THESIS

MECHANISN AND SUBPOPULATION SPECIFICITY OF MITOCHONDRIAL REACTIVE OXYGEN SPECIES RELEASE IN THE POST-ISCHEMIC HYPERTHYROID MYOCARDIUM

Submitted by

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ABSTRACT

MECHANISM AND SUBPOPULATION SPECIFICITY OF MITOCHONDRIAL ROS RELESASE IN THE POST-ISCHEMIC HYPERTHYROID MYOCARDIUM

Hyperthyroidism (HT) augments release of reactive oxygen species (ROS) from cardiac mitochondria following myocardial ischemia/reperfusion (I/R). The present study examined the mechanisms of this phenomenon and determined whether subsarcolemmal (SSM) and intermyofibrillar mitochondria (IFM) are differentially affected. Male SD rats received 10 daily injections of thyroid hormone (30ug/kg i.p.; HT) or vehicle (CON) before hearts were excised and exposed to a 20/25 min global I/R protocol ex vivo. Following I/R, ROS release was assessed in freshly isolated SSM and IFM using the Amplex Red assay with a variety of substrate and inhibitor combinations to examine sites and mechanisms of release. ROS release from SSM exceeded IFM in CON and HT hearts by 25–50% following I/R ($P < 0.01$). Surprisingly, HT augmented ROS release from SSM, but decreased ROS release from IFM ($P < 0.05$ for both). Blocking electron flow from respiratory complex 1 to 3 abolished the effect of HT on SSM, but not IFM. Inhibition of uncoupling proteins with GDP abolished the HT-induced reduction in IFM, but had little effect in SSM. Maximally uncoupling mitochondria with FCCP abolished effects of HT in IFM and SSM. Collectively, results indicate that 1) complex 3 in SSM is the primary source of mitochondrial ROS release following I/R in HT, and 2) enhanced activity of uncoupling proteins limits ROS release from IFM under these conditions. Funding: NHLBI

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Introduction

During myocardial ischemia tissue becomes devoid of oxygen. Myocardial ischemia may occur when blood supply to an area of the heart becomes interrupted due to occlusion to a coronary artery. The result of this ischemia to the heart is a myocardial infarction. Occlusion of coronary arteries may result from thromboses that develop when a region of unstable atherosclerotic plaque, built up over time, breaks free from the wall of an artery and subsequently becomes lodged within the distal coronary vessels (Mishra and Samanta, 2012). Reperfusion is the period of time during which tissue previously devoid of oxygen is subjected to oxygen. During reperfusion the electron transport chain restarts and this is a critical period of time in which oxygen free radicals can form. Thyroid hormone is a potent mediator of metabolism and results in increased oxygen consumption by myocardial mitochondria (Mishra and Samanta, 2012; Venditti and others, 2009). Under a hyperthyroid state there is increased electron flux through the electron transport chain relative to euthyroid, this creates an environment in which increased reactive oxygen species (ROS) may be generated following myocardial ischemia and subsequent reperfusion. Ischemia allows us to elucidate the mechanisms by which ROS generation occurs and assess potential mitochondrial damage or derangement that is the result of ischemic damage. When looking at the effect of thyroid hormone following established ischemia/reperfusion protocols, we are able to see if there is an additive effect on ROS production by driving the mechanical processes of the heart and ROS generation at a higher rate. By following up on previous work regarding thyroid hormone using an established ischemia/reperfusion protocol we are elucidating whether or not mitochondrial subpopulations are differentially effected by hyperthyroidism.

1.1 Reactive oxygen species (ROS)

Reactive oxygen species are highly reactive free radicals such as superoxide ($O2^{\cdot-}$) and hydroxyl ($^{\cdot}OH$), as well as the non-radical molecule hydrogen peroxide (H_2O_2) (Brand and others, 1992; Chen and others, 2006; Venditti and others, 2003a). Superoxide is produced by the univalent reduction by one-electron transfer of oxygen (Brand and Nicholls, 2011). Superoxide then undergoes dismutation by superoxide dismutase (SOD) to form H_2O_2. H_2O_2 can either be broken down by antioxidant enzymes such as glutathione peroxidase (GPx) or catalase (CAT) to form water, or H_2O_2 can undergoe single electron reduction via the Fenton reaction and form a hydroxyl radical (Brand and Esteves, 2005; Brand and Nicholls, 2011; Chen and others, 2006; Colombini, 2004; Venditti and others, 2003b). Superoxide and hydroxyl radicals are not readily diffusible across cell membranes, so they typically have oxidizable reactions that occur near their site of production (Mishra and Samanta, 2012). In contrast, H_2O_2 is diffusible across cell membranes and may be converted into a hydroxyl radical, having more potent oxidizing effects as $^{\cdot}OH$ rather than H_2O_2 at sites further from the site of production (Mishra and Samanta, 2012).

1.2 ROS damage

When ROS are generated they can damage: nearby lipids causing changes in membrane structure that result in aberrant cell function (Chen and Lesnefsky, 2006; Gredilla and others, 2001; Petrosillo and others, 2006); proteins, which can alter protein synthesis and function by altering protein structure (Gredilla and others, 2001); or DNA, resulting in the generation of DNA lesions or DNA strand breaks that if unrepaired in the cell can contribute to genomic

instability (Adluri and others, 2011; Gredilla and others, 2001; Lopez-Torres and others, 2000; Zhang and others, 2011). When ROS are formed in excess of compensatory antioxidant capabilities, oxidative stress ensues and contributes to the pathogenesis of multiple disease states including cardiovascular disease, diabetes mellitus, cancer, neurogenerative diseases and aging (Aasum and others, 2003; Azhar and others, 1999; Mishra and Samanta, 2012; Paradies and others, 2009; Suh and others, 2003; Venditti and others, 1997a; Willems and others, 2005).

1.3 Cell signaling

Along with oxidative damage within the cell, ROS have also been linked to playing important roles in cell signaling in relation to cell proliferation, death and aging (Carpi and others, 2009; Colombini, 2004; Kabir and others, 2006). The role that ROS play in signaling pathways supports the idea that ROS serve a physiologic purpose in addition to inducing oxidative damage, and their presence is necessary for normal cell function.

1.4 Mitochondria and mitochondrial subpopulations

Electron microscopy has since disproven the previously held belief that mitochondria were simple organelles contained as a bean shaped vesicle that supplies the cell with energy. The structure of the mitochondria is that of a fibrous network of interconnecting lattices of membranes and complexes which engage in electron transport and ATP generation. Intact mitochondrial structure is imperative for proper function of the organelle (Chen and Lesnefsky,

2006). A lipid bilayer, comprised mainly of tetra-linoleoyl cardiolipin, provides the framework for the mitochondria. The innermost part region of the mitochondrion is the matrix, separated from the outside of the mitochondrion by a membrane that is composed of an inner and outer membrane, separated by a region called the inner membrane space. Outside the inner membrane is the cytosol of the cell. There are five complexes within the mitochondria that are responsible for electron transport and proton flux. Four of these complexes, complexes I, III, IV, and V, are transmembrane complexes that span the inner membrane space and go from the matrix to the inner membrane. Complex II is not a transmembrane protein; it originates in the mitochondrial matrix and extends only to the inner membrane space. The five complexes working in coordinated effort to generate ATP is termed a mitochondrial supercomplex.

Mitochondria are essential to aerobic life and vital to all metabolic processes within the cell. Mitochondria are responsible for the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP), supplying the cell with the energy to carry out all metabolic processes. Mitochondria produce water, consume oxygen, and contribute to thermogenesis. Mitochondria are both generators of ROS as well as targets for ROS attack and ensuing oxidative damage (Chen and others, 2006; Paradies and others, 2004). Mitochondria therefore are not only responsible for supporting all aerobic cellular life; they can also function in the coordinated destruction of cells (Brand and Nicholls, 2011; Mishra and Samanta, 2012; Venditti and others, 1997a).

The term “mitochondria” refers to the membrane bound organelle that is responsible for energy and heat production. Information is lacking regarding the two spatially and potentially functionally distinct subpopulations of mitochondria that are present within the myocardium. The two subpopulations of mitochondria are the subsarcolemmal mitochondria (SSM), located beneath the sarcolemmal membrane, and the interfibrillar mitochondria (IFM) located within the contractile units of the myocardium. SSM and IFM are both responsible for the phosphorylation of ADP to ATP, therefore providing fuel for metabolic processes and generating heat and water via the movement of electrons and protons through the electron transport chain (ETC).

1.5 Ischemia/Reperfusion

During the course of ischemia mitochondrial complexes which are responsible for transporting electrons and moving protons between the mitochondrial matrix and the inner membrane become reduced, or gain electrons (Brand and Nicholls, 2011). In the reduced state mitochondrial complexes cease moving protons and electrons and the net result is that ATP generation ceases. Additionally, in the ischemic state there is no energy generation, oxygen consumption or water synthesis, and no ROS generation. Upon reperfusion the complexes become oxidized and the mitochondria begin generating ATP; during reperfusion ROS generation increases (Brand and Nicholls, 2011).

1.6 Thyroid Hormone

There are two main, biologically active thyroid hormones. Both thyroxine (3, 5, 3', 5'-tetraiodo-L-thyronine; T4) and triiodothyronine (3, 5, 3'-triiodo-L-thyronine; T3) are synthesized by the thyroid follicular cells and released from the thyroid gland. Thyroid hormones act by binding nuclear receptors that regulate transcription. Thyroid hormones act on multiple tissues in both genomic and non-genomic manners, and are necessary for growth and development as well as metabolic homeostasis (Barret, 2009; Biondi and others, 2002; Mishra and Samanta, 2012).

Thyroid hormone is a known cardiac inotrope that increases developed pressure and decreases action potential duration (APD) which leads to tachycardia (Biondi and others, 2002; Fadel and others, 2000; Mishra and Samanta, 2012).

Thyroid hormone synthesis is initiated with the sequestering of iodide (I^-) in the thyroid gland. Iodide is transported into the follicular cell by way of a sodium/iodide symporter (NIS). Iodide is then secreted from the follicular cell into the lumen by pendrin (an anion exchanger). Along with secreting iodide, the follicular cell also secretes thyroglobulin to which the iodide will bind. Thyroid peroxidase from thyroglobulin oxidizes iodide to iodine (I^o) where thyroid stimulating hormone (TSH) stimulates the iodination of thyroglobulin in the follicular lumen resulting in thyroglobulin molecules that have either one or two oxidized iodine atoms on tyrosyl residues. TSH can then stimulate the conjugation of two iodinated tyrosines to either form T4 (two tyrosines with two oxidized ions per tyrosine) or T3 (two tyrosines, one with one and 1 with two oxidized tyrosines) that are bound to thyroglobulin. Next TSH will stimulate the iodinated

thyroglobulin to be taken back up into the follicular cell from the thyroid colloid in the lumen where it will then undergo hydrolysis to form T3 and T4, which can be secreted into circulation.

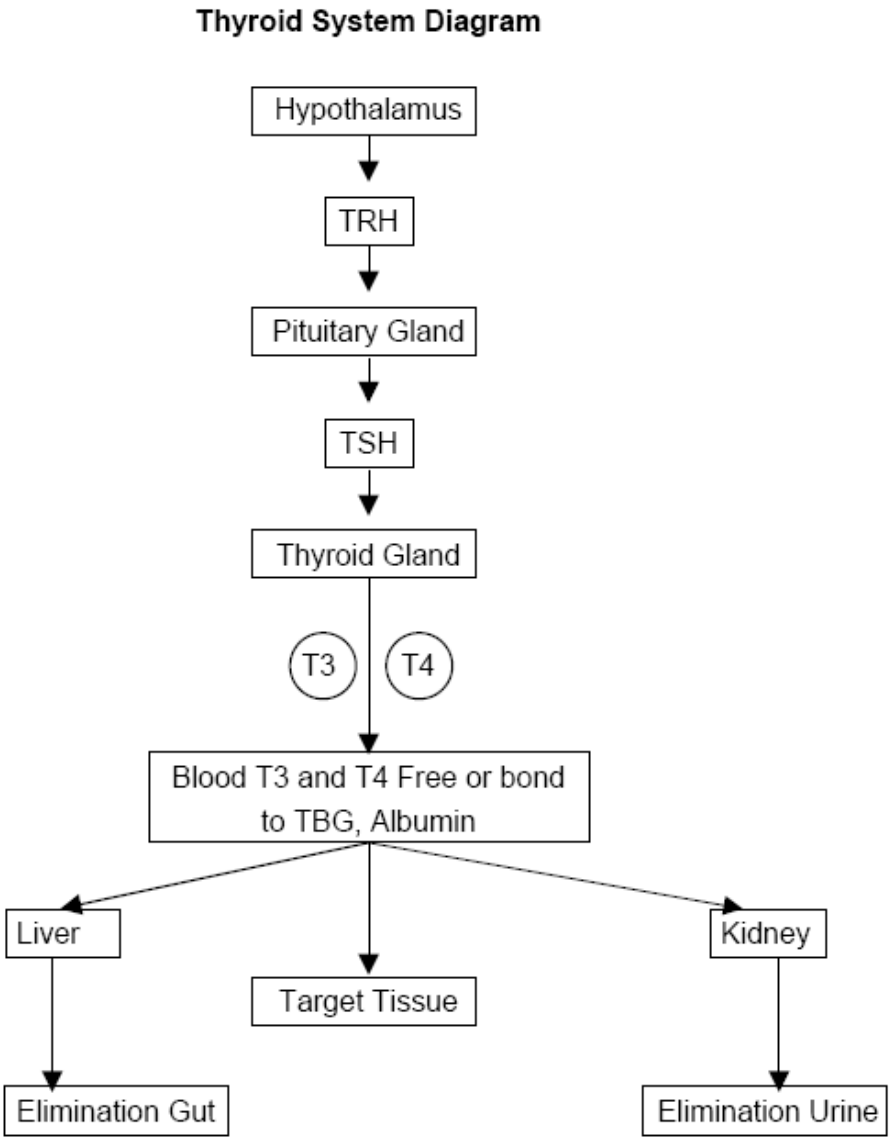


Figure 1.1 basic representation of Thyroid Hormone synthesis, transport, and excretion.

THYROID HORMONES AND ANTITHYROID DRUGS
RAVICHAND DM, SHESHAYAMMA V, KAMESHWARI LV, CHAKRADHAR T
Calicut Medical Journal 2005;3(4):e3
URL: http://www.calicutmedicaljournal.org/2005/3/4/e3

Approximately 90% of secreted thyroid hormone is in the form of T4 and 10% is released as T3; yet T3 is the more biologically active form of thyroid hormone. T4 can be de-iodinated to T3 peripherally, predominately in the liver and kidneys. In circulation both T3 and T4 are bound to either thyroid-binding globulin (TBG), transthyretin (TTR), or albumin. Only unbound thyroid hormone is responsible for thyroid hormone action on target tissue and this is referred to either as free T4 or free T3. Approximately 99.98% of T4 and 99.57% of T3 in circulation is bound, leading to more free T3 in circulation relative to free T4. While the amount of bound T3 and T4 can fluctuate in circulation, free T3 and T4 remain relatively stable in the absence of disease or other external stimulation. Protein binding serves multiple functions: to provide a pool to replace free thyroid hormone, and to increase the half-life of thyroid hormones. Bound T4 has a half-life in the range of 6- 8 days while the half-life of T3 is around 24 hours. The unbound forms of thyroid hormone have significantly shorter half-life durations (Barret, 2009; Norman, 2010).

1.7 Hyperthyroidism

Hyperthyroidism is an important disease because of the widespread action of thyroid hormones on most tissues in the body. Specifically, hyperthyroidism increases mitochondrial ROS release in myocardial tissues following ischemia/reperfusion (Baskin and others, 2002; Lopez-Torres and others, 2000; Venditti and others, 2009).

1.8 Actions of thyroid hormones: Genomic

Thyroid hormones leave the plasma and enter target tissues either by diffusing through the cell membrane or via carrier-mediated transport. Once in the cell, thyroid hormones act by binding to thyroid receptors (TRs). Once thyroid hormone binds to TRs in a target tissue they either activate or repress transcription (Barret, 2009; Bauer and Whybrow, 2001; Marin-Garcia, 2010; Venditti and others, 2009).

1.9 Actions of thyroid hormones: Non-genomic

Thyroid hormones have been shown to have actions that do not involve transcription and involve increasing mitochondrial oxidative phosphorylation (Barret, 2009). In addition to altering mitochondrial function, thyroid hormone has been shown to affect ion channel function, second messengers, and protein kinases (Barret, 2009; Mishra and Samanta, 2012).

1.10 Regulation

Thyroid hormone synthesis and secretion is regulated by the hypothalamic-pituitary-thyroid axis. Thyrotopin-releasing hormone (TRH) is released from the arcuate nucleus and the median eminence of the hypothalamus into the hypophyseal portal system, where it travels to the thyrotrophs located in the anterior pituitary. Here the TRH binds a TRH receptor and stimulates both the synthesis and release of TSH. TSH then binds to the TSH receptor on the follicular cell of the thyroid gland leading to the synthesis and secretion of both T4 and T3. Once T3 and T4

are secreted from the thyroid gland they provide feedback to the arcuate nucleus and the median eminence, decreasing TSH secretion. Additionally, T3 and T4 stimulate the thyrotrophs of the anterior pituitary, decreasing TSH secretion and thereby limiting additional hormone synthesis (Barret, 2009; Norman, 2010).

1.11 Thermogenesis

Thyroid hormones have an effect on both metabolism and thermogenesis through uncoupling of the mitochondrial oxygen phosphorylation system from ATP production (Barret, 2009). In brown adipose tissue (BAT) mitochondrial uncoupling proteins can disassociate oxygen phosphorylation from ATP generation, leading to oxygen consumption and heat generation independent of energy production.

1.12 Contractility

In the rodent heart thyroid hormone increases the expression of specific forms of myosin heavy chain that contributes to faster fiber shortening and increased contractility (Gustafson and others, 1986; Mishra and Samanta, 2012).

T3 binds to TRs in the nucleus, which leads to the binding of the protein complex to a response element, leading to promoter activation and initiation of transcription. Because of the effect on increased transcription of cardiac genes such as myosin heavy chain alpha, SERCA,

Na/K/ATPase/β-adrenergic receptor, cardiac troponin I and ANP (Averyhart-Fullard and others, 1994; Bahouth, 1991; Mishra and Samanta, 2012; Orlowski and Lingrel, 1990; Tsika and others, 1990; Zarain-Herzberg and others, 1994) It is believed that thyroid hormones may also function as key regulators in cardiovascular disorders that contribute to impaired cardiac function (Kisso and others, 2008; Portman, 2008).

1.13 Na^+/K^+ pump activity

T3 increase Na^+/K^+ pump activity in the plasma membrane by stimulating the synthesis of new transporters being inserted into the membranes. In a hyperthyroid state there is no significant change in plasma electrolyte levels so the increase in Na^+/K^+ pump activity is potentially compensated for by increasing Na^+ and K^+ leak across the membrane (Barret, 2009).

1.14 Oxidative Stress in myocardial I/R Injury

ROS are normal by-products of necessary metabolic processes. During aerobic cellular respiration superoxide is produced and converted to H_2O_2 on a regular basis. The cells, including the mitochondria, have built in enzymatic pathways in place to deal with the formation of ROS; these are antioxidants. Common antioxidants are Glutathione Peroxidase (GPx), Thioredoxin (TRx), Vitamins E and C, and Manganese Superoxide Dismutase (MnSOD). The mitochondrial electron transport chain is the main source of ROS generation throughout normal metabolic processes (Chen and Lesnefsky, 2006). It has been previously shown that mitochondrial

complex III is the main source of ROS generation and release into the inner membrane (Chen and Lesnefsky, 2006).

1.15 Electron transport through the mitochondrial ETC

Complex I substrates are malate and pyruvate, that together form NADH within the Kreb Cycle. NADH is then oxidized at complex I, depositing 2 electrons which are then transported from complex I to complex III by ubiquinol. Ubiquinol then deposits the electrons from the oxidation of complex I substrates onto the Q_0 site of complex III (cytochrome oxidase). Here electrons are transported via cytochrome C to complex IV, where 4 electrons in combination with 2 protons from the intermembrane space will undergo 4 electron reduction of oxygen and form water.

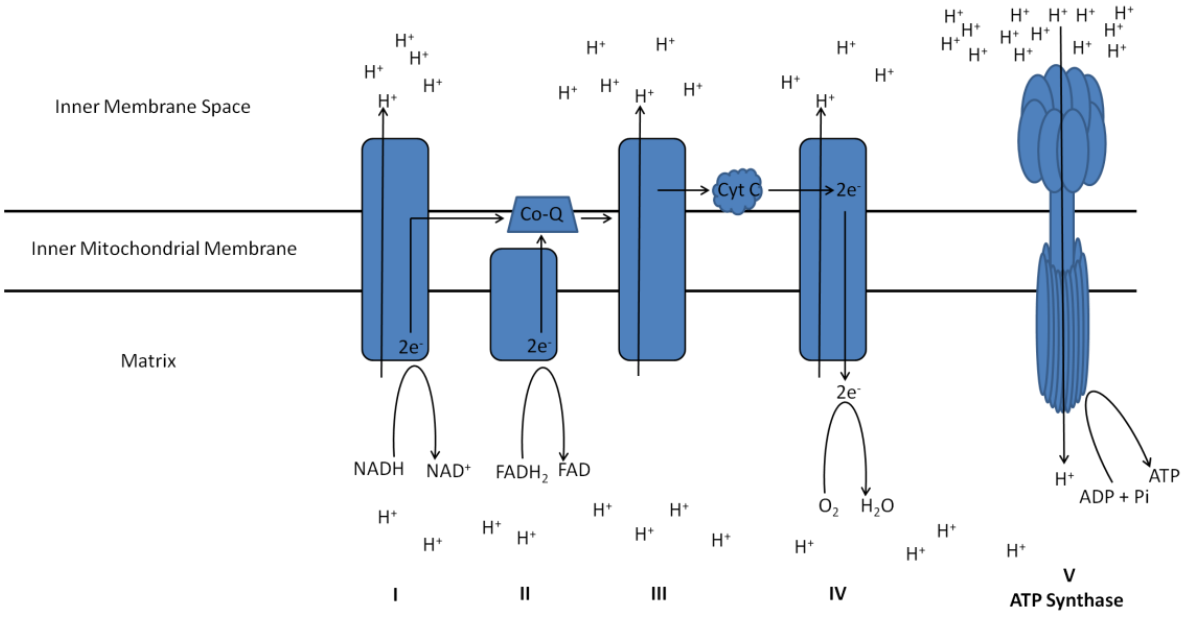


Figure 1.2 Location of mitochondrial subcomplexes within the mitochondrial membrane. The above figure shows the oxidation of NADH and FADH2 at complexes 1 and 2 respectively and the subsequent transfer of electron through the ETC culminating in the generation of water at complex IV and the phospohorylation of ADP to ATP at complex V.

Complex II substrate succinate is oxidized at complex II, again depositing 2 electrons which will be transported by ubiquinol to the Q_0 site of complex III and again onto complex IV via cytochrome C where it will reduce oxygen to form water.

When electrons are released from the electron transport chain, either via complex I, the Q_0 or Q_i sites of complex III, they undergo univalent reduction of oxygen and form superoxide ($O2^{-\cdot}$) (Chen and Lesnefsky, 2006). Superoxide released into the matrix are reduced by the antioxidant mechanisms described, and form H_2O_2 which can either diffuse across the membrane or undergo additional reduction by GPx or CAT to form water and H^+. There is not an antioxidant defense system to reduce ROS in the inner mitochondrial membrane; thus, ROS has the potential to induce oxidative damage to lipids, proteins, and DNA in this location (Chen and Lesnefsky, 2006; Mishra and Samanta, 2012)

Oxidative stress is a physiological state defined as ROS generation that exceeds antioxidant capacity (Brand and Esteves, 2005; Mishra and Samanta, 2012; Suh and others, 2003; Venditti and others, 1997a).

1.16 Membrane potential

Each time NADH or $FADH_2$ are oxidized by complex I or II, two protons are transported from the matrix to the inner membrane. The accumulation of protons in the inner membrane generates the membrane potential that will both inhibit the flow of electrons through the ETC as it

increases, and generate a large proton motive force which will drive the protons back into the matrix either through complex V, the inner membrane space via proton leak, or uncoupling proteins which allow for protons to diffuse across the membrane (Brand and Esteves, 2005; Brand and others, 1992; Chen and others, 2006).

1.17 Stoichiometry

During aerobic respiration and steady-state conditions the number of protons being moved outward is equal to the number of protons being moved inward (Brand and others, 1992). During these steady state conditions, the majority of proton influx occurs through complex V (ATP synthase) providing the energy for ADP phosphorylation to ATP. In non-steady state conditions there is an increase in the distribution of protons moving through the membrane either as leak due to kinks or blocks in the membrane, or due to the presence of uncoupling proteins that transport protons across the inner membrane space independent from complex V activity (Chen and Lesnefsky, 2006).

1.18 ETC Substrates

Complex I substrates: pyruvate and malate. Pyruvate is a product of glycolysis and when combined with CoA at pyruvate dehydrogenase, yields Acetyl CoA which is a main substrate of (Chen and Lesnefsky, 2006) the citric acid cycle. Malate is responsible for transporting electrons via the malate-aspartate shuttle, which takes electrons from glycolysis into the inner

membrane of the mitochondria. The inner membrane of the mitochondria is impermeable to NADH which supplies complex I (NADH dehydrogenase) with the electrons necessary to reform NADH from NAD^+ in the matrix. Therefore Malate is necessary in transporting reducing equivalents for NADH into the matrix to form new NADH from NAD^+.

Complex II substrate: succinate. Complex II, also known as succinate dehydrogenase, removes electrons from succinate for use depositing the electrons on ubiquinol. Succinate is produced during the citric acid cycle when succinyl-CoA synthase removes the CoA from succinyl-CoA. Succinate dehydrogenase is both a citric acid enzyme as well as an ETC enzyme.

1.19 ETC Inhibitors

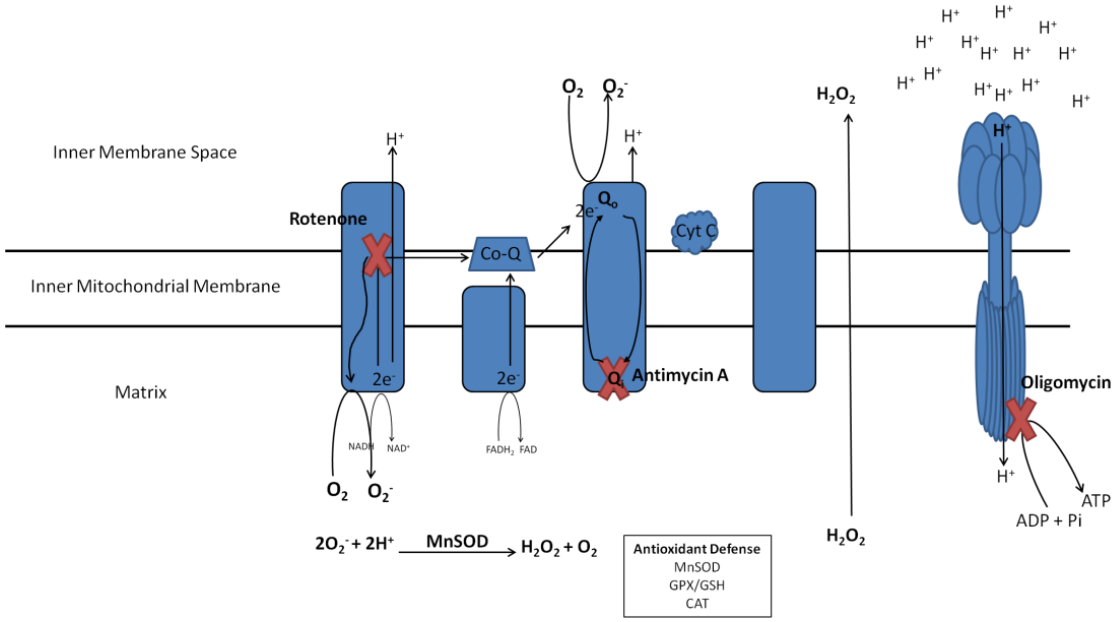


Figure 1.3 Shows the site of action of various inhibitors within the ETC and the location of ROS generation as well as diffusion of H_2O_2.

Rotenone: complex I inhibitor (Chen and Lesnefsky, 2006). Block the flow of electrons from complex I to ubiquinol. Assures that any electrons deposited into the ETC upstream of complex I (via complex II) move to complex III and blocks the movement of electrons from the inner membrane space back into complex I. Directs ROS generation through complex III.

Antimycin A: Blocks the Q_i site of complex III and increases ROS generation through the Q_o site (Chen and Lesnefsky, 2006). This selective blockade of complex III drives ROS release away from the antioxidant rich matrix and into the intermembrane space.

Oligomycin: Blocks phosphorylation of ADP and Pi to ATP at complex V (ATP synthase) (Penefsky, 1985).

1.20 ETC Uncouplers

Uncoupling proteins (UCP1, UCP2, UCP3) act by transporting protons from the inner membrane space to the matrix and subsequently decreases membrane potential allowing electrons to flow through the ETC more readily. Proton leak through the membrane as a result of uncoupling proteins uncouples substrate oxidations at complexes I and II from the phosphorylation of ADP to ATP at complex V (Brand and Esteves, 2005). Uncoupling increases oxygen consumption at complex IV, increases substrate utilization, increases heat production, and potentially decreases ROS release. UCP3 is believed to be found in low concentrations in heart tissue and may contribute to increasing proton conductance. The mechanism by which UCP3 is activated and

regulated in myocardial tissue has not been confirmed (Brand and Esteves, 2005; Divakaruni and Brand, 2011).

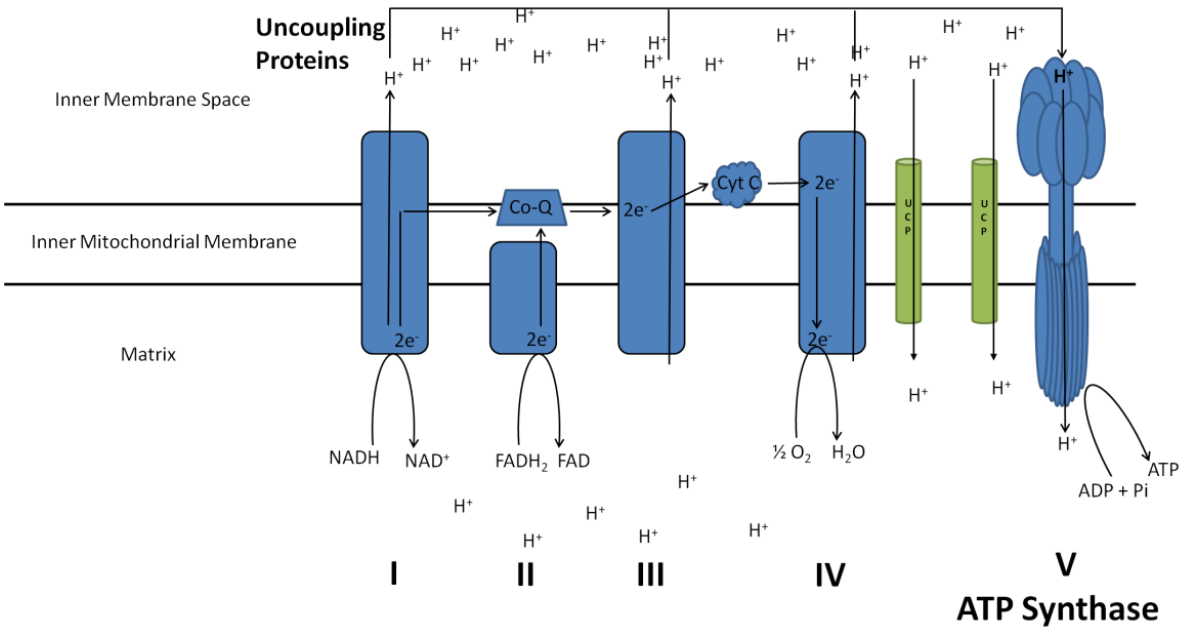


Figure 1.4 Shows the action of uncoupling proteins within the mitochondrial membrane and a proposed mechanism for dissipating the membrance potential. Exogenous GDP added into solution negates the effect of UCP's.

UCP3: uncoupling protein 3 present in low levels in myocardial tissue and is responsible for transporting protons from the inner membrane to the matrix independent of complex V activity.

Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone [FCCP]: Protonophore responsible for dissipating the membrane potential by allowing the membrane to become permeable to protons. As protons flow freely from the inner membrane to the matrix, electron flow through the ETC is maximized because electrons are not being inhibited by a membrane potential.

GDP: Responsible for blocking the transport of anions across the mitochondrial membrane and blocking the action of uncoupling proteins. Any increase in ROS release seen with the exogenous application of GDP implicates uncoupling proteins in the dissipation of the membrane potential that was modifying ROS release.

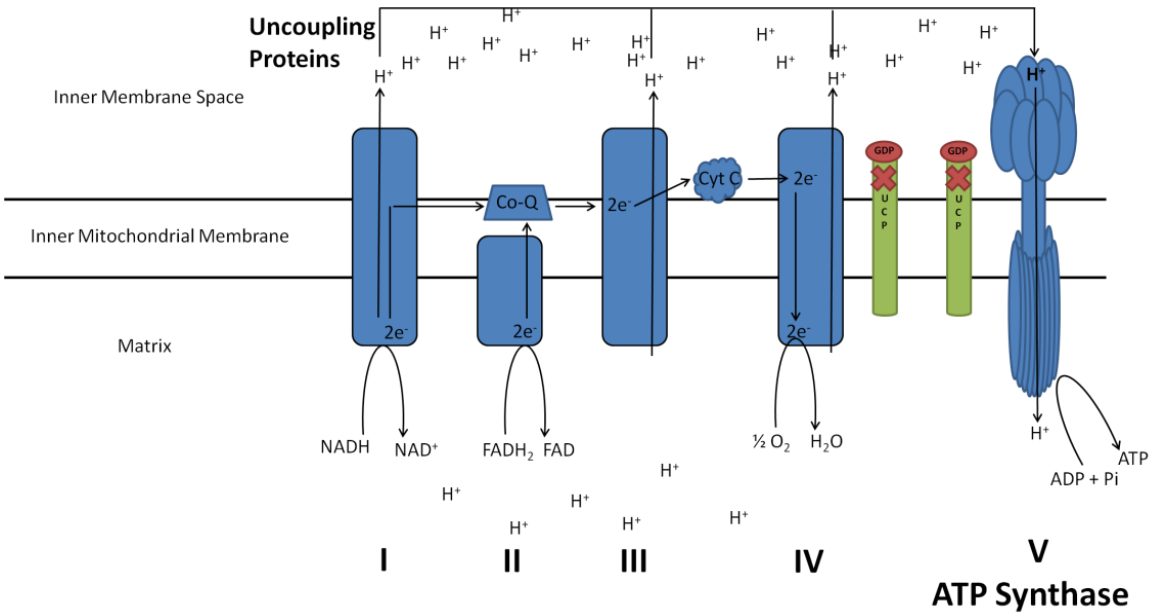


Figure 1.5 Illustration representing the action of GDP on UCP's.

1.21 Electron Carriers

Cytochrome C: Responsible for the transport of electrons from complex III to complex IV. Cytochrome C is bound to the inner membrane; therefore, changes in membrane composition can be identified by assessing cytochrome C presence. The loss of Cytochrome C from the membrane is a marker of lipid peroxidation of the membrane and is a marker of apoptosis (Chen and Lesnefsky, 2006).

Coenzyme Q (Ubiquinone/ubiquinol): Transports electrons from complex I and II to complex III

1.22 Mechanisms of mitochondrial ROS production and release

Ischemia impairs the mitochondrial electron transport chain, which augments mitochondrial ROS production (Azhar and others, 1999; Chen and Lesnefsky, 2006; Kabir and others, 2006; Katakam and others, 2007; Paradies and others, 2004; Venditti and others, 2002; Venditti and others, 2000). Previous studies have shown that 20 minutes of global ischemia damages the ETC by decreasing complex III activity, decreasing cytochrome C content, and decreasing cytochrome oxidase activity in both SSM and IFM populations (Becker, 2004; Chen and others, 2006; Masullo and others, 2000; Venditti and others, 2002; Venditti and others, 2008). During myocardial ischemia there is a decrease in oxidation occurring through cytochrome oxidase; this results in fewer electrons being transported from complex III to complex IV and an increase in electrons being released via the Q_0 site into the intermembrane space.

Hypoxia decreases myocyte pH and increases calcium uptake (Chen and others, 2006; Iverson and Orrenius, 2004; Lesnefsky and others, 2001; McMillin and Dowhan, 2002). Increased calcium uptake by the cell is indicative of mitochondrial permeability transition pore (MTPT) opening, potentially a marker of cellular dysfunction or a marker of cellular apoptosis.

1.23 Membrane Potential

The membrane potential is made up of a proton motive force that is established by the continuous movement of protons from the matrix to the intermembrane space (Brand and Nicholls, 2011). Protons are transported across the membrane using the energy generated from the movement of electrons. Protons return to the matrix through ATP synthase (complex V) and

the energy generated as the protons move down their concentration gradient drives ADP phosphorylation to ATP. As protons accumulate in the intermembrane space, they increase the membrane potential. The membrane potential is a necessary driving force in energy production, but when the membrane potential becomes too large it begins inhibiting the flow of electrons through the ETC. Electron transport and ATP synthesis are coupled mechanisms. Uncoupling proteins, specifically UCP3 found in the cardiac mitochondria, play a role in uncoupling ATP synthase from the flow of electrons through the ETC (Bobyleva and others, 1998; Brand and Esteves, 2005; Gong and others, 1997; Harper and Seifert, 2008; Silvestri and others, 2005; Venditti and Di Meo, 2006; Wang and others, 2010). Uncoupling proteins are transmembrane proteins that are inserted within the mitochondrial inner membrane and they allow for the dissipation of the membrane potential by allowing protons to flow from the intermembrane space to the matrix.

1.24 Antioxidants

Mitochondrial antioxidants play a central role in managing oxidative stress in cardiac myocytes. A high concentration of antioxidants is found within the myocardial mitochondrial matrix. Superoxide produced in the matrix undergoes dismutation by superoxide dismutase, specifically MnSOD found in the matrix to generate H_2O_2. H_2O_2 can then be broken down by either catalase or glutathione peroxidase to form water (Mishra and Samanta, 2012). When H_2O_2 undergoes single electron reduction via the Fenton or Haber-Weiss reaction it forms a hydroxyl radical, $^{\cdot}OH$.

Other potent antioxidants found in the mitochondria are Vitamins E and C which are responsible for reducing ROS and limiting oxidative damage (Venditti and others, 1997a; Venditti and others, 1997b).

It has been previously shown that levels of the antioxidants MnSOD, GPx, and TRx do not change in response to ROS. Some studies have shown that mitochondrial Vitamin E levels may increase in the matrix in response to increasing levels of ROS (Gredilla and others, 2001; Venditti and others, 2002; Venditti and others, 1997a).

1.25 Thyroid hormone and antioxidant defense

Because thyroid hormone is a potent mediator of metabolic processes and increases respiration of the ETC, it has been proposed that a hyperthyroid state leads to enhanced ROS generation, increased consumption of cellular antioxidants, and the inactivation of specific antioxidant defense mechanisms; all processes that lead to a state of oxidative stress (Lopez-Torres and others, 2000; Mishra and Samanta, 2012; Venditti and others, 2003a). Overall antioxidant capacity of the cardiac mitochondria with hyperthyroidism has been shown to decrease, which enhances the overall oxidative stress of the system and increases susceptibility to oxidants (Mishra and Samanta, 2012).

1.26 Aim

The aim of this study is to implicate thyroid hormone as a potent mediator of ROS production in ischemia/reperfusion injury and in the course of doing so, show that mitochondrial subpopulations are differentially affected by both a hyperthyroid state and during the course of ischemic damage.

Methods

2.1 Animals, protocol, equipment

Twenty-seven male Sprague-Dawley rats (4-6 months of age) purchased from Harlan Laboratories (Indianapolis, IN.) They were maintained by University animal facilities at room temperature of 22^{C} on a 12 hour light dark cycle and fed ad libitum commercial rodent chow #2918 purchased from Teklad Global Rodent Diets (Harlan North America). All experiments were performed in accordance with local and national guidelines governing animal experimentation.

Animals were randomly assigned to one of two groups: euthyroid control rats (CON) exposed to ischemia/reperfusion protocol (20 minutes ischemia / 25 minutes reperfusion) (Masullo and others, 2000; Venditti and others, 2002; Venditti and others, 2008; Venditti and others, 2000; Xi and others, 1999) and hyperthyroid rats (HT), made hyperthyroid via daily IP injection of T3 30µg/100g of body weight (Paradies and Ruggiero, 1988) soluble in 0.5mM NaOH (Brand and others, 1992; Lopez-Torres and others, 2000) pH 10 for 10 consecutive days. Control rats were injected daily with vehicle (0.5mM NaOH pH 10). Rats were anesthetized 24 hours post final injection with 0.2mL Sodium Pentobarbital and 0.1mL Heparin. Immediately upon confirmed surgical plane of anesthesia hearts were excised via midline thoracotomy and immediately placed in ice cold Krebs-Heinslet buffer for 30 seconds. Aortas were then cannulated and secured to the isolation apparatus. The pulmonary artery was left uncannulated. Integrity of the system was confirmed by flow rate measured from the pulmonary artery. Aortic sinuses

were determined to be distal to the site of cannula placement when flow rate was > 15mL/min. The pressure head was maintained at 115 mmHg and KH buffer was maintained at 37^{C}.

Upon cannulation hearts were allowed to equilibrate during a 10 minute period. Following 10 minutes, flow to the heart was discontinued and ventricles were submerged in KH buffer maintained at 37^{C} for 20 minutes. Subsequently flow was returned to the heart for 25 minutes of reperfusion. Flow rates were recorded at 0, 15, and 25 minutes. Following the reperfusion protocol hearts were cut down and placed in ice cold CP1 buffer. Hearts were weighed and the right and left atria were removed. Ventricles and septum were sectioned with a constant amount of tissue used for mitochondrial isolations.

2.2 Mitochondrial Isolation

Mitochondria was isolated into respective SSM and IFM subpopulations (Huq and Palmer, 1978). Trypsin was used to free IF mitochondria from surrounding myosin.

2.3 Mitochondrial H_2O_2 production

BCA Protein Assay (Thermo Scientific) was performed to determine isolated mitochondrial protein content.

The rate of mitochondrial H_2O_2 production was measured using a predetermined protocol of substrate and inhibitor pairings and measuring the linear fluorescence of the oxidation of cytosolic H_2O_2. 10µg of mitochondrial subpopulation protein was then added to each substrate-inhibitor pairing to elucidate the location of H_2O_2 release in to the cytosol.

2.4 Substrates and Inhibitors

The following substrates and inhibitors were used to investigate sites of ROS release within the mitochondrial ETC. Both abbreviations and working concentrations used are shown.

Complex I substrates

Malate and Pyruvate (MP): [1M]

Complex II substrate

Succinate (S): [1M]

Complex I inhibitor

Rotenone (R): [0.24mM]

Complex III inhibitor (site of Qo inhibition)

Antimycin A (AA): [0.2mM]

Complex V inhibitor

Oligomycin (OA): [0.5mg/mL]

Uncoupling

Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP): [100µM]

Guanosine diphosphate (GDP): [10mM]

2.5 Protein content blotting

Prepared mitochondrial subpopulation samples were prepped using lysis buffer (citation) and sonicated. Samples were exposed to 2 freeze-thaws prior to protein loading. Samples were assayed for UCP3, OXPHOS, MnSOD, and GPx1.

2.6 Chemicals

Amplex Ultra Red Assay Chemicals		
Chemical	**Manufacturer**	**Catalog Number**
Oligomycin A	Sigma-Aldrich	579-13-5
Rotenone	Sigma-Aldrich	83-79-4
Antimycin A	Sigma-Aldrich	642-15-9
Guanosine 5'-diphosphoglucose sodium salt (GDP)	Sigma-Aldrich	103301-72-0
Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)	Sigma-Aldrich	370-86-5
Sodium succinate dibasic hexahydrate (Succinate)	Sigma-Aldrich	6106-21-4
Diethyl malate (Malate)	Sigma-Aldrich	7554-12-3
Sodium pyruvate (Pyruvate)	Sigma-Aldrich	113-24-6
Horseradish Peroxidase (HRP)	Sigma-Aldrich	P8250
Amplex Red	Invitrogen	A12219
Pierce BCA Protein Assay Kit	Thermo Scientific	23225

Additional Chemicals		
Chemical	**Manufacturer**	**Catalog Number**
Sodium Pentobarbitol	Cardinal Health	4597126
Heparin	Hospira	0409-2720-01

Western Blotting Primary and Secondary Antibodies							
Primary Antibody	**Concentration**	**Manufacturer**	**Catalog Number**	**Secondary Antibody**	**Concentration**	**Manufacturer**	**Catalog Number**
UCP3	1:500	Abcam	Ab3477	GAR-AP	1:5000	Bio-Rad	170-5011
OXPHOS	1:2000	Mitosciences	M5604	GAM-AP	1:5000	Sigma-Aldrich	A7434
GPx1	1:2500	Abnova	Mab0845	GAM-AP	1:5000	Sigma-Aldrich	A7434
MnSOD	1:10,000	Thermo Scientific	PA1-31072	GAR-AP	1:5000	Bio-Rad	170-5011

Additional Western Blotting Chemicals		
Strep-Tacin	Bio-Rad	161-0382
BCIP/NBT Blue Liquid Substrate	Sigma-Aldrich	041M1785

2.7 Statistical Analysis

All data obtained is presented using mean values +/- standard variation based on the sample. For visual purposes some data is presented as percent change relative to CON SSM values while other data is presented as mean values relative to CON SSM or in absolute nmol of H_2O_2 produced / mg of mitochondrial protein / 30 minutes. Data were analyzed using the two-tailed T.TEST method and two-sample equal variance. Significance was set as $P<0.05$.

2.8 Experimental Protocols

Krebs-Henseleit Buffer

Adapted from Dr. Adam Chicco's Mouse Heart Perfusion Solution protocol, December 2010. Krebs-Henseleit (KH) buffer is a modified recipe of Ringer's solution which is used to maintain tissue ex vivo. It contains necessary electrolytes to be a source of energy during cell maintenance and is capable of transporting oxygen to metabolically active tissue.

10x Krebs Stock

Begin by calculating the amount of KH buffer that will be used throughout the course of the experiment. Make 10x Krebs stock in a volume that will support all experiments. KH buffer can be made in advance and stored at 4^{c}. All 10x KH stock should be made in 1 vessel to ensure identical KH buffer is used throughout the experiment.
(Total amount of KH buffer needed / 10) = amount of 10x KH stock

	1L	**2L**	**4L**	**6L**
NaCl (g)	68.61	137.22	274.44	411.66
KCL (g)	3.5	7	14	21
$CaCl_2$ (g)	2.75	5.5	11	16.5
$MgSO_4$ (g)	2.96	5.92	11.84	17.76
KH_2PO_4 (g)	1.63	3.26	6.52	9.78

1x Krebs-Henseleit Buffer

Step 1

	For final volume of:			
	1L	2L	3L	4L
Put into:	**500mL flask**	**1L flask**	**2L flask**	**2L flask**
5mM pyruvate (g)	0.55	1.1	1.65	2.2
11mM glucose (g)	1.98	3.96	5.94	7.92
Milli-Q H_2O	250 mL	500 mL	1 L	2 L
10x KH stock	100 mL	200 mL	300 mL	400 mL
100mM EDTA	5 mL	10 mL	15 mL	20 mL
Fill with Milli-Q H_2O to:	**500 mL**	**1 L**	**2 L**	**2 L**

- Mix until all solutes are full dissolved
- Filter solution through a fresh 0.8µM filter into a clean flask
- Transfer filtered solution to an Erlenmeyer flask, have 95/5% O_2/CO_2 bubbling

Step 2

	For final volume of:			
	1L	2L	3L	4L
Put into:	**500mL flask**	**1L flask**	**2L flask**	**2L flask**
25mM NaHCO3 (g)	2.075	4.15	6.225	8.3
1200 [units / L Heparin] add 1000 units / mL	1.2 mL	2.4 mL	3.6 mL	4.8 mL
Fill with Milli-Q H_2O to:	**500 mL**	**1 L**	**2 L**	**2 L**

- Mix until fully dissolved
- Filter solution through a fresh 0.8µM filter into a clean flask
- Transfer the filtered solution into the same Erlenmeyer flask used in step 1. Continue to have 95/5 O_2/CO_2 bubbling
- Place on stir plate and mix for 5 minutes
- Once solution has been sufficiently mixed begin filling the clean and rinsed perfusion reservoir with the KH buffer.

Ex vivo Heart Isolation Protocol

1. Rinse perfusion apparatus x 2 with ddH_2O on day of experiment and ensure proper function of all tubes, connections, and that water bath is set at 37^c
2. Fill perfusion reservoir with fresh KH buffer to marked line that provides the desired pressure for the isolation. Allow for entire perfusion apparatus to warm to 37^c prior to beginning experiments
3. Ensure that 95/5% O_2/CO_2 is bubbling at a constant level both in the Erlenmeyer flask and the perfusion reservoir
4. Set out all necessary surgical equipment for removing the heart tying to the perfusion apparatus
5. Set out 2 properly cut sutures and tie them on the cannula in anticipation of hanging the heart
6. Set cannula to very slow drip to ensure proper function of cannula and ensure warm perfusate will be present when heart is hung
7. Anesthetize rat with intraperitoneal injection of 0.2mL Sodium Pentobarbital and 0.1mL Heparin. Wait until reflexes are absent and rat is appropriately anesthetized
8. Excise heart via midline thoracotomy, immediately place heart onto petri dish containing ice cold CP-1 while the heart is placed under the cannula
9. Secure the aorta to the cannula with a mini-hemostat and initiate perfusion by opening flow to the heart. Immediately upon perfusing the heart open the top of the stock-cock to remove any air that was present in the line to avoid air thrombosis to the chamber of the heart
10. Ensure proper placement of the cannula in the aorta (cannula should be distal to the aortic sinuses) so that proper perfusion can occur
11. Secure heart to perfusion apparatus by tying a suture around the aorta to the cannula. Do not over tighten as the suture can cut through the vascular tissue
12. Once heart is secure initiate protocol
    a. Protocol used called for a 10 minute equilibration period followed by 20 minutes of ischemia and then a 25 minute reperfusion period
13. To initiate ischemia stop drainage of the tissue bath so that it fills halfway with warm perfusate
14. Next close the stopcock of the cannula to stop flow to the heart, bring the warm tissue bath up around the heart but do not submerge the heart in the perfusate. This serves to keep the tissue warm during ischemia
15. To initiate reperfusion of the heart following global ischemia open the top of the stopcock to again ensure there is no air in the tubing and that warm perfusate will be delivered to the heart

16. Lower the tissue bath away from the heart and open the stopcock to begin delivering warm perfusate to the heart. Make sure to restart the tissue bath drainage to remove used perfusate
17. Following reperfusion or the completion of the desired protocol securely grab the heart with hemostats just a the base of the cannula in the aorta and cut through the atria to remove the heart
18. Immediately place the heart in ice cold CP-1 solution
19. Trim the atria and excess tissue from the heart, weight it and begin the mitochondrial isolation protocol

Cleaning of the perfusion apparatus

1. Drain all KH buffer from the perfusion apparatus and the perfusion reservoirs
2. Drive all KH buffer from the lines by plugging the air exit and building up pressure in the reservoirs
3. Once all KH buffer is removed fill the reservoirs and lines with ddH_2O and drain
4. Repeat the ddH_2O rinse a minimum of 2 times. 95/5% O_2/CO_2 should be on during the entire cleaning protocol
5. Remove cannula and stopcock and rinse and set to dry
6. Make sure all the air has been removed from the lines and turn off the gas and the water bath
7. Wipe up work station and make sure all glassware used has been cleaned and is returned to a secure area

Heart Mitochondrial Isolation Protocol

Protocol Developed by Catherine Le
Integrative Cardiac Biology Lab, 2011

1. Excise heart and immediately place in cold CP-1 on ice in tissue coulter
    - After tissue has cooled remove from CP-1 and remove excess blood and buffer
    - Remove adipose tissue, excess vasculature, right and left atria
    - Weigh remaining intact tissue
    - Section tissue into RV, Septum, and LV. Weight each individual tissue section
    - Place sectioned LV portion to be used in mitochondrial isolation into appropriately labeled coulter vial with CP- 1 (5mL)
2. Mince tissue with 2 scissors in coulter vial w/5mL CP-1
    - Do not warm tissue or CP-1
    - Return buffer with tissue and scissors back to ice bath immediately after mincing
3. Transfer tissue and CP-1 to 15mL conical, store on ice
4. Wash x 1 with CP-1 (3-5mL), leave tissue in conical, remove CP-1
5. Bring volume in conical up to 7mL with CP-2
6. Polytron at 2/3 speed for **3 seconds**
7. Transfer tissue to glass homogenizing tube
8. **2 strokes** with loose pestle
9. Transfer homogenate into labeled low speed spin tube

Centrifuge Homogenate @ 580g for 10 minutes at 4C

- During centrifuge time return polytron and pestle to ice bath
- Set up a new coulter vial labeled "SSL" for each sample. Cover with double layered cheesecloth
    - Keep on ice

10. Pour supernatant (SN) of each tube through double layered cheesecloth into corresponding SSL coulter vial
    - The SN in the SSL coulter vial contains the SSL mitochondria
11. Add 2-3 mL CP-2 to remaining pellets in the low speed spin tube
    - Resuspend using the vortex at high speed
    - Polytron at 2/3 speed x **3 seconds**

Centrifuge Homogenate @ 580g for 10 minutes at 4C

- During centrifuge time mix Trypsin for the IF mitochondria
- For each mitochondrial sample: 5mg Trypsin + 1.25mL CP-1 in 15mL conical
    - 8 samples = (8*5mg Trypsin) + (1.25mL CP-1 x 8) = 40mg Trypsin in 10mL CP-1

12. Pour SN through double layered cheesecloth into corresponding SSL coulter vial on ice
13. Transfer SSL SN from coulter vial into appropriately labeled SSL centrifuge tube
    - Keep on ice

14. Add 1.25mL CP-1 to remaining IF pellet in low speed spin tube
15. Resuspend IF pellet with loose pestle x **1 stroke**
16. Add 1.25mL (1/2 squirt) of Trypsin/CP-1 solution to each IF tube
17. Polytron at 2/3 speed x **2 seconds**
18. Transfer IF sludge to 15mL conical tube, place on ice, put conicles on the rocker for 6 minutes
    - This frees up the IF mitochondria from the myofibrils. DO NOT leave in the Trypsin longer than 6 minutes as it will degrade the protein

Centrifuge SSL Mitochondria @ 3000g for 10 minutes at 4C

19. When timer for IF mitochondria goes off immediately add 2.5mL (1 squirt) of CP-2 to the IF sample
    - CP-2 serves to deactivate the Trypsin
20. Homogenize IF sample **2 strokes** with the loose pestle and **1 stroke** with the tight pestle
21. Transfer IF mitochondria back into the corresponding low speed centrifuge tube
22. Remove SSL tubes from the centrifuge, save 2mL of the SSL SN as ER SN. Return to ice

Centrifuge IF Mitochondria @ 580g for 10 minutes at 4C

23. Pour remaining SSL SN out as waste
24. Resuspend SSL pellets in CP-2 (~1mL), return to ice
25. Pour SN of IF mitochondria into labeled coulter vial through double layered cheesecloth
    - The SN contains the IF mitochondria
26. Transfer the IF SN into the appropriately labeled centrifuge tube

Centrifuge SSL and IF Mitochondria @ 3000g for 10 minutes at 4C

27. Dump SSL and IF SN as waste
28. Add CP-2 (~1mL) to IF mitochondria and resuspend by vortex
29. Add KME (~1mL) to SSL mitochondria and resuspend by vortex

Centrifuge SSL and IF Mitochondria @ 3000g for 10 minutes at 4C

30. Pour SSL and IF SN out as waste
31. SSL mitochondria are done unless they require an additional clarifying spin, set tubes to dry
32. Add KME (~1mL) to IF mitochondria and resuspend by vortex

Centrifuge IF Mitochondria @ 3000g for 10 minutes at 4C

33. Pour IF SN out as waste
34. IF mitochondria are done unless they require an additional clarifying spin, set tubes to dry
35. Add approximately 200mL of KME to SSL and IF pellets

- The size of the pellet determines the amount of KME to add. Smaller pellets require less KME
- Record amount of KME used to resuspend pellet and use this in calculating protein yield

36. Resuspend pellets by gently scraping pellet and homogenizing with pipette tip
37. Transfer homogenate into glass homogenizing tube
38. Resuspend with loose pestle until no chunks are visible
39. Transfer homogenized mitochondria into appropriately labeled Eppendorf tubes
    - Keep on ice. This homogenate will be used for OxPhos experiments
40. In a new, labeled Eppendorf tube dilute to mitochondria homogenate to 1:100
    - Use 25mM KH_2PO_4 pH 7.2 for protein assay
        - Ex: 5µL mitochondria + 495µL KH_2PO_4
    - Keep diluted mitochondria on ice. Following protein assay this will be stored at -80C along with other mitochondrial samples
41. Run protein assay to calculate mitochondrial yield
42. Calculate the amount of mitochondria needed to yield 250ug of mitochondrial protein necessary for OxPhos and respiration experiments

PROTEIN ASSAY

Sample Prep:
Add 5uL homogenized sample to 495uL 25mM KH2PO4 (in long box in 4C slider)

BCA Prep:
*****RUN IN TRIPLICATE*****
Prep 200uL per well
Need: 200uL x Sample Number
If you have 22 samples multiply x 3 (ie 66) = *13,200*uL total
BCA Reagent B: 13,200uL ÷ 50 = 264uL
BCA Reagent A: 13,200uL – 264uL = 12,936uL

Plate Prep:
On a clear 96 well plate, load 10uL BCA Standards in Column 1-3 from lowest [ ] (A) to highest [ ] (H)
Add 10uL of prepped samples onto plate
Using a repeat pipette, add 200uL prepped BCA Reagent to each well
Briefly shake plate on plate reader
Incubate for 30 min @ 37C

Read Plate:
Read Absorbance @ 562 nm

Analyze Data:
Copy and paste results into Excel file
Move data into template to determine protein concentration

Western Blotting Protocol

1. Remove frozen mitochondrial samples from -80^{F} freezer and collect approximately 30µg of desired sample
2. 1:10 dilution with MPER lysis buffer and phosphatase inhibitor
3. Sonicate tissue on output setting of 4 in 1 second pulses x 10. Return to ice.
4. Transfer sonicated protein, lysis buffer, and phosphatase inhibitor to 1.5mL micro centrifuge tube. Label as pellet.
5. Place in -80^{F} freezer
6. Remove from -80^{F} and place on ice. Allow samples to thaw.
7. Spin samples at 10,000g for 10 minutes at 4^{C}
8. Pull of supernatant and place in new micro centrifuge tubes labeled SN. Save pellet.
9. Complete a protein assay on the diluted SN protein
10. Complete a Western Blot Template including all samples along with protein concentrations
11. Load the same amount of protein for each sample into a new, labeled, locking micro centrifuge tube
12. Add β-merkapto ethanol (BME, loading buffer) to the sample. This is responsible for breaking disulfide bonds and denaturing protein.
13. After adding 7.5µL of BME, add mitochondrial protein. Add milli-Q H_2O to bring total volume in tube up to 15µL.
14. Heat samples for 10 minutes at 100^{C} (or 37^{C} for UCP3)
15. Make 1x tris running buffer (900mL milli-Q H_2O + 100mL Tris with SDS)
16. Rinse wells with 1x running buffer to remove air bubbles
17. Place wells in the electrophoresis tray
18. Fill well loading station with 1x running buffer
19. Remove samples from the heat block, complete a rapid spin to consolidate sample in the tube
20. Fill electrophoresis wells with appropriate sample according to the Western Blot Template
21. Load 5µL of ladder after samples have been loaded
22. Fill the remaining electrophoresis chamber with 1x loading buffer
23. Run at 100V until the lanes begin to separate, once separated can increase to 200V and run for approximately 1 hour.
24. While gel is running make 1500mL of transfer buffer (150mL 10x transfer buffer + 300mL Methanol + 1050mL milli-Q H_2O
25. Cover with parafilm and place in the 4^{C} refrigerator to cool

26. Once protein separation is complete open the tray and remove the gel. Cut the wells off the gel.
27. Place gel directly into transfer buffer, place on a shaker for 15 minutes
28. Dispose of 1x transfer buffer (non-hazardous waste)
29. Soak the pads for the transfer in transfer buffer for 5 minutes
30. Cut transfer membrane to size and remove filter paper. Label membrane with primary antibody and date.
31. Place transfer membrane in Methanol to activate the membrane, rinse to keep ink from bleeding
32. Transfer membrane into transfer buffer and submerge to dilute the methanol
33. Create the transfer setup by placing the black side of the casing down followed by a foam pad, filter paper, get, transfer membrane, filter paper, foam pad, and then the red casing. Current will travel from the black to the red side of the casing.
34. Place casing in transfer case and fill with 1x transfer buffer. Add ice to the case.
35. Run at 100V for 60 minutes. Do not exceed 1 amp during the transfer as this can cause damage to the gel
36. Once transfer is complete remove the membrane and immediately place the membrane in 1x TBS buffer
37. Place the membrane in Ponceau for 5 minutes to visualize the bands. Once this is complete rinse away the remaining Ponceau with milli-Q H_2O.
38. Place the gel in fixing solution for 15 minutes on the shaker
39. Block membrane in 5% non-fat dry milk (NFDM) for 1 hour
40. Once blocking is complete add primary antibody in 5% NFDM and leave overnight in the 4C for the antibody to bind the protein of interest
41. Remove the primary antibody and rinse the membrane with TTBS x 6 for 5 minutes each
42. Add secondary antibody diluted in 1% NFDM and incubate at room temperature for 1 hour
43. Dispose of the secondary antibody and repeat wash protocol (5 minutes in TTBS x 6)

AP Chemiluminesence

1. Slightly dry the membrane and add AP chemiluminesence if not using HRP
2. Cover the membrane in the AP solution and let sit for 5 minutes
3. Remove excess AP solution and transfer to a clean laminated sheet and cover
4. Develop and take a picture

Results

The hyperthyroid state of the T3 treated animals was reflected in higher heart weight/body weight ratios expressed as heart weight (g) / body weight (kg) (Table 1). Additionally, it has been shown that T3 treated rats are tachycardia relative to control rats (Masullo and others, 2000; Venditti and others, 2002; Venditti and others, 2008); this was confirmed in our study.

Table 3.1

Parameter	Group	
	Euthyroid	Hyperthyroid
	n = 14	n = 13
BW	435 +/- 15	371 +/- 9 *
HW	1.50 +/- 0.05	1.86 +/- 0.06 *
HW/BW	3.48 +/- 0.11	5.00 +/- 0.96 *

Values are shown as average +/- s.e.m. of 7 days of experiments. BW: body weight (g); HW/BW: heart weight/body weight (g/kg)
*Significance ($p<0.05$) vs. euthyroid control rats. TTest used for statistical analysis.

3.1 ROS release from the ETC

In both malate/pyruvate and succinate stimulated ROS release, IFM release less ROS than SSM in post ischemic euthyroid and hyperthyroid hearts. During succinate stimulated ROS release thyroid hormone augments the release of ROS from the SSM while reducing ROS release from the IFM. In malate/pyruvate stimulated ROS release, no change in ROS release is noted in the SSM population however similar to succinate stimulated ROS release; thyroid hormone treatment appears to limit ROS release in IFM relative to CON. Malate/pyruvate induced ROS release is lower than the ROS release measured in the inner membrane than when succinate is used as a substrate.

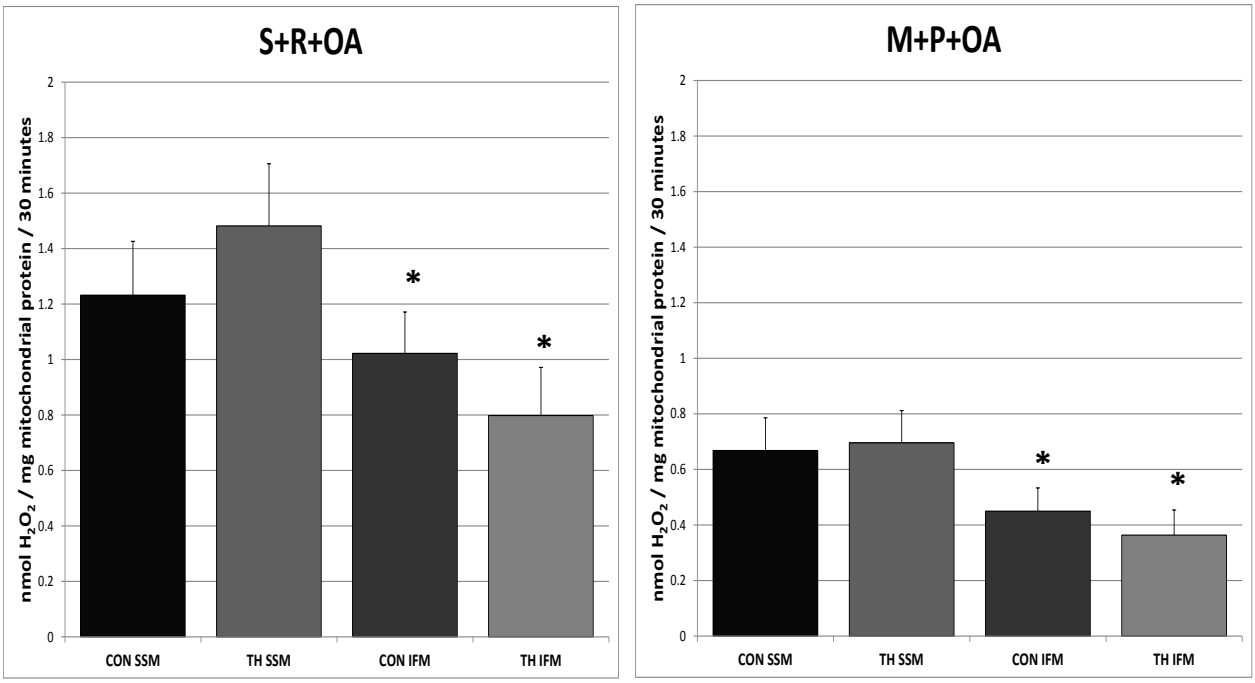


Figure 3.1: State 4 ROS release from Complex I and Complex II. Succinate (S) is a complex II substrate administered in the presence of rotenone (R) to prevent the backflow of electrons into complex I and ROS release directed into the mitochondrial matrix. Experiments were conducted in the presence of oligomycin (OA) to ensure state 4 conditions. * represents statistically significant subpopulation difference. $P<0.05$

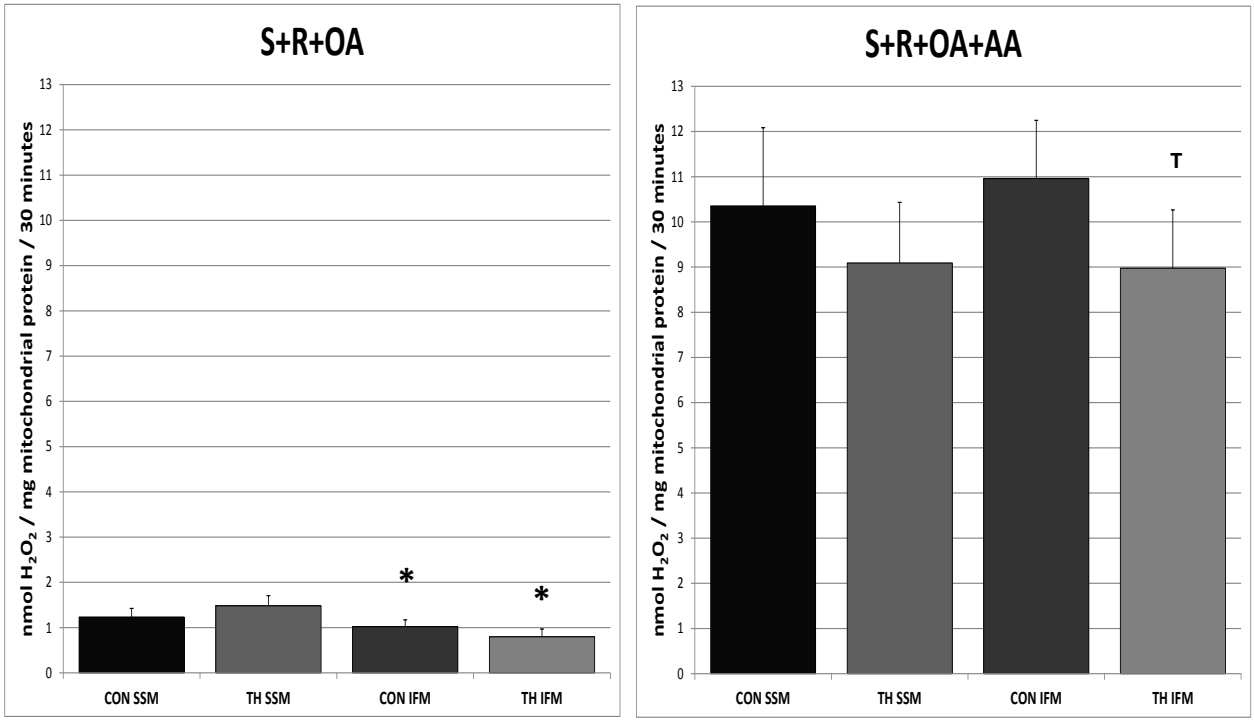
When the ability of complex I to produce ROS is examined independently of electron transport to complex III by the application of rotenone, there is an increase in ROS release from complex I by approximately 5-17 fold, with a greater increase in ROS release seen in the IFM vs. the SSM populations. By maximizing ROS release into the matrix via complex I, it shows that IFM posses a greater ability to produce ROS than SSM. When maximizing ROS release by complex I no effect of thyroid hormone is seen on the ability of either SSM or IFM to produce ROS via complex I.

Table 3.2

Absolute nmol of H_2O_2 released / mg mitochondrial protein / 30 minutes				
	CON SSM	**TH SSM**	**CON IFM**	**TH IFM**
M+P+OA	0.67 +/- 0.12	0.70 +/- 0.12	0.45 +/- 0.08	0.36 +/- 0.09
M+P+OA+R	5.15 +/- 0.85*	4.61 +/- 0.97*	7.28 +/- 0.70* ^{T}	6.36 +/- 0.78* ^{T}

Values shown are absolute amounts of H_2O_2 produced, measured as nmols of H_2O_2 produced / mg of mitochondrial protein / 30 minutes via Amplex Red fluourometric assay. * represents statistically significant difference compared to M+P+OA . ^{T} represents subpopulation difference within a treatment group. No effect of thyroid hormone treatment is seen. $P<0.05$

When complex III ROS production is maximized during succinate driven ROS release there is a 6-10 fold increase in ROS release in both SSM and IFM subpopulations. As seen in figures 2 and 3, ROS release increases significantly with the addition of antimycin a (AA), which is responsible for blocking the Q_o site of complex III and maximizing ROS release into the inner membrane. When complex III activity is maximized the IFM exhibit a greater increase in ROS release through complex III than the SSM, figure 4. This indicates that thyroid hormone does not affect the capacity of complex III to release ROS in SSM, but that thyroid treatment may be attenuating ROS release in the IFM population. Therefore the trend for IFM treated with thyroid hormone to produce less ROS relative to the euthyroid animals is maintained.



Figures 3.2 and 3.3 Absolute values of ROS release measured in the inner membrane. * represents statistically significant difference between subpopulations. ^{T} shows an affect of thyroid hormone within a subpopulation relative to CON. P<0.05

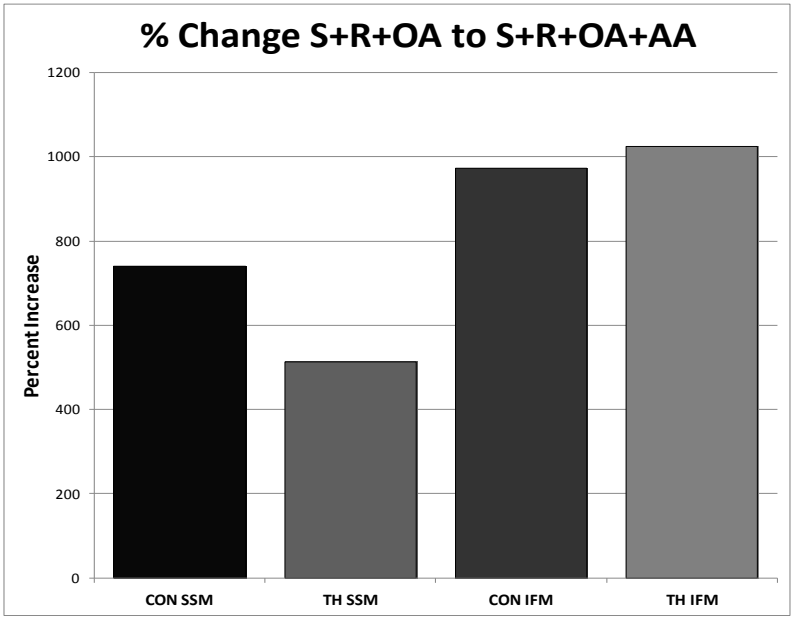


Figure 3.4 Percent increase in ROS release measured in the inner membrane with the addition of antimycin a to block electron flow from complex III to complex IV and maximize ROS production at complex III. The increase in ROS release from the IFM, both control and with thyroid treatment is greater than in SSM. This points to complex III IFM containing a greater ability to release ROS than SSM but that there is a mechanism that regularly limits the production/release of ROS selectively in IFM.

In the presence of FCCP, a previously mentioned protonophore, and succinate, ROS increases 2-8 fold in both the SSM and IFM subpopulations. The presence of FCCP increases the effect of thyroid hormone in both SSM and IFM with Succinate as in both SSM and IFM populations thyroid hormone treatment leads to significantly lower ROS release. A slight trend is seen that when maximally uncoupled, IFM have the ability to produce more ROS relative to the SSM demonstrating a greater capacity of IFM to release ROS relative to the SSM. Malate/pyruvate driven ROS release shows a significant increase with the addition of FCCP over baseline state 4 functions in both thyroid treated and subpopulations. There is no clear effect of thyroid hormone on ROS release with the addition of FCCP to malate/pyruvate ROS release.

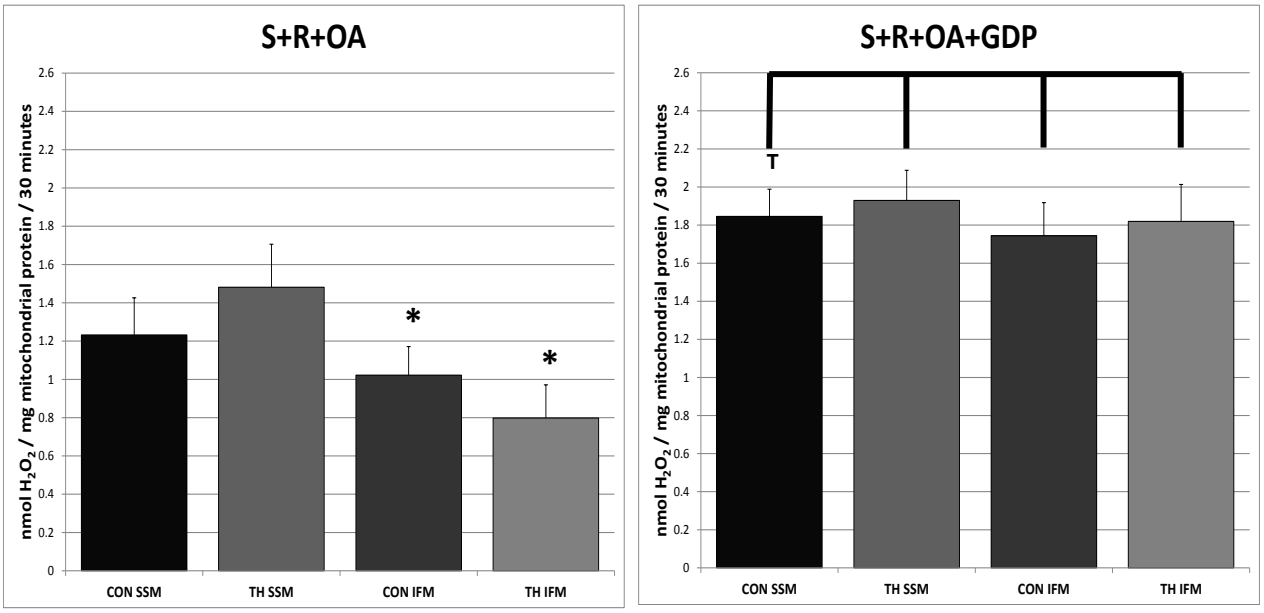
Table 3.3

Absolute nmol of H_2O_2 released / mg mitochondrial protein / 30 minutes				
	CON SSM	**TH SSM**	**CON IFM**	**TH IFM**
S+R+OA	1.23 +/- 0.19	1.48 +/- 0.22	1.02 +/- 0.15	0.80 +/- 0.17
S+R+OA+FCCP	7.82 +/- 2.04 *	3.36 +/- 2.0 *	8.83 +/- 1.99 *	5.56 +/- 2.12 *
M+P+OA	0.67 +/- 0.12	0.70 +/- 0.12	0.45 +/- 0.08	0.36 +/- 0.09
M+P+OA+FCCP	1.03 +/- 0.36 *	1.20 +/- 0.56 *	0.74 +/- 0.22 *	0.49 +/- 0.11 *

$P<0.05$. * shows significant increase in ROS release with the addition of FCCP.

When the effect of uncoupling proteins are inhibited, specifically UCP3 present in the cardiac mitochondria while using succinate, an increase in ROS release from both SSM and IFM subpopulations is evident. IFM ROS release increases to a greater extent than SSM, abolishing any subpopulation difference present and suggesting that uncoupling proteins may play a role in limiting membrane potential and ROS production in IFM while playing a lesser role in SSM. UCP3 dissipates the proton gradient that develops as protons build up in the inner membrane

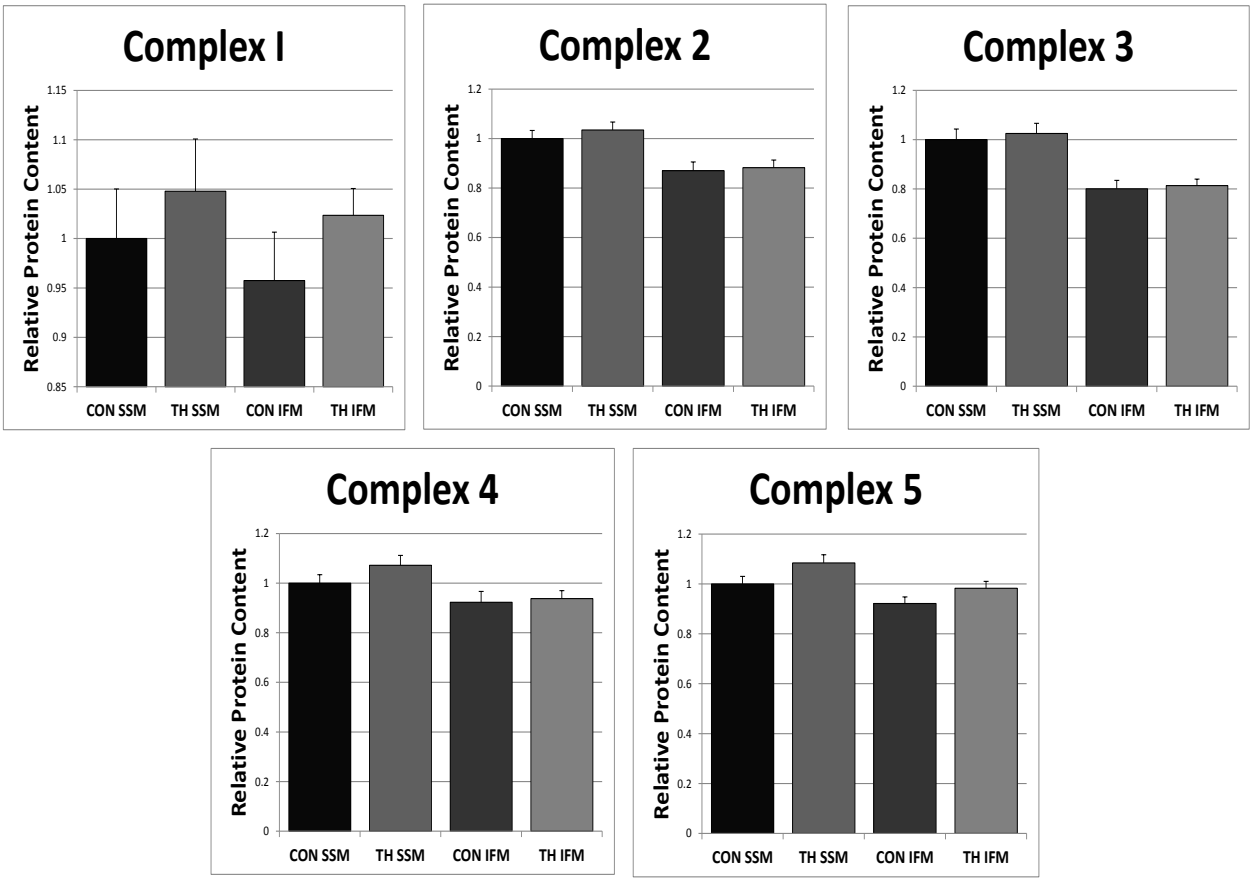
space, therefore allowing electrons to move through the ETC at a higher rate. When electrons are able to move more freely through the ETC, ROS release decreases as fewer electrons are being released onto oxygen at places other than the complex IV. See figure 1.3.



Figures 3.5 and 3.6 Show the change in absolute ROS release with the addition of GDP. By blocking the effect of uncoupling proteins membrane potential will increase and inhibit electron flow and increase ROS production/release. * represents previously shown subpopulation effects, ^{T} represents a lack of significance between subpopulations or thyroid treatment on any of the groups shown thereby implicating uncoupling proteins in limiting ROS production/release in IFM and thyroid treated mitochondria.

3.2 Oxidative phosphorylation (OXPHOS) protein content

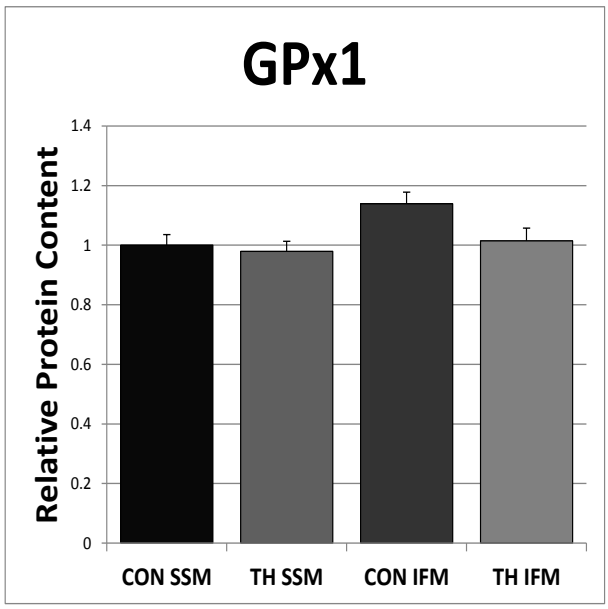
In probing for electron transport chain complexes I-V there was no difference in the presence of sub-complexes between euthyroid and hyperthyroid animals or between mitochondrial subpopulations.

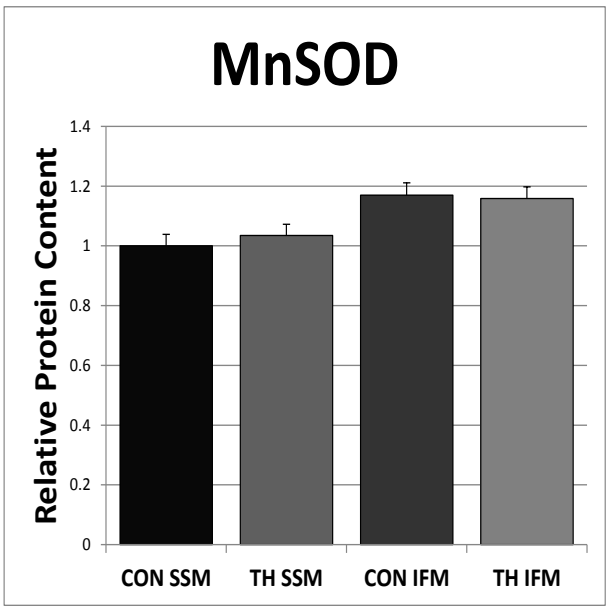


Figures 3.7 – 3.11 Show mitochondrial oxygen phosphorylation complexes I-V protein density expressed relative to CON SSM. No change was seen in protein content for any given protein due to either subpopulation or thyroid treatment. $P<0.05$

3.3 Presence of antioxidant enzymes

No difference was found in the presence of antioxidant enzyme content of Manganese superoxide dismutase (MnSOD) or glutathione peroxidase (GPx1) between mitochondrial subpopulations or as an effect of thyroid treatment.





Figures 3.12 and 3.13 No difference in either MnSOD content or GPx1 content shown, $P<0.05$.

3.4 Uncoupling protein content

The presence of uncoupling protein 3 (UCP3) which has been previously shown to be present in cardiac myocytes (Boss and others, 1997) was elevated both in IFM relative to SSM and again in thyroid treated animals relative to euthyroid though not significant. This potential increase in protein content of UCP3 could show that IFM contain increased UCP3 content relative to SSM

and that thyroid hormone increases UCP3 content in both SSM and IFM subpopulations in addition to a subpopulation effect.

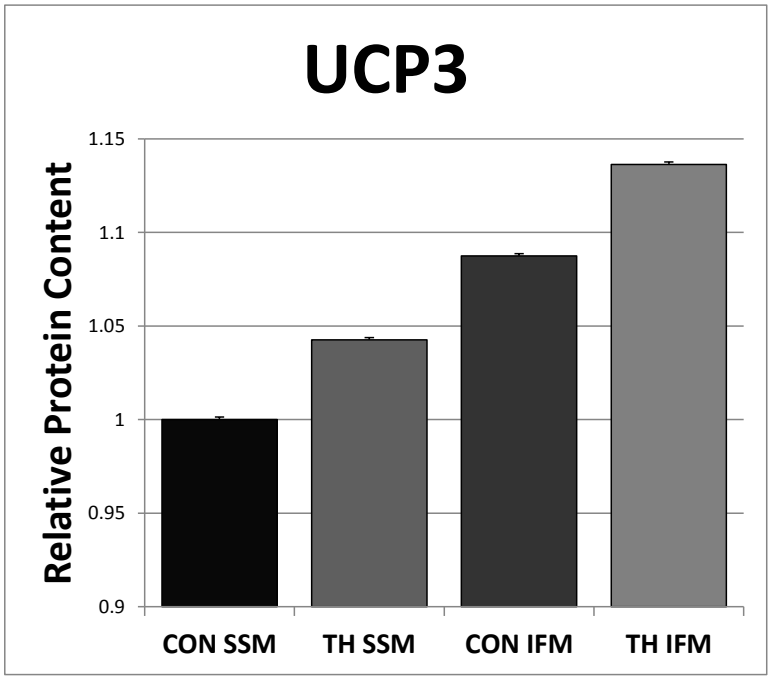


Figure 3.14 Relative content of UCP3 which has previously been identified as being present in cardiac tissue. No significant change was found in UCP3 content $P<0.05$ however data does indicate that there is a correlation between UCP3 content, mitochondrial subpopulations, and thyroid hormone treatment.

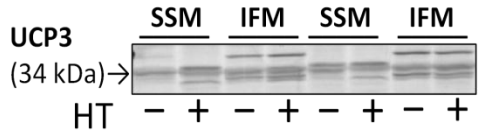


Figure 3.15 Shows the results of Western Blotting for UCP3. The presence of an additional bands of proteins present specifically in IFM populations are shown. Statistical analysis of UCP3 content did not take these additional bands outside 34 kDa into consideration.

Discussion

It is evident that mitochondrial subpopulations deserve further investigation into the roles that they may play in contributing to and modulating different disease states. Because mitochondria are an essential organelle for all aerobic cellular life, it would make sense that spatially distinct subpopulations could potentially serve similar yet diverse functions. Because of the vast array of mitochondrial function and roles in disease, elucidating the relevance of distinct population is imperative.

The present study has determined that SSM and IFM subpopulations are potentially structurally different to accommodate the spatially distinct metabolic needs of surrounding tissue. By isolating mitochondrial subpopulations and exposing those to similar conditions of global ischemia and hyperthyroidism, we are elucidating those potential differences.

4.1 IFM release less ROS than SSM

By measuring ROS release from different places within the ETC using a set protocol of substrates and inhibitors, it is clear that despite a greater capacity to produce and release ROS, IFM release less ROS that SSM in the post-ischemic hyperthyroid myocardium. This ability of IFM to limit ROS release was consistent throughout the experiments in the absence of ETC inhibitors under state 4 conditions in which additional stimuli to increase ROS were not used. Previous studies have suggested that decreased ROS release from IFM is not a result of lower electron flux through the IFM ETC. In fact, it would appear to be the opposite, that IFM release

less ROS while experiencing increased electron flux relative to SSM. One might note that there is a trend for higher MnSOD in IFM vs. SSL, but would this necessarily reduce ROS in response to global ischemia and thyroid hormone treatment it leads us to believe that the differences in ROS release are attributed to other factors, potentially related to changes in membrane potential or the structural integrity of the inner membrane.

4.2 Subspecies dimorphic effect of HT on mitochondrial ROS release

In the present study there was a strong trend for increased ROS release from the SSM, and decreased ROS release selectively in the IFM, in response to reperfusion hyperthyroidism. This potential opposite effect in ROS release that is subpopulation specific could be due to impairment of electron flow through complexes I and III that is specific to the SSM. As seen in figure 1, there is a trend in the S+R+OA group that is also present to a lesser degree in the M+P+OA group for ROS release to be increased in the SSM over the control. The trend for decreased ROS release seen in the IFM mitochondria treated with thyroid hormone could be the result of increased uncoupling due to UCP3 that is up-regulated in response to thyroid hormone (Gong and others, 1997; Harper and Seifert, 2008; Silvestri and others, 2005; Venditti and Di Meo, 2006). It appears that while thyroid hormone increases uncoupling in both SSM and IFM populations, it decreases ROS release in IFM while increasing ROS release in SSM. The idea that the uncoupling proteins modulate IFM ROS release in the present study is supported by increased UCP3 content in IFM vs. SSM.

When assessing mitochondrial uncoupling protein content, additional bands of protein were identified near the 34 kDa marker for known UCP3 that were selectively present only in the IFM samples (Figure 15). It is possible that this is a previously unidentified isoform or splice variant of UCP3 or another uncoupling protein present in IFM that serves to reduce ROS release by augmenting dissipation of the proton gradient that inhibits electron flow and contributes to increased ROS production and release.

In summary, subpopulation specificity of mitochondria is an interesting and potentially important field of study in mitochondrial function. Clearly there are functional differences that may contribute to the development and progression of certain disease states by modulating the effect of certain cellular processes, such as ROS generation and oxidative stress.

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