggesting that young male rats may have improved fatty acid oxidation capacity[119]. There is a paucity of information examining the effects of exercise training on cardiac metabolism and mitochondrial gene expression changes in aged

hearts and even less is known regarding the extent to which the effect exercise has on glucose and fatty acid metabolism, and mitochondrial metabolism/biogenesis.

SPECIFIC AIMS AND HYPOTHESES

Myocardial contractile function is diminished with age and contributes to cardiac morbidity and mortality in the elderly[50,123]. A number of factors contribute to the pathogenesis of cardiac aging such as; action potential prolongation, altered myosin heavy chain isoform expression, mitochondrial defects and free radical accumulation, and Ca^{2+}-handling dysregulation[50,123]. Accumulating evidence points to a role of altered cardiac energy metabolism in the onset and development of cardiac aging[24–26,124,125], but the precise nature of how these changes in cardiac fuel utilization impacts the aging heart has not been clearly determined. Aging appears to be associated with changes in glucose versus fatty acid metabolism, but it is unclear how changes might contribute to altered contractile function.

Most studies examining the effects of variations in substrate utilization have been performed in whole heart preparations. Thus, it is difficult to surmise the impact substrates have on altered contractile function. The study of cellular contractile function may yield additional insights into the mechanisms and consequences of age-related changes in cardiac metabolism.

Mitochondrial dysfunction has been observed with aging[14,22,46,126–128]. For example, a few of these mitochondrial defects are mitochondrial deletions and enzymatic abnormalities[14], reduced ADP-stimulated respiration[126,128], and cytochrome c release[22]. The connection between substrate metabolism and mitochondrial function resides in the fact that almost all of the myocardial cellular ATP is derived from oxidative phosphorylation by the oxidation of pyruvate and fatty acids[27]. However, most studies involved with age or pressure-overload hypertrophy have demonstrated decreases in mitochondrial function and contractile function in parallel[14,23], no studies to date have directly explored the relationship between contractile function and substrate or mitochondrial metabolism.

Previous studies have determined some of the possible molecular mechanisms involved with altered mitochondrial function, and changes to glucose and fatty acid metabolism, specifically altered levels of PPARα and PGC-1α [40,107] and changes in AMPK signaling that may impact glucose and fatty acid metabolism [40,108,109,129]. However, few studies have addressed molecular mechanisms associated with age-related alterations in cardiac metabolism.

The specific aims and hypotheses are outlined below:

1) Determine the impact that altered mitochondrial function has on contractile function in single isolated young and old rat cardiac myocytes
    - *Hypothesis 1:* After ten minutes of continuous stimulation there will be lower NADH levels in old cardiomyocytes compared to young
    - *Hypothesis 2:* The change in shortening properties over ten minutes of continuous stimulation will be directly related to NADH levels
    - *Hypothesis 3:* There will be a greater change in shortening properties with rotenone in old cardiomyocytes as compared to young.
2) Examine substrate effects on cardiomyocyte shortening properties in isolated, single cardiomyocytes from young and old rats
    - *Hypothesis 1:* There will be no substrate-specific differences on shortening properties in young cardiomyocytes
    - *Hypothesis 2:* Shortening properties will be attenuated in old cardiomyocytes perfused with either substrate compared to young
    - *Hypothesis 3:* Oleate –induced decreases in shortening properties will be exacerbated in old cardiomyocytes as compared to glucose
3) Determine the mechanisms of substrate effects on cardiomyocyte shortening properties

- *Hypothesis 1:* There will be a decrease in genes involved in fatty acid oxidation in old rat left ventricular tissue compared to young rats
- *Hypothesis 2:* There will be no differences in genes involving glucose metabolism between young and old rat left ventricular tissue
- *Hypothesis 3:* There will be a decrease in genes involving AMPK signaling in old rat left ventricles compared to young
- *Hypothesis 4:* There will be a decrease in genes involved with mitochondrial biogenesis and mitochondrial energy metabolism in old rat left ventricles compared to young
- *Hypothesis 5:* Exercise training in old rats will ameliorate the age-related declines in genes involved in fatty acid oxidation, AMPK signaling, and mitochondrial biogenesis.

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LINKING METABOLIC AND CONTRACTILE DYSFUNCTION IN AGED CARDIAC MYOCYTES

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INTRODUCTION

Myocardial contractile function is diminished with age and contributes to cardiac morbidity and mortality in the elderly[1,2]. A number of factors contribute to the pathogenesis of cardiac aging such as; action potential prolongation, altered myosin heavy chain isoform expression, mitochondrial defects and free radical accumulation, and Ca^{2+} handling dysregulation[2,3]. Recent evidence points to a role of altered cardiac energy metabolism in the onset and development of cardiac aging[4–8], but the precise role of changes in cardiac metabolism in the aging heart has not been clearly delineated. Aging hearts have a demonstrated reduction in enzymes involved in both the TCA cycle and the electron transport chain, and these changes are coupled to a reduction in oxygen consumption and ATP formation[9,10].

Age-related changes in metabolic or mitochondrial function presumably are detrimental in that they lead to changes in myocardial contractile performance, but this link has not been explored in the aging heart. Myocardial work is exquisitely coupled to ATP turnover[11,12].The mammalian heart is capable of increasing energy flux with minimal changes in the concentrations of ATP and ADP associated with energy metabolism. This occurs despite the myocardial capacity to increase pump work 3-fold and the limited capacity of the cells to store chemical energy[13,14]. At high workloads the ATP pool can turn over in as little as 2 seconds[12]. ATP pool turnover is related to the mitochondrial respiratory rate. Therefore, modest changes in cardiac metabolism may have a significant impact on contractile function. Studies in pressure overload hypertrophy demonstrated that changes in energy metabolism occur in parallel or precede contractile dysfunction in models of pressure-overload hypertrophy[15–17]. Furthermore, enhancing mitochondrial substrate metabolism, either pharmacologically or genetically, has been shown to mitigate the decline in contractile function in pressure-overload hypertrophy[16,17]. However, there

is very little work describing the impact of altered energetics on contractile function in aging alone, without the presence of other pathologies.

In this study we used contractile performance of intact isolated myocytes to determine the relationship between contractile changes and metabolic changes. In order to focus specifically on contractile consequences of altered metabolic function, we measured contractile properties (peak shortening, maximum velocity of shortening +dL/dt, and diastolic cell length) of cells during ten minutes of continuous stimulation. We hypothesized that declines in cellular contraction over this stimulation period would be reflective of metabolic changes occurring in the cell.

To determine metabolic changes over this time period we measured changes in NADH autofluorescence in contracting myocytes. Measuring NADH fluorescence is a method for evaluating mitochondrial function in intact cells. Mitochondrial respiratory rate has a linear relationship with NADH levels, thus increases in NADH formation result in increases in respiratory rate[12]. Since NADH levels are linearly related to mitochondrial respiratory rate, any decrement in NADH levels would result in a reduction in ATP formation by the mitochondria and an inability to maintain an appropriate ATP pool for the maintenance of cardiac work. In this study we used changes in NADH fluorescence during ten minutes of continuous stimulation to monitor changes in metabolic status over that time. Our overall hypothesis was that contractile performance would decline to a greater extent during continuous stimulation in old myocytes compared to young myocytes, and this decline would be related to decreased metabolic function as measured by NADH autofluorescence.

METHODS

Animals. 6 month & 33 month old male Fischer 344 x Brown Norway hybrid rats (FBN) were obtained from the National Institute on Aging colony at Harlan Industries (Indianapolis, IN). The FBN hybrid rat is a long-lived strain with a median life-span of 33 months and a maximum life-span of 40 months. The FBN rat is considered a 'healthy aging model' widely used and highly recommended for gerontological research. All rats were confined to standard size rodent cages and housed 2 rats per cage. Rats had access to food and water *ad libitum* and were acclimated to reverse daylight (12h dark, 12h light). Animal housing and handling was carried out under the guidelines of the University of Wisconsin-Madison Institutional Animal Care and Use Committees and conducted in pathogen-free facilities that are accredited by the American Association of Accreditation of Laboratory Animal Care.

Cardiomyocyte Isolation. Rats were terminated using isoflurane/ pheumothorax euthanasia. Hearts were rapidly removed from the rats and mounted onto a temperature-controlled (37° C) Langendorff system[18–20]. Hearts were perfused with Ringer's solution in mM: 118 NaCl, 4.8 KCl, 1.2 NaH_2PO_3, 25 HEPES, 11 glucose, 1.2 $MgCl_2$, 1 $CaCl_2$, at pH 7.4 and gassed with 5% CO_2 – 95% O_2. After perfusing with Ca^{2+} Ringers for 3 min, the hearts were digested with Ringers (Ca^{2+} free) containing type II collagenase and hyaluranidase for 25 min. The digested heart was removed from the cannula, and the ventricles cut into smaller pieces and placed in a 37° C water bath for another 25 minutes of digestion in Ringers (Ca^{2+} free) containing type II collagenase and hyaluranidase. Tissue pieces were gently triturated and the suspension centrifuged to pellet the cells. Extracellular Ca^{2+} was added incrementally back to 1mM by centrifugation in Ringers containing first 200 μM Ca^{2+}. Only rod-shaped myocytes with clear sarcomere striations were chosen for contractile studies.

Cell shortening and mitochondrial NADH measurement. Simultaneous cell shortening and NADH fluorescence measurements were performed at 37° C. Cells were placed into a Warner chamber and superfused with glucose Ringers solution (2mM Ca^{2+}) and stimulated at 4 Hz with platinum electrodes. Changes in cell length during shortening were acquired at time 0 (T0) and after ten minutes (T10) of continuous contraction. The change in contractile properties over time was assessed by the arithmetic ratio of (T10/T0). Contractile shortening was assessed using a SoftEdge MyoCam® system (IonOptix Corp., Milton, MA, USA). The myocyte being studied was displayed on a computer monitor using an IonOptix MyoCam camera. An IonOptix SoftEdge software was used to capture changes in cell length during shortening and relengthening. Cell shortening was assessed using the following indices: peak shortening (distance shortened/diastolic cell length * 100), an indication of peak ventricular contractility. The maximal velocity of shortening (+ dL/dt) was calculated as the maximal slope derivative of the shortening phase and is an indication of maximal velocity of ventricular pressure increase.

Mitochondrial NADH autofluorescence $[NADH]_m$. NADH autofluorescence was measured in cells in which contractile measurements were made by using methods similar to those described elsewhere[21–27]. Briefly, cells were illuminated with 340 nm light through a Plan-Flour 40x objective on an Eclipse Ti microscope (Nikon). Similarly, emitted light was passed through the same Plan-Flour 40x objective, and 460-nm emission was measured using IonOptix hardware and software. Both contractile data and emission data were digitized at 250 Hz. To minimize photobleaching and the artifact due to cell contraction, the excitation light was applied for only 10 seconds during contractile measurements at T0 and then again at T10. Since intracellular NADH cannot be calibrated in absolute terms, the intensity of NADH fluorescence was expressed as a percentage of full scale change in the fluorescence in the presence of 5 mM

NaCN to give maximum NADH fluorescence and then in the presence of 5 μM carbonyl cyanide 4 – (trifluoromethoxy) phenylhydrazone (FCCP) to give minimum NADH fluorescence, respectively, at the end of each experiment[22]. NaCN inhibits complex III of the electron transport chain (ETC) and this will result in reduced flux through the ETC and subsequently increase NADH fluorescence measurement. FCCP is a mitochondrial uncoupler which will stimulate flux through the ETC and result in a minimal NADH fluorescence measurement. We used the following equation to calculate NADH levels: % NADH = 100 x (Fmaximum - F)/ (Fmaximum - Fminimum), where F is the fluorescence intensity measured. A representation of the simultaneous measurement of cell length changes and NADH fluorescence is shown in Figure 1.

In order to determine contractile consequences of decreased mitochondrial function, we measured single cell contractile properties in some cells following administration of rotenone, a known inhibitor of mitochondrial oxidative phosphorylation. For these experiments contractile properties of cardiac myocytes were assessed by visualizing the cell through a Hitachi camera, with the video image collected at 60 frames/second. Cell shortening was measured using edge detection software created in LabView (National Instruments, Austin, TX). Cells were placed in a Warner chamber mounted on a stage of an inverted microscope (Olympus, CK 40). After cardiac myocytes contracted initially (T0) in glucose Ringers solution at 2 Hz, we rapidly perfused with a glucose Ringers solution containing 1 μM rotenone in 0.15% DMSO using the MP Series Chamber Manifold (Warner Instruments, Inc.). Rotenone concentration was chosen and modified based on studies of rotenone effects in intact hearts[28]. Cell shortening measurements were recorded (T10) after ten minutes of continuous contraction in rotenone.

For isoproterenol exposure experiments, cells were superfused (1ml/min at 37 ˚C) with glucose Ringers containing 100nM isoproterenol. Isoproterenol concentration was modified from

previous experiments on cardiac myocytes[29–31]. Cells were allowed to incubate in the isoproterenol buffered solution for 5 minutes prior to the acquisition of cell shortening measurements. The cells were field stimulated at a frequency of 2 Hz and changes in cell length were measured at time 0 (T0) and after 5 minutes (T5) of contraction.

Statistical Analysis. Between - group means were analyzed by the Kruskal-Wallis one-way analysis of variance. Correlation analyses were performed using Pearson's rho on cell shortening data. Significance was determined at $P < 0.05$.

RESULTS

Contractile measurements. Contractile properties for young and old cardiac myocytes are shown in Table 1. All contractile properties measured significantly ($p<0.05$) declined over ten minutes of continuous stimulation in both young and old myocytes and there were no differences in the peak shortening, rate of shortening, or diastolic cell length between young and old myocytes at either T0 or T10. The change over time in these contractile properties (T10/T0) was also not different between young and old myocytes.

Mitochondrial NADH fluorescence $[NADH]_m$. $[NADH]_m$ levels in young and old myocytes are shown in figure 2. At T0, there was a significantly higher $[NADH]_m$ in old myocytes compared to young myocytes (Old = 54% of max vs YG = 40% of max). After ten minutes of continuous stimulation, $[NADH]_m$ levels significantly declined in old myocytes while $[NADH]_m$ levels in young myocytes were maintained.

Relationship between NADH and contractile properties. In order to determine the relationship between changes in contractile function and changes in $[NADH]_m$ in myocytes, we plotted the various contractile properties (peak shortening, rate of shortening and diastolic cell length) versus NADH fluorescence. These data are shown in figure 3. In figure 3, we looked at the relationship between NADH at T10 and the change in diastolic cell length over time relative the diastolic length relative to T0 (T10/T0). $[NADH]_m$ during ten minutes of stimulation correlates with peak shortening (Figure 3A) and the rate of shortening (Figure 3B) in old ($p < 0.05$) but not young myocytes. Diastolic cell length declined over the 10 minutes in both groups, however, only old myocytes demonstrate significant relationships ($p < 0.05$) between T10 $[NADH]_m$ and the change in diastolic cell length (Figure 3C).

Effects of rotenone on myocyte contractile properties. Rotenone is an irreversible inhibitor of complex I in the electron transport chain, and we assayed the impact that this metabolic stressor

had on contractile function in old and young myocytes. Rotenone had a greater effect ($p < 0.05$) on the change from the pre-rotenone measurement in the + dL/dt in old myocytes compared to young (Old = 0.76 vs YG = 0.88) when stimulated at 2Hz (Figure 4). The decline in peak shortening (Figure 4) trended towards a greater decrement in old myocytes compared to young (Old = 0.72 vs. YG = 0.85) but this difference was not significant ($p = 0.09$).

Isoproterenol effects on myocyte shortening properties. Isoproterenol (ISO) is a non-selective β – adrenergic agonist, and we administered 100nM ISO to young and old cardiac myocytes throughout the duration of 5 minutes of continuous electrical stimulation in order to increase the contractile workload. Old myocytes demonstrated greater declines in peak shortening and diastolic cell length ($p < 0.05$) over time in the presence of ISO compared to young myocytes (Figure 5). The decline in the + dL/dt over time was not different between young and old myocytes.

DISCUSSION

The primary findings of this study were that a decline in contractile performance over ten minutes of continuous stimulation correlated with a decrease in $[NADH]_m$ levels in old myocytes but not in young. We hypothesized that contractile properties would decline to a greater extent in old compared to young, but found no significant difference between young and old myocytes (Table 1). However, we did find that when the cells were under increased strain, through either the manipulation of decreased mitochondrial function with rotenone or through increased workload with isoproterenol, there was a significant decrease in contractile performance in old compared to young myocytes (Figures 4-5). These results suggest that metabolic dysfunction may play a role in stress-induced contractile dysfunction in aged myocytes.

Baseline contractile performance was not different between old and young myocytes at either T0 or T10, suggesting there is no age-related alteration in fundamental properties of contraction. Contractile properties of isolated myocytes have been studied previously in old versus young myocytes with mixed results. Some studies have demonstrated that aged myocytes have increases in time to peak shortening[18,32], decreased velocity of sarcomere shortening[20], and a greater decrement in peak shortening with step increases in stimulation frequency[33]. In contrast, other reports have demonstrated no age-related differences in contractile function (peak shortening and rate of shortening) in rodents[18,32,33]. Some of the discrepancies in the results of contractile function with age can be explained in part by differences in temperature, stimulation frequency, and the contractile property being studied. We chose to stimulate the cells at a frequency of 4 Hz because it was a stimulation frequency that mimicked resting heart rate in the FBN strain during in vivo cardiac functional analysis[34], and previous studies on intact aging myocytes used subphysiological stimulation frequencies[18,33].

Despite the lack of age-related changes in baseline contractile properties in our study, it seems clear from our data that when the cell is put under metabolic or workload stress, contractile performance declines to a greater extent in aged myocytes compared to young cells. Rotenone is known to have a high affinity for complex I of the electron transport chain and is an irreversible inhibitor of complex I. In one previous study, rotenone was administered to an isolated buffer-perfused rabbit heart and developed pressure declined to 60% of control values[28]. In our study, we found that old myocytes demonstrated increased sensitivity to rotenone treatment as shown by a decreased +dL/dt in compared to young myocytes , suggesting that, in old myocytes contractile function is more sensitive to metabolic disruption.

Isoproterenol is a non-selective β-adrenergic agonist. Observed effects of isoproterenol on cardiac myocytes include: increases in peak shortening/tension[30,35–37], increased rate of shortening/force development[30,35–37] and rate of relaxation[30,35,37], and protein phosphorylation of cardiac TnI[30], phospholamban[30], myosin binding protein C[30], ryanodine receptor (RyR2)[38], and L-type Ca^{2+} channel[39,40]. The net effect of these changes is to increase the work output of the cell. We took advantage of these changes to study contractile performance over time with increasing workload. Aged myocytes showed an augmented decline in peak shortening and diastolic cell length over time compared to young in the presence of isoproterenol. Previous studies have demonstrated reduced myocardial contractile performance during β-adrenergic agonist stimulation with age[30,32,41], but this is the first study to examine differences in contractile performance during continuous stimulation. Taken together, we found contractile functional decline in old versus young myocytes when treated with rotenone to increase metabolic stress and by administering isoproterenol to induce a workload stress on myocytes. These results suggest that metabolic processes may be unable to keep up with the increased demand.

We utilized steady-state NADH autofluorescence measurements to assay mitochondrial function in single cardiac myocytes. The fluorescence of single cells has been previously used as a measure of mitochondrial NADH[22,24–27]. Although single cell fluorescence cannot distinguish between cytosolic and mitochondrial NADH, over 80% of cell autofluorescence in myocytes has been shown to originate from mitochondrial NADH[21,22]. Previous NADH measures in single myocytes focused on changes in NADH with acute changes in workload[22–24,26]. These experiments demonstrated that with an increase in workload, NADH fluorescence initially decreased but then slowly recovered to a new, higher, steady-state[22,25]. Our results demonstrated that steady-state $[NADH]_m$ levels declined in old myocytes over ten minutes of continuous stimulation, whereas, young myocytes maintained steady-state $[NADH]_m$ levels over that same duration. Although, most previous studies have looked at acute changes in NADH, there is one earlier study that did demonstrate that, in young healthy myocytes, steady-state $[NADH]_m$ levels are maintained at a given workload for up to thirty minutes[22]. We also observed a maintenance of [NADH]m levels in young myocytes but not old, suggesting that the decline in steady-state $[NADH]_m$ levels in old myocytes reflects altered mitochondrial function with age.

Most importantly, our results show that, while there were no differences between young and old myocytes in the decline in contractile performance over ten minutes, in old myocytes changes in contractile performance was significantly correlated with changes in $[NADH]_m$ levels. In contrast, young myocytes exhibited no decrease in $[NADH]_m$ levels over time and there was no significant relationship between contractile performance and $[NADH]_m$ levels. Changes in contractile performance of isolated, intact myocytes have been used to study basic properties of numerous cellular components[18–20,32,42,43], but this is the first report of changes over the course of long duration stimulation and their correlation to changes in metabolic status

(NADH).These results suggest that contractile performance decrements is coupled to mitochondrial dysfunction in aged myocytes.

Mechanisms and consequences of altered energetics and mitochondrial function with age

Mitochondrial functional changes have been observed in the aging heart, with changes ranging from altered substrate metabolism[4,6] to mitochondrial DNA deletions and enzymatic abnormalities[44]. Mitochondrial dysfunction has been demonstrated in parallel with age and pressure-overload hypertrophy[15,44], but our study is the first to our knowledge to demonstrate the relationship between mitochondrial function (as measured by $[NADH]_m$) and contractile function by simultaneously measuring both parameters in aged cardiac myocytes. We found that there were significant correlations between declining $[NADH]_m$ levels and decrements to both systolic and diastolic function in aged myocytes. Our results may in part be explained by previous work demonstrating age-related reductions in the enzyme activity of Krebs cycle dehydrogenases (isocitrate, malate, and α-ketoglutarate) in rat myocardium[10]. Previous works suggest that the slow recovery of NADH to steady-state levels is due to the upregulation of NADH formation through the increase in mitochondrial $[Ca^{2+}]$[22,25]. Although we did not measure mitochondrial Ca^{2+} levels, Ca^{2+} uptake into the mitochondria is important for the activation of several mitochondrial dehydrogenases, particularly pyruvate dehydrogenase (PDH), isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase[45]. O'Donnell et al[45] demonstrated that Ca^{2+} influx into the mitochondria is important for favoring oxidation and continued formation of reducing equivalents. Thus, one possible explanation for decreased $[NADH]_m$ in aged myocytes over ten minutes of continuous contraction, could be due to an inability to maintain appropriate mitochondrial Ca^{2+} mediated activation of calcium-dependent dehydrogenases in the mitochondria. In hypertrophic cardiac myocytes, mitochondrial Ca^{2+} levels has been shown to be

reduced which resulted in a decrement in NADPH levels in the mitochondria[46]. The proposed mechanism by these authors describing the decline in mitochondrial Ca^{2+} levels and reduced levels of mitochondrial NADPH is: low mitochondrial Ca^{2+} results in blunted upregulation of Krebs cycle dehyodrogenases, and subsequent reduction in NADH levels. Mitochondrial NADH is known by transhydrogenation to maintain NADPH levels in the mitochondria[46]. However, little is known about the effect of age on mitochondrial Ca^{2+} uptake, thus more research is needed in this area.

One study exploring the mechanisms linking energy metabolism and contractile reserve in isolated perfused rat hearts, demonstrates that graded inhibition of creatine kinase (CK) activity and subsequent reduction of [ATP], reduced the increase in the rate pressure product (RPP) from baseline during high calcium perfusion[47]. Since contractile function is exquisitely coupled to ATP formation and mitochondrial oxidative phosphorylation[11,12], declines in mitochondrial NADH are likely to impact ATP formation, hence, contractile function in aged myocytes.

Tian et al[48] studied the relationship between energetics and diastolic function and demonstrated that left ventricular end diastolic pressure increased as a function of [ADP] in pressure-overload hypertrophy rat hearts. The proposed mechanism for this observation is thought to be reduced velocity of cross-bridge cycling in cardiac myocytes[49,50]. Our results showed that diastolic cell length decreased during the ten minute contraction period and this change in diastolic cell length was correlated with $[NADH]_m$ values after ten minutes in old myocytes. An elevation of [ADP] could provide a potential link between $[NADH]_m$ decrements and decreases in diastolic cell length.

Metabolic reserve is defined as the ability to access any available potential energy in response to increased cardiac work[14]. A decrement of metabolic reserve has been observed as altered fuel

utilization[4,7,51] and a decrease in the [ATP] along with a decrease in phosphocreatine (PCr) - the primary energy buffer in the heart. In the hypertrophic heart, increased workload leads to a decrease in the concentrations of ATP and PCr[52], and this decrease was related to declining contractile function. A similar observation has been demonstrated in aging hearts, where decreases in [ATP] and [PCr], along with attenuated fatty acid oxidation during increases in workload, was associated with a decline in function in the working heart[4]. These findings suggest that the aging heart may be unable to access available potential energy in the form of fuel substrates which may lead to decrements in the concentrations of ATP and PCr, and thus a decrement in the metabolic reserve. Our data suggests that this reduced metabolic reserve in old cardiac myocytes may result in the inability to maintain $[NADH]_m$ during continuous stimulation, and this reduction in mitochondrial function results in a decrease in contractile function.

Conclusion. In summary, old myocytes have similar contractile function under resting conditions compared to young cardiac myocytes. However, old myocytes demonstrate impaired contractile function to a workload (ISO) and metabolic (rotenone) stress compared to young myocytes. Even under a stimulation frequency closely resembling resting heart rate, old myocytes demonstrate decrements in $[NADH]_m$ over time, and these decrements were correlated to contractile decline. These data suggest that when old cardiac myocytes are stressed they demonstrate contractile dysfunction which may be related to altered metabolism or mitochondrial function.

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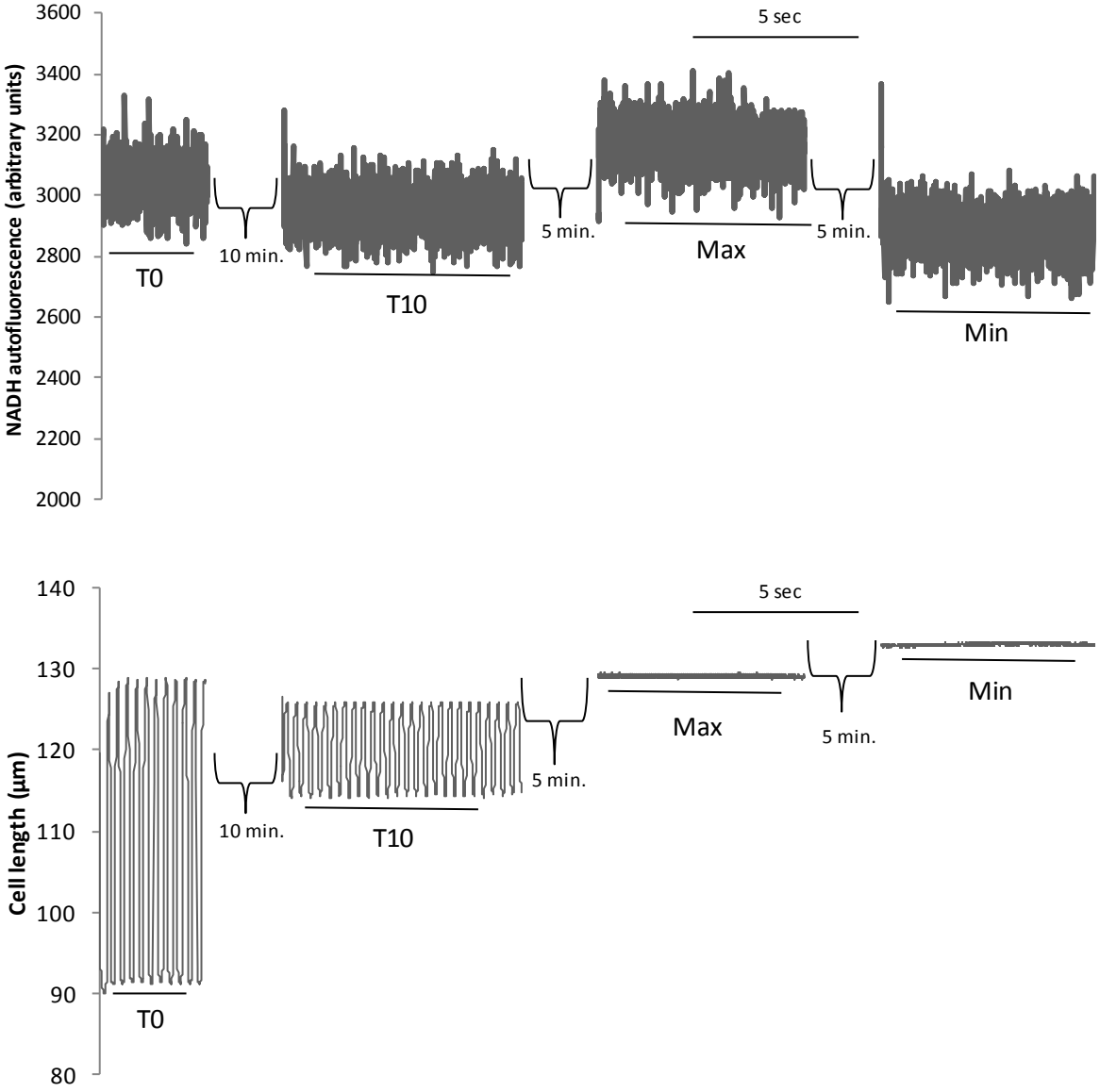

Figure 1. Representative trace of an old cardiac myocyte during simultaneous contractile and NADH measurements. T0 measurements occur after 30 s of contraction in order to ensure steady-state NADH tracings and T10 measurements occurred after 10 minutes of continuous stimulation. The Max and Min, represent the maximum and minimum NADH fluorescence with cyanide and FCCP, respectively. The Max and Min measures were performed after the 10 minutes of continuous stimulation while the myocyte was quiescent. In this case, T0 and T10 NADH levels were 63% and 23% of max, respectively.

Table 1. Contractile properties in young and old myocytes at 4 Hz

	YG	Old
peak shortening (% of DL)		
T0	10.39 ± 0.51	11.11 ± 0.80
T10	6.59 ± 0.33	7.04 ± 0.37
T10/T0	0.66 ± 0.03	0.68 ± 0.04
+ dL/dt (µm/s)		
T0	271 ± 18.16	264 ± 17.46
T10	157 ± 10.15	165 ± 9.43
T10/T0	0.62 ± 0.04	0.67 0.05
Diastolic cell length (µm)		
T0	111 ± 3.62	113 ± 3.51
T10	104 ± 3.95	104 ± 4.02
T10/T0	0.936 ± 0.01	0.916 ± 0.01

Values reflect Means ± S.E.M.

Peak shortening is defined as the percentage of: (distance shortened / diastolic cell length * 100)

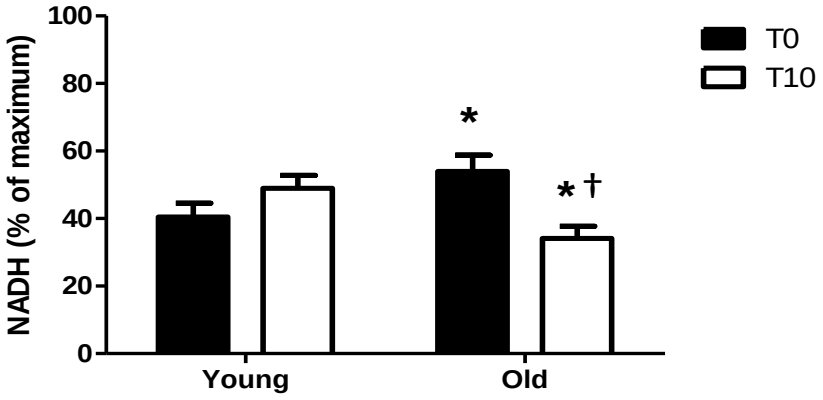

Figure 2. Values reflect Mean ± S.E.M. Single cell NADH values at T0 and after ten minutes (T10) of continuous stimulation). NADH values are expressed as a percent of maximum NADH values as described in Methods. * $p < 0.05$ Old vs Young at same time point. † $p < 0.05$ T10 vs. T0 within group.

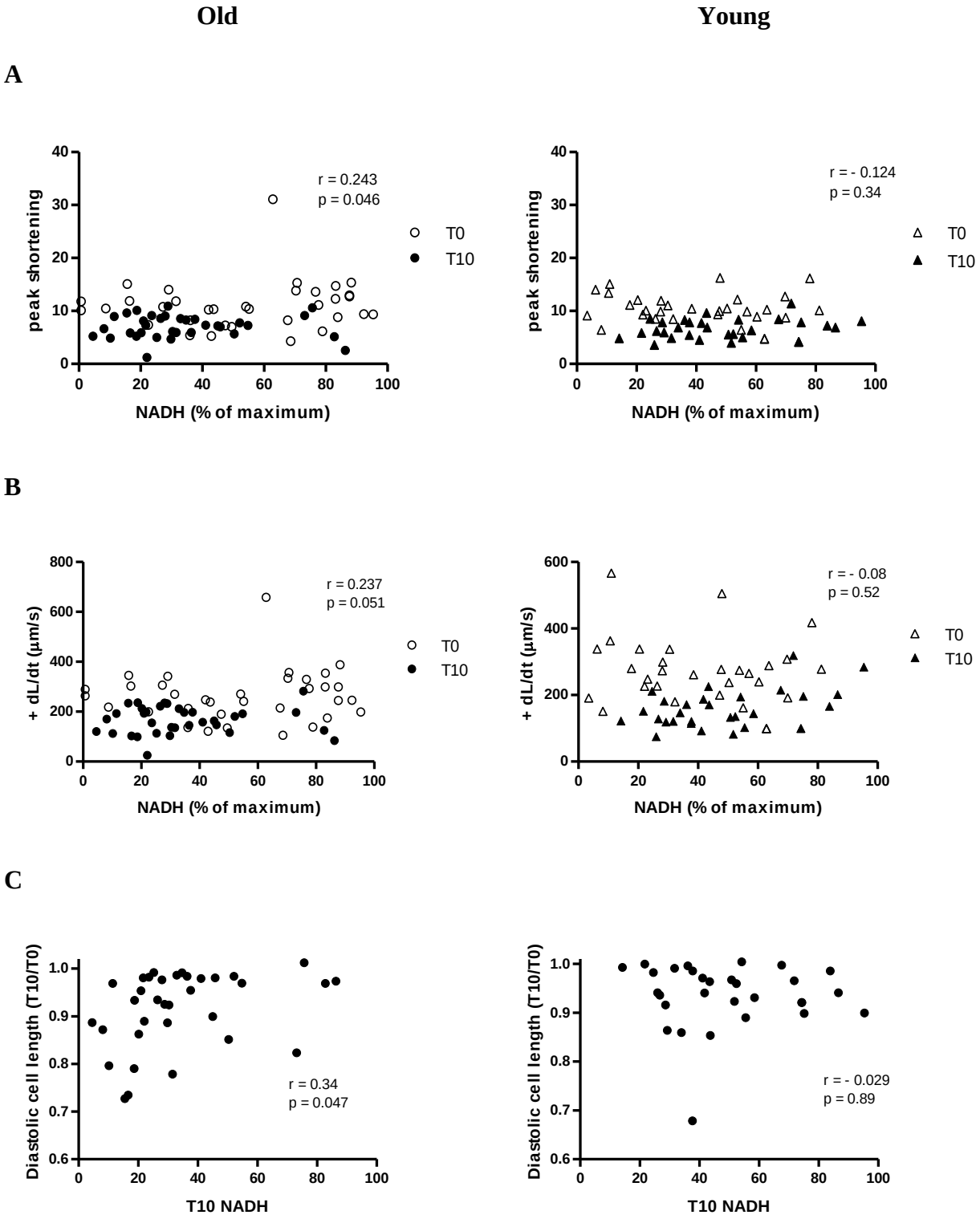

Figure 3. Contractile properties are correlated with $[NADH]_m$ in old myocytes but not in young. A) peak shortening (as percent of cell length) versus $[NADH]_m$, B) Rate of Shortening (+ dL/dt) versus $[NADH]_m$, C) Change in diastolic cell length (at T10 relative to T0) versus $[NADH]_m$ at T10. n = 34 cells and 30 cells in old myocytes versus young myocytes, respectively. Both T0 and T10 values for each cell are represented in A and B.

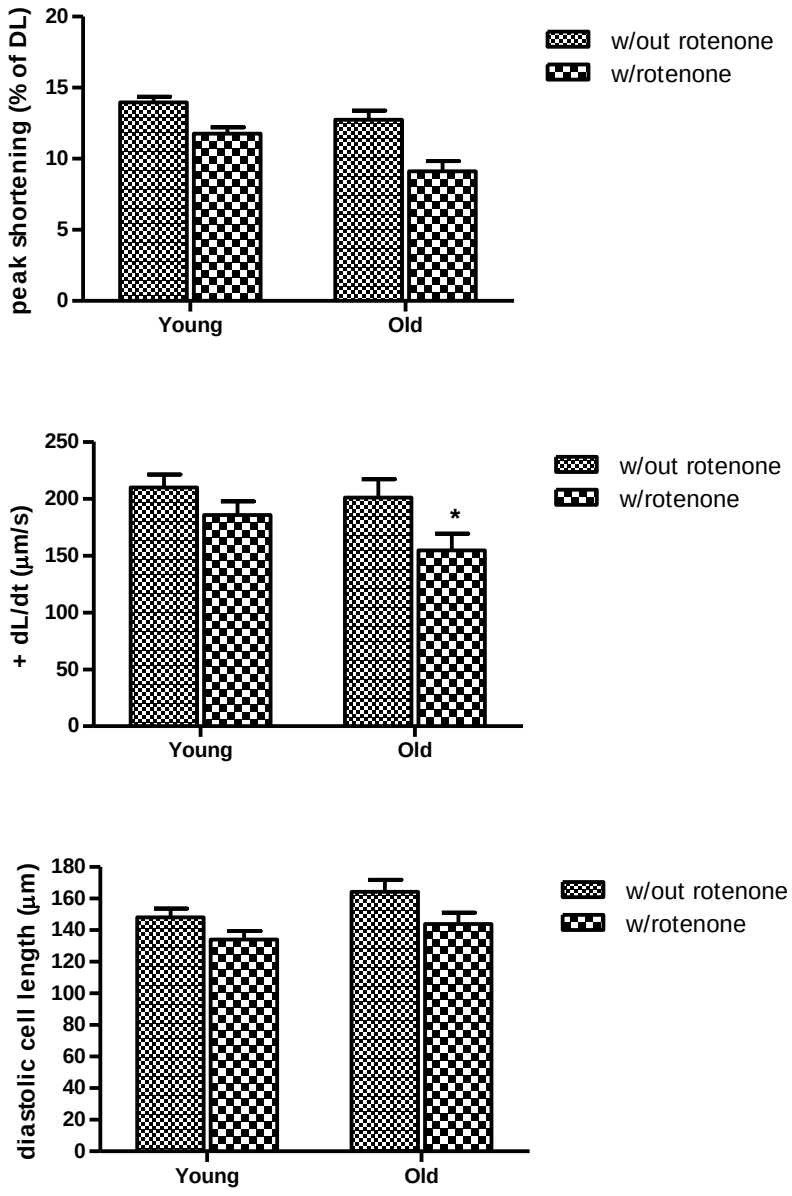

Figure 4. Rotenone was used to alter mitochondrial function. Contractile properties are expressed as mean absolute values before and after rotenone administration. Statistical analyses were performed on the change in contractile properties before and after rotenone administration (rotenone/without rotenone) between young and old myocytes. n = 28 cells and 26 cells in old myocytes versus young myocytes, respectively. * $p < 0.05$ old vs young

Peak shortening is defined by the distance shortened relative to diastolic length.

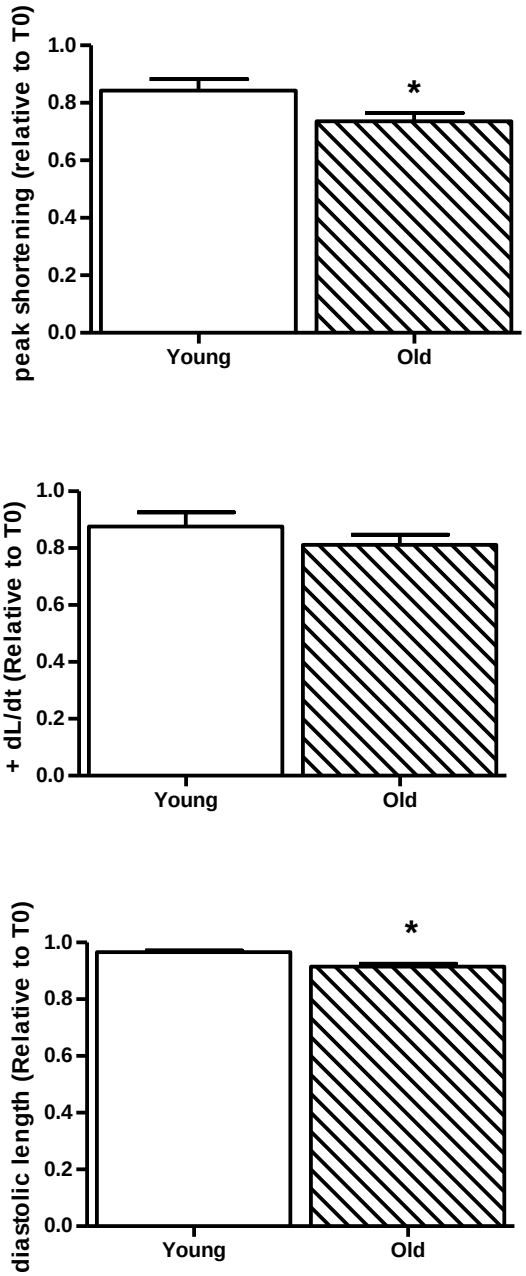

Figure 5. Values represent Means ± S.E.M. Isoproterenol was administered to increase cellular stress on young and old myocytes. Contractile properties are expressed as a ratio of the value after 5 minutes of isoproterenol exposure and the initial value (T5/T0). n = 40 cells in old myocytes and young myocytes, respectively ***** - $p < 0.05$ Old vs. Young

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SUBSTRATE SPECIFIC EFFECTS ON CONTRACTILE FUNCTION IN YOUNG AND OLD CARDIAC MYOCYTES

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INTRODUCTION

It is predicted that by the year of 2035, twenty-five percent of the United States population will be 65 years of age or older[1]. Aging is associated with a decline in cardiac function in humans as well as rodents[1]. The incidence of heart failure is directly related to age [2]. The aging rat heart shows several structural and functional changes in both systolic and diastolic function[3–6]. Recent evidence points to a role of altered cardiac substrate metabolism in the onset and development of cardiac aging[7–11], however, the role that changes in cardiac energy fuel utilization in the aging heart has not been clearly delineated.

There is substantial evidence that cardiac metabolism changes with age [7,9,10,12], but there is very little work describing the metabolic effects of aging alone, without the presence of other pathologies. In a recent study comparing young and old (29 v 69 yrs of age) subjects, Positron Emission Tomography (PET) was used to demonstrate that aged hearts exhibit reductions in fatty acid uptake and oxidation without an increase in glucose utilization[12]. These results indicate that older hearts have an attenuated ability to utilize fatty acids, and imply that old hearts rely more heavily on glucose, yet glucose utilization itself did not increase. Studies in the aged rats have indicated similar effects to these studies of human aging[7,9]. Specifically, in isolated perfused aged rat hearts there was a decrease in cardiac efficiency along with reduced fatty acid oxidation when normalized to heart tissue mass [7]. A decrease in palmitoylcarnitine oxidation, CPT-1 activity, and carnitine exchange in isolated cardiac mitochondria has been reported in aged Fischer 344 rat hearts[9]. Another important finding from this study was that in young hearts the normal Randle cycle was observed (fatty acid inhibition of glucose uptake), and in the aged hearts there was no decrease in glucose utilization seen when fatty acids were present[9]. In this study, these findings suggest reduced β-oxidation and thus a decrease in the Randle cycle. Findings of age associated decreases in fatty acid oxidation with an increased reliance on glucose

are by no means universal. One study on aged Wistar rats, demonstrated an increase in fatty acid oxidation in old compared to young with no change to glucose oxidation. Lactate oxidation was significantly reduced in old rats in this study[10]. These data confound the widely assumed notion of reduced fatty acid oxidation with age, and demonstrates the need for further exploration of age-related changes in substrate utilization.

While it appears that with age there is altered cardiac substrate utilization, the effects of this altered substrate utilization on cardiac contractile function has not been clearly delineated. However, there is evidence to suggest substrate-specific effects on contractile performance of the myocardium in young animals and in models of disease. A few studies have demonstrated that cardiac efficiency (cardiac power/MV_{o2}) is greater when the heart is oxidizing more glucose and lactate, and less fatty acids [14–17]. There is also some cellular evidence for substrate-specific effects on cardiomyocyte shortening properties and Ca^{2+} handling, as an acute switch to palmitate perfusion of contracting cardiomyocytes produced a decrease in peak shortening as well as a reduction in the Ca^{2+} amplitude. Mechanisms for these effects are thought to be increasing reactive oxygen species[18] and augmenting repolarization K^{+} currents[19], respectively. While these results suggest altered contractile properties in the presence of different substrates, there are few studies that address altered contractile consequences of altered substrate utilization by the cell, and the effect of aging on these substrate effects.

Therefore, the aim of this study was to examine the effect of altered substrate utilization on single, isolated cardiomyocyte shortening properties in young and old rats. In order to determine the role of altered utilization of substrate rather than acute effects due to the presence of different substrates, we examined contractile performance over the course of ten minutes of continuous stimulation. By examining contractile properties over time while being perfused with different

substrates: 1) this eliminates age-related changes that may be due to Ca^{2+} handling[20] and the myofilament[21,22], 2) places the emphasis on energy availability, 3) allows us to assay differences in substrate utilization, and 4) allows for control of potential direct effects of substrates on contractile properties. We hypothesized that old cardiomyocytes would exhibit reduced shortening properties compared to young. Secondly, we hypothesize that when fatty acid was the sole substrate shortening properties in old cardiomyocytes will be decreased compared to when glucose is the substrate.

METHODS

Animals. 6 month & 33 month male Fischer 344 x Brown Norway hybrid rats (FBN), were obtained from the National Institute on Aging colony at Harlan Industries (Indianapolis, IN). The FBN hybrid rat is a long-lived strain with a median life-span of 33 months and maximum life-span is 40 months. The FBN rat is considered a 'healthy aging model' widely used and highly recommended for gerontological research. All rats were confined to standard size rodent cages and housed 2 rats per cage. Rats had access to food and water *ad libitum* and were acclimated to reverse daylight (12h dark, 12h light). Animal housing and handling was carried out under the guidelines of the University of Wisconsin-Madison Institutional Animal Care and Use Committees and conducted in pathogen-free facilities that are accredited by the American Association of Accreditation of Laboratory Animal Care.

Cardiomyocyte Isolation. Rats were euthanized using isoflurane /pneumothorax. Hearts were rapidly removed from FBN male rats and mounted onto a temperature-controlled (37° C) Langendorff system[23–25]. Hearts were perfused with Ringer's solution in mM: 118 NaCl, 4.8 KCl, 1.2 NaH_2PO_3, 25 HEPES, 11glucose, 1.2 $MgCl_2$, 1 $CaCl_2$, at pH 7.4 and gassed with 5% CO_2 – 95% O_2. After perfusing with Ca^{2+} Ringers for 3 min, the hearts were digested with Ringers (Ca^{2+} free) containing type II collagenase and hyaluranidase for 25 min. The digested heart was removed from the cannula, and the ventricles cut into smaller pieces and placed in a 37° C water bath for another 25 minutes of digestion. Tissue pieces were gently triturated and the suspension centrifuged. Extracellular Ca^{2+} was added incrementally back to 1mM. Only rod-shaped myocytes with clear sarcomere striations were chosen for contractile studies.

Cell Shortening. For these experiments contractile properties of cardiac myocytes were assessed by visualizing the cell through a digital camera (Hitachi, KP-FD30CL), with the video image collected at 60 frames/second. Cell shortening was measured using edge detection software

created in LabView (National Instruments, Austin, TX). Cells were placed in a perfusion chamber (Warner Instruments) mounted on the stage of an inverted microscope (Olympus, CK 40) and superfused (1ml/min at 37 °C) with a buffer containing (in mM): 118 NaCl, 4.8 KCl, 1.2 NaH_2PO_3, 25 HEPES, 11 glucose, 1.2 $MgCl_2$, 2 $CaCl_2$, at pH 7.4. Cells bathed in oleate were superfused with the above solution minus glucose; but containing 0.4 mM oleate and 12.8 mg/ml BSA to give a non-esterified fatty acids NEFA/albumin ratio of 2:1[26]. The cells were field stimulated at a frequency of 2 Hz or 4 Hz for 5 msec pulse duration, using a pair of platinum wires on opposite sides of the chamber. Edge Detection software was used to capture changes in cell length during shortening at time 0 (T0) and after 10 minutes (T10) of continuous contraction. Cell shortening properties were assessed using the following indices: peak shortening (distance shortened/diastolic cell length * 100) – an indication of peak ventricular contractility, as well as maximal velocity of shortening (rate of shortening), i.e., the maximal slope derivative of shortening phase – related to the maximal velocity of ventricular pressure increase.

For isoproterenol (ISO) exposure experiments, cells were superfused (1ml/min at 37 °C) with glucose or oleate Ringers containing 100nM isoproterenol[27–30]. Cells were allowed to incubate in the isoproterenol buffered solution for 5 minutes prior to the acquisition of cell shortening measurements. The cells were field-stimulated at a frequency of 2 Hz and changes in cell length were measured at time 0 (T0) and after 5 minutes (T5) of contraction. Representative traces of a cardiac myocyte cell length changes under each experimental condition is given in Figure 1.

Statistical Analysis. Statistical analysis was performed on the change in contractile properties over time (T10/T0) for 2 Hz and 4 Hz stimulation, and for the isoproterenol study (T5/T0). We analyzed the independent samples at 2 Hz versus 4 Hz, as well as 2 Hz versus 2 Hz + ISO. We utilized a two-way between groups ANOVA to look for the main effects of workload, age, and

substrate between groups. We also assessed the workload-substrate interaction by age group which allowed us to determine if the change in contractile properties upon changing workloads is substrate-dependent in each age group. We also analyzed the age-workload interaction by substrate, which allowed us to determine if the change in contractile properties are different between age groups utilizing a specific substrate. When there were significant interaction effects, main effects were not interpreted. Significance was determined at $P < 0.05$. All statistical analysis was performed using SAS software.

RESULTS

Comparison of 2 Hz and 4 Hz stimulation. Figure 1 shows representative length tracings of myocytes stimulated under different conditions. These tracings show the decline in shortening performance during ten minutes of continuous stimulation. We used the ratio of contractile properties at T0 versus those at T10 (T10/T0) as a measure of contractile performance. Table 1 gives values for peak shortening and rate of shortening for myocytes under a variety of conditions. There was a main effect for workload on peak shortening between 2 Hz and 4 Hz (Table 1 and Figure 2A), indicating that regardless of substrate or age; there was a workload-dependent decline in peak shortening.

When analyzing the changes in the rate of shortening, there was a significant workload-age interaction when glucose was the substrate (Table 1 and Figure 2B), which suggests older myocytes saw a greater workload-dependent decline in the rate of shortening, but the young myocytes demonstrated no decrement. In old myocytes, there was a main effect for workload, indicating when old myocytes use either glucose or oleate, the rate of shortening declines similarly in a workload-dependent manner. In young myocytes, the workload-substrate interaction was not significant ($p = 0.07$) in the rate of shortening (Table 1 and Figure 2B). There was also a close substrate main effect ($p = 0.06$). These data suggests that young myocytes demonstrate a greater workload-dependent decline in the rate of shortening when oleate is the sole substrate compared to glucose.

We found with young myocytes that the change in diastolic cell length (Table 2 and Figure 2C) by increasing workload was dependent on the substrate used ($p < 0.01$). This is characterized as a workload-dependent decline in diastolic cell length in oleate, but no workload-dependent change in diastolic cell length when glucose was the substrate. In old myocytes we found a workload main effect ($p < 0.01$) but not a significant interaction. This indicates that diastolic cell

length declined in a workload-dependent fashion when old myocytes where perfused with either glucose or oleate. Lastly, when analyzing workload-age interaction by substrate used, we found no significant interaction effects, but found significant workload main effects ($p < 0.01$) for both glucose and oleate. This indicates that the workload-dependent decrement in diastolic cell length is not dependent upon age group.

Comparison of 2 Hz vs 2 Hz + ISO. In young myocytes, there were no substrate or workload-dependent differences when comparing the time-dependent change in contractile properties between 2 Hz stimulation and 2 Hz + ISO (Figure 3). In old myocytes we found significant workload-substrate interaction effects for peak shortening ($p < 0.01$), diastolic cell length ($p < 0.05$) and the rate of shortening ($p = 0.059$) was close but not quite significant. This suggests that when increasing workload with ISO in old myocytes, they demonstrate a substrate-dependent decline in contractile function. This was manifested as a significant workload-dependent decline in contractile function when glucose was the sole substrate, and no workload-dependent decrement with oleate (Figure 3).

When glucose was the sole substrate, we found a significant interaction between workload and age for peak shortening and diastolic cell length. This was characterized by old myocytes experiencing a workload-dependent decline in peak shortening ($p < 0.01$), and diastolic cell length ($p = 0.01$), while young myocytes demonstrate no change in contractile function with increasing workload. There was a trend towards a workload-age interaction for the rate of shortening ($p = 0.054$) in old myocytes compared to young myocytes. When oleate was the substrate, we found there was a significant age effect for diastolic cell length, indicating when workload is held constant, old myocytes observe a greater decline in diastolic cell length compared to young when oleate is the sole substrate.

DISCUSSION

The major findings from this study are myocytes from young hearts versus old hearts had different contractile responses to different substrates (glucose vs. oleate); 1) young myocytes had better contractile function when glucose was the sole substrate, compared to when oleate was the only substrate (Figure 2 and Table 1), 2) aged myocytes, demonstrated greater peak shortening and rate of shortening when oleate was the substrate compared to glucose, and 3) old myocytes demonstrated declines in contractile performance compared to young myocytes when glucose was the fuel substrate (Figure 2B and 3). We hypothesized that old cardiac myocytes would demonstrate greater contractile property decrements when oleate was the sole substrate compared to glucose, based on previous reports of age-related decreases in fatty acid oxidation. We found the opposite was true. These data are the first to our knowledge to assay substrate effects on contractile function in young and old cardiac myocytes. Our data suggests that contractile performance is substrate-dependent and these substrate effects on contractile function are different between young and old myocytes. Specifically, young myocytes are better able to utilize glucose to support contractile function in the face of increased workload compared to oleate. Old myocytes demonstrate increased contractile performance when workload is increased with oleate as the substrate compared to glucose.

The mammalian heart is characterized by its metabolic plasticity, switching its preferred substrate depending upon the environmental conditions[31]. The loss of metabolic flexibility has been seen in conditions of pathology[32–37] as well as aging[7,9,10,12]. In most cases the change in substrate metabolism in pressure overload hypertrophy has also been observed with aging; that is a reduction in fatty acid oxidation with an increased reliance on glucose metabolism[7,9,33,35]. However, one study assessed substrate metabolism in the isolated perfused heart in aging Wistar rats, demonstrated an increase in fatty acid oxidation and a reduction in lactate oxidation[10]. This

study was unique in that, in the majority of studies assaying substrate metabolism in the whole heart, the hearts are generally perfused with both a fatty acid moiety and glucose, but this particular study also included lactate in the perfusate. Taken together, most studies on aging corroborate the findings of pathological settings in respect to reduced fatty acid oxidation and subsequent increased dependence on glucose metabolism, although the effects on cardiac metabolism with aging alone is less clear. One aspect to altered substrate metabolism that has not been determined, is whether this change in substrate metabolism is deleterious to cardiac cellular contractile function. No study to date has assayed substrate effects on cellular contractile function in isolated contracting myocytes from aged hearts. We found that different substrates had different effects on contractile function in young compared to old myocytes. Specifically, aged myocytes demonstrated better contractile function when oleate was the substrate compared to glucose. While young myocytes displayed greater contractile performance when glucose was the sole substrate compared to oleate.

Contractile function in isolated myocytes is often defined by variables such as: peak shortening, rate of shortening, time to peak shortening, among many other variables. Reports of the effect of aging on these properties have been disparate[23,25,38,39]. Some studies have demonstrated that time to peak shortening declined with age, however in these same studies peak shortening and the rate of cell shortening was not different[23,38]. We used a measure of contractile function in this study that assayed changes in contractile function over time by measuring contractile properties at two different time points during continuous stimulation and analyzing the change in a given contractile property relative to the T0 time point. We performed this determination of the cell's ability to maintain contractile performance over time as a way of assaying cellular energetic differences between young and old myocytes. Furthermore, we

assayed how contractile performance changed with a change in workload as a way to exacerbate substrate-specific effects. We utilized changes in workload because; in our study baseline contractile properties (T0 and T10 at 2Hz) were not different between young and old myocytes. A stimulation frequency below 4 Hz is lower than resting heart rate in vivo in the FBN rat[4]. Previous work on aged cardiac myocytes has demonstrated that at a subphysiological workload, there were no differences in contractile performance between young and old myocytes[39]. Age-related differences in this study were only manifest when workload was increased and the change in contractile function was compared back to baseline contractile function at the subphysiological workload. Interestingly, all of the previous data supporting age-related declines in baseline contractile function in isolated intact cardiac myocytes utilized glucose in the perfusion medium[23,25,38,39], and we are the first to study contractile function in aged myocytes with both glucose and oleate as sole substrates. We hypothesized that old myocytes would exhibit reduced shortening properties compared to young. Our hypothesis was partially supported by a greater decline in contractile properties when glucose was the sole substrate in old myocytes compared to young (Figure 1B & 2). We did not observe a difference between young and old myocytes when oleate was the substrate, thus, illustrating that contractile dysfunction with age may be substrate-dependent.

Oleate mitigates the decrement in contractile function in old myocytes

Our findings (Figure 2) that old cardiac myocytes demonstrate better contractile function with increasing workload when oleate was the sole substrate compared to glucose refuted our hypothesis. One potential explanation for our findings and the findings from Sample et al[10], that fatty acids are the preferred fuel of the aging myocytes, is the tendency for aged mammals to develop insulin resistance and glucose intolerance[37,40–43]. A number of studies in whole hearts

have demonstrated increased fatty acid uptake and oxidation along with a marked reduction of glycolysis and glucose oxidation in models of insulin resistance[36,44–47]. A few studies in isolated intact cardiac myocytes have looked at substrate effects on contractile function in models of insulin resistance. Contractile performance in insulin resistant cardiac myocytes was blunted when glucose was the sole substrate compared to healthy myocytes, but this contractile dysfunction was mitigated when palmitate was introduced to the insulin resistant cardiac myocytes[18,48]. Insulin resistance has been observed in the elderly population[42,43] and aged rodents[40] along with recent data indicating that ~ 40% of U.S. adults aged > 60 years of age meet current criteria for insulin resistance syndrome[49]. We did not measure any aspects of insulin resistance in our aged rats but future works that measure glucose tolerance in aged rats along with cardiac myocyte function will provide greater insight into the relationship between aging, insulin resistance, and cardiac myocyte function.

Oleate exacerbates the decline in contractile function in young myocytes

Our study showed that young myocytes have a greater decline in contractile function when workload was increased and when oleate is the sole substrate compared to glucose (Figure 1). Adverse effects of oleate has been observed in young adult cardiac myocytes in previous works[50,51]. One study demonstrated that not only does oleate increase resting metabolic rate, but also leads to a depolarization of the mitochondrial membrane potential[51]. Of particular importance in this study[51], they saw these effects at 0.4mM oleate, which is the same concentration of oleate we used in our study during the continuous stimulation. The postulated mechanism by which oleate dissipates the mitochondrial membrane potential and produces heat, is translocation of the fatty acid anion through uncoupling proteins (UCPs)[52]. Another report demonstrated that oleic acid reduces the open probability of ryanodine receptors (RyRs) in

artificial bylayers within 5 minutes of cytosolic application[50]. This would lead to reductions in contractile properties by reducing the amount of calcium-induced calcium release during stimulation. However, if either of these potential mechanisms were to account for our results, we should have observed differences between glucose and oleate at baseline (T0) in young myocyte contractile function and these differences would have continued to persist despite time and workload effects. However, our results demonstrate the attenuation of young myocyte contractile function in oleate was a function of increased workload and contractile changes over time. Our data suggests that the decline in contractile function in young myocytes perfused with oleate may be due to an energy supply/demand mismatch compared to glucose.

Conclusion. We showed for the first time myocytes from young hearts had better contractile function when glucose was the substrate, with a smaller workload-dependent decline in function, compared to when oleate was the substrate. In aged myocytes, the contrary was true, with old myocytes showing less workload-dependent decline in peak shortening and diastolic cell length when oleate was the substrate. Lastly, young myocytes clearly displayed greater contractile function compared to old myocytes when glucose was the fuel substrate. This data suggests that when forced to use only one substrate, myocytes from young hearts demonstrate greater contractile performance when perfused with glucose, while contractile performance is greater in cells from old hearts when perfused with oleate.

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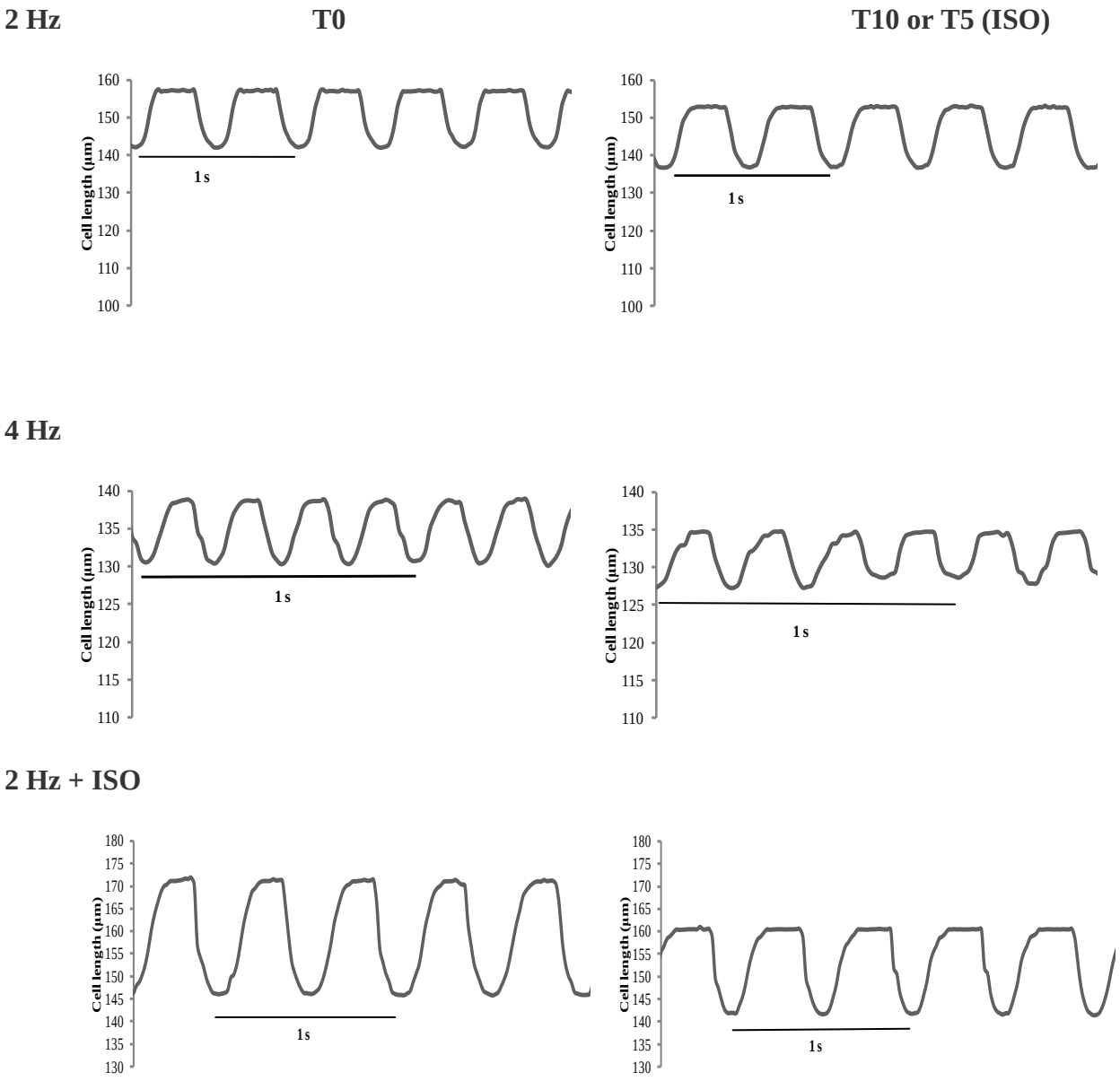

Figure 1. A representative trace of cardiac myocyte cell length changes. Both T0 and T10 traces are represented for a given aged cardiac myocyte within each experimental condition. After we analyzed these traces, we measured the change over time as a ratio of (T10/T0) for a given contractile property.

Table 1. Workload and substrate-dependent changes in shortening properties in Young and Old cardiac myocytes

2 Hz Peak Shortening					
Young			**Old**		
	Glucose	**Oleate**		**Glucose**	**Oleate**
T0	10.69±0.36	11.34±0.36	**T0**	9.79±0.49	10.61±0.52
T10	8.93±0.29	9.37±0.25	**T10**	8.85±0.45	8.48±0.37
T10/T0	0.87±0.03	0.85±0.02	**T10/T0**	0.93±0.03	0.84±0.03
4 Hz Peak Shortening					
Young			**Old**		
	Glucose	**Oleate**		**Glucose**	**Oleate**
T0	8.99±0.47	9.79±0.37	**T0**	9.93±0.50	9.32±0.59
T10	6.48±0.32	6.66±0.27	**T10**	7.48±0.40	6.70±0.31
T10/T0	0.75±0.04†	0.70±0.03†	**T10/T0**	0.79±0.04†	0.77±0.04†

2 Hz Rate of Shortening (µm/s)					
Young			**Old**		
	Glucose	**Oleate**		**Glucose**	**Oleate**
T0	135.28±5.51	135.97±5.82	**T0**	118.87±9.97	119.21±7.66
T10	115.96±4.86	116.72±4.62	**T10**	116.31±11.08	102.93±7.53
T10/T0	0.89±0.03	0.89±0.03	**T10/T0**	0.97±0.04	0.89±0.04
4 Hz Rate of Shortening (µm/s)					
Young			**Old**		
	Glucose	**Oleate**		**Glucose**	**Oleate**
T0	154.64±13.85	155.36±8.18	**T0**	181.43±14.60	163.7±14.72
T10	126.86±8.69	113.62±6.58	**T10**	134.87±8.60	117.84±8.62
T10/T0	0.89±0.05	0.75±0.04†,#	**T10/T0**	0.81±0.04†,ª	0.79±0.04†

Values indicate Means ± S.E.M.

† Indicates main effect of workload, # Indicates workload-substrate interaction by age, ª Indicates workload-age interaction by substrate. All of these differences are for a $P < 0.05$.

Table 2. Changes in Diastolic Cell length are workload and substrate-dependent

2 Hz Diastolic Cell Length (µm)					
Young			**Old**		
	Glucose	**Oleate**		**Glucose**	**Oleate**
T0	133.27±3.94	133.88±3.94	**T0**	150.85±9.14	140.51±5.50
T10	129.45±3.99	129.74±3.92	**T10**	146.66±9.28	133.72±5.66
T10/T0	0.97±0.005	0.97±0.004	**T10/T0**	0.97±0.006	0.95±0.009
4 Hz Diastolic Cell Length (µm)					
Young			**Old**		
	Glucose	**Oleate**		**Glucose**	**Oleate**
T0	137.17±6.00	135.82±6.33	**T0**	157.54±7.51	147.43±6.83
T10	131.31±5.79	124.63±5.77	**T10**	147.61±7.38	135.37±6.13
T10/T0	0.96±0.007†	0.92±0.01†	**T10/T0**	0.94±0.01†	0.92±0.01†

Values indicate Means ± S.E.M.

† Indicates main effect of workload $P < 0.05$.

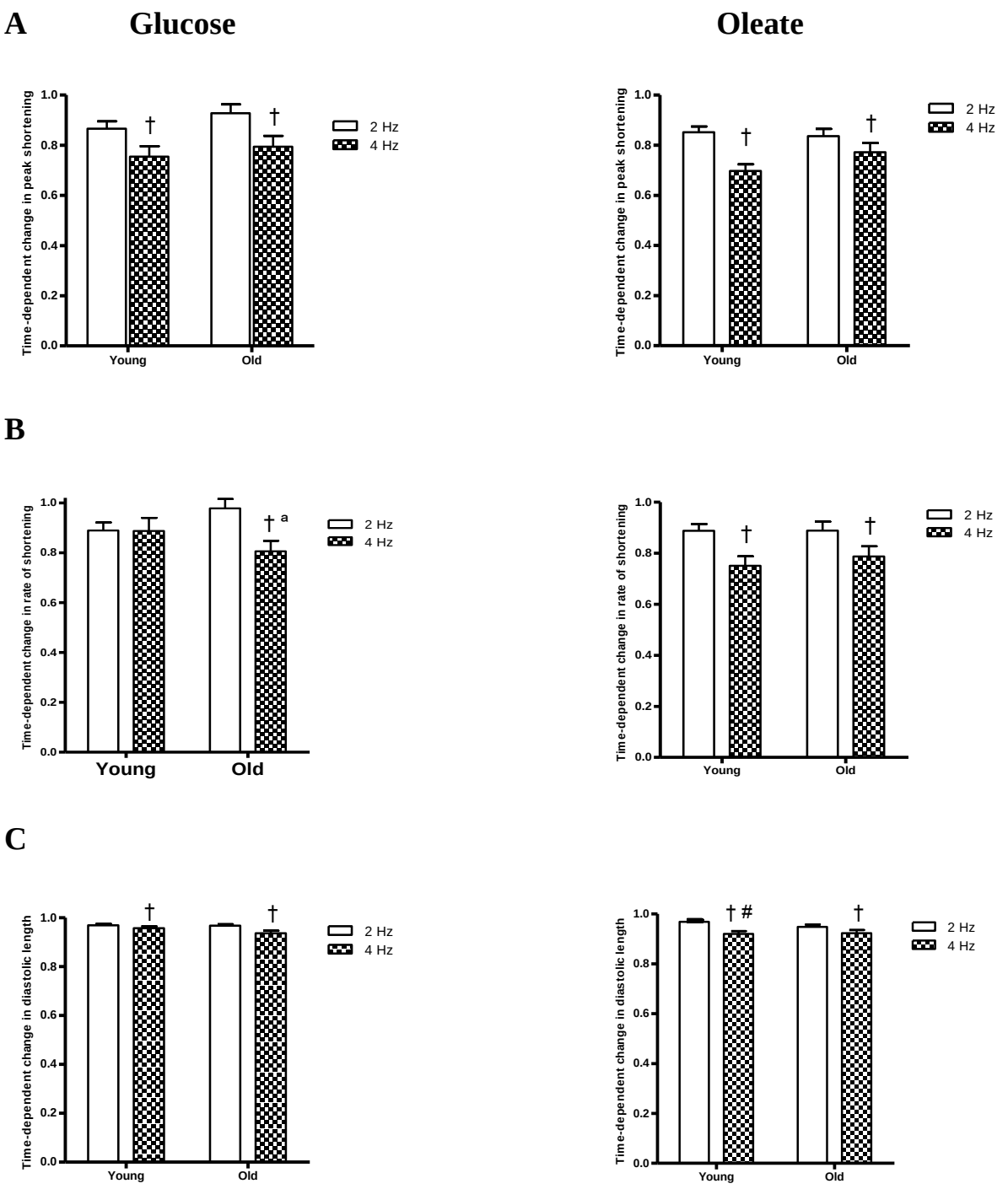

Figure 2: Values indicate Means ± S.E.M. Workload-dependent changes in contractile properties over 10 minutes of continuous contraction. All values expressed are a function of changes in contractile properties over time (T10/T0). 2A - peak shortening; 2B – rate of shortening; 2C – diastolic cell length
† Indicates main effect of workload, # Indicates workload-substrate interaction by age group, ª Indicates workload-age interaction by substrate. These differences are for a $P < 0.05$.

Table 3. Age and Substrate Effects on Contractile Properties with Isoproterenol

	YG		Old	
peak shortening				
	Glucose	**Oleate**	**Glucose**	**Oleate**
T0	11.25 ± 0.59	12.82 ± 0.44	14.30 ± 0.45	13.41 ± 0.56
T5	9.16 ± 0.54	10.76 ± 0.46	10.52 ± 0.53	10.56 ± 0.49
T5/T0	0.84 ± 0.04	0.85 ± 0.03	0.74 ± 0.03$^{\#,a}$	0.81 ± 0.03
Rate of shortening (µm/s)				
T0	165 ± 12.5	188 ± 11.0	144 ± 7.3	161 ± 9.4
T5	136 ± 8.8	166 ± 10.3	115 ± 6.8	135 ± 7.8
T5/T0	0.88 ± 0.05	0.89 ± 0.03	0.81 ± 0.03	0.85 ± 0.03
Diastolic cell length (µm)				
T0	140 ± 6.8	141 ± 5.9	140 ± 5.0	156 ± 6.3
T5	135 ± 6.7	136 ± 5.7	128 ± 4.5	146 ±5.7
T5/T0	0.97 ± 0.01	0.96 ± 0.01	0.91 ± 0.01$^{\#,a}$	0.93 ± 0.01‡

Values indicate Means ± S.E.M. Statistical comparisons were performed comparing 2 Hz stimulation versus 2 Hz stimulation + ISO

‡ Indicates main effect of age, # Indicates workload-substrate interaction by age, ᵃ Indicates workload-age interaction by substrate. All of these differences are for a $P < 0.05$.

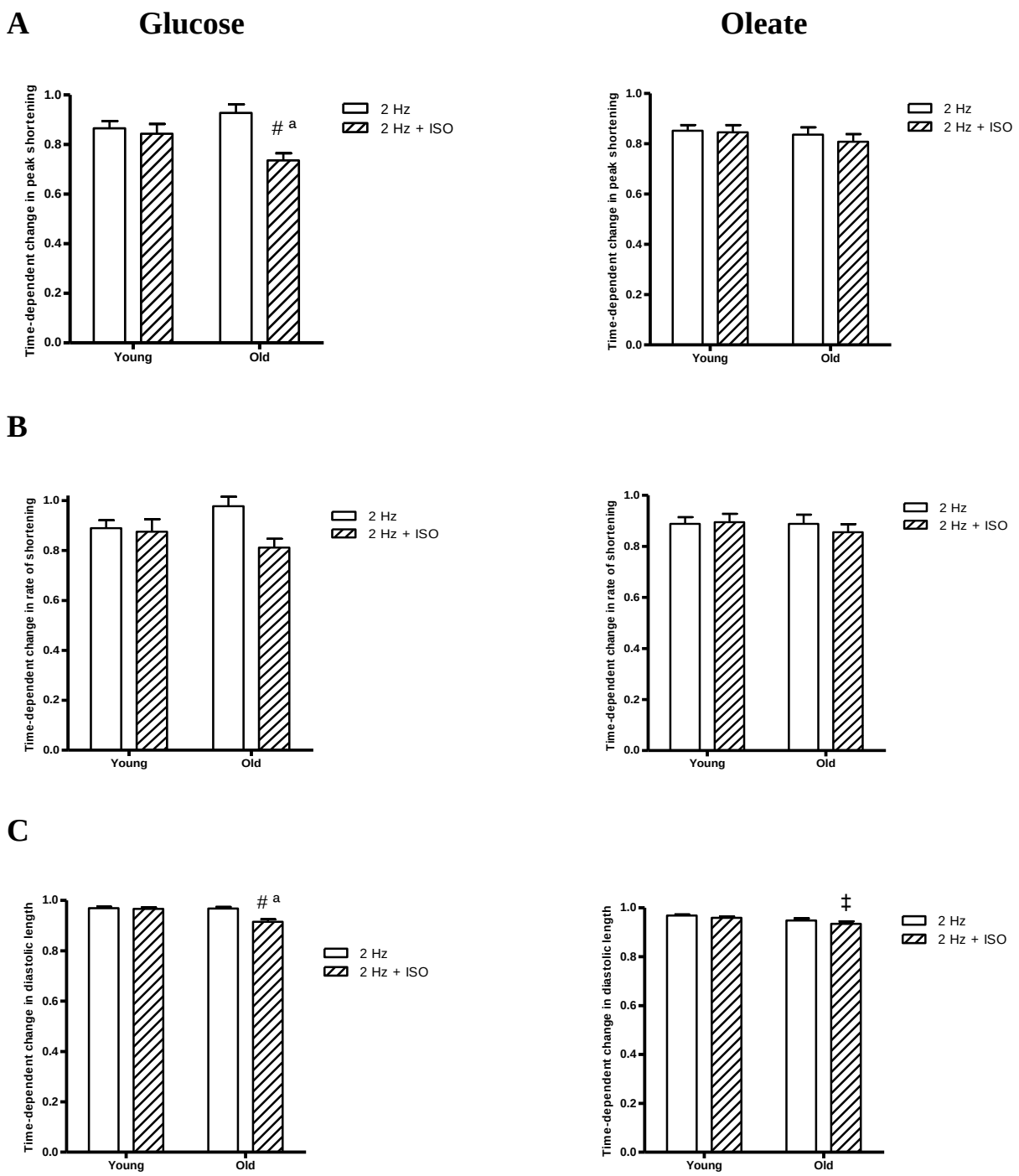

Figure 3: Values indicate Means ± S.E.M. The effects of increasing workload using isoproterenol on changes in contractile properties in young and old myocytes. All values expressed are a function of changes in contractile properties over time (T10/T0 for 2Hz) (T5/T0 for 2Hz + ISO) 3A - peak shortening; 3B – rate of shortening; 3C – diastolic cell length.

‡ Indicates main effect of age, # Indicates workload-substrate interaction by age group, ª Indicates workload-age interaction by substrate. All of these differences are for a $P < 0.05$.

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MITOCHONDRIAL AND METABOLIC GENE EXPRESSION CHANGES IN THE AGED MYOCARDIUM

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INTRODUCTION

The prevalence of cardiovascular disease is as high as ~ 70% between the ages of 60-79 and almost 85% for individuals 80 + years of age[1]. The incidence of heart failure is directly related to age[2]. Aging is associated with a decline in cardiac function in humans as well as rodents[3]. The decline in cardiac function with age is associated with changes in mitochondrial function and energy metabolism. Since the myocardium derives nearly all of its energetic needs from the oxidation of pyruvate and fatty acids within the mitochondria, mitochondrial changes have great potential to contribute to cardiac dysfunction with age. Indeed, mitochondrial energetic deficiency has been widely documented in heart failure in both human patients and rodent models[4]. The mechanisms of this mitochondrial dysfunction may include biogenesis that is inadequate to match the increasing demand[5], as well as increased mitochondrial uncoupling and decreased substrate availability[6]. Several studies have documented age-dependent impairment in a decline in mitochondrial respiratory capacity (state 3) due to diminished activity of complexes I and IV, with complexes II, III, and V relatively unaffected[7,8].

Along with mitochondria functional changes is a change in cardiac substrate utilization during the aging process. At rest, fatty acids are the primary substrate utilized for ATP supply in the myocardium and glucose uptake and oxidation supplies the remainder of the carbon substrates[9]. There is some evidence that cardiac substrate utilization changes with age[10–12], but there is very little work describing the metabolic effects of aging alone, without the presence of other pathologies. Changes to cardiac substrate utilization have also been seen in cardiac hypertrophy and models of heart failure and these changes appear to be somewhat similar to age-related changes. Therefore, changes in cardiac substrate metabolism observed in these disease models are often used to provide some insight into what may be occurring with age and make up for the paucity of information on aging *per se*. The prevailing metabolic phenotype in the aging heart

and age-related pathologies is a reduction in fatty acid oxidation, with the implication of increased reliance on glucose. Interestingly, it has been determined that glucose utilization itself does not increase[10,11,13]. However, in at least one study an age-related increase in fatty acid oxidation was observed, with no change to glucose utilization, and it was lactate oxidation that was significantly reduced with age[12]. This study illustrates that age-related changes in cardiac metabolism, mitochondrial function, and energy metabolism are poorly understood.

The molecular mechanisms underlying age-related changes in mitochondrial function or substrate energy metabolism is largely unknown. There is some molecular evidence to back the reduction in fatty acid oxidation and mitochondrial function. In one study, decreases in peroxisome proliferator-activated receptor (PPARα), and some its downstream targets (i.e. CPT-1, β-hydroxyacyl dehydrogenase) mRNA[14] were observed. Furthermore, it has been shown that peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α) is reduced in the aging left ventricle[15]. PPARα is a transcription factor for genes involved with fatty acid transport and β-oxidation[16]. PGC-1α, a co-transcription factor is known to stimulate mitochondrial DNA replication and the coding for genes involved with oxidative phosphorylation[16]. Both PPARα and PGC-1α have many downstream effects on energy metabolism and mitochondrial function, yet very little information exists on the effects of aging alone on the expression of genes downstream from these two proteins. Another mechanism for age-related changes in substrate metabolism may be related to AMP-activated protein kinase (AMPK) activity. Dually activated by AMP and by upstream phosphorylation, this kinase promotes fatty acid oxidation, glucose uptake, and glycogenolysis while it inhibits anabolic processes such as fatty acid synthesis[17–21]. There is evidence that AMPK activity declines with age[15,22], but what role gene expression changes play on AMPK in the myocardium is currently unknown. While there is some evidence of age-related

changes to specific molecular markers that help explain some of the altered metabolism and mitochondrial function in the aged myocardium, these specific molecular markers exist in extensive pathways, but there are few studies examining comprehensive changes in gene expression. Lastly, previous work has used a variety of rat and mouse species, but there has been little work examining age-related metabolic changes in the Fischer 344 x Brown-Norway F1 (FBN) rat strain, which was introduced by the National Institute of Aging as an improved model for the study of aging because of the lower occurrence of comorbidity in the FBN rodents[23].

Exercise training is known to improve indices of cardiac function in humans[24,25] and rodents[14,26]. Specifically, these functional improvements range from; increased rate of isometric contraction[26], enhanced fractional shortening[14], increases in stroke volume[27,28], increased ejection fraction[24], as well as increased maximal oxygen uptake (VO_{2max})[24,27,28]. While there is much more evidence of the beneficial effects of exercise on contractile function, there is some evidence that exercise can alter the metabolic phenotype of the aging heart. Exercise training in aging Wistar rats has demonstrated to increase AMPK activity, PPARα mRNA and protein content, and proteins involved in fatty acid oxidation in the young and aged heart[14,29,30]. Also, lifelong voluntary wheel running in mice increased complex IV gene expression[31]. While there is some evidence of specific changes to genes associated with metabolism and mitochondrial function, there is a lack of study of aging and exercise on a comprehensive list of metabolic genes.

Therefore, the aim of this study was to determine the effects of age and exercise on the expression of specific genes related to glucose and fatty acid metabolism, and mitochondrial function. We hypothesize that gene expression associated with fatty acid metabolism, AMPK

signaling, and mitochondrial function will decrease with age and, exercise in aged-rats will mitigate the attenuation.

METHODS

Animals. Male Fischer 344 x Brown Norway hybrid rats (FBN), were obtained from the National Institute on Aging colony at Harlan Industries (Indianapolis, IN). The FBN hybrid rat is a long-lived strain with a median life-span of 33 months and maximum life-span is 40 months. The FBN rat is considered a 'healthy aging model' widely used and highly recommended for gerontological research. All rats were confined to standard size rodent cages and housed 2 rats per cage. Rats had access to food and water *ad libitum* and were acclimated to reverse daylight (12h dark, 12h light). Body weights and average food intake were monitored through the course of the study. Five rats were randomly assigned to one of three groups: (1) Young (6 mo), (2) Old (33 mo) and (3) Old + Exercise (Old+EXE) (34 mo). Nine Old + Exercise (Old+EXE) (34 mo) and Old Sedentary (Old SED) (34 mo) rats were used for the exercise training part of this study. The Old SED rats were a part of the exercise training study, but not included in the gene expression and protein analysis.

Animal housing and handling was carried out under the guidelines of the University of Wisconsin-Madison Institutional Animal Care and Use Committees and conducted in pathogen-free facilities that are accredited by the American Association of Accreditation of Laboratory Animal Care.

Training Protocol. Trained animals were exercised on a motor driven treadmill during their 12 h dark cycle. Before training began, maximal exercise tests were performed and lactate measurements were taken at each successive speed on the treadmill during the test. The endurance trained rats began exercise training at 30 months of age with a training program that consisted of 30 min/day, five days per week at a speed that corresponded to each animal's lactate threshold. Thus, the training speed was adjusted each month based on the results of the maximal exercise tests (below). All exercise training sessions included a 3 minute warm-up period at

5m/min. No negative stimuli (electric shock) were used during the daily exercise training of the animals to minimize stress involved in exercise for these aging animals.

Maximal Exercise Testing. Assessment of peak exercise capacity occurred each month beginning at 30 months until 34 months of age. The exercise test started with a treadmill speed at 5m/min and progressively increased by 3m/min at each exercise stage. For the exercise test, animals ran at each new treadmill speed for 3 minutes to assure steady-state values. To encourage the rats to run, the treadmill was equipped with an electric shock grid at the rear of the treadmill. The shock grid was set to deliver a 0.2 mA current, which gives an uncomfortable shock but does not physically harm or injure the rat. The test was terminated when animals were no longer able to maintain position on the treadmill, and the highest speed was recorded as peak exercise capacity. Blood lactate levels were measured during the graded exercise tests. Lactate accumulates in the blood in an exercise-intensity dependent manner and thus blood lactate levels can be used to quantify relative exercise intensity. At the end of the 3 minute interval at each treadmill speed during the max test, animals were briefly removed from the treadmill, immobilized with the tail extended, the lateral tail vein pierced, and a drop (~25μL) of blood was collected. The drop of blood was placed on a lactate strip which was inserted into a lactate meter (Lactate Plus Meter, Nova Biomedical). Blood lactate was measured at each exercise intensity level during the graded test.

RNA isolation and cDNA preparation. Total RNA from each rat was isolated from the left ventricular free wall using RNeasy® Microarray Tissue Mini Kit (Qiagen) according to the manufacturer instruction. Immediately after elution RNA concentration and purity was measured spectrophotometrically (Beckman-Coulter). For each sample 700 ng of total RNA was reversed transcribed using the RT^2 First Strand Kit (Qiagen). The reaction was performed at 42°C for 15

min followed by a termination step at 95°C for 5 min. cDNA was stored at – 20°C until qRT-PCR.

qRT-PCR data analysis. A total of 84 genes of interest for each of three PCR arrays from Qiagen were assayed for this study (252 genes in total from Glucose Metabolism RT^2 Profiler PCR Array, Fatty Acid Metabolism RT^2 Profiler PCR Array and RT^2 Custom PCR Array for mitochondrial function). There was one gene that was in both the fatty acid metabolism and custom array, so 251 different genes were assayed. A list of all of the genes contained in each array can be found inTables 1 - 3. PCR was performed on Step-One Plus PCR system (Applied Biosystems), according to the manufacturer's instructions. For data analysis, the ΔΔCt method was used with the aid of a Microsoft Excel spreadsheet containing algorithims and a student's t-test was used to analyze differences in fold-changes in gene expression provided by the manufacturer[32–34]. The ΔΔCt method for calculating differences in gene expression are as follows: ($\Delta Ct = Ct^{GOI} - Ct^{AVG\ HKG}$, where GOI is the gene of interest and HKG is the housekeeping gene selected. ΔΔCt = (ΔCt (Experimental group) - ΔCt (Control)). The housekeeping gene selected for data analysis was Ribosomal protein, large P1 (Rplp1). Fold-changes were then calculated and expressed as log-normalized ratios of values from Old/Young, Old + EXE/Young and Old + EXE/Old heart tissues.

Western Blotting. Whole cell lysate from isolated left ventricle was prepared using CelLytic™ MT Cell Lysis Reagent and 1:100 dilution of Protease Inhibitor Cocktail (Sigma, St. Louis, MO). The protein concentration was determined using Bio-Rad Protein Assay (BioRad, Hercules, CA). Thirty micrograms of whole cellular protein per lane was separated by SDS-PAGE with a 4-12% Bis-Tris Criterion™ XT gel (XT MOPS running buffer) and blotted onto a nitrocellulose membrane. The membrane was incubated with blocking buffer (5% non-fat dry milk/TBS/0.1%

Tween 20) at room temperature for 1 hour. The membrane was then probed with the primary antibodies diluted in blocking buffer overnight at 4°C. Subsequently, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody diluted 1:2000 in blocking buffer. Blots were developed with Clarity™ Western ECL substrate (BioRad, Hercules, CA) and imaged with GE ImageQuant LAS-4000 (GE Healthcare Bio-Sciences, Pittsburgh, PA). The image of the blots was uploaded and densitometry analysis was done with Image Studio Lite (LI-COR Biosciences, Lincoln, NE).

Antibodies. Rabbit anti-PGC-1α, AMPKα_2, and Vinculin antibodies were acquired from Cell Signaling Technology, Inc., Beverly, MA. Rabbit anti- PPARα antibody was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA.

Citrate Synthase activity. Maximal citrate synthase (CS) activity was determined using (Citrate Synthase Assay Kit, Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol with absorbance kinetically measured at 412 nm at baseline and after addition of oxaloacetate (Sigma-Aldrich). CS activity was normalized to protein content, with tissue protein determined using the Bio-Rad protein assay.

Statistical Analysis. A two-tailed Student's t-test was used for analyzing differences in gene expression and exercise performance. A one-way ANOVA was utilized to analyze differences in protein content and citrate synthase activity. Post-hoc analysis was performed when the one-way ANOVA was significant using Tukey's LSD to assess between group differences. Significance was determined at $p < 0.05$.

RESULTS

Effects of exercise training on exercise performance. The effect that four months of exercise training had on exercise performance in 34 month old sedentary (Old SED) and trained (Old + EXE) FBN rats is seen in Figure 1. We measured exercise capacity in two different ways; 1) treadmill speed at lactate threshold (LT) and 2) peak exercise capacity was measured as the final treadmill speed achieved during the maximal exercise test. Exercise training significantly improved lactate threshold ($p < 0.01$) in the Old + EXE rats as they were able to run faster on the treadmill at the point of reaching lactate threshold (Figure 1A). Peak exercise capacity was also greater ($p = 0.01$) in the Old + EXE rats compared to Old SED rats (Figure 1B).

Gene expression changes with age. In order to determine the effects age has on left ventricular gene expression changes in relation to glucose and fatty acid metabolism as well as mitochondrial function we performed qRT-PCR using three different PCR arrays. Table 4 shows all genes for which expression was significantly different between Old and Young rats. Between the three arrays there were a total of 44 genes that were significantly different ($p < 0.05$) in Old compared to Young. All but two of these genes decreased in Old compared to Young. Notably, three important aspects of energy production demonstrated altered gene expression with age; fatty acid oxidation (FAO), mitochondrial biogenesis, and AMPK signaling. There were no significant changes involved with glucose metabolism with age. Figure 2 shows changes in expression with age and exercise in key genes involved in the pathways of FAO, mitochondrial biogenesis, and AMPK signaling.

Effects of Exercise Training on gene expression in aged hearts. Table 5 summarizes the genes whose expression was different between old and old - exercise rat hearts. Table 5 shows a significant ($p < 0.05$) difference in 70 genes associated with glucose metabolism, FA metabolism

and mitochondrial function were altered with exercise training in old rats. Of these 70 genes, only three were upregulated while the other 67 genes were downregulated with exercise training in old rats compared to old sedentary rats. Of the 67 genes that demonstrated a decline in gene expression were from metabolic processes involving: Kreb's cycle, glucose transport (Glut4), fatty acid oxidation, and mitochondrial electron transport chain.

Western Blot analysis. In order to determine if the gene expression changes with age and exercise training were associated with altered protein content, we selected one protein from fatty acid oxidation (PPARα), AMPK signaling ($AMPK\alpha_2$), and mitochondrial biogenesis/function (PGC-1α) categories shown in Figure 2. Each of these genes showed decreased mRNA expression but, found no differences in the protein content of PPARα, $AMPK\alpha_2$, and PGC-1α between young and old (Figure 3). PGC-1α content was increased in the Old + EXE compared to both Old and Young, but PPARα and $AMPK\alpha_2$ protein content was decreased in the Old + EXE rat hearts compared to Young and Old rat hearts.

Citrate Synthase Activity. To determine functional consequences of changes in gene expression/protein content we assayed citrate synthase activity in left ventricular homogenates. We found that Old rat hearts had increased citrate synthase activity compared to Young hearts, and the Old + EXE hearts demonstrated no differences in citrate synthase activity compared to Young or Old hearts (Figure 4).

DISCUSSION

To our knowledge this is the first study to determine the effects of age and exercise on changes in expression of a comprehensive group of genes related to cardiac substrate metabolism and mitochondrial function. We hypothesized that genes associated with fatty acid oxidation, AMPK signaling, and mitochondrial biogenesis/function would be decreased with age and that exercise would mitigate these changes in aged rat hearts. We found that aging results in the decreased expression of many genes involved with energy metabolism and mitochondrial function and exercise did not mitigate these changes. Genes associated with glucose metabolism were unaffected in old compared to young. The decline in gene expression are profound in that we demonstrated decreased expression in 42 genes involved with fatty acid metabolism and mitochondrial function. However, we found no age-related differences in the protein levels of PGC-1α, PPARα and AMPKα_2 and in fact, observed that citrate synthase activity (a common measure of mitochondrial volume) was increased with age. These results suggest that significant disruption of cardiac metabolism signaling pathways with age, but molecular mechanisms of age-related changes in cardiac metabolism and mitochondrial function are complex and controlled at a variety of levels including transcriptional, translational, and post-translational.

Mitochondrial structure and number is not static, rather changes in response to increased energy demands or physiological stimuli. Changes in mitochondrial number and structure are associated with a variety of chronic diseases, including heart failure, neurodegenerative diseases, and aging[35–40]. These changes in mitochondrial number and structure are driven by a complex network of nuclear transcription factors that synchronize the expression of nuclear and mitochondrial genes encoding mitochondrial proteins. PGC-1α is a member of a family of transcriptional coactivators that includes the closely related PGC-1β[41–43]. PGC-1 coactivators are regulated at the level of mRNA and protein expression in response to a variety of signaling

pathways, namely, AMPK and CaMK involved in growth, differentiation, and energy metabolism[44]. However, signaling through PGC-1α takes places, in part, through post-translational modifications. For example, PGC-1α is directly phosphorylated by AMPK and deacetylated by SIRT1 in response to energy depletion[45,46]. Studies involving forced overexpression of PGC-1α have shown that this transcriptional regulator is capable of driving virtually all aspects of mitochondrial biogenesis, including activation of respiratory chain and fatty acid oxidation genes, increased mitochondrial number and mitochondrial respiratory capacity[41,47,48]. PGC-1α exerts these effects through interaction with, and coactivation of PPARs, estrogen related receptors (ERRs), and nuclear respiratory factors 1 & 2 (NRF-1/NRF-2)[49,50]. Along these lines PPARα, not only can be activated by PGC-1α, but is widely known to exert transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes[51,52]. The complexity involving mitochondrial biogenesis and function suggest changes in gene expression play an important, albeit incomplete role in understanding mitochondrial function and energy metabolism. In our study we found that a large number of genes involved with fatty acid metabolism and mitochondrial energy metabolism / biogenesis were downregulated with age. These results are consistent with previous reports suggesting age-related reductions in fatty acid oxidation[10,11,13], AMPK activity[15,22,53], and mitochondrial function[7,40,54–57]. Interestingly, we found that the protein levels of PGC-1α, PPARα, and AMPKα_2 were not different between young and old hearts even though expression of each of these genes was decreased. This indicates that decreases in mRNA expression did not result in declines in protein expression. Lastly, we saw that protein activity in the form of citrate synthase was increased with age, which seems counter to a previous report of age-related changes in cardiac metabolism and mitochondrial function[12].

However, a number of previous studies support the idea that functional changes do not always coincide with coordinate changes in gene expression or protein content[58–60]. A recent study that utilized transcriptomic and metabolomic profiling to understand energy metabolism reprogramming in the hypertrophied heart demonstrated that metabolite levels distinguished the cardiac hypertrophic phenotype and that post-transcriptional regulatory events contributed to the metabolic reprogramming that occurs in hypertrophic hearts[60]. In addition, many of the changes in gene expression did not correlate with changes in metabolite pool size or cardiac dysfunction[60]. Notably, a previous report demonstrated in the aging heart that gene expression changes may or may not coincide with changes in that gene's protein product[54]. This previous study also demonstrated decreases in mitochondrial oxygen consumption and decreased expression of genes associated with mitogenesis and mitochondrial energy metabolism. In spite of those findings, mitochondrial number was increased and the mitochondrial marker, citrate synthase was not different in the senescent hearts compared to young[54]. This is consistent with our results of increased citrate synthase activity in old hearts compared to young hearts despite decreased gene expression of a large number of genes associated with mitochondrial biogenesis. This may be related to findings by Wanagat et al[40], which demonstrated mitochondrial dysfunction is associated with an increase in the expression of nuclear encoded genes, while genes encoded by mitochondrial DNA, such as cytochrome c oxidase (COX) are mutated and less abundant[40]. These authors postulated that as levels of mitochondrial encoded COX decline, the nucleus responds by upregulating mitochondrial volume[40]. This idea could explain how mitochondrial dysfunction can persist despite higher levels of citrate synthase activity found in aged hearts of our rats.

There is little to no information with regard to gene expression and protein content changes in exercise-trained aged rat hearts with regard to fatty acid oxidation, AMPK signaling or mitochondrial function, but functional studies led us to hypothesize that exercise training would mitigate age-related declines in metabolic gene expression. Surprisingly, we found that exercise training did not attenuate the age-related downregulation in the expression of genes involving fatty acid oxidation, AMPK signaling, and mitochondrial function. Specifically, in old exercise-trained hearts compared to old hearts we found a significant decline in the expression of PPARα (Ppara), which is a transcriptional activator of many genes involved with fatty acid oxidation. Genes regulated by PPARα that were also downregulated by exercise were Acyl CoA dehydrogenases (Acad), CD36, CPT1b and CPT2. There were no differences in AMPK signaling genes (Figure 2B) between Old + EXE and Old rats; however, there was an upregulation of AMPKα_1 in the exercise trained rats. AMPKα_1 is ubiquitously expressed in cells and has lower levels of expression in the myocardium compared to AMPKα_2 [61].We also found that compared to old rat hearts, exercise trained rats demonstrated further downregulation of many genes involved with glucose transport (Glut4), Kreb's cycle and mitochondrial function (complex I and III in the electron transport chain).

In our western blot analysis we found that in the old-exercise trained rats PPARα and AMPKα_2 protein content was decreased compared to young and old hearts, which was similar to our findings of decreased gene expression of these two genes. However, PGC-1α content was increased compared to young and old hearts despite the decrease in PGC-1α gene expression compared to young. Citrate synthase activity was not different in old-exercise trained hearts compared to young or old hearts. These results are in conflict with a report demonstrating that exercise training attenuated the age-related decrement in PPARα mRNA expression and protein

content in aged Wistar rat myocardium[14]. However, a previous study demonstrated that exercise training can elicit adaptations that lead to increases in exercise capacity along with augmented glucose and fatty acid oxidation in the myocardium despite no changes in the protein content of several proteins involved with glucose and fatty acid metabolism[58]. Strikingly, we found that exercise training improved functional exercise capacity (Figure 4) despite either no changes in gene expression compared to old hearts or in some cases further reductions in the expression of genes associated with energy metabolism and mitochondrial function. These data suggest that exercise training may impact other aspects of energy metabolism and mitochondrial function downstream of gene expression. Also, exercise is known to induce adaptations systemically, such as, skeletal muscle[62,63], which may have been responsible for the increased exercise capacity in our old exercise-trained rats.

Conclusion. This study was the first to assay a comprehensive list of genes involved with substrate metabolism, AMPK signaling, and mitochondrial function in the aged FBN rat heart and the effects that exercise training has on these age-related changes. We found aging results in the downregulation of a large number of genes associated with fatty acid metabolism, AMPK signaling and mitochondrial function, and exercise in aged-rats did not attenuate these changes. We also found that gene expression changes may or may not be reflective of changes at the level of protein expression. Future work including measurements of protein activity may help determine the molecular underpinnings of the effect that age and exercise training in aged hearts has on fuel metabolism and mitochondrial function.

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Table1. Gene list Glucose metabolism array

Symbol	Description
Acly	ATP citrate lyase
Aco1	Aconitase 1, soluble
Aco2	Aconitase 2, mitochondrial
Agl	Amylo-1,6-glucosidase, 4-alpha-glucanotransferase
Aldoa	Aldolase A, fructose-bisphosphate
Aldob	Aldolase B, fructose-bisphosphate
Aldoc	Aldolase C, fructose-bisphosphate
Bpgm	2,3-bisphosphoglycerate mutase
Cs	Citrate synthase
Dlat	Dihydrolipoamide S-acetyltransferase
Dld	Dihydrolipoamide dehydrogenase
Dlst	Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)
Eno1	Enolase 1, (alpha)
Eno2	Enolase 2, gamma, neuronal
Eno3	Enolase 3, beta, muscle
Fbp1	Fructose-1,6-bisphosphatase 1
Fbp2	Fructose-1,6-bisphosphatase 2
Fh	Fumarate hydratase 1
G6pc	Glucose-6-phosphatase, catalytic subunit
G6pc3	Glucose 6 phosphatase, catalytic, 3
G6pd	Glucose-6-phosphate dehydrogenase
Galm	Galactose mutarotase (aldose 1-epimerase)
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Gapdhs	Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic
Gck	Glucokinase
Gpi	Glucose phosphate isomerase
Gsk3a	Glycogen synthase kinase 3 alpha
Gsk3b	Glycogen synthase kinase 3 beta
Gys1	Glycogen synthase 1, muscle
Gys2	Glycogen synthase 2
H6pd	Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
Hk2	Hexokinase 2
Hk3	Hexokinase 3 (white cell)
Idh1	Isocitrate dehydrogenase 1 (NADP+), soluble
Idh2	Isocitrate dehydrogenase 2 (NADP+), mitochondrial
Idh3a	Isocitrate dehydrogenase 3 (NAD+) alpha
Idh3b	Isocitrate dehydrogenase 3 (NAD+) beta
Idh3g	Isocitrate dehydrogenase 3 (NAD), gamma
Mdh1	Malate dehydrogenase 1, NAD (soluble)
Mdh1b	Malate dehydrogenase 1B, NAD (soluble)
Mdh2	Malate dehydrogenase 2, NAD (mitochondrial)
Ogdhl	Oxoglutarate dehydrogenase-like
Pc	Pyruvate carboxylase
Pck1	Phosphoenolpyruvate carboxykinase 1 (soluble)
Pck2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)
Pdha2	Pyruvate dehydrogenase (lipoamide) alpha 2
Pdhb	Pyruvate dehydrogenase (lipoamide) beta
Pdhx	Pyruvate dehydrogenase complex, component X
Pdk1	Pyruvate dehydrogenase kinase, isozyme 1
Pdk2	Pyruvate dehydrogenase kinase, isozyme 2
Pdk3	Pyruvate dehydrogenase kinase, isozyme 3
Pdk4	Pyruvate dehydrogenase kinase, isozyme 4
Pdp2	Pyruvate dehyrogenase phosphatase catalytic subunit 2
Pdpr	Pyruvate dehydrogenase phosphatase regulatory subunit
Pfkl	Phosphofructokinase, liver
Pgam2	Phosphoglycerate mutase 2 (muscle)
Pgk1	Phosphoglycerate kinase 1
Pgk2	Phosphoglycerate kinase 2
Pgls	6-phosphogluconolactonase
Pgm1	Phosphoglucomutase 1
Pgm2	Phosphoglucomutase 2
Pgm3	Phosphoglucomutase 3
Phka1	Phosphorylase kinase, alpha 1
Phkb	Phosphorylase kinase, beta
Phkg1	Phosphorylase kinase, gamma 1
Phkg2	Phosphorylase kinase, gamma 2 (testis)
Pklr	Pyruvate kinase, liver and RBC
Prps1	Phosphoribosyl pyrophosphate synthetase 1
Prps111	Phosphoribosyl pyrophosphate synthetase 1-like 1
Pygl	Phosphorylase, glycogen, liver
Pygm	Phosphorylase, glycogen, muscle
Rbks	Ribokinase
Rpia	Ribose 5-phosphate isomerase A
Sdha	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
Sdhb	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
Sdhc	Succinate dehydrogenase complex, subunit C, integral membrane protein
Sdhd	Succinate dehydrogenase complex, subunit D, integral membrane protein
Sucla2	Succinate-CoA ligase, ADP-forming, beta subunit
Suclg1	Succinate-CoA ligase, alpha subunit
Suclg2	Succinate-CoA ligase, GDP-forming, beta subunit
Taldo1	Transaldolase 1
Tkt	Transketolase
Tpi1	Triosephosphate isomerase 1
Ugp2	UDP-glucose pyrophosphorylase 2

Table 2. Gene list for the Fatty Acid metabolism array

Symbol	Description
Acaa1a	Acetyl-Coenzyme A acyltransferase 1A
Acaa2	Acetyl-Coenzyme A acyltransferase 2
Acad10	Acyl-Coenzyme A dehydrogenase family, member 10
Acad11	Acyl-Coenzyme A dehydrogenase family, member 11
Acad9	Acyl-Coenzyme A dehydrogenase family, member 9
Acadl	Acyl-Coenzyme A dehydrogenase, long-chain
Acadm	Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain
Acads	Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain
Acadsb	Acyl-Coenzyme A dehydrogenase, short/branched chain
Acadvl	Acyl-Coenzyme A dehydrogenase, very long chain
Acat1	Acetyl-coenzyme A acetyltransferase 1
Acat2	Acetyl-Coenzyme A acetyltransferase 3
Acot12	Acyl-CoA thioesterase 12
Acot2	Acyl-CoA thioesterase 2
Acot3	Acyl-CoA thioesterase 3
Acot7	Acyl-CoA thioesterase 7
Acot8	Acyl-CoA thioesterase 8
Acot9	Acyl-CoA thioesterase 9
Acox1	Acyl-Coenzyme A oxidase 1, palmitoyl
Acox2	Acyl-Coenzyme A oxidase 2, branched chain
Acox3	Acyl-Coenzyme A oxidase 3, pristanoyl
Acsbg1	Acyl-CoA synthetase bubblegum family member 1
Acsbg2	Acyl-CoA synthetase bubblegum family member 2
Acsl1	Acyl-CoA synthetase long-chain family member 1
Acsl3	Acyl-CoA synthetase long-chain family member 3
Acsl4	Acyl-CoA synthetase long-chain family member 4
Acsl5	Acyl-CoA synthetase long-chain family member 5
Acsl6	Acyl-CoA synthetase long-chain family member 6
Acsm2a	Acyl-CoA synthetase medium-chain family member 2
Acsm3	Acyl-CoA synthetase medium-chain family member 3
Acsm4	Acyl-CoA synthetase medium-chain family member 4
Acsm5	Acyl-CoA synthetase medium-chain family member 5
Aldh2	Aldehyde dehydrogenase 2 family (mitochondrial)
Bdh1	3-hydroxybutyrate dehydrogenase, type 1
Bdh2	3-hydroxybutyrate dehydrogenase, type 2
Cpt1a	Carnitine palmitoyltransferase 1a, liver
Cpt1b	Carnitine palmitoyltransferase 1b, muscle
Cpt1c	Carnitine palmitoyltransferase 1c
Cpt2	Carnitine palmitoyltransferase 2
Crat	Carnitine acetyltransferase
Crot	Carnitine O-octanoyltransferase
Decr1	2,4-dienoyl CoA reductase 1, mitochondrial
Decr2	2,4-dienoyl CoA reductase 2, peroxisomal
Echs1	Enoyl Coenzyme A hydratase, short chain, 1, mitochondrial
Eci2	Enoyl-Coenzyme A delta isomerase 2
Ehhadh	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
Fabp1	Fatty acid binding protein 1, liver
Fabp2	Fatty acid binding protein 2, intestinal
Fabp3	Fatty acid binding protein 3, muscle and heart
Fabp4	Fatty acid binding protein 4, adipocyte
Fabp5	Fatty acid binding protein 5, epidermal
Fabp6	Fatty acid binding protein 6, ileal
Fabp7	Fatty acid binding protein 7, brain
Gcdh	Glutaryl-Coenzyme A dehydrogenase
Gk	Glycerol kinase
Gk2	Glycerol kinase 2
Gpd1	Glycerol-3-phosphate dehydrogenase 1 (soluble)
Gpd2	Glycerol-3-phosphate dehydrogenase 2, mitochondrial
Hadha	Hydroxyacyl-Coenzyme A dehydrogenase[1]
Hmgcl	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase
Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)
Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)
Lipe	Lipase, hormone sensitive
Lpl	Lipoprotein lipase
Mcee	Methylmalonyl CoA epimerase
Mut	Methylmalonyl-Coenzyme A mutase
Oxct2a	3-oxoacid CoA transferase 2A
Pecr	Peroxisomal trans-2-enoyl-CoA reductase
Ppa1	Pyrophosphatase (inorganic) 1
Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit
Prkaa2	Protein kinase, AMP-activated, alpha 2 catalytic subunit
Prkab1	Protein kinase, AMP-activated, beta 1 non-catalytic subunit
Prkab2	Protein kinase, AMP-activated, beta 2 non-catalytic subunit
Prkaca	Protein kinase, cAMP-dependent, catalytic, alpha
Prkacb	Protein kinase, cAMP dependent, catalytic, beta
Prkag1	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit
Prkag2	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit
Prkag3	Protein kinase, AMP-activated, gamma 3 non-catalytic subunit
Slc27a1	Solute carrier family 27 (fatty acid transporter), member 1
Slc27a2	Solute carrier family 27 (fatty acid transporter), member 2
Slc27a3	Solute carrier family 27 (fatty acid transporter), member 3
Slc27a4	Solute carrier family 27 (fatty acid transporter), member 4
Slc27a5	Solute carrier family 27 (fatty acid transporter), member 5
Slc27a6	Solute carrier family 27 (fatty acid transporter), member 6

[1] Hydroxyacyl-Coenzyme A dehydrogenase 3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit

Table 3. Gene list for the Custom array

Symbol	Description
Ppargc1	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
Nrf1	Nuclear respiratory factor 1
Tfam	Transcription factor A, mitochondrial
Sir2	Sirtuin (Silent mating type information regulation 2 homolog) 2
Esrra	Estrogen related receptor, alpha
Prkaa2	Protein kinase, AMP-activated, alpha 2 catalytic subunit
CREB1	cAMP response element binding protein 1
CaMK4	Calcium/calmodulin-dependent protein kinase IV
SOD2	Superoxide dismutase 2, mitochondrial
UCP2	Uncoupling protein 2 (mitochondrial proton carrier)
UCP3	Uncoupling protein 3 (mitochondrial proton carrier)
Bax	Bcl2 – associated X protein
Bcl2	B-cell CLL/lymphoma 2
Casp3	Caspase 3
Gabpa	GA binding protein transcription factor (alpha subunit) (i.e. NRF-2a)
UCP1	Uncoupling protein 1 (mitochondrial proton carrier)
Ppara	Peroxisome proliferator activated receptor alpha
Slc2a4	Solute carrier family 2 (facilitated glucose transporter) member 4
CD36	CD 36 molecule (thrombospondin receptor)
Acacb	Acetyl-Coenzyme A carboxylase beta
Mlycd	Malonyl-CoA decarboxylase
Ndufa1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1
Ndufa10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10
Ndufa11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 11
Ndufa2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2
Ndufa5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5
Ndufa6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6
Ndufa7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7
Ndufa8	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8
Ndufa9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9
Ndufab1	NADH dehydrogenase (ubiquinone) 1 alpha/beta subcomplex, 1
Ndufb2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2
Ndufb3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3
Ndufb5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5
Ndufb6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6
Ndufb7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7
Ndufb8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8
Ndufb9	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9
Ndufc2	NADH dehydrogenase (ubiquinone) 1 subcomplex unknown, 2
Ndufs1	NADH dehyrogenase (ubiquinone) Fe-S protein 1
Ndufs2	NADH dehyrogenase (ubiquinone) Fe-S protein 2
Ndufs3	NADH dehyrogenase (ubiquinone) Fe-S protein 3
Ndufs4	NADH dehyrogenase (ubiquinone) Fe-S protein 4
Ndufs6	NADH dehyrogenase (ubiquinone) Fe-S protein 6
Ndufs7	NADH dehyrogenase (ubiquinone) Fe-S protein 7
Ndufs8	NADH dehyrogenase (ubiquinone) Fe-S protein 8
Ndufv1	NADH dehydrogenase (ubiquinone) flavoprotein 1
Ndufv2	NADH dehyrogenase (ubiquinone) flavoprotein 2
Bcs1l	BCS1-like yeast
Cyc1	Cytochrome c-1
Uqcrb	Ubiquinol-cytochrome c reductase binding protein
Uqcrc1	Ubiquinol-cytochrome c reductase core protein 1
Uqcrc2	Ubiquinol-cytochrome c reductase core protein 2
Uqcrfs1	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1
Uqcrh	Ubiquinol-cytochrome c reductase hinge protein
Uqcrq	Ubiquinol-cytochrome c reductase, complex III subunit VII
Cox15	COX 15 homolog, cytochrome c oxidase assembly protein (yeast)
Cox17	COX 17 cytochrome c oxidase assembly homolog (S. cerevisiae)
Cox4i1	Cytochrome c oxidase subunit IV isoform 1
Cox4i2	Cytochrome c oxidase subunit IV isoform 2
Cox5a	Cytochrome c oxidase subunit Va
Cox5b	Cytochrome c oxidase subunit Vb
Cox6a1	Cytochrome c oxidase subunit VIa polypeptide 1
Cox6a2	Cytochrome c oxidase subunit VIa polypeptide 2
Cox6c	Cytochrome c oxidase subunit VIc
Cox7a2	Cytochrome c oxidase subunit VIIa polypeptide 2
Cox7a2l	Cytochrome c oxidase subunit VIIa polypeptide 2 like
Cox7b	Cytochrome c oxidase subunit VIIb polypeptide
Cox8a	Cytochrome c oxidase subunit VIIIa
Cox8c	Cytochrome c oxidase subunit VIIIc
mfn1	Mitofusin 1
mfn2	Mitofusin 2
fis1	Fission 1 (mitochondrial outer membrane) homolog (S. cerevisiae)
lonp1	Lon protease
Aifm2	Apoptosis-inducing factor, mitochondrial-associated 2
Bcl2l1	Bcl2-like 1
Clpb	ClpB caseinolytic peptidase B homolog (E. coli) (i.e. HSP 78)
pnpt1	Polyribonucleotide nucleotidyltransferase 1
Me1	Malic enzyme 1, NADP(+)-dependent, cytosolic
Foxo3	Forkhead box O3
Camkk2	Calcium/calmodulin-dependent protein kinase kinase 2,beta
Stk11	Serine/threonine kinase 11 (i.e. LKB1)
Ppargc1b	Peroxisome proliferator-activated receptor gamma, coactivator 1 beta
Tp53	Tumor protein p53

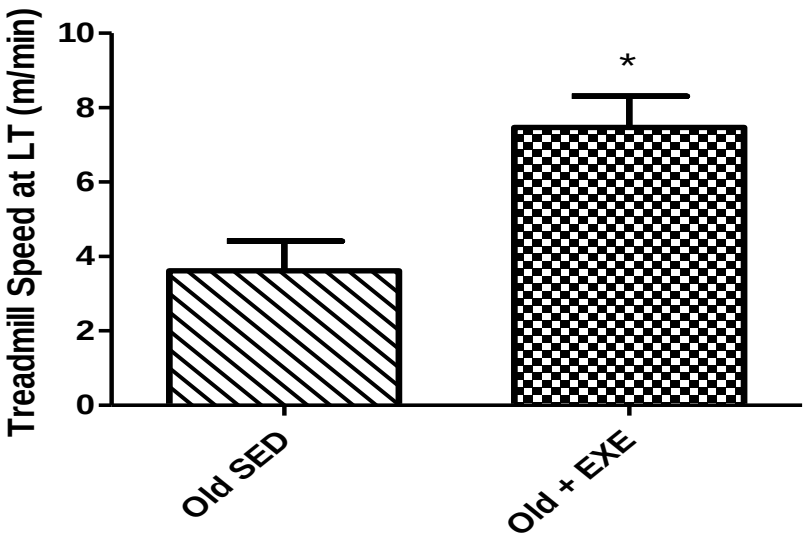
Table 4: Age-related gene expression changes in the left ventricle

FA Metabolism			
Gene name	**Gene symbol**	**Fold Regulation**	**p-value**
Acyl-CoA thioesterase 12	Acot12	2.88	0.026
Acyl-CoA thioesterase 7	Acot7	-1.86	0.017
Acyl-CoA thioesterase 8	Acot8	-1.44	0.02
Acyl-Coenzyme A oxidase 1, palmitoyl	Acox1	-2.1	0.032
Acyl-CoA synthetase bubblegum family member 1	Acsbg1	2.09	0.034
Acyl-CoA synthetase long-chain family member 6	Acsl6	-1.98	0.029
3-hydroxybutyrate dehydrogenase, type 1	Bdh1	-2.55	0.036
Carnitine palmitoyltransferase 1c	Cpt1c	-1.49	0.027
Carnitine palmitoyltransferase 2	Cpt2	-2.02	0.001
Carnitine acetyltransferase	Crat	-2.69	0.011
2,4-dienoyl CoA reductase 1, mitochondrial	Decr1	-2.05	0.03
Enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	Echs1	-2.39	0.004
Enoyl-Coenzyme A delta isomerase 2	Eci2	-1.79	0.049
Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh	-3.29	0.034
Fatty acid binding protein 3, muscle and heart	Fabp3	-2.84	0.017
Glutaryl-Coenzyme A dehydrogenase	Gcdh	-1.97	0.013
Glycerol-3-phosphate dehydrogenase 2, mitochondrial	Gpd2	-2.64	0.028
Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit	Hadha	-2.23	0.035
3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase	Hmgcl	-1.51	0.03
Lipase, hormone sensitive	Lipe	-2.14	0.03
Lipoprotein lipase	Lpl	-2.31	0.025
Peroxisomal trans-2-enoyl-CoA reductase	Pecr	-1.67	0.013
Pyrophosphatase (inorganic) 1	Ppa1	-1.78	0.03
Protein kinase, AMP-activated, alpha 2 catalytic subunit	Prkaa2	-2.09	0.034
Protein kinase, AMP-activated, beta 1 non-catalytic subunit	Prkab1	-3.96	0.041
Protein kinase, AMP-activated, beta 2 non-catalytic subunit	Prkab2	-1.7	0.041
Protein kinase, cAMP-dependent, catalytic, alpha	Prkaca	-2.03	0.005
Protein kinase, cAMP dependent, catalytic, beta	Prkacb	-1.65	0.031
Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	Prkag1	-1.77	0.002
Solute carrier family 27 (fatty acid transporter), member 1	Slc27a1	-2.02	0.03
Mitochondrial Function			
Peroxisome proliferator - activated receptor gamma, coactivator 1 alpha	Ppargc1	-2.14	0.03
NADH dehydrogenase (ubiquinone) Fe-S protein 7	Ndufs7	-2.24	0.015
Ubiquinol - cytochrome c reductase, complex III subunit VII	Uqcrq	-1.48	0.05
COX17 cytochrome c oxidase assembly homolog (S. cerevisiae)	Cox17	-1.68	0.011
Cytochrome c oxidase subunit IV isoform 1	Cox4i1	-1.72	0.02
Cytochrome c oxidase subunit VIIa polypeptide 2 like	Cox7a2l	-1.35	0.049
Mitofusin 1	mfn1	-2.41	0.01
Mitofusin 2	mfn2	-3.21	0.028
Clpb caseinolytic peptidase B homolog (E. coli) (i.e. HSP78)	clpb	-2.3	0.039
Calcium/calmodulin-dependent protein kinase kinase 2, beta	Camkk2	-1.77	0.017
Serine/threonine kinase 11 (i.e. LKB1)	Stk11	-1.86	0.029
Peroxisome proliferator - activated receptor gamma, coactivator 1 beta	Ppargc1b	-2.69	0.012
Tumor protein p53	Tp53	-2.19	0.025

Table 5: Exercise-related changes in gene expression in Old FBN rats

Glucose Metabolism			
Gene name	**Gene symbol**	**Fold Regulation**	**p-value**
Amylo-1,6-glucosidase, 4-alpha-glucanotransferase	Agl	-6.15	0.024
Aldolase A, fructose-bisphosphate	Aldoa	-5.28	0.036
Enolase 1, (alpha)	Eno1	-4.10	0.042
Enolase 2, gamma, neuronal	Eno2	-3.27	0.019
Glucose phosphate isomerase	Gpi	-5.59	0.005
Glycogen synthase kinase 3 alpha	Gsk3a	-3.17	0.046
Isocitrate dehydrogenase 3 (NAD+) beta	Idh3b	-7.70	0.040
Isocitrate dehydrogenase 3 (NAD), gamma	Idh3g	-5.41	0.027
Malate dehydrogenase 2, NAD (mitochondrial)	Mdh2	-6.31	0.009
Pyruvate dehydrogenase kinase, isozyme 1	Pdk1	-9.29	0.004
Pyruvate dehydrogenase kinase, isozyme 2	Pdk2	-6.48	0.006
Phosphoglucomutase 3	Pgm3	-1.70	0.037
Phosphorylase kinase, gamma 1	Phkg1	-2.78	0.011
Ribose 5-phosphate isomerase A	Rpia	-2.38	0.018
Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	Sdha	-3.93	0.038
Succinate-CoA ligase, ADP-forming, beta subunit	Sucla2	-5.64	0.021
Solute carrier family 2 (facilitated glucose transporter), member 4	Slc2a4	-10.45	0.025
FA Metabolism			
Acetyl-Coenzyme A acyltransferase 1A	Acaa1a	-9.79	0.001
Acetyl-Coenzyme A acyltransferase 2	Acaa2	-11.70	0.029
Acyl-Coenzyme A dehydrogenase family, member 10	Acad10	-7.02	0.002
Acyl-Coenzyme A dehydrogenase family, member 11	Acad11	-14.38	0.002
Acyl-Coenzyme A dehydrogenase family, member 9	Acad9	-8.81	0.008
Acyl-Coenzyme A dehydrogenase, long-chain	Acadl	-21.18	0.002
Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	Acadm	-15.17	0.009
Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	Acads	-17.09	0.001
Acyl-Coenzyme A dehydrogenase, short/branched chain	Acadsb	-7.75	0.007
Acyl-Coenzyme A dehydrogenase, very long chain	Acadvl	-9.49	0.002
Acetyl-coenzyme A acetyltransferase 1	Acat1	-14.79	0.004
Acetyl-Coenzyme A acetyltransferase 3	Acat2	-6.25	0.003
Acyl-CoA thioesterase 2	Acot2	-18.92	< 0.001
Acyl-CoA thioesterase 3	Acot3	-3.52	0.024
Acyl-CoA thioesterase 7	Acot7	-4.43	0.027
Acyl-CoA thioesterase 8	Acot8	-2.68	0.002
Acyl-CoA thioesterase 9	Acot9	-5.41	0.001
Acyl-Coenzyme A oxidase 1, palmitoyl	Acox1	-5.16	0.007
Acyl-Coenzyme A oxidase 3, pristanoyl	Acox3	-4.47	0.015
Acyl-CoA synthetase long-chain family member 1	Acsl1	-18.06	< 0.001
Acyl-CoA synthetase long-chain family member 3	Acsl3	-3.60	0.008
Acyl-CoA synthetase long-chain family member 4	Acsl4	-2.46	0.011
Acyl-CoA synthetase long-chain family member 5	Acsl5	-2.23	0.001
Acyl-CoA synthetase long-chain family member 6	Acsl6	-5.54	0.010
3-hydroxybutyrate dehydrogenase, type 1	Bdh1	-10.48	0.040
3-hydroxybutyrate dehydrogenase, type 2	Bdh2	-5.03	0.008
Carnitine palmitoyltransferase 1a, liver	Cpt1a	-3.04	0.033
Carnitine palmitoyltransferase 1b, muscle	Cpt1b	-7.93	0.003
Carnitine palmitoyltransferase 2	Cpt2	-7.02	0.004
Carnitine acetyltransferase	Crat	-4.27	0.018
Fatty acid binding protein 3, muscle and heart	Fabp3	-7.31	0.034
Protein kinase, AMP-activated, alpha 1 catalytic subunit	Prkaa1	1.83	0.042
Protein kinase, cAMP-dependent, catalytic, alpha	Prkaca	-2.89	0.041
Protein kinase, cAMP dependent, catalytic, beta	Prkacb	-1.43	0.047
Solute carrier family 27 (fatty acid transporter), member 1	Slc27a1	1.73	0.033
Solute carrier family 27 (fatty acid transporter), member 4	Slc27a4	3.15	0.002
CD36 molecule (thrombospondin receptor)	CD36	-2.97	0.001
Peroxisome proliferator activated receptor alpha	Ppara	-9.28	0.029
Mitochondrial Function			
Sirtuin (silent mating type information regulation 2 homolog) 1 (S. cerevisiae)	Sirt1	-3.27	0.036
NADH dehyrogenase (ubiquinone) 1 alpha subcomplex, 1	Ndufa1	-2.24	0.040
NADH dehyrogenase (ubiquinone) 1 alpha subcomplex, 6 (B14)	Ndufa6	-4.70	0.041
NADH dehyrogenase (ubiquinone) 1 alpha subcomplex, 7	Ndufa7	-5.67	0.033
NADH dehyrogenase (ubiquinone) 1 alpha subcomplex, 8	Ndufa8	-10.41	0.005
NADH dehyrogenase (ubiquinone) 1 alpha subcomplex, 9	Ndufa9	-5.71	0.018
NADH dehyrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	Ndufab1	-5.67	0.001
NADH dehyrogenase (ubiquinone) 1 beta subcomplex, 2	Ndufb2	-4.49	0.006
NADH dehyrogenase (ubiquinone) 1 beta subcomplex, 5	Ndufb5	-4.72	0.032
NADH dehyrogenase (ubiquinone) 1 beta subcomplex, 8	Ndufb8	-4.92	0.010
NADH dehyrogenase (ubiquinone) Fe-S protein 6	Ndufs6	-2.94	0.047
Cytochrome c-1	Cyc1	-5.47	0.033
Ubiquinol-cytochrome c reductase core protein 2	Uqcrc2	-5.38	0.016
Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	Uqcrfs1	-5.11	0.045

A



B

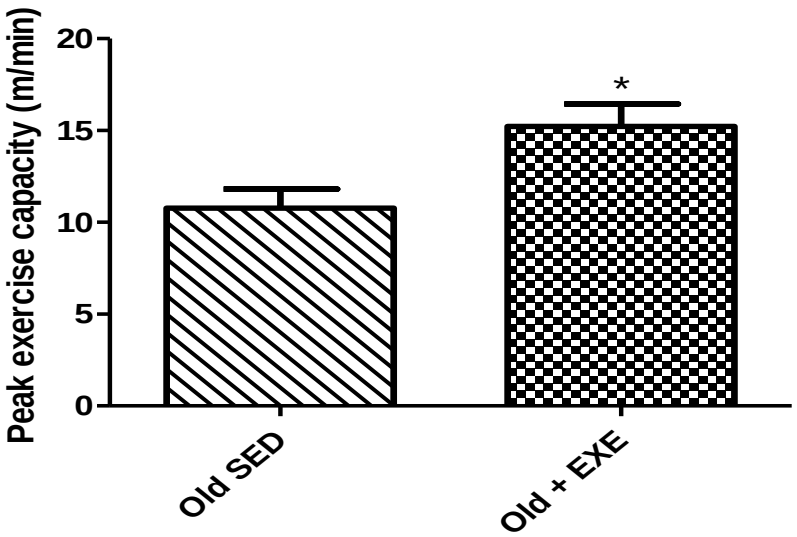


Figure 1. Values indicate Means ± S.E.M. Effects of exercise training on peak exercise capacity in 34 month old FBN rats. 1A.We measured exercise capacity as a function of treadmill speed at lactate threshold (LT) 1B. Peak exercise capacity was measured as the maximal speed at the end of the maximal exercise testing. Old SED (n = 9), Old + EXE (n = 9) * $P < 0.05$

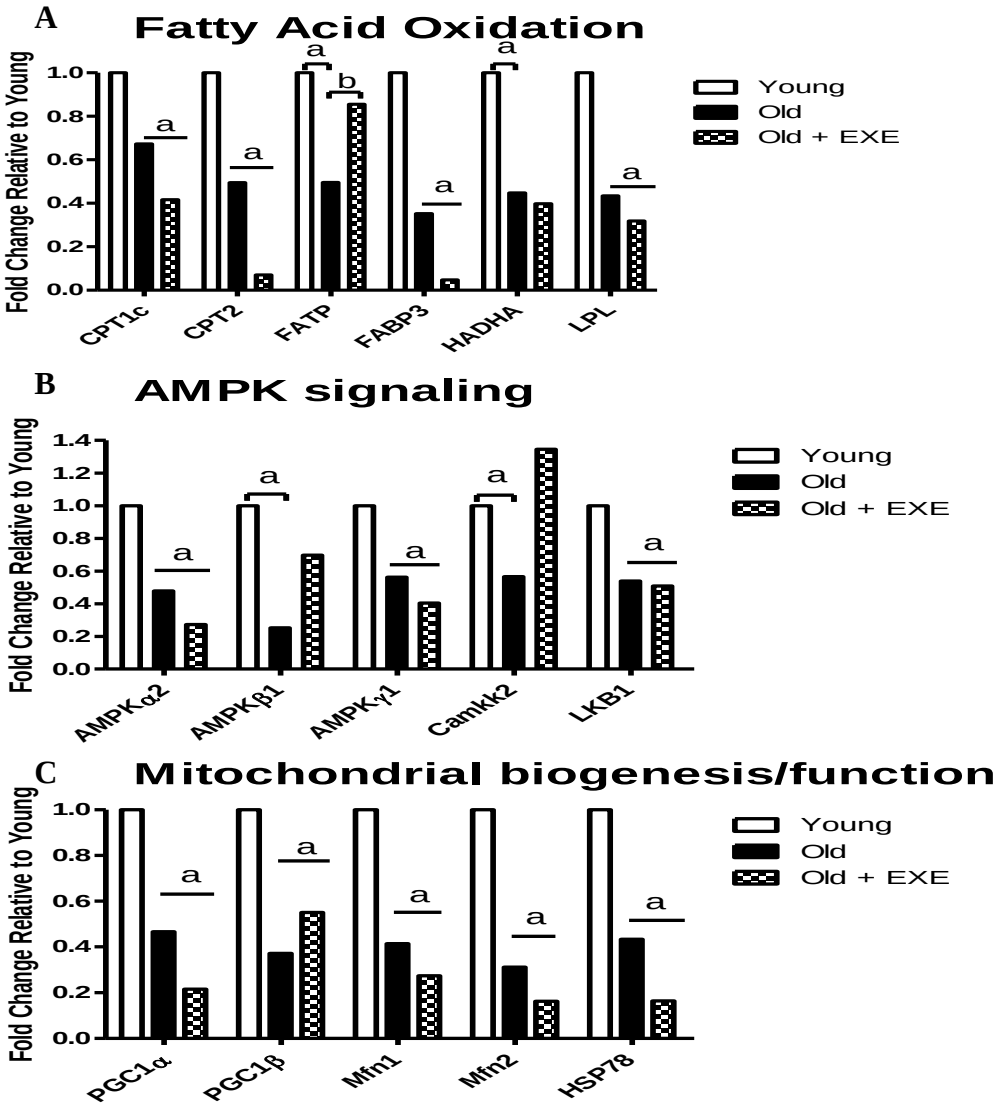


Figure 2. Fold changes in gene expression in Old and Old + EXE hearts relative to young hearts. 1A. Genes involved in fatty acid transport and oxidation decline with age and this attenuation is not mitigated with exercise. (CPT1c – Carnitine-palmitoyl transferase 1c, CPT2 – Carnitine palmitoyl transferase 2, FATP – Fatty acid transporter, FABP3 – Fatty acid binding protein 3, HADHA – Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-CoA hydratase (trifunctional protein), LPL – Lipoprotein lipase). 1B. AMPK signaling decrements with age involve changes in the gene expression of AMPK subunits and upstream kinases. (AMPKα2 – Protein kinase, AMP – activated alpha 2 catalytic subunit, AMPKβ1 - Protein kinase, AMP – activated beta 1 non-catalytic subunit, AMPKγ1 - Protein kinase, AMP – activated gamma 1 non-catalytic sununit, Camkk2 – Calcium/calmodulin-dependent protein kinase kinase 2 beta, LKB1 – Liver kinase B 1 1C. Mitochondrial biogenesis and maintenance of mitochondrial function decline with age and exercise does not appear to improve the attenuation. (PGC1α – Peroxisome proliferator – activated receptor gamma, coactivator 1 alpha, PGC1β – Peroxisome proliferator – activator receptor gamma,coactivator 1 beta, Mfn1 – Mitofusin 1, Mfn2 – Mitofusin 2, HSP78 – Heat shock protein 78).

[a] $P < 0.05$ compared to Young, [b] $P < 0.05$ compared to Old + EXE

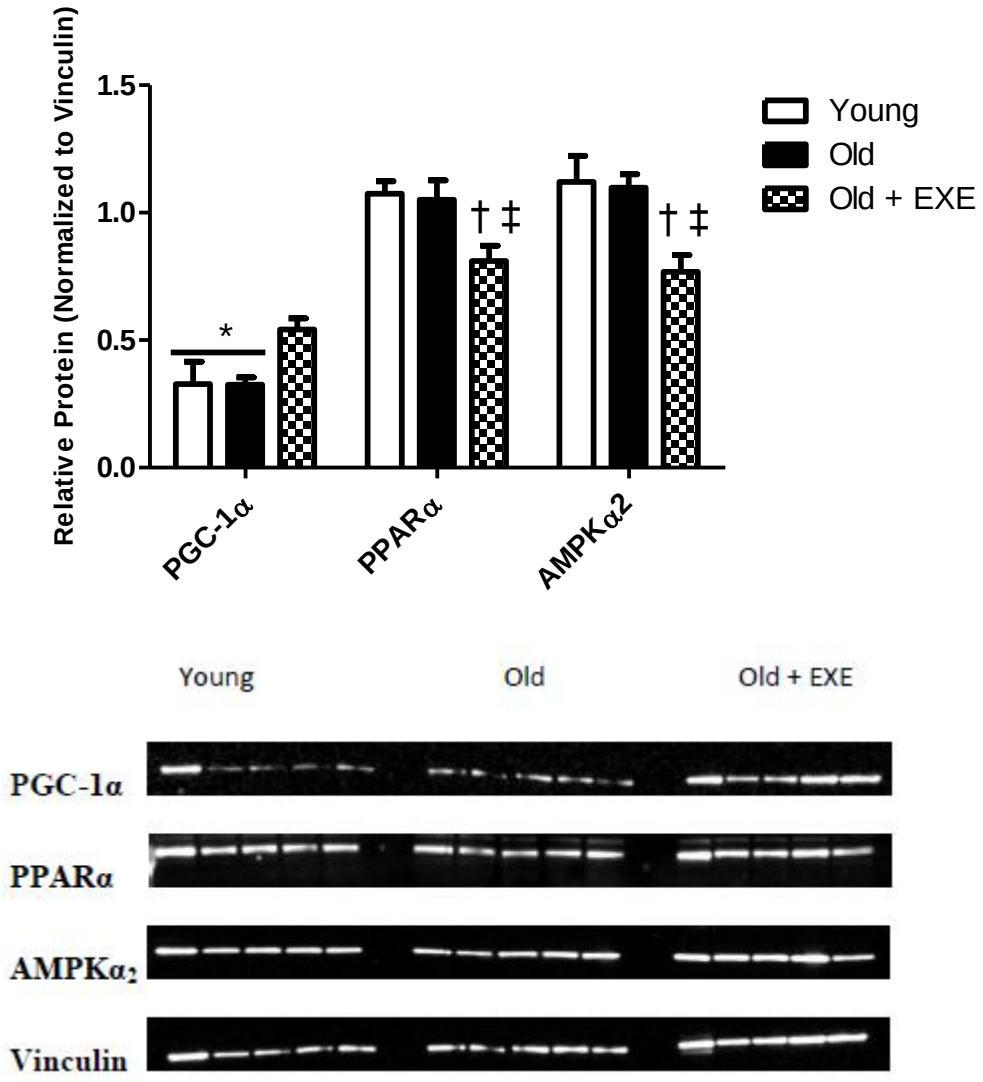


Figure 3. Values indicate Means ± S.E.M. Relative protein content in Young, Old, and Old + EXE groups. Old + EXE demonstrate increases in PGC-1α but decreased PPARα and AMPKα_2 protein content compared to Young and Old, respectively.

* $P < 0.05$ vs. Old + EXE, † $P < 0.05$ vs. Young, ‡ $P < 0.05$ vs. Old

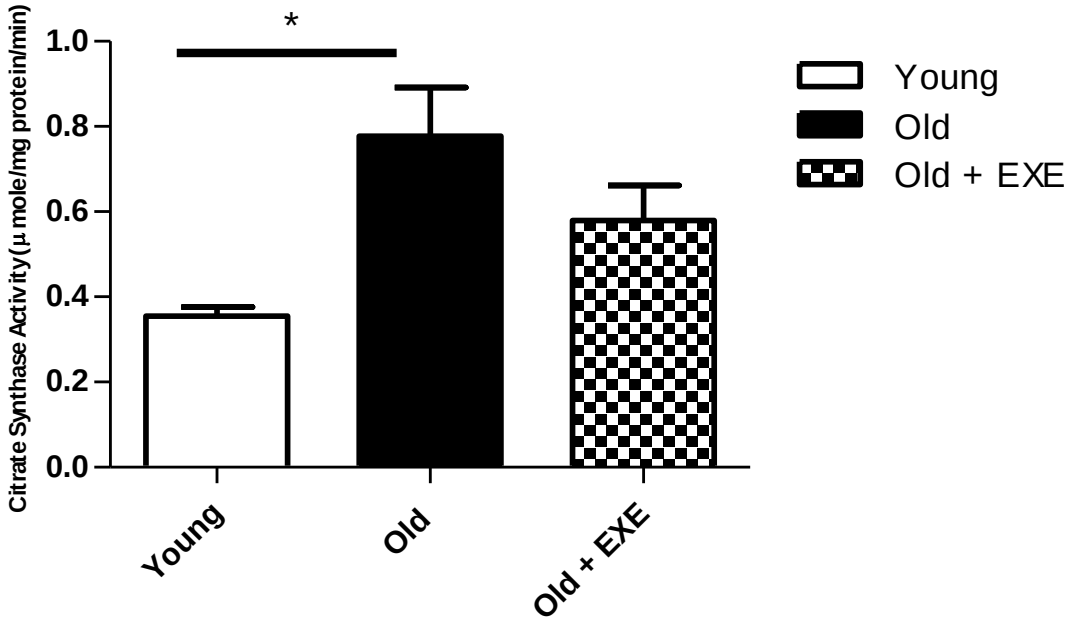


Figure 4. Values indicate Means ± S.E.M. Citrate synthase activity in left ventricular homogenates in young, old, and old exercise-trained hearts.

* $P < 0.05$

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SUMMARY AND CONCLUSIONS

Aging is associated with declining contractile function, but the cellular and molecular mechanisms are not completely understood. Along with declining contractile function with age, there is altered substrate metabolism and mitochondrial dysfunction. Most studies examining the effects of variations in substrate utilization have been performed in whole heart preparations. Thus, it is difficult to surmise the impact substrates have on altered contractile function. The study of cellular contractile function may yield additional insights into the mechanisms and consequences of age-related changes in cardiac metabolism. The role that altered substrate metabolism and mitochondrial dysfunction have on the age-related contractile dysfunction has yet to be defined. Previous studies have explored contractile function and substrate metabolism or mitochondrial function in parallel. In order to fundamentally determine the impact that altered energetics has on contractile function with age there is an increased need for studies that include simultaneous measurements in order to better understand the relationship between contractile dysfunction and altered energetics in the aged myocardium. In this study, we have addressed age-related changes in mitochondrial energetics and potential substrate-specific effects on contractile function in aged cardiac myocytes. In addition, we have addressed molecular mechanisms that may explain the changes. The significant conclusions outlined in this dissertation are as follows:

1) In Chapter II, we describe simultaneous measurements of steady-state $[NADH]_m$ levels and cell shortening properties in cardiac myocytes from the hearts of young adult and old rats. Although there were no differences in contractile function at 4 Hz, $[NADH]_m$ levels significantly declined in old cardiac myocytes during ten minutes of continuous stimulation, while young myocytes maintained NADH levels throughout the duration of

the stimulation protocol. Along with age-related decreases in $[NADH]_m$ levels, we demonstrated a significant correlation between $[NADH]_m$ and contractile properties in old myocytes that was not observed in young myocytes. Secondly, we described measurements of cell shortening properties when cardiac myocytes from young and old FBN rats were administered rotenone to induce a metabolic stress or isoproterenol to increase the workload on the cell. Old myocytes demonstrated significant declines in the rate of shortening compared to young myocytes when administered rotenone or isoproterenol. Previous work has described altered mitochondrial or metabolic function in the aged myocardium, but this is the first description of age-related altered metabolic function in contracting myocytes which was correlated with altered contractile function. These results suggest that aged myocytes demonstrate contractile dysfunction under increased stress which may be related to altered metabolism or mitochondrial function.

2) In Chapter III, we describe, for the first time, cellular contractile properties when young and old cardiac myocytes from FBN hearts were perfused with either glucose or oleate as substrates. We also describe the effect that increasing workload had on contractile properties in the presence of these two substrates. Young myocytes demonstrated better contractile function when perfused with glucose as compared to oleate. Whereas, with increasing workload, old myocytes demonstrated functional decrements in contractile properties when glucose was the sole substrate compared to oleate. Oleate-induced declines in contractile function in young myocytes were observed with increasing workload. This decline in contractile function with increasing workload with oleate in young myocytes may have been due to a decline in mitochondrial membrane potential[1]. The fact that contractile function in old myocytes was better with oleate as a substrate

was surprising, given that previous work suggested that aged hearts demonstrate decreased fatty acid utilization. This result could be explained by the increased prevalence of insulin-resistance with age[2–5]. Insulin resistance results in a change in substrate metabolism in the heart, demonstrated by decreases in glycolysis and glucose oxidation and an increase in fatty acid oxidation[6–10]. There have been a few studies on isolated cardiac myocytes from insulin-resistant rodents that demonstrated that palmitate perfusion enhanced cell shortening properties compared to glucose [11,12]. One report demonstrated that contractile dysfunction was not observed in insulin-resistant cardiac myocytes until workload stress was induced, but this contractile dysfunction was mitigated by palmitate perfusion [12]. Taken together, we concluded that young myocytes demonstrate better contractile function when glucose is the sole substrate compared to oleate, but old myocytes demonstrate augmented contractile function when oleate is the sole substrate as compared to glucose. Future works investigating the mechanisms behind these substrate-specific effects with age will clarify how metabolic processes impact cardiac contractile function.

3) In Chapter IV, we describe potential molecular mechanisms describing the functional changes we observed in Chapters II and III. We also examined whether exercise training in aged FBN hearts could mitigate the age-related changes in gene expression. In old FBN hearts we saw declines in the expression of genes involved with fatty acid oxidation, AMPK signaling, and mitochondrial function/biogenesis compared to young FBN hearts. Exercise training did not mitigate these age-related decrements in gene expression. We also measured the relative protein content of a select number of proteins to assay whether changes in gene expression had an impact on the levels of protein

content for PPARα, AMPKα_2, and PGC-1α. Interestingly, we found that in the Old and Old + EXE hearts that gene expression changes and the protein content are not necessarily in agreement with each other. Specifically, PPARα, PGC-1α, and AMPKα_2 protein content was not different in young and old hearts even though the expression of these three genes was decreased in old compared to young. However, we found in exercise trained hearts that PGC-1α protein content was greater compared to young and old hearts, but PPARα and AMPKα_2 protein content was diminished. Notably, despite diminished gene expression and protein content of important proteins involved in energy metabolism in the exercise trained rat hearts, these rats did demonstrate increased exercise capacity compared to old sedentary rats at 34 months of age. In the exercise-trained rats, these results indicate that decreased gene expression and protein content of genes involved with mitochondrial energetics is not reflective of their ability to produce external work. Taken together, these results suggest that differences in protein activity at the level of post-translational modifications may play a strong role in functional changes at the cellular level with age.

Conflicting results

4) In Chapter II, we found that under basal conditions, contractile function between young and old myocytes were not different but NADH levels declined in old myocytes. Further, we described a significant correlation between NADH and contractile function in old myocytes, suggesting that old myocytes contractile function is dependent upon NADH production. We also demonstrated when the myocytes were administered rotenone or isoproterenol; old myocytes demonstrated declining contractile function compared to young myocytes. From our gene expression analysis in Chapter IV, we found that there

were no age-related changes to the expression of genes for glucose metabolism and all of the myocytes in Chapter II were perfused with glucose. This suggests that altered glucose metabolism may not be the culprit behind that contractile dysfunction or altered metabolism observed in Chapter II. It is important to note that we did not measure sarcolemmal Glut4 protein content, and it is feasible that decreases in glucose transport played a role in our observed results. However, we did demonstrate that the expression of genes involving AMPK signaling, and mitochondrial biogenesis and function, particularly PGC-1α and genes involved with the electron transport chain (Complex I, III, and IV) were decreased with age. Thus, it appears that the age-related altered mitochondrial energetics and contractile dysfunction could be due to downregulation of genes involving mitochondrial energetics and reductions in the cellular energy sensor, AMPK. The complicating matter is that we found no differences in the protein content of PGC-1α and AMPKα_2 between young and old hearts. Taken together, we can conclude that the molecular underpinnings describing our results in Chapter II are not completely understood, and changes in protein activity at the level of posttranslational modifications likely play a role in age-related decrements in mitochondrial energetics. More research in the area of protein activity measurements may better elucidate the molecular mechanisms describing the declining NADH levels and contractile dysfunction in aged myocytes.

5) In Chapter III, we found that young myocytes are better able to use glucose compared to oleate, while old myocytes appear to maintain contractile function better when oleate was the sole substrate compared to glucose. The results found in young myocytes do not appear to be related to gene expression changes or protein content that we assayed in Chapter IV, but is most likely due to substrate-specific effects of oleate on cell contractile

function. Specifically, previous research has demonstrated in young healthy myocytes that oleate perfusion resulted in a degradation of the mitochondrial membrane potential, possibly by inducing uncoupling protein activity[1,13]. In old myocytes, we found that contractile function was augmented when oleate was the sole substrate compared to glucose, yet our gene expression changes would indicate fatty acid oxidation is decreased. However, we did not see any observed changes in PPARα protein content between young and old, suggesting that fatty acid oxidation may not necessarily be impaired. One future study to better clarify the mechanism behind how oleate perfusion in aged myocytes results in better contractile performance compared to glucose, would be to assay PPARα DNA binding activity and perform a protein activity assay on hydroxyacyl CoA dehydrogenase (rate-limiting step of β-oxidation) to better understand fatty acid metabolism in the aged myocardium.

Summary

Myocardial aging results in altered metabolic function and contractile dysfunction. This altered metabolic function with age is correlated with altered contractile function. Changes in substrate utilization appear to be a culprit in the altered metabolism and contractile dysfunction with age. Aging hearts demonstrate widespread decreases in the expression of genes involved with substrate metabolism and mitochondrial function, but these changes may not coincide with the level of protein for a specific gene. Thus, the molecular mechanisms involved with altered substrate utilization and mitochondrial function are complex and may be due to changes in the activity of proteins involved with substrate utilization and mitochondrial function.

Limitations

Selection bias. Selection bias is inherent in any study of isolated myocytes, as only those cells that withstand the rigorous isolation procedure can be studied. This limitation is exacerbated in aging studies as myocyte function is diminished. Unlike the myocytes from young animals, many old myocytes were unable to last the entire ten minutes of the stimulation protocol, which was even more pronounced during the administration of rotenone or isoproterenol due to the development of arrhythmias or cessation of contraction. There was increased variability in contractile property responses in aged myocytes compared to young. This may imply that myocytes are not a heterogeneous pool and the adaptation during the aging process occurs heterogeneously. One possible method to counter this increased variability with age would be to measure a greater number of cells, however, this was not possible in these current studies because the number of cardiac myocytes are reduced with aging [14,15] and aging accelerates cell death even more [16]. This could be a limitation of the current study in that cardiac myocyte contractile data may only be collected from relatively more robust and healthy cells, thus eliminating from the pool of myocytes cells that may have had greater functional declines but could not survive the isolation process. Regardless of the limitation, contractile function was diminished in old myocytes compared to young when a metabolic and workload stress was induced by the administration of rotenone or isoproterenol, respectively.

NADH measurements. We investigated steady-state NADH autofluorescence and simultaneous cellular contractile properties. We assayed changes in steady-state NADH autofluorescence as a measure of mitochondrial function. The fluorescence of single cells has been previously used as a measure of mitochondrial NADH [17–21]. Although single cell fluorescence cannot distinguish between cytosolic and mitochondrial NADH, over 80% of cell

autofluorescence in myocytes has been shown to originate from mitochondrial NADH [20,22]. However, we cannot rule out the possibility of the decline in $[NADH]_m$ in old myocytes that we demonstrated in Chapter II, could in part be due to the oxidation of NADH by lactate dehydrogenase (LDH). One experiment that could be performed in the future would be to measure NADH autofluorescence in old myocytes while being perfused with lactate, which can only be metabolized via pyruvate oxidation. This experiment would be able to begin to delineate if indeed, the changes in NADH may be reflective of increased activity of LDH. Although, NADH oxidation via LDH is a possible explanation for our declines in NADH levels over time in old myocytes, it is not the most likely explanation. A previous study in single cardiac myocytes, demonstrated the intricate relationship between mitochondrial Ca^{2+} uptake and changes in NADH fluorescence with an increase in workload [20]. These investigators demonstrated that when mitochondrial Ca^{2+} uptake was inhibited, NADH autofluorescence did not increase with an increase in workload, suggesting that changes in NADH fluorescence in single cardiac myocytes is most likely due to changes in NADH levels in the mitochondria. One future experiment that would strengthen our results would be to measure NADH fluorescence, cell shortening and mitochondrial Ca^{2+} simultaneously in old cardiac myocytes.

Remaining Questions

Mitochondrial Ca^{2+}uptake and its relationship to $[NADH]_m$ in aged myocytes. Several investigations have attempted to identify cellular components that link mitochondrial respiration and cardiac demand. Ca^{2+} release by the sarcoplasmic reticulum (SR) appears to be a central player in modulating the metabolic rate of the myocardium in response to changes in energetic demand[23,24]. Ca^{2+} enters the mitochondria through the mitochondrial calcium uniporter[25], which is driven by the membrane potential across the inner mitochondrial membrane[25] which is

dependent upon electron transport activity and mitochondrial metabolism[26,27]. Furthermore, mitofusin 2 is a protein that tethers the sarcoplasmic reticulum to the mitochondrial outer membrane[28] and the loss of mitofusin 2 severely limits mitochondrial Ca^{2+} uptake[23]. We demonstrated declining $[NADH]_m$ levels during continuous stimulation in the old, and these changes in NADH were correlated with contractile function in old myocytes. We also demonstrated that mitofusin 2 gene expression was downregulated in aged hearts compared to young hearts. Previous research has implicated mitochondrial Ca^{2+} uptake to the activation of mitochondrial dehyodrogenases and subsequent NADH production within the Kreb's cycle in response to a change in cardiac workload[19,20,29]. Indeed, one study demonstrated that inhibition of the Ca^{2+} uniporter in the mitochondrial membrane assuaged the increase in mitochondrial NADH in response to a stepwise augmentation in workload in young healthy myocytes[20]. Our results of declining mitochondrial NADH levels in old myocytes could be explained by an inability of aged myocytes to maintain appropriate mitochondrial Ca^{2+}-uptake during continuous stimulation. Reductions in mitochondrial Ca^{2+}-uptake would lead to reduced activation of mitochondrial dehydrogenases and subsequently, reductions in NADH production. A future study, measuring mitochondrial Ca^{2+}, $[NADH]_m$, and contractile function simultaneously would provide more insight into the role mitochondrial Ca^{2+} uptake has on $[NADH]_m$ and contractile function in aged myocytes.

Insulin-insensitivity in aged rodents. We found that oleate mitigated the contractile dysfunction with increasing workload in old cardiac myocytes compared to the contractile deficits observed when glucose was the sole substrate. Our results are similar to results in single cardiac myocytes from insulin-resistant hearts [11,12]. Along with the mitigating effect of free fatty acids on contractile function in insulin-resistant cardiac myocytes, one study demonstrated that

the mechanism behind free fatty acid- induced improvements in contractile function could be due to reduced reactive oxygen species and improved redox balance in cardiac myocytes from insulin-resistant myocytes [12]. However, we did not measure glucose tolerance to determine whether our old FBN rats do demonstrate insulin insensitivity. Measuring glucose tolerance along with measurements of contractile function, NADH levels and mitochondrial ROS during continuous stimulation when single cardiac myocytes are perfused with either glucose or oleate, with and without isoproterenol, would provide insight into substrate metabolic effects and whether these substrate effects are due to insulin resistance in aged myocyte contractile function.

Oleate-induced decrements in contractile function in young myocytes. We demonstrated that young cardiac myocytes show reduced contractile function when perfused with oleate as compared to glucose when workload was increased. Since a previous study demonstrated oleate-induced decrements in the mitochondrial membrane potential [1] in cardiac myocytes, one future experiment that would allow us to determine the mechanism behind these oleate-induced decrements in contractile function would be to perform simultaneous measurements on contractile function and mitochondrial membrane potential during continuous stimulation in single cardiac myocytes from young FBN rats.

More analysis of changes in protein content. 42 genes associated with fatty acid oxidation, mitochondrial energetics/ mitogenesis, and AMPK signaling was decreased in aged hearts. We measured a protein that represents fatty acid oxidation (PPARα), mitochondrial biogenesis (PGC-1α), and AMPK signaling ($AMPK\alpha_2$). We found that the protein levels of PPARα, PGC-1α, and $AMPK\alpha_2$ were not different between young and old hearts, even though the expression of each of these genes was decreased in old hearts. This indicates that decreases in mRNA expression did not result in declines in protein expression. However, we only measured the

protein content of three proteins representing each of the three major areas above. In order to determine the relationship between gene expression changes and protein changes, measuring the protein content from many more of the 42 genes that demonstrated decreased expression would provide greater insight into the relationship between gene expression and protein expression.

Measurements of protein activity. While gene expression changes and relative protein content assays are a great starting point to understanding the molecular mechanisms involved in the relationship between age-related cardiac energetic and contractile changes, our results suggest that there is an increased need for the study of protein activity. Among the many levels of regulation of a biological system with age, measurements of protein activity may be the area of research that illuminates the mechanisms driving age-related alterations to cellular energetics and contractile function.

Exercise training effects in aged myocytes. We demonstrated that aged rats do respond to exercise training by increases in the lactate threshold and peak exercise capacity (maximal running speed achieved during the maximal test). What we did not measure in these hearts were cardiomyocyte shortening properties, and previous work in permeabilized cardiac myocytes from aged rats demonstrated that exercise training results in increases in power output[30]. This study indicates exercise-induced adaptations at the level of the myofilament, but to our knowledge what is currently unknown, is whether exercise training elicits adaptations in cellular metabolic function that could result in potentially improved contractile function. A future experiment studying substrate-specific effects on exercise-trained aged cardiomyocyte shortening properties, similar to our work in Chapter III, would provide insight into whether exercise training induces cellular metabolic adaptations and whether these adaptations impact aged cardiomyocyte contractile function.

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