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Effects of the Mitochondrial Uncoupling Protein 3 On Fuel Substrate Oxidation and Reactive Oxygen Species Formation in Rat L6 Muscle Cells

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James Darcy MacLellan

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master of Science

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TABLE OF CONTENTS

i. ACKNOWLEDGEMENTS

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ii. LIST OF FIGURES AND ILLUSTRATIONS

iv. ABSTRACT

Uncoupling protein 3 (UCP3) is an integral mitochondrial membrane protein thought to disassociate fuel substrate oxidation by allowing proton re-entry into the mitochondrial matrix. Expression of UCP3 has been correlated with fatty acid and glucose metabolism, and reactive oxygen species (ROS) formation. To improve our understanding of the potential involvement of UCP3 in such pathways we investigated the effects of a UCP3 overexpression (2.2-2.5 fold) in the L6 muscle cell line. These findings were compared to those of UCP2 overexpression and DNP exposure. Palmitate oxidation was significantly increased by overexpressing UCP3 but unaffected by the other treatment conditions. Both glucose oxidation and oxygen consumption were unaffected by UCP2 and UCP3 overexpression but were significantly increased by DNP treatment. ROS production was decreased by UCP2, UCP3 and DNP treatment. These findings suggest a role for UCP3 in the regulation of fatty acid oxidation and ROS formation but not in glucose oxidation

1. INTRODUCTION

Mitochondrial uncoupling proteins (UCPs) are integral proteins of the mitochondrial inner membrane that separate fuel substrate (*e.g.* glucose and fatty acid) oxidation from ATP generation by permitting the re-entry of protons into the mitochondrial matrix. The archetypal UCP, UCP1, is abundantly expressed specifically in brown adipose tissue, where it functions in thermogenesis by allowing the energy destined for storage in ATP to be released as heat. A similar function was also attributed to the more recently discovered UCPs, UCP2 and UCP3. However, mounting evidence indicates that these proteins have alternative functions. There is intense debate as to the exact mechanism of action and biological function of UCP2 and UCP3 as several hypotheses have been proposed. Strong arguments have been made for a role of UCP3 in lipid metabolism and reactive oxygen species production (ROS). Less convincing evidence has been presented for a role of UCP3 in glucose metabolism. Identification of the function of UCP3 has been hindered by the artifactual uncoupling induced by supraphysiological concentrations of UCP3 and the potential for compensatory mechanisms occurring in the absence of UCP3. We hypothesize that physiological increases in UCP3 will increase fatty acid oxidation and mitigate the formation of ROS but will have no effect on the oxidation of glucose. We also propose that the effects of UCP3 will not be mimicked by chronic low dose exposure to a chemical mitochondrial uncoupler, dinitrophenol (DNP), or by UCP2. The ability of UCP3 to regulate lipid metabolism and ROS production has significance in a number of metabolic diseases which will be subsequently discussed

1.1 Significance of Research

The prevalence of obesity, a disease characterized by an imbalance between energy intake and energy expenditure, is increasing dramatically worldwide. In Canada, adult obesity has more than doubled (Katzmarzyk, 2002) and childhood obesity has more than tripled in the last 20 years (Spurgeon, 2002). Obesity is associated with significant morbidity (McLaughlin et al., 2004; Pan et al., 2004; Morse et al., 2005) and mortality (Katzmarzyk and Arden, 2004; McGee, 2005). Birmingham and colleagues (1999) have estimated that the direct health care cost of obesity in Canada was nearly $2 billion in 1997. This large increase in adult and childhood obesity is likely a major contributor to the concomitant increase in type 2 diabetes mellitus (T2DM), as obese Canadians are six times more likely to develop T2DM than their lean counterparts (Chauhan, 2003). Similar to obesity, T2DM is associated with numerous comorbidities and has been shown to have significant economic burden. Estimated Canadian health care costs associated with T2DM in 1998 were reported to be approximately $5 billion U.S. dollars (Dawson et al., 2002).

The strong link between obesity and the development of T2DM is due to a decrease in pancreatic islet cell mass, beta cell function, and progressive resistance to the action of insulin in obese individuals. Insulin resistance, defined as an inability of insulin to sufficiently stimulate glucose transport and metabolism in peripheral tissues and to sufficiently inhibit glucose production by the liver, is observed in all obese individuals with T2DM. Skeletal muscle is the principal tissue responsible for insulin-stimulated glucose uptake and metabolism (Klip and Paquet, 1990) and as a result is the major site of peripheral insulin resistance. The underlying cause of insulin resistance in skeletal

muscle is unclear. However, intramuscular triglyceride content has been shown to be a robust predictor of skeletal muscle insulin sensitivity (Pan et al., 1997; Perseghin et al., 1999), except in endurance trained athletes. The paradoxical increase in muscle triglyceride stores in these individuals is associated with their elevated capacity to oxidize fat, with elevated intramuscular fat stores increased in muscle to provide a readily available source of fuel for oxidative metabolism (van Loon, 2004). In non-endurance trained athletes, a decreased capacity to oxidize fat in skeletal muscle is thought to be responsible for triglyceride accumulation and loss in insulin sensitivity (Kelley et al., 1999). In support of this proposal, obese individuals have reduced fat oxidation rates and enzyme activities (Kim et al., 2000) and increased muscle triglyceride stores (He et al., 2001).

Oxidation of fatty acids occurs principally within mitochondria. Therefore, it is not unexpected that mitochondrial dysfunction is also associated with triglyceride accumulation and insulin resistance. In fact, muscle mitochondria oxidative capacity is more strongly correlated with insulin sensitivity than muscle triglyceride content (Bruce et al., 2003). Key genes involved in skeletal muscle mitochondrial fuel substrate oxidation are decreased in insulin resistant individuals (Simoneau et al., 1999; Patti et al., 2003). Skeletal muscle insulin sensitivity, mitochondrial function and oxidative capacity all decrease concomitantly with age (Petersen et al., 2003). It is also interesting to note that insulin resistant offspring of patients with T2DM have reduced mitochondrial oxidative activity (Petersen et al., 2004). Together, these data suggest that impairment in mitochondrial fuel handling and oxidation is responsible for the development of skeletal muscle insulin resistance observed in prediabetic individuals.

1.2 Mitochondrial Bioenergetics: Oxidative Phosphorylation and Proton Leak

The primary function of mitochondria is to produce ATP for energy- dependent cellular processes. Aerobic metabolism of fuel substrates, such as glucose and fatty acids, is coupled to the synthesis of ATP by mitochondrial oxidative phosphorylation (Figure 1.1). This process involves the transfer of electrons from fuel substrates by electron carriers, nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD), to a series of enzyme complexes (I-IV) within the mitochondrial inner membrane. The complexes collectively are referred to as the electron transport chain. The passage of electrons down the electron transport chain through a succession of reduction/oxidation reactions to the final electron acceptor, oxygen, is linked to the transport of protons from the mitochondrial matrix to the intermembrane space. The proton electrochemical potential gradient generated by the pumping of protons across the inner membrane is referred to as the protonmotive force. Protons re-enter the matrix via ATP synthase with the potential energy of the protonmotive force coupled to the phosphorylation of ADP.

Fuel oxidation is not perfectly coupled to ATP synthesis. Isolated mitochondria continue to consume oxygen in the absence of ADP (*i.e.* state 4 respiration) or in the presence of inhibitors of ATP synthase. Initially, it was believed that these results were an artifact of mitochondrial damage during isolation. However, a non-ohmic (*i.e.* non-linear) relationship between respiratory rate and protonmotive force is also observed in intact cells (Nobes et al., 1990). These findings indicate that protons can move back into

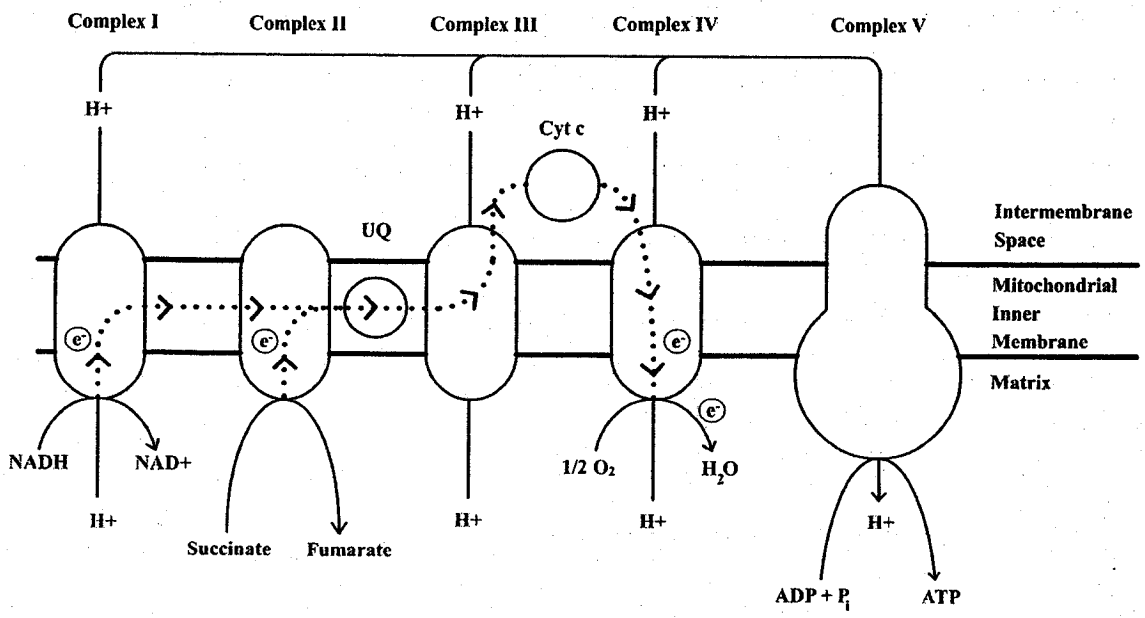

Figure 1.1 Mitochondrial oxidative phosphorylation

Fuel substrate oxidation generates electron carrying intermediates, NADH and $FADH_2$, which are oxidized at complexes I and II of the electron transport chain, respectively. Subsequent reduction/oxidation reactions within the members of the electron transport chain (dotted line) are coupled to the transport of protons across the mitochondrial inner membrane by complexes I, III, and IV. Accumulation of protons in the intermembrane space creates a proton gradient (protonmotive force) across the mitochondrial inner membrane. ATP synthase (complex V) allows proton re-entry into the mitochondrial matrix and utilizes the free energy stored in the proton gradient to generate ATP.

the matrix through an alternative pathway, not involving ATP synthase. The proportion of cellular or mitochondrial oxygen consumption that is due to proton leak is variable. When ATP synthase is active and protons are moving rapidly through its ATP synthase 'motors', protonmotive force is relatively low and the return of protons to the matrix through other means (*i.e.*, leak) is low. This situation predominates during periods of energy demand (*i.e.* state 3 respiration) when ATP generation and oxygen consumption are high. On the other hand, when ATP synthase activity is low, and protonmotive force is thus high, leak is increased. This situation predominates during resting conditions (*i.e.* state 4 respiration) when ATP and oxygen demand are low. The proportion of total oxygen consumption that is due to leak is much higher in the latter metabolic state.

It is now widely recognized that proton leak is an inherent part of oxidative phosphorylation. However, the mechanisms by which proton leak occurs are poorly understood. It is generally believed that proton leak involves both protein and non-protein components of the mitochondrial inner membrane. Non-protein proton leak may occur non-specifically through membrane pores and protein-lipid interfaces and is thought to be a characteristic of the membrane, related to its surface area and fatty acid composition (Porter et al., 1996). Whereas, protein-mediated uncoupling involves integral membrane proteins such as UCP1 and possibly the more recently discovered homologues, UCP2 and UCP3. These alternative pathways of proton leak have great implications in cellular energy expenditure as approximately 35-50% of the resting metabolic rate of rat skeletal muscle (Rolfe and Brand, 1996; Rolfe et al., 1999) and as much as 25% of whole body standard metabolic rate is accounted for by mitochondrial proton leak (Stuart et al., 1999a).

1.3 Mitochondrial Uncoupling Proteins

UCPs belong to a superfamily of anion carrier integral membrane proteins that facilitate the transfer of metabolic intermediates across the mitochondrial inner membrane. Due to the selective impermeability of the inner membrane, members of this superfamily are essential for communication between cytosolic (*e.g.* glycolysis, pentose phosphate pathway) and mitochondrial pathways (*e.g.* citric acid cycle, urea cycle, fatty acid oxidation, gluconeogenesis). Mammals express five UCP homologues, UCP1-5, and orthologues have been identified in a number of animal and plant species (Maia et al., 1998; Stuart et al., 1999b; Raimbault et al., 2001). UCP1 is the archetypal UCP from which all other members of the family have been identified. Of the five UCPs identified in mammals, UCP1-3 have a high sequence similarity with each other while UCP4 and UCP5, found predominantly in the brain, are more similar to other members of the anion carrier superfamily. Due to this phylogenetic divergence between UCP1-3 and UCP4-5, Nedergaard and Cannon (2003) have suggested that UCP4 and UCP5 not be considered part of the UCP family. UCP4 and UCP5 will thus not be discussed further in this thesis.

UCPs are thought to dissociate respiration from ATP synthesis by allowing protons to leak back into the matrix, releasing the potential energy of the protonmotive force as heat (nonshivering thermogenesis) (Figure 1.2). Results from a number of *in vitro* studies have suggested that proton leak mediated by UCPs is regulated by fatty acids and purine nucleotides (Jaburek et al., 1999; Echtay et al., 2001 Zackova et al., 2003). Many believe that fatty acids are obligatory for UCP activity but this has been disputed (Rial et al., 2004). Ubiquinone (coenzyme Q) has also been shown to be a potent activator of UCP-mediated proton leak (Echtay and Brand, 2001) although it may

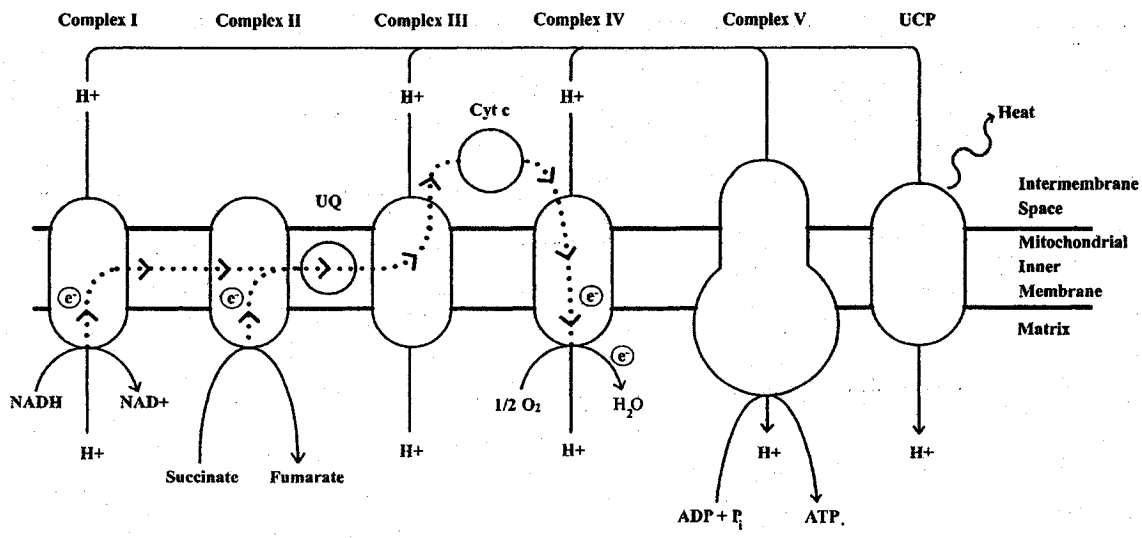

Figure 1.2 Disassociation of oxidative phosphorylation by UCP

UCP provides an alternative route for proton re-entry into the mitochondrial matrix, bypassing ATP synthase (complex V). Proton transport by UCP results in release of the free energy of the protonmotive force as heat rather then stored in the high energy phosphate bonds of ATP.

not be necessary for UCP activity (Esteves et al., 2004). Whereas, UCP1 clearly functions in the manner described above (*i.e.* proton translocation activated by fatty acids, and inhibited by purine nucleotides), the physiological functions of UCP2 and UCP3 remain unclear.

1.3.1 Uncoupling Protein 1

Brown adipose tissue (BAT) functions primarily in thermoregulation by oxidizing fatty acids to produce heat (Smith and Horwitz, 1969). This is accomplished by UCP1, which allows the energy stored in fuel substrates to be released as heat, instead of being used for ATP synthesis. The latter is referred to as nonshivering thermogenesis and is clearly distinct from the heat that is produced as a normal byproduct of biochemical processes and work (*e.g.* muscular contraction). UCP1 provides an efficient and regulated mechanism for temperature- and diet-induced thermogenesis (Rothwell and Stock, 1979; Himms-Hagen, 1990; Liu et al., 2003).

UCP1 is exclusively expressed in brown adipocytes and is responsible for the high sensitivity of brown adipocyte mitochondria to fatty acid-induced uncoupling (Rafael et al., 1969). Expression and activity of UCP1 is tightly regulated. Purine nucleotides were the first known regulators of UCP1 activity and were integral in its discovery (Nicholls, 1976; Heaton et al., 1978). Di- and triphosphates bind with high affinity and inhibit UCP1 activity, while monophosphates bind with low affinity (Echtay et al., 1999). Later, UCP1-mediated uncoupling was shown to be activated by fatty acids (Locke et al., 1982). Fatty acids compete with purine nucleotides for UCP1 activation. The competitive regulation of UCP1 by fatty acids and purine nucleotides was initially

thought to be due to a shared binding site but more recent evidence indicates that the regulatory sites are separate (Shabalina et al., 2004).

Definitive evidence of the thermogenic activity of UCP1 in BAT is demonstrated by the inability of UCP1-ablated mice to maintain their body temperature when cold challenged (Enerback et al., 1997). Paradoxically, these mice do not have increased susceptibility to obesity, despite having lost a major pathway of energy expenditure (Kopecky et al., 1995; 1996). Later work on congenic UCP1-ablated mice suggested that they had a reduced metabolic efficiency and some resistance to diet-induced obesity, the opposite of what was expected (Liu et al., 2003). These authors postulated that in the absence of UCP1, other less efficient pathways of heat production are activated in an attempt to maintain body temperature (*e.g.* shivering). UCP2 and UCP3 mRNA are highly expressed in the BAT of UCP1-ablated mice (Enerback et al., 1997; Matthais et al., 1999; Nedergaard et al., 1999) and may therefore provide a compensatory mechanism for mitochondrial proton leak. However, stimulation of BAT cells or mitochondria, from UCP1 null mice, with norepinephrine or fatty acids does not increase mitochondrial uncoupling (Matthais et al., 2000). Moreover, mitochondrial proton leak of UCP1-ablated mice is insensitive to purine nucleotides (Monemdjou et al., 1999), indicating that BAT UCP2 and UCP3 are not involved. Work by Monemdjou and colleagues (2000) demonstrated an increased mitochondrial proton leak in skeletal muscle of UCP1-ablated mice suggesting that compensatory mechanisms in tissues other then BAT may be responsible for the lack of obesity observed in UCP1-ablated mice. UCP2 and UCP3 are expressed in tissues other then BAT, such as skeletal muscle, and therefore remain prime candidates to explain the lean phenotype of UCP1-ablated mice.

1.3.2 Uncoupling Protein 2 and 3: The Novel UCPs

UCP2 and UCP3 share 55% and 57% homology with UCP1, respectively, and 73% homology with each other (Boss et al., 1997a; Gimeno et al., 1997; Vidal-Puig et al., 1997). UCP3 is predominantly expressed in skeletal muscle, a major tissue in human nonshivering thermogenesis, with lower levels in BAT, cardiac muscle and in areas of the brain (Boss et al., 1997a; Vincent et al., 2004). UCP2 is ubiquitously expressed in mammalian tissues, and has highest levels of expression in cells of the immune system (*e.g.* macrophages, spleen), lung, intestine, white adipose tissue, kidney, BAT, brain, pancreatic islets, heart, and detectable levels in skeletal muscle and liver (Fleury et al., 1997; Gimeno et al., 1997; Horvath et al., 1999; Chan et al., 2001; Rippe et al., 2000). In liver, Kupffer cells rather then parenchymal cells are believed to express UCP2 mRNA (Larrouy et al., 1997). UCP2 protein expression in tissues does not closely correspond to UCP2 mRNA expression patterns (Pecqueur et al., 2001). UCP2 protein has been detected in relatively few tissues, including spleen, lung, white adipose tissue, and pancreas (Pecqueur et al., 2001; Joseph et al., 2004). The lack of correlation between UCP2 mRNA and protein content has complicated interpretation of work where mRNA alone has been measured. Moreover early antibodies used for immunodetection work were not highly specific for UCP2 protein.

Initial work on the novel UCPs focused heavily on their potential thermogenic activity. This was not unexpected considering the strong similarity of UCP2 and UCP3 to UCP1 and a tissue distribution in metabolically active tissues (*i.e.* UCP3 in skeletal muscle). Moreover, the novel UCPs showed similar patterns of activation to various physiological stimuli as that for UCP1, including activation by fatty acids and inhibition

by purine nucleotides (Zackova et al., 2003). Both UCP2 and UCP3 gene expression are elevated by norepinephrine (Nagase et al., 2001) and thyroid hormone stimulation (Barbe et al., 2000) in skeletal muscle, similar to UCP1 gene expression in BAT. Cold exposure, which causes a strong induction of UCP1 expression and activity, also stimulates UCP2 mRNA in several tissues and UCP3 mRNA expression in skeletal muscle (Boss et al., 1997b; Yamashita et al., 1999; Masaki et al., 2000). Simonyan and colleagues (2001) observed a 3 fold increase in UCP3 mRNA expression and a decreased mitochondrial membrane potential in skeletal muscle mitochondria in response to cold exposure. However, this increase in expression and activity of the novel UCPs does not appear to provide a thermogenic function as the thermogenic capacity of UCP2- and UCP3-ablated mice is unchanged from wild type mice (Arsenijevic et al., 2000; Gong et al., 2000; Vidal-Puig et al., 2000). Although it could be argued that in the absence of one of the novel UCPs the other is compensating no such activity has been demonstrated to date. Further, cold sensitive UCP1-ablated mice remain cold intolerant, despite having increased mRNA expression of UCP2 and UCP3. Therefore, it is unlikely that the novel UCPs function as regulators of thermogenesis.

Studies conducted in numerous *in vitro* model systems have, however, provided evidence for an uncoupling activity by UCP2 and UCP3. Introduction of UCP2 or UCP3 into yeast mitochondria results in decreased mitochondrial membrane potential and increased proton leak and respiratory rate (Fleury et al., 1997; Gimeno et al., 1997; Paulik et al., 1998; Hinz et al., 1999; Rial et al., 1999; Zhang et al., 1999). Expression of UCPs in *Escherichia coli* results in protein accumulation in inclusion bodies that can be extracted and reconstituted in proteoliposomes. These proteoliposomes demonstrate fatty

acid-dependent mitochondrial proton flux that is absent in proteoliposomes lacking UCPs (Jaburek et al., 1999). Overexpression of UCP3 in cultured human muscle cells (Garcia-Martinez et al., 2001) and MCF7 breast cancer cells (Mao et al., 1999) lowers the mitochondrial membrane potential of these cells. UCP3 overexpression, in the physiological range, also reduces the membrane potential of C2C12 myoblasts (Boss et al., 1998b). Adenoviral overexpression of UCP2 and UCP3 in INS1 insulinoma cells results in increased respiration and proton leak (Hong et al., 2001). Similarly, Fink and colleagues (2002) have demonstrated that adenoviral overexpression of UCP1 and UCP2 in INS1 insulinoma cells increases proton flux.

The physiological relevance of these studies has been put into question. UCP overexpression has been shown to be not regulated by purine nucleotides (Heidkaemper et al., 2000) or, in certain model systems, localized to the mitochondrial inner membrane (Winkler et al., 2001). Further, Martin Brand's group has demonstrated artifactual uncoupling induced by supraphysiological levels of UCP1-3 (Stuart et al., 2001a; Stuart et al., 2001b; Harper et al., 2002). This is postulated to result from improper folding and insertion in the mitochondrial inner membrane.

Studies with mutant and transgenic mice have provided conflicting results. In support of an uncoupling function, UCP3 knockout mice show evidence of decreased mitochondrial proton leak, increased membrane potential, and increased respiratory control ratio (ratio of oxygen reduction to ATP phosphorylation) (Gong et al., 2000; Vidal-Puig et al., 2000; Bezaire et al., 2001). These mice also have increased affinity for ATP production (Cline et al., 2001). However, there are no differences in susceptibility to obesity or to cold-intolerance (Gong et al., 2000; Vidal-Puig et al., 2000). Transgenic

mice in which there is 66 fold (Clapham et al., 2000), or an 18 fold (Son et al., 2004) increase in UCP3 mRNA expression demonstrate a dramatic or a moderate protection from diet-induced obesity, respectively. These mice also show signs mitochondrial uncoupling in the form of decreased respiratory control ratios, increased state 4 respiration rates, and decreased mitochondrial membrane potential (Clapham et al., 2000). The biological relevance of these mice has been criticized on the basis of the high and non-physiological levels of gene expression, as physiological interventions in mice and humans are in the 4-8 fold range. Although Cadenas and colleagues (2002) have demonstrated increased mitochondrial uncoupling in mice overexpressing UCP3, they concluded that it was not due to the native function of UCP3. The increase in uncoupling was not proportional to the increase in UCP3 concentration and was not inhibited by purine nucleotides. The authors attributed the uncontrolled mitochondrial uncoupling to improper folding and insertion of UCP3 into the mitochondrial inner membrane.

To date there are no murine transgenic models of UCP2 overexpression. UCP2 knockout mice however, have been studied by a number of research groups. Initial reports on the phenotype of the UCP2 knockout mouse indicated that, like UCP3 knockout mice, they have normal body weights, food intake patterns, and adapt normally to the cold (Arsenijevic et al 2000). Arsenijevic et al (2000) however found that UCP2 knockout mice are resistant to bacterial infection and concluded that UCP2 plays a role in ROS production and macrophage-mediated immunity. Later studies indicated that UCP2 knockout mice have increased mitochondrial membrane potential and ATP levels (Zhang et al., 2001; Krauss et al., 2002; 2003). Finally, a series of elegant studies in UCP2 knockout mice and corresponding studies of cell lines has demonstrated quite

clearly that UCP2 acts as a negative regulator of insulin secretion from pancreatic beta cells (review: Chan et al., 2004). Well controlled immunodetection methods (immunohistochemistry and western blotting) have clearly demonstrated UCP2 protein expression in beta cells (Chan et al., 1999; Zhang et al., 2001)

A lack of firm evidence linking the novel UCP homologues with thermogenesis and whole body energy expenditure, as is observed with UCP1, has led researchers to propose alternative functions. The widely distributed expression of UCP2 has made it difficult to conclude a specific biological function. However, as indicated above, it appears that protein levels of UCP2 expression are much less widespread than is mRNA expression. Measurable levels of UCP2 protein have been found in immune associated tissues and cells, stomach, lung, thymocytes, and pancreatic beta cells but are noticeably absent from skeletal and cardiac muscle, kidney, and BAT (Pecqueur et al., 2001; Zhang et al., 2001; Krauss et al., 2002; Nishio et al., 2005). These findings were surprising considering the high level of UCP2 mRNA in some of the tissue where protein was found to be absent. As well, the identification of UCP2 in macrophages has made it difficult to verify the protein is from the specific tissue and not from resident macrophages. Further, it has been shown that UCP2 can be induced under certain circumstances in cell types where it was previously undetectable, yielding a measurable level of UCP2. For instance, Ruzicka and colleagues (2005) have demonstrated that lipopolysaccharide exposure can induce UCP2 expression and protein in liver parenchymal cells, cells that were previously thought to be devoid of UCP2 mRNA. Despite all of these complications there remain two primary hypothesized functions for UCP2. Many believe that UCP2 is involved in the regulation of ROS production. A

second hypothesis suggests that by regulating ROS production UCP2 alters glucose-stimulated insulin secretion in pancreatic beta cells (Negre-Salvayre et al., 1997; Zhang et al., 2001).

The temporal expression pattern of UCP3 in skeletal muscle has led researchers to propose roles in lipid metabolism and ROS production, and this is the subject of the subsequent sections of the introduction. As dysregulation of skeletal muscle lipid metabolism and ROS production are implicated in the development of insulin resistance and T2DM the identification of physiological function of UCP3 may provide some important insights into these metabolic pathologies.

1.4 Role of Uncoupling Proteins 3 in Lipid Metabolism

Fatty acids appear obligatory for UCP3 function and also likely stimulate UCP3 expression via peroxisome proliferative activated response α (PPARα) or PPARδ and their association with PPAR responsive elements in the UCP3 gene (Silva and Rabelo, 1997; Acin et al., 1999). Moreover, the nonmetabolizable fatty acid derivative, α-bromopalmitate, stimulates increased UCP3 gene expression in L6 myotubes (Son et al., 2001). In light of these findings it is not surprising that UCP3 gene expression is positively correlated with extracellular free fatty acid levels *in vitro* (Son et al., 2001) and *in vivo*. *In vivo*, UCP3 is increased under conditions of elevated fatty acid metabolism, such as fasting (Samec et al., 1998; Boss et al., 1998b), high fat diets (Matsuda et al., 1997), and lipid infusion (Khalfallah et al., 2000). Further, UCP3 expression and protein are downregulated during conditions when circulating fatty acids are decreased, such as lactation (Pedraza et al., 2000) and when hypolipidemic agents

are administered (Dusquet et al., 2001). In newborn mice, UCP3 expression is not observed in skeletal muscle until suckling is initiated and free fatty acid levels rise in the circulation (Brun et al., 1999).

In human studies examining the potential link between UCP3 and obesity and T2DM, several populations with polymorphism in the 5' proximal noncoding region of the UCP3 gene have been associated with disorders in fat accumulation (Cassell et al., 2000). Humans heterozygous for an exon 6 donor splice mutation to their UCP3 gene have elevated respiratory quotients (ratio of carbon dioxide produced to oxygen consumed), indicative of reduced fat oxidation, and reduced fatty acid oxidation rates (Argyropoulos et al., 1998). However, in another human population unaltered respiratory quotients have been observed in individuals that are hetero- and homozygote for the exon 6 splice site mutation (Chung et al., 1999a; 1999b). This mutation was shown to prevent the full length isoform of UCP3 from being expressed. Expression of the short form of UCP3 (UCP3S) was elevated in these individuals and the authors of this report postulated that it may have compensated in the absence of the long isoform of UCP3.

Studies of lipid metabolism in transgenic mice have provided conflicting results. Bezaire and colleagues (2001) have shown increased respiratory quotients in UCP3-ablated mice. Two other studies have demonstrated no changes in respiratory quotients (Gong et al., 2000; Vidal-Puig et al., 2000) and no changes in muscle lipid oxidation in UCP3-ablated mice (Vidal-Puig et al., 2000). This discrepancy may be due to the fact that the mice used in the latter studies were from the F1 generation whereas Bezaire and colleagues used inbred F10 generation mice, and conducted their indirect calorimetry

over longer periods of time. Recently, this same group provided evidence that UCP3 overexpression at physiological levels in mice increases fatty acid uptake, mitochondrial transport and oxidation in skeletal muscle (Bezaire et al., 2005). These mice had increased preference for fatty acid oxidation, significantly lower intramuscular triglyceride levels and elevated activity of key enzymes in fatty acid transport, (*e.g.* carnitine palmitoyltransferase 1), and beta oxidation (*e.g.* beta hydroxyacyl CoA dehydrogenase). In two other studies using UCP3 overexpressing mice, lipid oxidation rates were significantly elevated (Clapham et al., 2000; Wang et al., 2003). These mice however, expressed UCP3 at supraphysiological levels. Thus, the latter findings may have resulted from artifactual uncoupling (discussed more extensively above).

Much of the work demonstrating a role of UCP3 in lipid metabolism has also provided data inconsistent with a role in energy expenditure and uncoupling. Overexpression of UCP3 in INS-1 insulinoma cells does not have a significant effect on the ADP/O ratio or proton leak yet still improves lipid oxidation rates (Hong et al., 2001). Increased expression of UCP3 in response to fasting, a period of energy conservation when thermogenic and catabolic activity is decreased, strongly suggests that UCP3 has an alternate function. It has been suggested that the fasting-induced increase in UCP3 may be an adaptive mechanism to provide a source of nonshivering thermogenesis when other thermogenic pathways have been downregulated. However, mitochondrial proton leak in skeletal muscle is unaltered by fasting (Cadenas et al., 1999; Bezaire et al., 2001).

1.5 Mitochondrial Reactive Oxygen Species and Uncoupling Protein 3

Mitochondrial oxidative phosphorylation utilizes a major proportion (approximately 90%) of cellular oxygen uptake in order to fully oxidize fuel substrates, such as glucose and fatty acids, and generate ATP. This process does not occur without its costs. As a byproduct of oxidative phosphorylation reactive oxygen species (ROS), such as superoxide, are produced. Approximately 0.2-2% of total oxygen uptake is converted to ROS within mitochondria (Chance et al., 1979; Hansford et al., 1997). Complex I (*i.e.* NADH-dehydrogenase flavin-semiquinone) and complex III (*i.e.* ubiquinol:cytochrome c reductase) of the electron transport chain are the primary sources of mitochondrial superoxide. Superoxide generated at these sites enters the matrix rather than the cytosol (Turrens, 1997; Kudin et al., 2005). However, there is some evidence that superoxide generated at complex III enters the intermembrane space (Han et al., 2001). Generation of superoxide from these sites is strongly dependent on the protonmotive force, which itself is dependent on the metabolic state of the cell (Papa and Skulachev, 1997). During resting conditions (*i.e* state 4 respiration) when ATP and oxygen demand are low the protonmotive force and production of superoxide are elevated. Whereas, when ATP generation and oxygen consumption are high (*i.e.* state 3 respiration), protonmotive force and superoxide production are reduced. The increased formation of superoxide when the protonmotive force is elevated occurs as the result of increased leak of electrons from single electron carrying intermediates, predominantly ubisemiquinone, to oxygen (Turrens et al., 1985; Gonzalez-Flecha and Boveris, 1995).

Mitochondria contain a number of antioxidant defence systems to combat the toxic effects of superoxide. Superoxide is rapidly converted to hydrogen peroxide by

superoxide dismutase which is then converted to oxygen and water by catalase and glutathione peroxides. When ROS escape these and other mitochondrial antioxidants (*e.g.* vitamins E and C) they accumulate and form more reactive ROS (*e.g.* hydroxyl radical) that are able to react with biological macromolecules. Due to the proximity of membrane fatty acids and proteins to the electron transport chain, the main source of superoxide, these macromolecules are particularly vulnerable to ROS-mediated damage. The interaction of ROS with membrane phospholipids (and other unsaturated fatty acid derivatives) initiates a chain reaction of lipid peroxidation that causes membrane damage and instability (Strassburger et al., 2005). ROS can also react directly or indirectly with mitochondrial proteins causing chemical modification and catalytic deactivation (Giron-Calle and Schmid, 1996). Although mitochondrial DNA is not in direct proximity to the electron transport chain, it is not protected by histones and is highly sensitive to ROS-mediated damage and mutations (de Grey, 2005). This damage to mitochondrial macromolecules can lead to further ROS production and eventual cell death (Kujoth et al., 2005).

The strong relationship between the protonmotive force and ROS production indicates that mechanisms that increase mitochondrial proton leak can prevent superoxide formation. The first evidence suggesting that UCP3 may be involved in the regulation of ROS production came when purine nucleotide inhibition of UCP1 or UCP2 was shown to increase hydrogen peroxide production (Negre-Salvayre et al., 1997). Subsequent work revealed that UCP3 (as well as UCP1 and UCP2) exposed to superoxide generates a mild GDP-sensitive proton conductance in skeletal muscle mitochondria of wild type mice but not UCP3 knockout mice (Echtay et al., 2002b).

These findings were demonstrated using an exogenous source of superoxide, produced by xanthine and xanthine oxidase. Identical findings have been demonstrated by generating endogenous superoxide using succinate (Talbot et al., 2004). In UCP3 knockout mice, where superoxide is unable to stimulate a mild uncoupling, ROS concentrations and oxidative damage are increased (Vidal-Puig et al., 2000; Brand et al., 2002). Overexpression of UCP3 mitigates oxidative stress in neurons exposed to high levels of glucose (Vincent et al., 2004).

The mechanism by which superoxide stimulates UCP3 is unclear. Some insight has been provided by Echtay and colleagues (2003) who showed that 4-hydroxyl-2-nonenal (HNE), a product of lipid peroxidation, can stimulate proton leak by UCP3 (Echtay et al., 2003) suggesting that downstream products of ROS damage are responsible for UCP3 activation. A later study by Murphy and colleagues (2003) demonstrated that [4-[4-[[(1,1-Dimethylethyl)-oxidoimino]methyl]phenoxy]butyl] triphenylphosphonium bromide (mitoPBN), which inhibits carbon-centered radical formation but does not directly influence superoxide or lipid peroxidation inhibits UCP3 activation. These findings are consistent with a model whereby superoxide reacts with membrane phospholipids generating carbon-centered radicals that initiate lipid peroxidation and HNE production that in turn activates UCP3. This creates a feedback loop in which ROS activates a mild UCP3-mediated proton leak that reduces the mitochondrial membrane potential and future ROS production. Maintaining a low level of ROS prevents cellular oxidative damage at the cost of only a minor decrease in the efficiency of oxidative phosphorylation.

1.6 Role of Uncoupling Protein 3 in Glucose Metabolism

Little is known about the potential involvement of UCP3 in glucose homeostasis as much of the focus has been on roles for UCP3 in lipid handling and ROS production. However, there is increasing evidence that UCP3 may influence skeletal muscle glucose uptake and metabolism. Transgenic mice overexpressing UCP3 have lower fasting plasma glucose and insulin levels, and increased glucose clearance (Clapham et al., 2000; Wang et al., 2002; Son et al., 2004). Similar results are observed in mice ectopically expressing UCP1 in skeletal muscle (Li et al., 2000; Han et al., 2004). These mice have decreased plasma glucose levels as the result of increased insulin-stimulated glucose uptake and GLUT4 protein, and increased enzyme activity of AMP-activated protein kinase (AMPK) and hexokinase. In a study that is of direct relevance to the project described herein, Huppertz and colleagues (2001) demonstrated that the overexpression of UCP3 in rat L6 myotubes using an adenoviral construct has no effect on the total cellular content of GLUT4 protein but does increase GLUT4 translocation to the plasma membrane and glucose uptake. This group also demonstrated that a chronic low dose of DNP could mimic these effects. This has led to speculation that the results with UCP3 may be an artifact of improper folding and insertion in the membrane, leading to uncontrolled proton leak. A similar argument has been made for the results observed in transgenic mice (discussed above). In fact, chemical mitochondrial uncouplers, such as DNP, which induce uncontrolled proton leak, have been shown to have the same positive effects on glucose homeostasis (Khayat et al., 1998a, 1998b).

Despite a lack of firm evidence linking UCP3 with glucose metabolism, several studies have demonstrated that UCP3 expression often correlates with that of GLUT4.

During cold exposure and acute exercise GLUT4 and UCP3 expression are both transiently increased (Neufer and Dohm, 1993; Lin et al., 1998; Tsuboyama-Kasaoka et al., 1998). Fasting also increases mRNA and protein content of both GLUT4 and UCP3 in skeletal muscle. However, their expression patterns differ between muscle fiber types. Expression of UCP3 is greater in glycolytic then in oxidative muscle fibers (Schrauwen et al., 2001a; Jimenez et al., 2002). GLUT4 expression is unchanged in glycolytic muscle and increased in oxidative muscle (Neufer et al., 1993). Other situations, in which UCP3 expression is elevated, such as lipid infusion, increase the expression of UCP3 but not GLUT4 in rat skeletal muscle (Vettor et al., 2002).

Thus, the correlation between UCP3 expression and skeletal muscle glucose uptake, as measured by GLUT4 expression and protein, appears to be weak. Interestingly however, overexpression of GLUT4 in mice causes a 2-4 fold increase in UCP3 mRNA expression (Tsuboyama-Kasaoka et al., 1999). The authors and others suggest that the increase in UCP3 expression is the result of elevated glucose flux. In support of these findings, several conditions in which glucose uptake is elevated have been shown to be positively correlated with UCP3 expression. In T2DM patients, where UCP3 expression is decreased 2 fold (Schrauwen et al., 2001a), UCP3 expression is associated with improved insulin-mediated glucose utilization (Krook et al., 1998). Similar decreases in glucose utilization and UCP3 expression are observed in obese diabetic rats (Matsuda et al., 1998). During exercise, when both carbohydrate and lipid metabolism are elevated, UCP3 expression was found to parallel changes in glucose uptake (Tsuboyama-Kasaoka et al., 1998). Other conditions when glucose flux is increased do not induce changes in UCP3 expression (Millet et al., 1997) suggesting the

changes observed in the aforementioned studies are due to increased circulating fatty acids levels or production of ROS (by increased glucose flux).

1.7 Summary and Research Questions

Summary:

Dysregulation of skeletal muscle mitochondrial fuel oxidation and ROS generation have been linked to the development of a number of pathological diseases, including obesity, insulin resistance, and T2DM. Improvements in muscle glucose and lipid metabolism, as well as reduced formation of ROS have been demonstrated with increased expression of UCP3 protein. Support for roles in lipid oxidation and ROS production are strong. Whereas, a role for UCP3 in glucose metabolism is weak, as much of the evidence favouring such a function is believed to be artifactual or is based on correlative data. We will further explore the putative roles of UCP3 in fuel substrate metabolism and ROS production and verify that the results we observe are not due to artifactual uncoupling, a problem in other model systems. Moreover, we will compare these results to those of UCP2 and the chemical mitochondrial uncoupler, DNP.

Research Questions:

1. What effect does UCP3 overexpression have on L6 muscle cell palmitate oxidation?
2. What effect does UCP3 overexpression have on L6 muscle cell glucose oxidation?
3. What effect does UCP3 overexpression have on L6 muscle cell ROS formation?

2. Methods

2.1 Cell Line Maintenance

L6 rat myoblasts were maintained in α minimum Eagle's medium containing 10% (vol/vol) fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (growth medium). Myoblasts were grown in 5 cm^2 cell culture dishes at 37°C and 5% CO^2. Growth medium was replaced every 48 h. Myoblasts were passaged at 60-80% confluency by removing the growth medium and washing once with phosphate buffered saline (137 mmol/l NaCl, 2.7 mmol/l KCl, 10 mmol/l Na_2HPO_4, 1.8 mmol/l KH_2PO_4; PBS). Trypsin (0.25%; Invitrogen, Burlington, ON) was then added and myoblasts were incubated at 37°C for 3-5 min. After the incubation period, fresh growth medium was added and lifted myoblast were transferred to a 15 ml centrifuge tube (Sarstedt, Montreal, QC). Myoblasts were spun down, resuspended in fresh growth medium, and then transferred to sterile cell culture plates. The cells were passaged no more than 12 times.

2.2 Cell Differentiation

For experimental procedures, cells were reseeded into 6 cm^2 cell culture dishes, 10 cm^2 cell culture dishes, opaque 96 well plates, or 25 cm^2 flasks, depending on the experiment to be performed. Once cells reached confluency, differentiation was induced by lowering the serum concentration to 2% (differentiation medium). Differentiation medium was replaced every 48 h for 5 days, unless otherwise stated, allowing sufficient time for myotube formation. All experiments were initiated 5 days after differentiation had been induced.

2.3 Adenoviral Transfection

Transfection of L6 muscle cells with an adenoviral vector (1x 10^7 pfu/ml) containing recombinant UCP2 (Ad5CMV-UCP2) or UCP3 (Ad5CMV-UCP3) was performed 2 days after cell differentiation had been induced. The virus was removed after 48 h incubation period and fresh differentiation medium was added. The efficacy of infection was determined by transfecting parallel plates with an equivalent titer of an adenoviral vector containing green fluorescence protein (Ad-GFP). GFP transfected myotubes were also used to verify that experimental outcomes were not an artifact of adenoviral infection. To simultaneously study the effect of chemical uncoupling, separate plates were treated with 50 μmol/l DNP starting 2 days after cell differentiation had been initiated. Myotubes were treated with DNP for 72 h with the DNP being replaced every 24 h. This concentration of DNP was chosen based on results of preliminary studies in our laboratory and on the concentrations used chronically in the same cell line by others (Huppertz et al., 2001). Control myotubes were left untreated.

2.4 Protein Isolation

Myotubes, grown in 10cm^2 dishes, were incubation with 0.25% trypsin at 37°C, washed, and then total protein was isolated using Trizol (Invitrogen). Cells were lysed by the addition of 3 ml of Trizol to each dish for 5 min. The homogenate was transferred to 2 ml tubes (0.5 ml each) and 100 μl of chloroform was added. The tubes were then capped, shaken vigorously for 15 sec and then incubated at room temperature for 3 min. After the incubation period, the tubes were centrifuged at 10,000 g for 15 min at 4°C. The upper aqueous phase containing RNA was discarded. DNA was precipitated by

incubating the samples in 150 µl of 100% ethanol at room temperature for 3 min followed by spinning the samples at 2,000 g for 5 min at 4°C. The supernatant was transferred to fresh tubes leaving sedimented DNA behind. Protein was precipitated by the addition of 0.75 ml isopropanol to each tube, which was allowed to stand for 10 min. The tubes were then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was removed and the protein pellet was washed three times with guanidine hydrochloride in 95% ethanol by adding 1 ml to each tube, incubating for 20 min at room temperature, and then centrifuging at 7,500 g for 5 min at 4°C. After the final wash, the protein pellet was washed with 1 ml of ethanol using the same procedure as above. The protein pellet was then vacuum dried and dissolved in 1% SDS by pipetting at 50°C. Any insoluble material was removed by centrifuging each sample at 10,000 g for 10 min at 4°C and transferring the supernatant to fresh tubes. To increase the concentration of protein before western blotting, cell protein extracts were passed through Microcon YM-10 centrifugal filters (Millipore, Etobicoke, ON) following manufacturer's guidelines. The filters were spun at 14,000 g to exclude proteins having molecular weights less than 10 kDa and to concentrate protein for western blotting. To prevent increases in the concentration of SDS during the procedures, concentrated protein was diluted in PBS and centrifuged twice at 14,000 g.

2.5 Western Analysis of Uncoupling Protein 3

UCP3 protein content of L6 myotubes transfected with Ad5CMV-UCP3 was determined by Western blot. Samples were diluted in PBS and sample buffer (50 mmol/l Tris HCl pH 6.8, 25% glycerol, 2% SDS, 14.4 mmol/l 2-mercaptoethanol, 0.2%

bromophenol blue) at a ratio of 4:1 to obtain a final protein concentration of 2 μg/μl. Molecular weight markers (Santa Cruz Biotechnology, Santa Cruz, CA) and rainbow markers (Amersham Biosciences, Baie d'Urfe, QC) were diluted in PBS and sample buffer at a ratio of 1:15:4 and 1:3:1, respectively. Samples were then incubated at 70°C for 5 min. Protein samples (80 μg) and markers (25 μl) were loaded into each lane of a BioRad Minigel (12% polyacrylamide) system (BioRad, Mississauga, ON). Samples and markers were transferred to a nitrocellulose membrane by electrophoresis. The membrane was then incubated for 1 h in blocking buffer (PBS with 0.05% (v/v) Tween-20 and 20 mg/ml casein) followed by an overnight incubation at 4°C in blocking buffer containing primary antibody (rabbit anti-human UCP3, Chemicon, Temecul, CA) at a 1:1,000 dilution. The membrane was then washed and incubated in blocking buffer containing the secondary antibody, a peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) at a 1:500 dilution for 1 h at room temperature. The membrane was then washed briefly in PBS three times followed by three 10 min incubates in PBS with the buffer solution replaced with each incubation. For protein detection, blots were processed using the Enhanced Chemiluminescence Plus detection kit (Amersham Biosciences). As positive and negative controls, recombinant murine UCP3 protein (75 μg) and muscle mitochondrial protein from UCP3-ablated mice were used, respectively

2.5 Fuel Substrate Oxidation

Rates of palmitate and glucose oxidation in L6 myotubes cultured in 25 cm^2 flasks were quantified using methods similar to Roduit et al. (2004). Stock solutions of bovine serum albumin (BSA)-bound palmitate were prepared prior to experiments as

follows. A saturating quantity of sodium-palmitate (67 g/l) was dissolved (at 37°C with gentle mixing overnight) in Krebs Ringer bicarbonate (135 mmol/l NaCl, 3.6 mmol/l KCl, 0.5 mmol/l NaH_2PO_4, 0.5 mmol/l $MgCl_2$, 1.5 mmol/l $CaCl_2$, 2 mmol/l $NaHCO_3$) containing 10 mM Hepes (pH 7.4) (KRBH) and 5% (w/v) BSA. The solution was then filtered through a 0.2 μm filter. BSA-palmitate was quantified using a NEFA C kit (Wako Chemicals GmbH, Germany). The stock solution was then kept at -20°C until required to prepare the pre-incubation and oxidation solutions.

Control and treated myotubes were washed once and then pre-incubated for 30 min at 37°C in 2 ml of KRBH containing 5 mmol/l glucose and 0.5% BSA. For palmitate oxidation experiments, myotubes were incubated for 1 h at 37°C in KRBH containing 200 μmol/l palmitate/0.5% BSA (wt/vol), 0.2 μCi/ml 1-^{14}C palmitate (Perkin Elmer Life Sciences, Boston, MA), 1 mmol/l carnitine, and either 5.0 or 16.0 mmol/l glucose (oxidation medium). Glucose oxidation was measured under identical conditions but in the presence of 16 mmol/l glucose and 0.2 μCi/ml U-^{14}C glucose (Perkin Elmer Life Sciences), 1 mmol/l carnitine, and either 50 or 200 μmol/l palmitate. To collect liberated $^{14}CO_2$ produced by the oxidation of radiolabeled substrates, Whatman (Fischer Scientific, Pittsburg, PA) GF/B filter paper (1/3 of a 25 mm circle) was soaked in 150 μl of 5% KOH and placed in the lumen of a 3 cm piece of polyvinyl chloride (PVC) tubing (internal diameter 4.7 mm; Fischer Scientific). The tube was then inserted into the underside of a rubber stopper and the stopper was used to seal the flasks during the oxidation experiments with the tube facing inward. An empty flask containing no cells was also incubated with oxidation medium and sealed with the rubber stopper, tubing, and filter paper, to measure the background radioactivity picked up by the filter paper.

Further, as a positive control to verify that the assay was measuring glucose and palmitate oxidation, 100 nmol/l insulin (Sigma, Saint Louis, MO) was added into separate flasks during the 15 min preincubation period to influence their respective oxidation rates. Substrate oxidation was terminated by injecting 200 µl of 40% perchloric acid into the flask through the rubber stopper and gently rocking the flask. The flasks were then left overnight to allow the filter to collect the $^{14}CO_2$ released by the oxidation of radiolabeled substrates. The following day, the filter paper and 100 µl of the remaining oxidation medium were removed from each flask and placed in separate scintillation vials containing 4 ml of scintillation fluid. Vials were left overnight before counting to allow to $^{14}CO_2$ leech out of the filter paper. The specific activity of palmitate or glucose in the oxidation solution was determined by dividing the counts per minute (cpm) in 100 µl of the oxidation medium by the nmol of cold palmitate or glucose in 100 µl of the oxidation medium. To determine the rate of oxidation of palmitate or glucose, the cpm of the filter minus the cpm of a blank filter was divided by the specific activity of palmitate or glucose from the same flask.

2.6 Electrochemical Detection of Cellular Oxygen Consumption

Rates of oxygen consumption from individual L6 myotubes were determined using a self-referencing microelectrode system (Land et al., 1999; Figure 2.1). Oxygen microelectrodes were manufactured using the methods of Jung and colleagues (1999). Myotubes, cultured in a 6 cm^2 dish, were washed twice with Earle's basic salt solution containing 117 mmol/l NaCl, 5.4 mmol/l KCL, 1.5 mmol/l CaCl, 0.8 $MgSO^4$, 0.9 mmol/l NaH^2PO^4, 10 mmol/l HEPES, and 5.6 mmol/l glucose (BSS) and then placed in

6-10 ml of BSS. The dish was then placed under an Axiovert 200 (Ziess) inverted microscope that is housed within an insulated Faraday box equipped with temperature control equipment that allowed the internal environment to be maintained at 37°C. The tip of an oxygen microelectrode was placed approximately 2 μm above the surface of a myotube and oscillated (10 μm) along the z axis at a frequency of 0.3 Hz to collect alternating oxygen flux and reference measurements. Measurements were collected for 5-6 min. Translational movement of the microelectrode was accomplished with a translational motion control system mounted onto the head-stage of the microscope. Movement was driven by Ionview software developed, along with motion controllers and amplifiers, at the Biocurrent Research Center (Marine Biological Laboratory, Woods Hole, MA). The differential current outputs (in fA) obtained from three separate myotubes per plate were averaged and converted to oxygen flux using the Fick equation: $J = -D(\Delta C/\Delta r)$, where J is the flux rate ($mol \cdot cm^{-2} \cdot s^{-1}$), D is the diffusion coefficient ($cm^2 \cdot s^{-1}$) of oxygen in water, ΔC is the oxygen concentration difference (mol) between the flux and reference positions, and Δr is the distance of measurement (cm). ΔC was determined by multiplying the differential current obtained with the slope of an independently determined electrode calibration curve.

2.7 Mitochondrial Membrane Potential

The mitochondrial membrane potential of L6 myotubes was determined using tetramethylrhodamine ethyl ester (TMRE), a lipophilic fluorescent dye that accumulates in mitochondria based on the electrical potential difference across the inner membrane. Myotubes were grown in opaque 96 well plates to prevent interference from

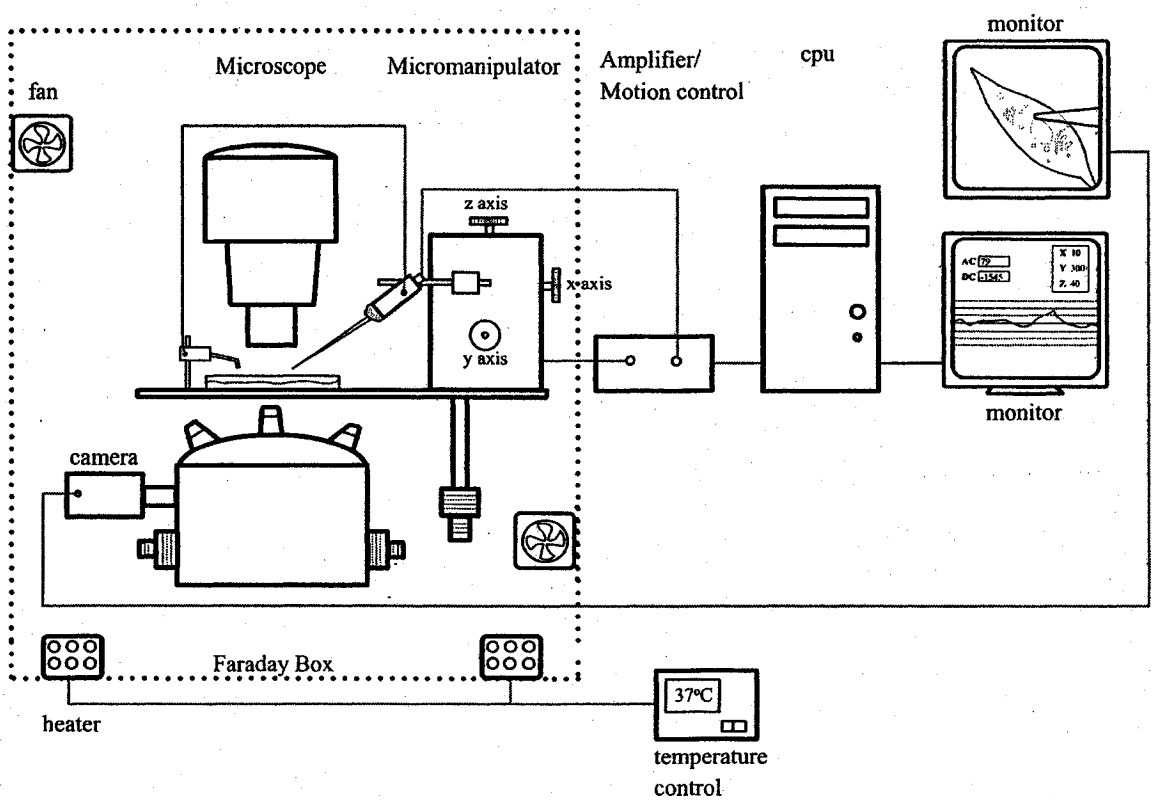

Figure 2.1 Self-referencing microelectrode system

Schematic representation of the self-referencing microelectrode system used to measure oxygen consumption of individual L6 myotubes.

neighbouring wells during fluorescence detection. Myotubes were incubated in differentiation medium containing 100 μmol/l TMRE for 15 min in the dark at 37°C. Fresh differentiation medium was then added after washing the myotubes twice with PBS to remove extracellular TMRE. Fluorescence intensity was detected spectrophotometrically with excitation and emission wavelengths of 544 and 590 nm, respectively.

2.8 Mitochondrial Reactive Oxygen Species Production

Myotubes were cultured in opaque 96 well plates, similar to that for mitochondrial membrane potential experiments to prevent interference. The assay was conducted using methods similar to those described by Esposti (2002). Control and treated myotubes were incubated in PBS containing 1 μmol/l 5-(and-6)-carboxy-2',7'-dichlorohydrofluorescein diacetate (carboxy-H_2DCFDA) for 10 min in the dark at 37°C. Carboxy-H_2DCFDA is a fluorescent probe sensitive to mitochondrial ROS. Before measurements were initiated, 16 mmol/l glucose was added to the myotubes incubated in PBS. This concentration of glucose is great enough to induce mitochondrial hyperpolarization and ROS production (Joseph et al., 2004). Fluorescence produced by ROS-mediated oxidation of carboxy-H_2DCFDA was followed spectrophotometrically for 1 h with measurements collected every 2 min (excitation = 490 nm; emission = 516 nm).

2.9 Statistical Analysis

Statistical analysis was performed using one-way ANOVA and Bonferroni post hoc tests. Results are presented as means ± SEM, unless otherwise stated. Analyses were conducted using Graphpad Prism 4 software (San Diego, CA).

3. RESULTS

3.1 Adenoviral Overexpression of UCP3 in L6 Myotubes

Protein expression of UCP3 in L6 myotubes was measured by Western blot. Quantification of UCP3 from cellular homogenates revealed a 2.2-2.5-fold increase in protein expression in L6 myotubes infected with Ad5CMV-UCP3 compared to uninfected control, Ad5-GFP infected, and DNP treated myotubes. (Figure 3.1). No differences in UCP3 protein content where found between control, GFP-transfected, and DNP treated cells. To reduce problems associated with cross reactivity of the antibody, a known issue with available polyclonal UCP3 antibodies, we used mouse recombinant UCP3 fusion protein as a positive control. It migrates at a molecular weight of 39 kDa due to a 5 kDa fusion peptide. As well, a negative control was used to identify UCP3 and non-UCP3 proteins that reacted with the antibody. Protein isolated from purified mitochondria of muscle from UCP3 ablated mice was used as the negative control. The immunoblotted proteins from muscle mitochondria of UCP3 ablated mice consistently showed that the middle bands of three bands was absent, indicating that it was UCP3. The identities of the two other bands are unknown but are both present in the immunoblots of cellular and muscle mitochondrial proteins.

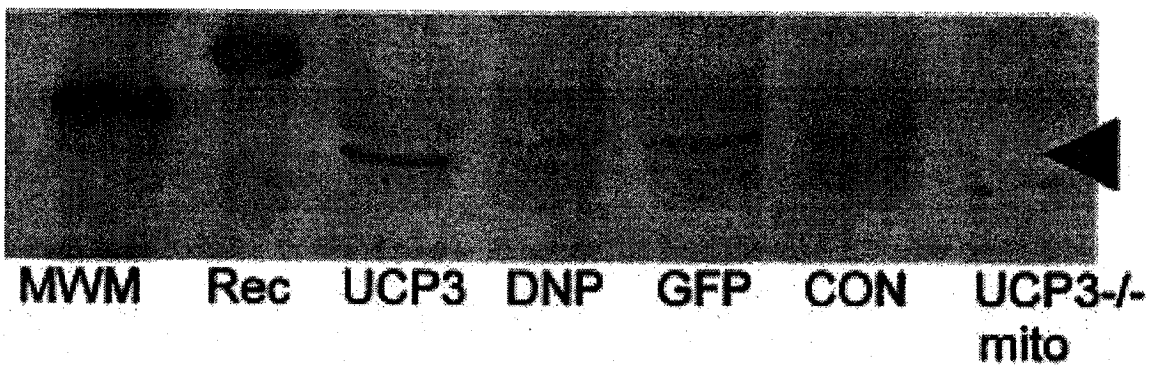

Figure 3.1 Effects ofAd5CMV-UCP3 transfection on L6 myotube UCP3 protein expression.

Representative Western blot for UCP3 expression in infected and uninfected L6 myotubes. *Lane 1:* Molecular weight markers (MWM). The 34 kDa marker is the prominent band. *Lane 2:* Recombinant mouse UCP3 (Rec) migrated to 39 kDa due to a 5 kDa fusion peptide. *Lane 3:* UCP3 infected L6 myotubes (UCP3). *Lane 4:* Myotubes chronically exposed to a low dose of DNP (DNP). *Lane 5:* GFP-infected myotubes (GFP). *Lane 6:* uninfected control myotubes (CON). *Lane 7:* Mitochondria isolated from hind limb skeletal muscle of UCP3 ablated mice (UCP3-/- mito). The antibody reacted with two unknown proteins having slightly higher and lower molecular weights that UCP3; these non-specific bands are present in mitochondria from UCP3 ablated mice. Arrow indicates 34 kDa marker.

3.2 Uncoupling Protein 3 Increases Palmitate Oxidation

Rates of myotube palmitate oxidation under normoglycemic (5.0mmol/l) and hyperglycemic (16.0 mmol/l) conditions were evaluated by measuring CO_2 release (Figure 3.2). Myotube oxidation of palmitate was unaffected by infection with GFP at both concentrations of glucose, indicating that adenoviral transfection had no effect on palmitate oxidation. Treatment with DNP also had no effect on the rate of palmitate oxidation at either glucose concentration when compared to controls. Transfection with UCP3 but not with UCP2 had a significant impact on the rate of myotube oxidation of palmitate. UCP3 transfection significantly increased palmitate oxidation at normal ($P<0.001$) and high ($P<0.01$) glucose concentrations compared to GFP-infected myotubes. UCP2 had no effect on palmitate oxidation at either concentration of glucose when compared to myotubes transfected with GFP. Differences between UCP2 and UCP3 transfected myotubes were significantly different at both normal ($P<0.05$) and high ($P<0.001$) glucose concentrations. UCP3 overexpression also resulted in a significant difference when compared to DNP at normal ($P<0.01$) and high ($P<0.001$) glucose concentrations. Myotubes exposed to high levels of glucose had significantly lower rates of palmitate oxidation when compared to myotubes exposed to normal levels of glucose ($P<0.05$). Pre-treatment with insulin showed a tendency for decreased palmitate oxidation but results were not statistically significant.

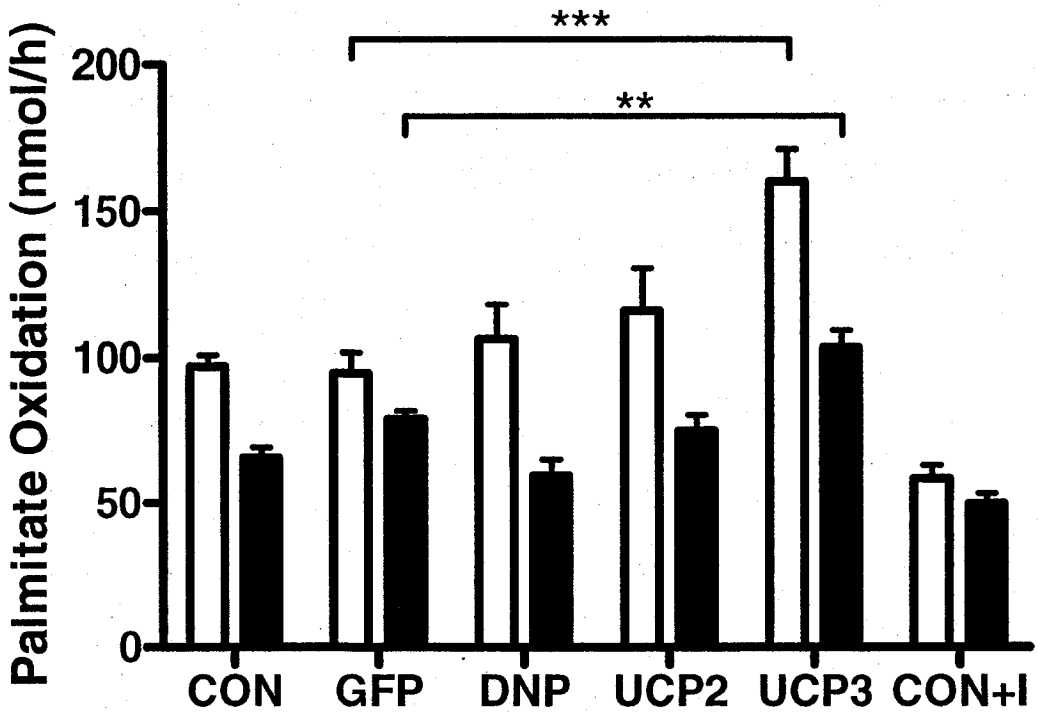

Figure 3.2 Effects of UCP3 overexpression on the rate of palmitate oxidation in L6 myotubes

Palmitate oxidation was measured in L6 myotubes incubated at normal (5mmol/l; white bars) and high (16.0 mmol/l; black bars) concentrations of glucose in the presence of 200 μmol/l palmitate. From left to right, results are shown for untreated control (CON) cells, GFP-transfected cells, DNP treated cells, UCP2- and UCP3-transfected cells, and control cells pretreated with insulin (100 nmol/l). Comparisons between treatments were analyzed by one-way ANOVA with a Bonferroni post hoc test. Results are presented as means ± SEM, n = 8-16 for each treatment condition. **P<0.01; ***P<0.001.

3.3 Uncoupling Protein 3 has no Effect on Glucose Oxidation

Rates of glucose oxidation were assayed using the same methods as those described for palmitate oxidation (Figure 3.3). Glucose oxidation rates were similar among many of the treatment groups. Myotubes transfected with GFP, UCP2, and UCP3 had no differences in glucose oxidation at either concentration of palmitate (50 and 200 μmol/l) when compared to untransfected control myotubes. There was no difference between GFP-, UCP2-, and UCP3-transfected myotubes. Glucose oxidation was reduced by approximately 50% in the presence of 200 μmol/l palmitate as compared to 0.05 mmol/l palmitate. Treatment with DNP on the other hand had a strong influence on glucose oxidation. DNP significantly increased glucose oxidation at both 0.05 mmol/l ($P<0.001$) and 200 μmol/l ($P<0.001$) palmitate concentrations. As well, the effects of DNP were also significantly increased when compared to the transfection groups ($P<0.001$). As was expected, insulin pretreatment also had a strong influence on the rate of glucose oxidation. At both concentrations of palmitate, pretreatment with insulin caused a significant increase in glucose oxidation (0.05 mmol/l palmitate, $P<0.001$; 200 μmol/l palmitate, $P<0.01$) when compared to control or transfected myotubes. Treatment with DNP or pretreatment with insulin caused comparable results at both concentrations of palmitate.

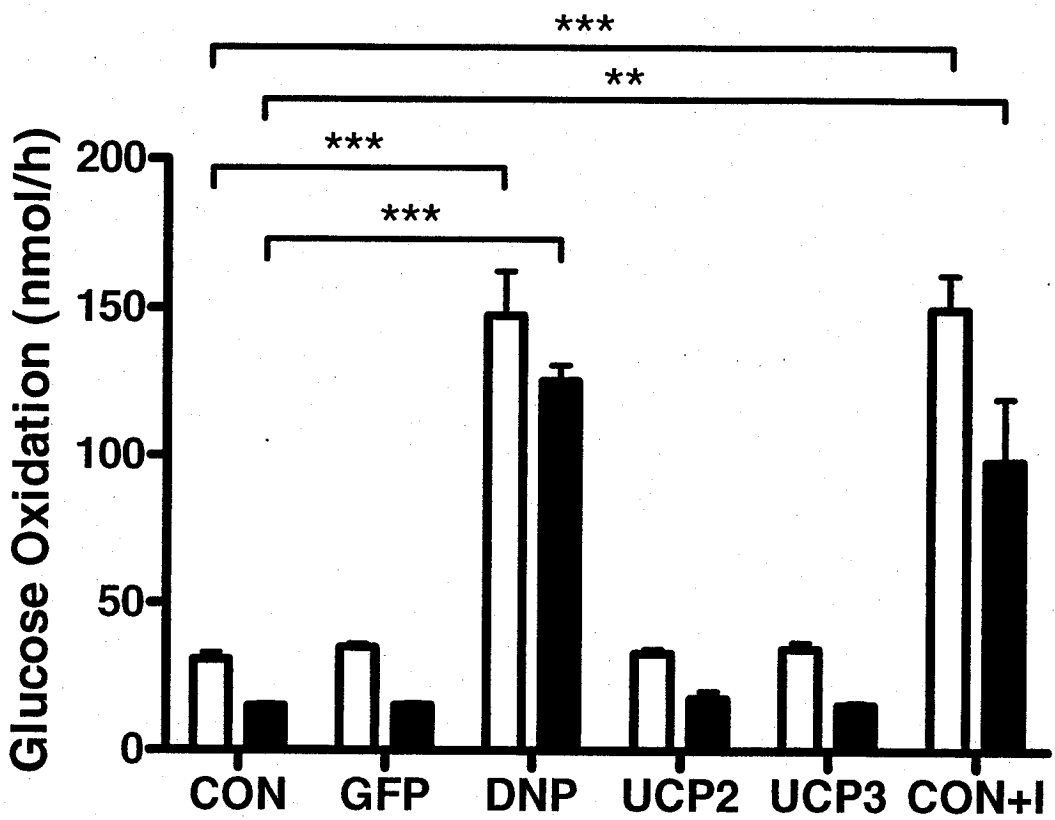

Figure 3.3 Effect of UCP3 overexpression on L6 myotube glucose oxidation

Glucose oxidation was measured in L6 myotubes incubated at low (50 μmol/l; white bars) and high (200 μmol/l; black bars) concentrations of palmitate in the presence of 16.0 mmol/l. From left to right, results are shown for control untreated (CON) cells, GFP-transfected cells, DNP treated cells, UCP2- and UCP3-transfected cells, and control cells that were pretreated with insulin (100 nmol/l). Comparisons between treatments were analyzed by one-way ANOVA with a Bonferroni post hoc test. Results are presented as means ± SEM, n = 5-15 for each treatment condition. **P<0.01; ***P<0.001.

3.4 Uncoupling Protein 3 has no Effect on Basal Oxygen Consumption

Basal cellular oxygen consumption in L6 myotubes was assessed *in situ* using a self-referencing microelectrode system (Figure 3.4). Myotubes transfected with GFP showed similar rates of oxygen consumption compared to control myotubes. Transfection with UCP2 or UCP3 had no effect on basal oxygen consumption compared to GFP-transfected myotubes. No differences were observed between UCP2 and UCP3 transfected myotubes. Myotubes chronically treated with DNP had highly elevated basal oxygen consumption rates compared to control myotubes ($P<0.001$). DNP-induced increase in basal oxygen consumption was also greater then that of GFP- ($P<0.01$), UCP2- and UCP3-transfected myotubes ($P<0.05$).

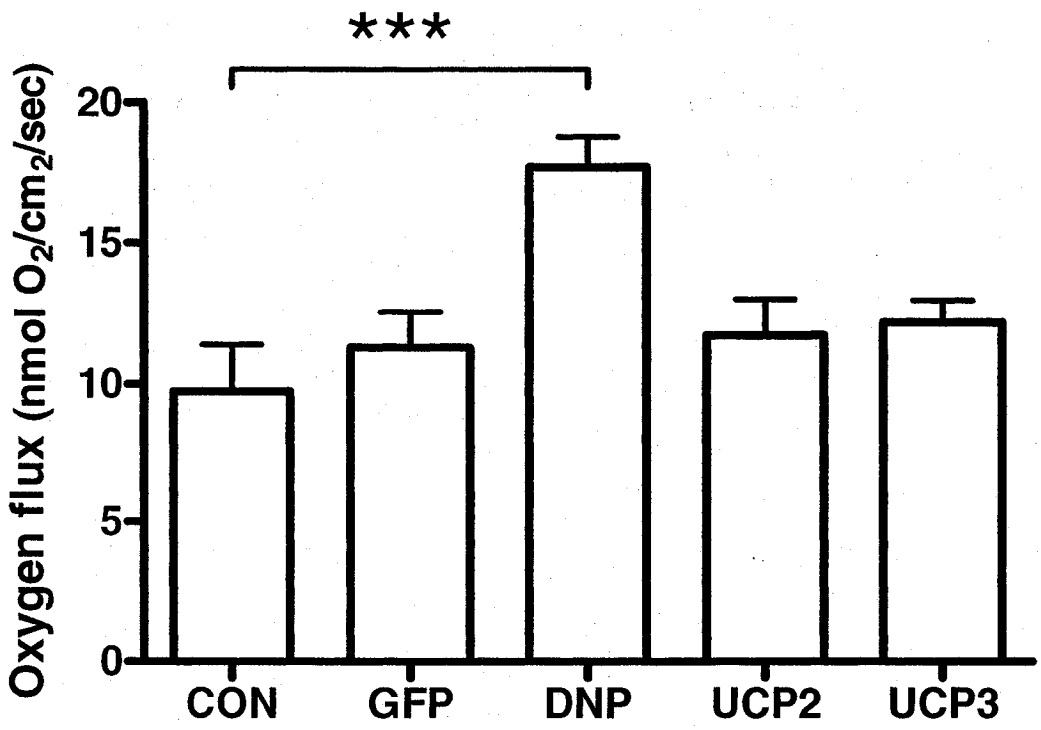

Figure 3.4 Effects of UCP3 overexpression on L6 myotube basal oxygen consumption

In situ oxygen consumption of L6 muscle cells was measured using oxygen-sensitive microelectrodes. From left to right, results are shown for control untreated (CON) cells, GFP-transfected cells, DNP treated cells, UCP2- and UCP3-transfected cells. Comparisons between treatments were analyzed by one-way ANOVA with a Bonferroni post hoc test. Results are presented as means ± SEM, n = 8-10 for each treatment condition. ***P<0.001.

3.5 Uncoupling Protein 3 has no Effect on Mitochondrial Membrane Potential

To determine if myotubes transfected with UCP2 or UCP3 had any inherent decrease in mitochondrial membrane potential, myotubes were treated with the mitochondrial probe, TMRE (Figure 3.5). Mitochondrial membrane potential was unchanged by the transfection of myotubes with GFP compared to control myotubes. Transfection with UCP2 or UCP3 had no effect on mitochondrial membrane potential compared to GFP-transfected myotubes. Differences between UCP2- and UCP3-transfected myotubes were also insignificant. Chronic treatment with DNP, however, strongly decreased mitochondrial membrane potential compared to controls ($P<0.001$). This difference in mitochondrial membrane potential was maintained when compared to GFP-, UCP2-, and UCP3-transfected myotubes ($P<0.001$).

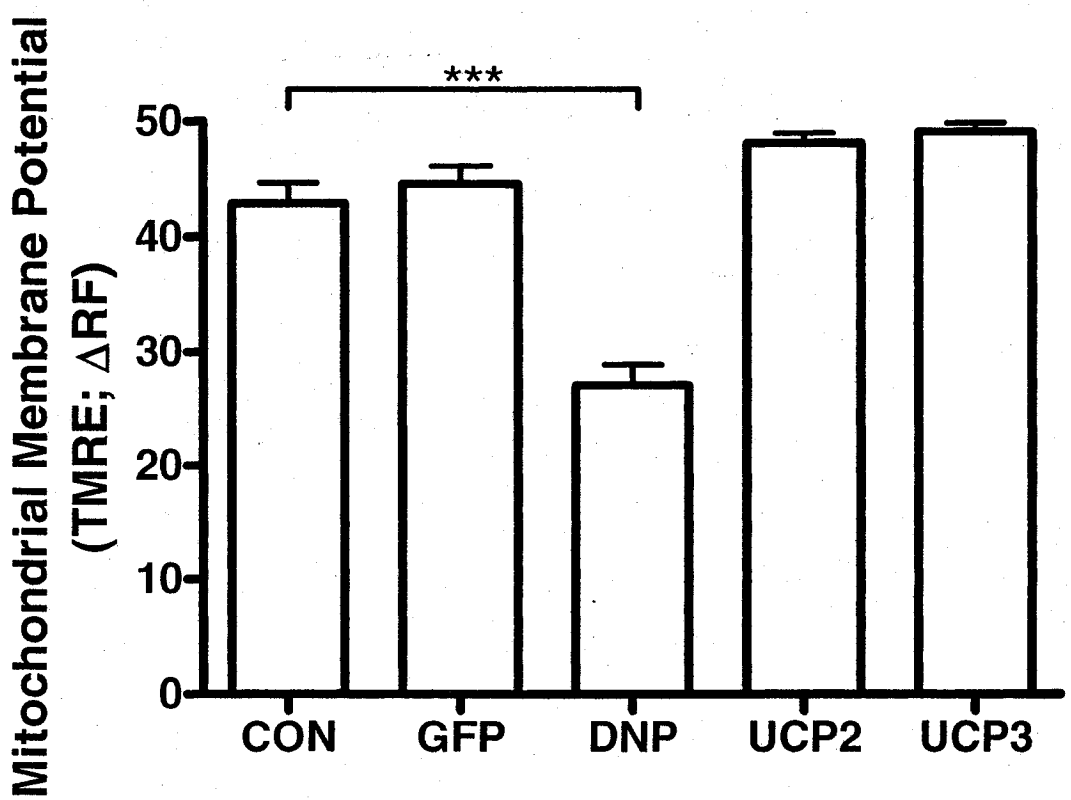

Figure 3.5 Effects of UCP3 overexpression on mitochondrial membrane potential

Mitochondrial membrane potential was assessed in intact L6 myotubes using the fluorescent probe TMRE. From left to right, results are shown for control untreated (CON) cells, GFP-transfected cells, DNP treated cells, UCP2- and UCP3-transfected cells. Comparisons between treatments were analyzed by one-way ANOVA with a Bonferroni post hoc test. Results are presented as means ± SEM, n = 8 for each treatment condition. ***P<0.001.

3.6 Uncoupling Protein 3 Mitigates the Formation of Reactive Oxygen Species

The rate of ROS production in L6 myotubes was unaffected by GFP-transfection when compared to control myotubes (Figure 3.6). Myotubes transfected with UCP2 ($P<0.05$) or UCP3 ($P<0.01$) had significantly decreased ROS production compared to GFP-transfected myotubes. Chronic DNP treatment also significantly mitigated ROS production compared to control myotubes ($P<0.001$). The decrease in ROS production observed in chronically DNP treated myotubes was similar to that observed in UCP2- and UCP3-transfected myotubes.

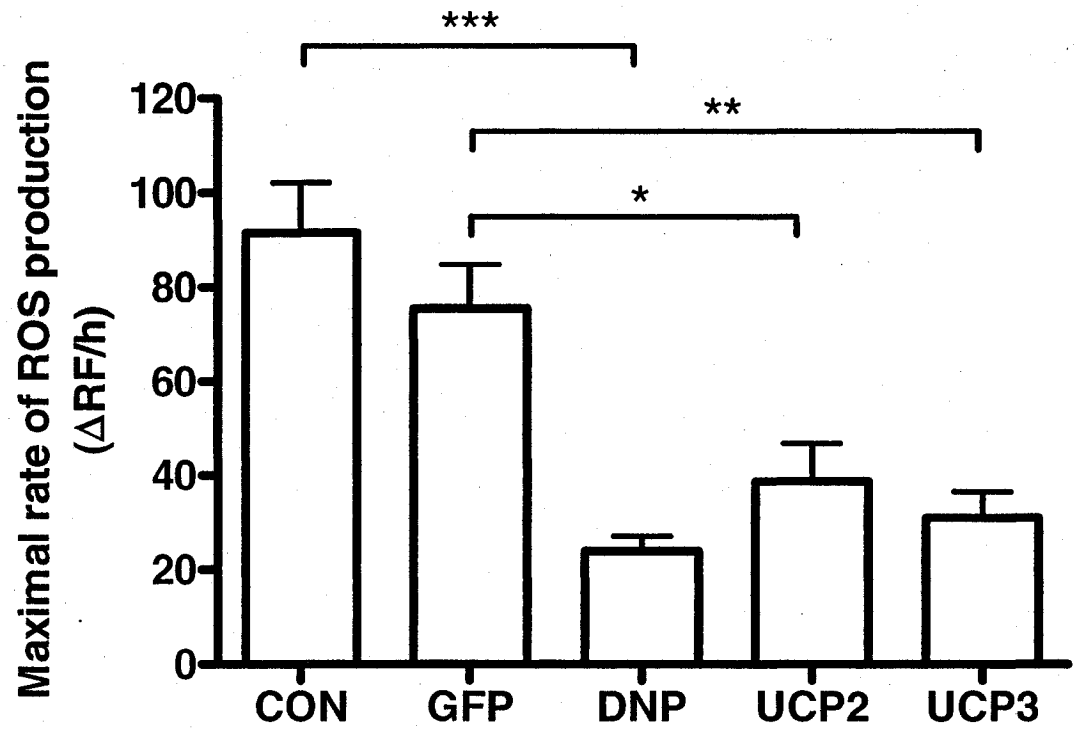

Figure 3.6 Effects of UCP3 overexpression on ROS production in L6 myotubes

ROS production was assessed in intact L6 myotubes using the fluorescent marker, carboxy-H_2DCFDA. From left to right, results are shown for control untreated (CON) cells, GFP-transfected cells, DNP treated cells, UCP2- and UCP3-transfected cells. Comparisons between treatments were analyzed by one-way ANOVA with a Bonferroni post hoc test. Results are presented as means ± SEM, n = 10-13 for each treatment condition. *P<0.05; **P<0.01; ***P<0.001.

4. DISCUSSION

To determine the effects of UCP3 overexpression on fuel homeostasis and ROS production in muscle we chose to use the L6 rat muscle cell line. Differentiated L6 myotubes maintain many of the functional and enzymatic characteristics of skeletal muscle (Shainberg et al., 1971; Klip et al., 1984), including those for glucose and fatty acid uptake and metabolism (Bilan et al., 1992; Tanaka et al., 2003). These cells show a high sensitivity to insulin (Mitsumoto et al., 1991) and respond to palmitate inhibition of insulin action (Perdomo et al., 2004). They have also been used widely in the study of lipid (Marra et al., 2002; Tanaka et al., 2003) and glucose (Khayat et al., 1998a; 1998b) uptake and metabolism, as well as ROS production and signalling (Kozlovsky et al., 1997). In the past, the L6 muscle cell line has been used extensively to study the functional characteristics of UCP2 (Hatakeyama and Scarpace, 2001; Hammarstedt and Smith, 2003) and UCP3 (Nagase et al., 1999; Huppertz et al., 2001; Nagase et al., 2001; Son et al., 2001; Guerini et al., 2002). Unlike some other model systems (*e.g.* yeast), L6 myotubes express endogenous UCP2 and UCP3 (Guerini et al., 2002; Shimokawa et al., 1998), albeit, at levels much lower than those observed in skeletal muscle *in vivo*. Based on these findings, L6 myotubes appear to provide an excellent model system to study the functional characteristics of UCP3 in skeletal muscle *in vitro*.

UCP3 is regulated at the posttranscriptional level resulting in protein levels that do not always correspond to mRNA levels (Cadenas et al., 1999; Sivitz et al., 1999). UCP3 mRNA, therefore, does not necessarily provide an accurate measure of UCP3 protein. This lack of correlation between UCP3 mRNA and protein means that immunodetection is required to accurately quantify mitochondrial UCP3 content. We

have done so in our study and produced a modest but significant increase in UCP3 protein (2.2-2.5 fold) by transfecting L6 myotubes with Ad5CMV-UCP3. This concentration of UCP3 is similar to what would be observed in rats fasted for 48 h (Moreno et al., 2003). A comparable increase in UCP3 protein expression has also been demonstrated in transgenic mice (Bezaire et al., 2005) and transfected mammalian cells (Guerini et al., 2002) without signs of artifactual uncoupling activity. Our findings also suggest that this physiological increase in UCP3 does not induce artifactual uncoupling.

We demonstrate that UCP3 overexpressing muscle cells have no changes in mitochondrial membrane potential or basal oxygen consumption. These findings indicate an absence of artifactual uncoupling and also suggest that under basal conditions UCP3 does not stimulate mitochondrial proton leak. It is necessary to measure both mitochondrial membrane potential and cellular oxygen consumption as alterations in these parameters on their own are not indicative of uncoupling activity. Decreased membrane potential could also result from inhibition of fuel substrate oxidation. As well, if a membrane potential-sensitive fluorescence probe is used, a decrease in the total fluorescence intensity could represent a decrease in cellular mitochondrial content. Similarly, increased oxygen consumption could represent increased cellular mitochondrial content or an increased capacity to oxidize fuel substrates. Only when both parameters are considered can changes be attributed to proton leak. Guerini and colleagues (2002) found that a more than 10 fold increase in UCP3 protein content in L6 muscle cells has no effect on basal oxygen consumption but did increase the portion of oxygen consumption due to respiratory uncoupling. The authors suggest that the uncoupling observed was not an inherit function of UCP3 but

rather due to improper folding and insertion in the mitochondrial membrane as lower levels of UCP3 transfection, similar to that found in our study, had no effect on proton leak-dependent oxygen consumption. Other groups have also acknowledged that non-physiological concentrations of UCP3 protein induce artifactual uncoupling for the same reason (Cadenas et al., 2001; Harper et al., 2002).

L6 myotubes were also transfected with AD5CMV-UCP2. We did not go so far as to quantify the amount UCP2 protein overexpression in these cells. Therefore, we have limited our interpretation of these results. Despite a lack of evidence showing increased UCP2 protein expression we have demonstrated differences in activity between UCP2 overexpressing and GFP-expressing myotubes, suggesting that the differences observed are the result of UCP2 activity specifically and not an artefact of adenoviral transfection. As well, we verified that basal oxygen consumption and mitochondrial membrane potential were unaltered by UCP2 transfection indicating that any increase in UCP2 that may have occurred was not causing uncontrolled, artifactual uncoupling.

An absence of uncoupling in our study does not indicate that UCP3 (or UCP2) is unable to mediate proton conductance under certain conditions. Exposure of palmitate to skeletal muscle mitochondria of hyperthyroid rats is associated with increased respiration rate and decreased membrane potential (Silvestri et al., 2005). These findings are absent in euthyroid rats where UCP3 levels are much lower. A similar increase in palmitate-induced uncoupling and UCP3 expression has been observed in cardiac muscle from hyperthyroid rats (Boehm et al., 2001). In a cohort of obese women, those who showed a reduced responsiveness to diet-induced weight loss have reduced skeletal

muscle UCP3 mRNA expression and proton leak-dependent respiration (Harper et al., 2002). Six weeks of endurance training decreases human muscle UCP3 protein content and mitochondrial uncoupling (Fernstrom et al., 2004). These findings only provide correlative evidence of UCP3-mediated uncoupling. Brand and colleagues have demonstrated in several recent studies that superoxide and its downstream products can specifically induce UCP3-mediated proton conductance (Echtay et al., 2002b; Echtay et al., 2003; Murphy et al., 2003). Overexpression studies have also demonstrated a link between UCP3 and proton leak (Clapham et al., 2000; Son et al., 2004) but many of these results have been attributed to artifactual uncoupling (Cadenas et al., 2002). Work in UCP3 ablated mice has provided the strongest argument for a role in proton conductance as the skeletal muscle proton leak of these mice is significantly reduced (Gong et al., 2000; Vidal-Puig et al., 2000). These findings are not consistent in all studies of UCP3 ablated mice. Cadenas and colleagues (2002) found no difference in respiratory coupling between UCP3 ablated mice and wild-type mice.

Several metabolic and physiological states that influence UCP3 expression have been shown to have no effect on mitochondrial proton leak kinetics. Changes in rat muscle UCP3 content during long term calorie restriction does not follow changes in mitochondrial proton leak (Bevilacqua et al., 2005). Rat heart chronically exposed to hypobaric hypoxia significantly reduces UCP3 mRNA but has no effect on mitochondrial respiratory coupling (Essop et al., 2004). Crescenzo and colleagues (2003) found that semistarvation and refeeding had differential effects on rat skeletal muscle mitochondria UCP3 protein content and state 4 respiration. Bezaire and colleagues (2001) demonstrated that fasting increases UCP3 protein expression but has

no effect on mitochondrial proton leak. Similarly, skeletal muscle from rats fasted for 48 h have increased UCP3 protein but no changes in respiratory rate or mitochondrial membrane potential (Moreno et al., 2003). Interestingly, administration of thyroid hormone or coenzyme Q to these rats during fasting stimulates UCP3-mediated uncoupling. Although physiological, these latter findings may not be significant, as thyroid hormone and coenzyme Q are normally depressed during fasting, they do demonstrate that UCP3-mediated uncoupling can be induced *in vivo*.

Collectively, the above findings suggest that under certain circumstances UCP3 may be able to stimulate proton conductance. However, a consistent relationship between UCP3 expression and proton leak is absent. Therefore, it is unlikely that the primary function of UCP3 involves significant proton leak. Alternative regulatory roles for UCP3 are not lacking. There are several lines of evidence supporting a role for UCP3 in lipid metabolism. Firstly, numerous studies have demonstrated an increased expression of UCP3 in response to conditions where plasma and cellular fatty acid concentrations are elevated. Fasting, high fat feeding, and lipid infusion have all been shown to correlate with UCP3 expression *in vivo* (Matsuda et al., 1997; Samec et al., 1998; Boss et al., 1998b; Khalfallah et al., 2000). Extracellular fatty acid levels have also been correlated with UCP3 expression *in vivo* (Son et al., 2001). Fatty acids are believed to be necessary for UCP3 activity and have been incorporated into all present hypothesized functions of UCP3. Further, changes in UCP3 expression have been linked with alterations in the preference and capacity of skeletal muscle mitochondria to oxidize fatty acids. Bezaire and colleagues (2001) found that UCP3 ablated mice have an increased respiratory quotient, indicative of a reduced preference for fatty acids as a

fuel source. In a later study by this same group, they demonstrated that mice overexpressing UCP3 at physiological levels have increased uptake of fatty acids into muscle cells and into mitochondria and increased capacity to oxidize fatty acids (Bezaire et al., 2005).

Other studies of UCP3 overexpression have also observed increased rates of lipid oxidation (Clapham et al., 2000; Wang et al., 2003). However the level of UCP3 overexpression in these mice is far greater then is seen *in vivo* and is believed to result in artifactual uncoupling. Similar to the *in vivo* findings of Bezaire and colleagues (2005) we have demonstrated increased rates of fatty acid oxidation when UCP3 is increased with the physiological range *in vitro*. These results strongly suggest that UCP3 is involved in lipid handling. What remains unclear is the pathway by which fatty acids stimulate UCP3 expression and activity.

The mechanism underlying fatty acid-induced expression of UCP3 has not been fully elucidated although strong support for the involvement of PPARs has been established. Fatty acids are endogenous ligands of PPARs and stimulate their activity directly (Nakamura et al., 2004). In skeletal muscle PPARα and PPARδ isoforms are expressed. Both of these isoforms are also found in L6 myotubes (Hatakeyama and Scarpace, 2001), although PPARα has not been observed by some groups (Nagase et al., 1999; Son et al., 2001) and is likely expressed at very low levels. This is not unexpected though because PPARδ is ubiquitously expressed at levels greater, in many cases, to those of PPARα or PPARγ (Ferre, 2004). UCP3 contains two PPAR response elements providing a putative pathway whereby fatty acids activate PPARα or PPARδ which, in turn, forms a heterodimer with retinoic X receptor and stimulates gene expression of

UCP3. Evidence favouring this pathway comes from studies demonstrating PPAR ligand activation of UCP3 expression when circulating fatty acid levels are low (Pedraza et al., 2000) and in tissues where UCP3 is not normally expressed (Lanni et al., 2002). Compelling evidence has also been provided by Solanes and colleagues (2003) who demonstrated that mutation of the PPAR binding site on the UCP3 gene prevents fatty acid-induced UCP3 gene transcription.

PPARs are known to activate transcription of genes involved in fatty acid uptake and oxidation (Kota et al., 2005) of which UCP3, as we have just discussed, is a member. Overexpression of PPARδ prevents genetic- and diet-induced obesity by stimulating fat metabolism (Tanaka et al., 2003; Wang et al., 2003). Another gene activated by PPARs is mitochondrial thioesterase 1 (MTE1) which catalyzes the cleavage of long chain fatty acyl CoA into a nonesterified fatty acid anion and free CoA. Like UCP3, MTE1 is expressed in tissues with high rates of fatty acid oxidation, such as BAT, cardiac and skeletal muscle (Svensson et al., 1995) and has been proposed to be involved in lipid handling and oxidation (Hunt et al., 1999). More specifically, MTE1 may regulate the relative levels of acyl CoA, acyl carnitine, and nonesterified fatty acids under various conditions of fatty acid load (Hunt et al., 2000), such as during fasting. Fasting is associated with increased fatty acid oxidation and acyl CoA levels in mitochondria. During maximal rates of beta oxidation as much as 95% of available intramitochondrial CoA is esterified to fatty acyl CoA (Garland et al., 1965). As a result, CoA is unavailable for other mitochondrial reactions, such as the conversion of acyl carnitine back to acyl CoA by carnitine palmitoyltransferase II during fatty acid

transport into the matrix. The lack of available CoA can lead to acyl carnitine accumulation and damage to mitochondrial membranes (Goni et al., 1996).

MTE1 could prevent acyl carnitine accumulation by increasing the available CoA, through acyl CoA hydrolysis. CoA is also required to maintain beta oxidation and the citric acid cycle. Thus, MTE1 may also improve rates of substrate flux through these pathways during elevated fatty acid oxidation. However, freeing CoA for these pathways occurs at the expense of generating deleterious fatty acid anions. In the cytosol, where other members of thioesterase family (*e.g.* CTE1) perform a similar function, acyl CoA synthetase is availably to re-esterify the fatty acid anion to CoA. In mitochondria, acyl CoA synthetase is absent, preventing further metabolism of fatty acid anions. Our group has proposed that UCP3 provides a pathway for removal of these deleterious metabolites by translocating them to the intermembrane space (Himms-Hagen and Harper, 2001) (Figure 4.1). Thus, under conditions when fatty acids exceed the oxidative capacity of skeletal muscle, MTE1 and UCP3 act in concert to liberate CoA. In doing so they are able to maintain high rates of fatty acid oxidation and remove fatty acid anions to the cytosol where they can be re-esterified to acyl CoA.

Much of the evidence to date supporting this hypothesis is based on positive correlations between UCP3 and MTE1 mRNA expressions. In fact, under all conditions tested UCP3 expression has been found to be mirrored by that of MTE1. Mice overexpressing UCP3 show a 3 fold induction of MTE1 gene expression while other genes involved in fat metabolism are unchanged (Moore et al., 2001). These appear to provide strong evidence of a link between UCP3 and MTE1 activity although it can not be ignored that these mice express supraphysiological concentrations of UCP3 which

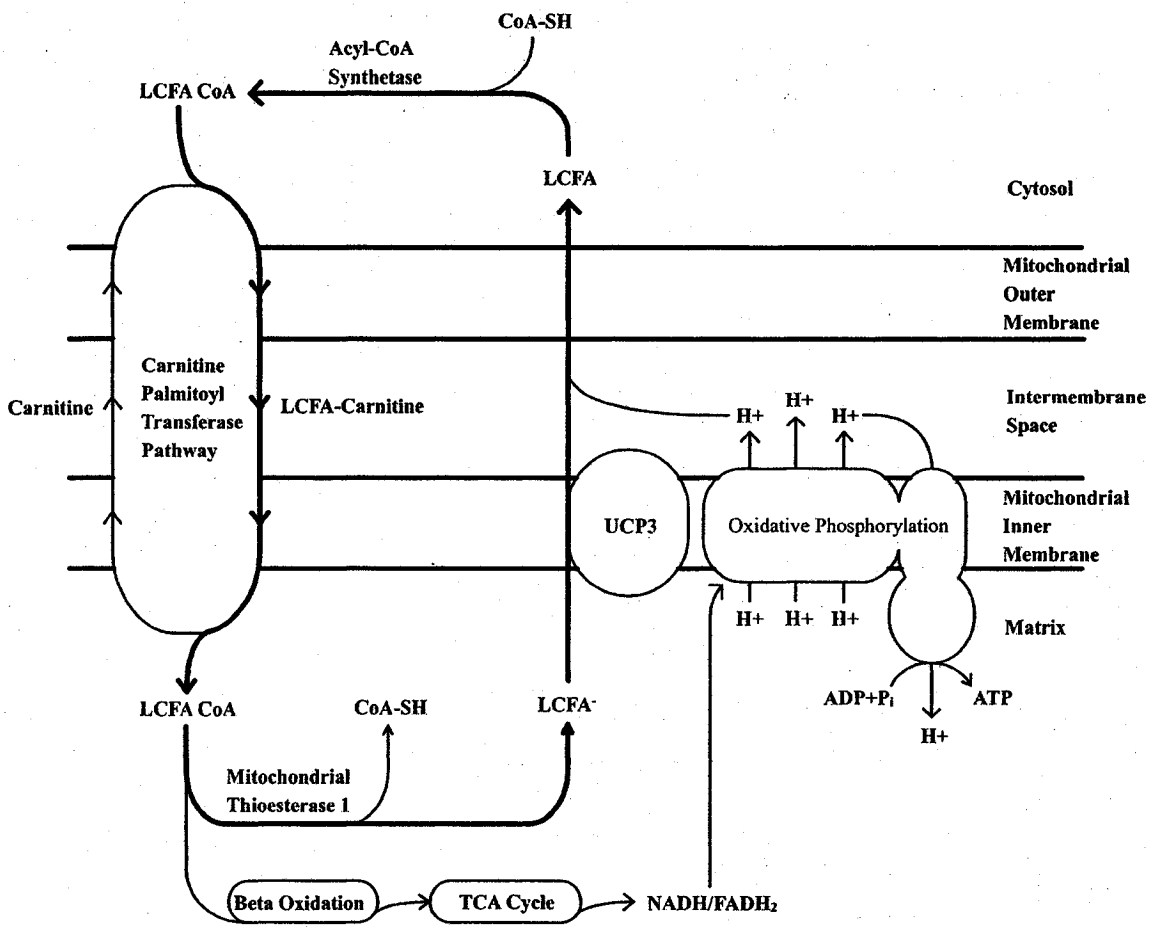

Figure 4.1 UCP3 translocation of acyl anions: Himms-Hagen and Harper Model

MTE1 and UCP3 are proposed to function in tandem to facilitate fatty acid oxidation. MTE1 liberates CoA from long chain fatty acids (LCFA) making it available for the carnitine palmitoyl transferase pathway, beta oxidation, and the TCA cycle. With increased CoA availability, fatty acid oxidation may be increased. Fatty acid anions generated by MTE1 are removed from the mitochondrial matrix by UCP3. Once in the intermembrane space, fatty acid anions are protonated and can be re-esterified to CoA by acyl-CoA synthetase in a reaction that requires 2 ATP equivalents.

may induce an artifactual increase in MTE1 expression. However, the lack of change in the expression of other genes involved in fatty acid metabolism would suggest otherwise as changes in these genes would also be expected. Clapham and colleagues (2001) also provided evidence that UCP3 and MTE1 were involved in the same metabolic pathway. They demonstrated that UCP3 expression more closely follows changes in MTE1 expression then UCP1 or UCP2 in white and brown adipose tissue and in skeletal muscle of db/db or db/+ mice untreated or treated with PPARα and γ agonists. An interesting study by Lanni and colleagues (2002) demonstrated not only that a PPARα agonist can stimulate both UCP3 and MTE1 expression but it could do so in liver, where UCP3 is normally absent. Further, this group found that fatty acid oxidation was increased by administration of PPARα agonist, favouring the putative role of UCP3 and MTE1 in a common lipid oxidation pathway. Stavinoha and colleagues (2004) followed up on this study by demonstrating that MTE1 is a PPARα-regulated gene in both cardiac and skeletal muscle, tissues where UCP3 is also expressed. Further evidence is required to definitively demonstrate a connection between MTE1 and UCP3 activity. However, it does provide an attractive mechanism for how UCP3 increases fatty acid oxidation.

A second, but not so different hypothesis by Schrauwen and colleagues (2001b) also proposes that UCP3 exports fatty acid anions. However, they suggest that these anions are derived from deprotonated nonesterified fatty acids that have crossed the mitochondrial inner membrane (Figure 4.2). The removal of these fatty acid anions by UCP3 prevents fatty acid accumulation within the mitochondrial matrix.

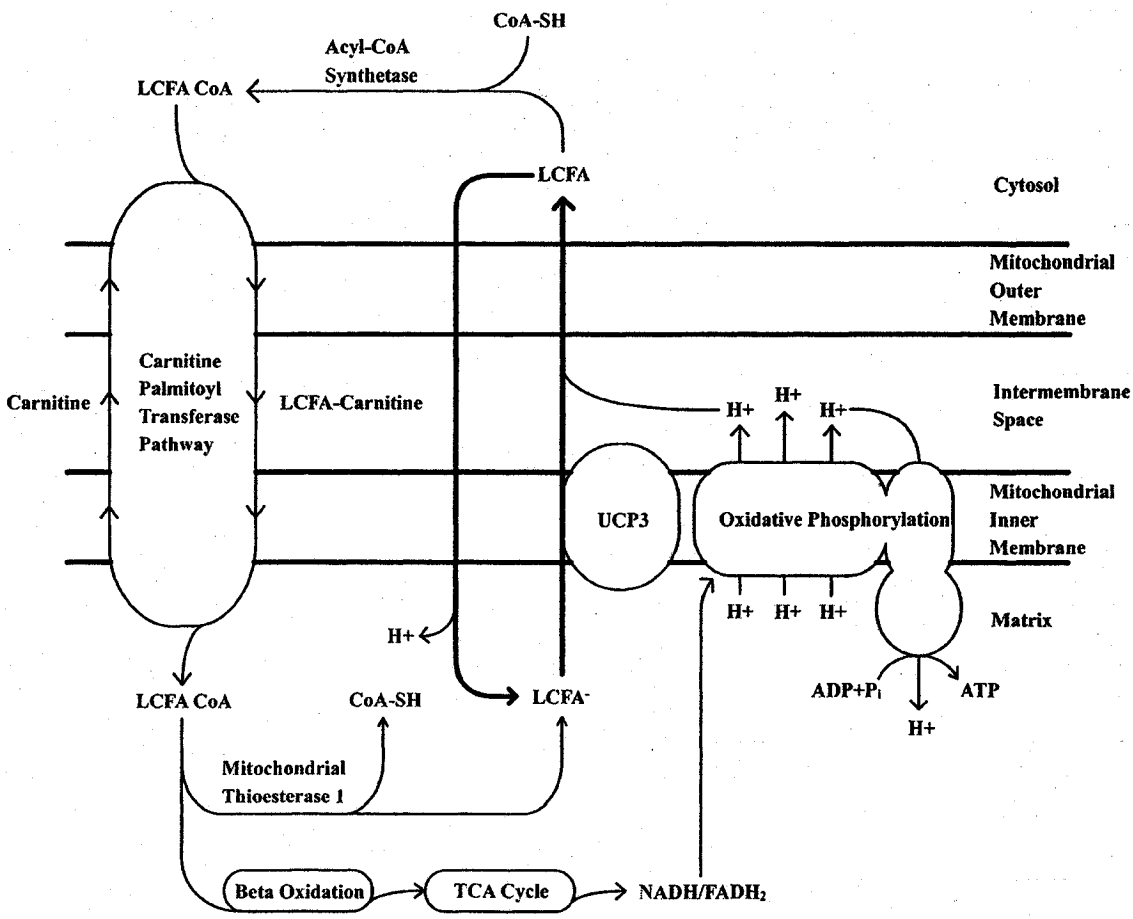

Figure 4.2 UCP3 Translocation of Acyl Anions: Schrauwen Model

UCP3 exports fatty acid anions generated by the flip-flop of nonesterified fatty acids into the mitochondrial matrix. In doing so UCP3 prevents the accumulation of fatty acids with the matrix.

This hypothesis is based predominantly on earlier findings that etomoxir, an inhibitor of fatty acyl-CoA entry into the mitochondrial matrix decreased fatty acid oxidation but increased UCP3 protein (Schrauwen et al., 2002). The authors believe that the increase in UCP3 protein represents a concomitant increase in UCP3 activity. They also believe that due to the blockage of fatty acyl-CoA entry the susbtrate for this increase in UCP3 activity (fatty acid anions) must come from nonesterified fatty acids that have flip-flopped across the mitochondrial membrane. Increased UCP3 protein does not necessarily represent increased UCP3 activity. The upregulation of UCP3 protein likely occurred as the result of increased PPAR activation of UCP3 gene expression, stimulated by the accumulation of fatty acids in the cytosol. Therefore our hypothesis of the function of UCP3 still remains the most likely explanation for the increase in fat oxidation observed with increased expression of UCP3.

We found no differences in fuel oxidation rates in myotubes transfected with UCP2. These data could indicate a lack of increase in UCP2 protein expression or a lack of involvement of UCP2 in these pathways. In a study by Hong and colleagues (2001), using the same adenoviral constructs as we have used, they found that both UCP2 and UCP3 increase INS1 insulinoma cell lipid oxidation. These are in opposition to our findings of a lack of influence of UCP2 on fatty acid oxidation. This discrepancy may be due to the different cell types used or differences in expression levels of UCP2. Unlike in our study, the authors found that increased UCP2 stimulated mitochondrial uncoupling, suggesting that the concentration of UCP2 protein may have been sufficient to cause artifactual uncoupling. There is little evidence supporting a role for UCP2 in fatty acid metabolism. Unlike UCP3, UCP2 expression is unrelated to that of MTE1

(Clapham et al., 2001; Moore et al., 2001). As well, whereas UCP3 overexpression in muscle increases beta oxidation, UCP2 overexpression in pancreatic beta cells lacking UCP2 decreases beta oxidation (Joseph et al., 2004). Further, UCP2 protein is also found in significant quantities in tissues with high rates of fuel metabolism.

Few studies have been conducted on the potential link between UCP3 activity and glucose homeostasis when compared to the vast amount of data on UCP3 and lipid handling. This may not be without reason as such a role for UCP3 remains controversial. However, if UCP3 was involved in glucose metabolism it would have major implications on whole body glucose homeostasis due to the importance of skeletal muscle in glucose metabolism (Baron et al., 1988). Several models of UCP3 overexpression have demonstrated a strong correlation between UCP3 and improved glucose uptake and metabolism. Transgenic mice overexpressing UCP3 (Clapham et al., 2000; Wang et al., 2002; Son et al., 2004) in skeletal muscle have decreased fasting plasma glucose levels as a result of increased glucose clearance. The supraphysiological levels of UCP3 in these studies are of concern because concentrations of mitochondrial UCPs outside of the physiological range can cause artifactual proton leak. Thus the effects observed in these mice may be stimulated by energy depletion, as proton leak reduces ATP production, rather then to a specific function of UCP3.

Mice and cultured human skeletal muscle cells overexpressing UCP3 have increased ATP/ADP ratios and fuel oxidation rates (Garcia-Martinez et al., 2001; Schrauwen et al., 2004). The increase in the ATP/ADP ratio is likely due to increased adenylate kinase activity which catalyzes the conversion of 2 ADP molecules to ATP and AMP in an attempt to compensate for reduced ATP production. Increased levels of

AMP generated by this reaction stimulate AMPK activity. AMPK enzyme activity is sensitive to alterations in the energy charge of the cell and responds by increasing fuel uptake and metabolism (Stoppani et al., 2002; Iglesias et al., 2004). Therefore, the increased glucose uptake and metabolism observed in UCP3 overexpressing studies may represent increased AMPK activity rather then increased UCP3 activity. Indeed, AMPK activity has been shown to be upregulated in mice overexpressing UCP1 and UCP3 in skeletal muscle (Han et al., 2004; Schrauwen et al., 2004).

We provide further evidence that UCP3 is not involved in glucose metabolism. Overexpression of UCP3 within the physiological range in L6 myotubes had no effect on glucose oxidation in the presence of 50 or 200 μmol/l palmitate. Only when myotubes were chronically treated with a low dose of DNP or preincubated with insulin were significant increases in glucose oxidation observed. An approximately 50% reduction in glucose oxidation in the presence of increasing palmitate (200 μmol/l as compared to 50 μmol/l) and strong induction of glucose oxidation by insulin indicate a high efficacy of the experiment. Our findings are in direct opposition to those of Huppertz and colleagues (2001). They found that UCP3 overexpression in L6 myotubes stimulates a 2 fold increase in glucose uptake and GLUT4 translocation to the plasma membrane in a manner similar to that of insulin, through activation of phosphoinositol 3 kinase. They postulated that UCP3 may stimulate glucose uptake through a ROS-dependent signalling cascade (Huppertz et al., 2001). In support of this hypothesis, ROS have been shown to modulate basal and insulin-stimulated glucose uptake in muscle through the same pathway described for UCP3 (Kozlovsky et al., 1997).

Glucose uptake is the rate limiting step in glucose metabolism therefore it would be expected that glucose oxidation would also be increased in L6 muscle cells overexpressing UCP3. However, we failed to observe any changes in the rate of glucose oxidation when UCP3 levels were increased. This disparity between our results and those of Huppertz and colleagues (2001) may be due to differences in UCP3 expression. In our study we clearly demonstrate physiological levels of UCP3 protein that has no effect on basal oxygen consumption or mitochondrial membrane potential. We also show that the effects of UCP3 on glucose oxidation are not mimicked by DNP which causes uncontrolled proton leak. Whereas, in the study by Huppertz and colleagues, no experiments were conducted to determine whether the level of UCP3 being expressed was causing artifactual proton leak. They also demonstrate that the effects of UCP3 on glucose uptake could be mimicked by chronic exposure to low doses of DNP (similar to the level used by our group). It is possible, therefore, that the results observed by Huppertz and colleagues are an artefact of improper folding and insertion of UCP3 in the membrane, leading to uncontrolled proton leak and activation of AMPK, as discussed above. However, because we did not measure glucose uptake directly, it remains possible that glucose uptake in our study was indeed increased and shuttled through non-oxidative pathways.

Interestingly, ROS have also been shown to signal the activation of AMPK (Toyoda et al., 2004; Hwang et al., 2005). Thus, there still remains the possibility that the findings of Huppertz and colleagues are mediated by UCP3 and not by an artifactual proton leak. However, it is difficult to understand how increased UCP3 activity, which is thought to mitigate the formation of mitochondrial ROS, could stimulate ROS-mediated

signalling pathways. As well, low levels of ROS have been shown to stimulate increased GLUT1 expression and decreased GLUT3 and GLUT4 expression in L6 myotubes (Kozlovsky et al., 1997). Based on these findings it appears unlikely that UCP3 is stimulating glucose uptake through a ROS-mediated pathway.

Huppertz and colleagues (2001) demonstrated that chronic exposure to a low dose of DNP could stimulate glucose uptake through a phosphoinositol 3 kinase-dependent pathway. Other groups have also shown that mitochondrial uncoupling by DNP stimulates glucose uptake (Khayat et al., 1998a, 1998b). In agreement with these findings we demonstrate that chronic exposure to DNP using a similar concentration as that used by Huppertz and colleagues (2001) results in a significant increase in glucose oxidation. The increase in glucose oxidation by DNP was similar to that observed by preincubating L6 myotubes with 100 nmol/l insulin for 15 min.

In a study similar to ours, conducted in pancreatic insulinoma (INS1) cells, UCP3 overexpression had no effect on the rate of glucose oxidation rate (Hong et al., 2001). This group used the same recombinant UCP3 adenovirus (Ad5CMV-UCP3) as that used by our group and in addition to studying glucose oxidation also found no difference in the ATP/ADP ratio or respiratory uncoupling of these cells. This complementary study to ours confirms that there is no relationship between UCP3 expression and glucose oxidation. Further, the findings of Hong and colleagues (2001) favour our view that the results observed by Huppertz and colleagues (2001) are the result of artifactual mitochondrial uncoupling. More data in support of our results are demonstrated in UCP3-ablated mice. Unlike in UCP3 overexpressing mice, those lacking any UCP3 show no changes in glucose homeostasis (Vidal et al., 2000; Gong et

al., 2000). As well, Bezaire and colleagues (2005) found no differences in serum glucose levels in UCP3-ablated, or mice overexpressing UCP3 within the physiological range, compared to controls. Huppertz and colleagues (2001) have argued that compensatory mechanisms occur in UCP3-ablated mice that mask any loss of function by UCP3. However, to date no such mechanisms have been identified.

Inconsistent with the findings demonstrated by our group, UCP3 expression is greater in glycolytic then in oxidative muscle (Samec et al., 2002). This would appear to point towards a role for UCP3 in glucose rather then lipid metabolism. However, based on our hypothesized function for UCP3 in lipid metabolism this muscle type expression would be expected. Glycolytic muscle has less of a capacity to handle fatty acid compared to oxidative muscle. Therefore, the increased expression of UCP3 in glycolytic muscle may represent a mechanism for increased lipid oxidation when fatty acid metabolism predominates. As well, based on the potential role of UCP3 in the regulation of ROS production increased expression in glycolytic muscle could provide a mechanism to decrease ROS production.

In concordance with our findings Hong and colleagues (2001) also found that glucose oxidation is unaffected by UCP2 and UCP3 overexpression. This was not unexpected as no studies have demonstrated a link between UCP2 expression and glucose oxidation. Rather than a role in fuel substrate metabolism, most studies of UCP2 function have demonstrated a role for UCP2 in the regulation of ROS formation.

A role for UCPs in the defence against mitochondrial ROS accumulation and damage has been demonstrated in multiple members of the UCP family. The addition of purine nucleotides to durum wheat and potato tuber mitochondria increases hydrogen

peroxide production in these plants (Kowaltowski et al., 1998; Pastore et al., 2000). The addition of fatty acids however, prevents the increase in ROS production. This regulation of ROS formation has been attributed to the activity of the plant UCP (PUMP), as purine nucleotides and fatty acids are known modulators of its activity. Similar findings have been demonstrated in rat BAT and nonparachymal liver cell (*e.g.* Kupffer cell) mitochondria expressing UCP1 and UCP2, respectively (Negre-Salvayre et al., 1997). When these mitochondria and those of hepatocytes are exposed to purine nucleotides hydrogen peroxide production is increased in the two former, but not in the latter mitochondria as they do not express any UCPs. Further, overexpression of avian UCP in yeast is protective against ROS-mediated damage (Criscuolo et al., 2005) and UCP2-ablated mice are resistant to *Toxoplasma gondii* infection due to an 80% increase in macrophage ROS production (Arsenijevic et al., 2000). Collectively, these findings lend strong support for a role of UCPs in the regulation of ROS formation.

In our study we have demonstrated that physiological increases in UCP2 and UCP3 can strongly inhibit ROS production in L6 myotubes. The antioxidant properties observed in UCP2 and UCP3 overexpressing muscle cells was similar to that of muscle cells exposed to chronic low levels of DNP. These data suggest that UCP2 and UCP3 was mitigating ROS formation by increasing the proton conductance across the inner mitochondrial membrane. Based on our measurements of basal oxygen consumption and mitochondrial membrane potential this would not appear to be the case. However, we can not exclude the possibility that under the experimental conditions used to assay the rate of ROS formation, UCP3 was stimulating a mild purine nucleotide sensitive, proton leak (to be discussed later). Further, due to the fact that we did not measure UCP2

protein concentrations we can not definitively link the lowered ROS formation in UCP2 transfected cells with UCP2 activity. Our work is supported by findings demonstrating reduced glucose-induced ROS production and damage in rat dorsal root ganglion neurons overexpressing UCP1 or UCP3 (Vincent et al., 2004). In the neurons overexpressing UCP1 or UCP3 glucose-induced hyperpolarization of the mitochondrial membrane potential was prevented

The mechanism underlying the regulation of ROS production by UCP3 remains somewhat hypothetical. However, Brand's group has provided strong evidence that superoxide and its downstream products have the ability to stimulate UCP3-mediated proton leak. Echtay and colleagues (2002b) have demonstrated that exogenously produced superoxide stimulates a purine nucleotide-sensitive, fatty acid dependent proton leak in mitochondria expressing UCP1, UCP2, and UCP3 but not from mitochondria expressing no UCPs. This exogenously generated ROS reaches the mitochondria and decreases the activity of aconitase (Echtay et al., 2002a), a matrix protein highly sensitive to superoxide (Gardner et al., 1995). Vidal-Puig and colleagues (2000) have shown similar results in the skeletal muscle mitochondria from UCP3-ablated mice. These mice produce more ROS and have more damaged aconitase. In a second study by Echtay and colleagues (2002a) they demonstrated that the uncoupling activity induced by exogenous ROS was also sensitive to the mitochondria-targeted antioxidants, 10-(6'-ubiquinonyl)-decyltriphenylphosphonium (mitoQ) and 2-[2-(triphenylphosphonio)ethyl]-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol bromide (mitoVitE). Further support for a ROS-mediated activation of UCP3 activity was shown by Echtay and colleagues (2003). They found that 4-hydroxyl-2-nonenal

(HNE), a product of lipid peroxidation, could stimulate proton leak by UCP3. These data suggested that downstream products of superoxide-induced damage and not superoxide itself were responsible for activating UCP3. Later, it was demonstrated that mitoPBN, an inhibitor of carbon-centered radical (*e.g.* HNE) formation inhibits UCP3 activation. MitoPBN has no effect on superoxide formation or lipid peroxidation indicating that the substrate activating UCP3 is downstream of these reactions and is most likely HNE.

Based on the above findings, Brand's group hypothesized that ROS react with membrane phospholipids causing lipid peroxidation and HNE production. HNE then activates UCP3-mediated proton re-entry into the matrix or is itself translocated across the membrane by UCP3 where it is protonated and able to flip back the matrix (Figure 4.3). By reducing the protonmotive force through either of these two mechanisms, UCP3 activity inhibits further production of ROS. A similar hypothesis has been proposed by Goglia and Skulachev (2003) who suggest that UCP3 acts as a translocator of fatty acid peroxides from the inner leaflet to the outer leaflet of the mitochondrial inner membrane (Figure 4.4). By removing these toxic fatty acid molecules from the matrix UCP3 prevents further oxidative damage of mitochondrial matrix-localized proteins and DNA. These authors also suggest that the low level of UCP3 as compared to UCP1 in mitochondria is a consequence of differences in substrate concentration (*i.e.* fatty acid peroxides versus fatty acids).

The similar reduction in the rate of ROS formation by UCP2, UCP3 and DNP in our study may represent similar levels of mild uncoupling. However, the uncoupling stimulated by UCP3 is induced specifically by increased ROS-mediated lipid peroxidation. The uncoupling present in myotubes chronically exposed to low levels of

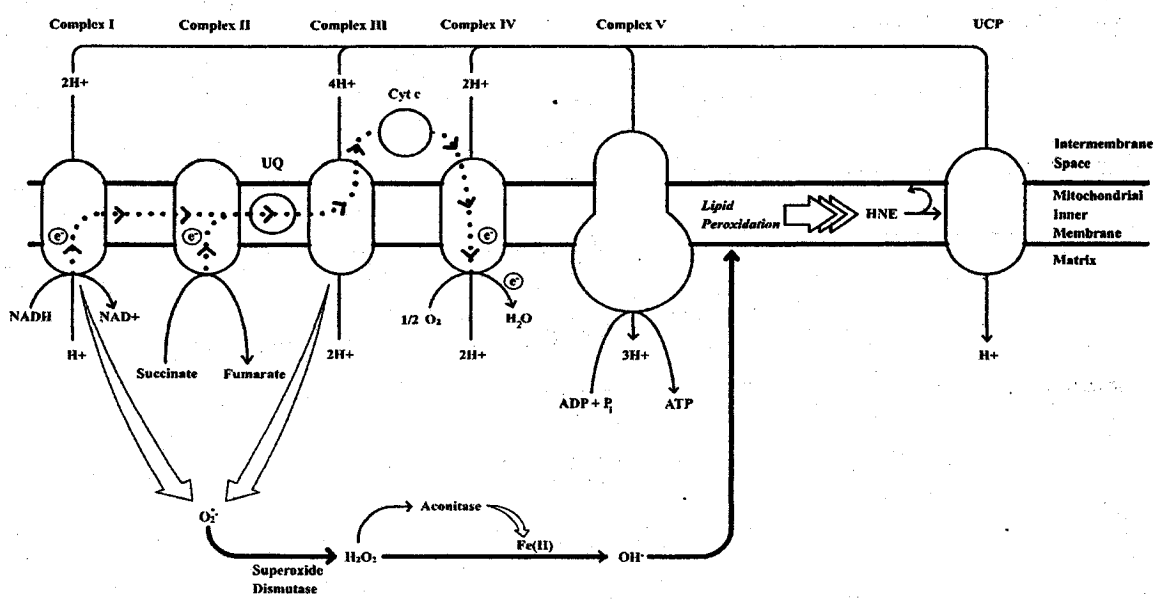

Figure 4.3 HNE Stimulates UCP3 Activity: Brand Model

Superoxide generated into the mitochondrial matrix by complexes I and III of the electron transport chain is converted to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide reacts with aconitase releasing ferrous iron which can in turn react with other hydrogen peroxide molecules generating hydroxyl radicals. Highly reactive hydroxyl radicals can interact with membrane phospholipids, forming carbon-centered radicals and initiating lipid peroxidation. Lipid peroxidation leads to the formation of HNE which is believed to directly activate UCP3.

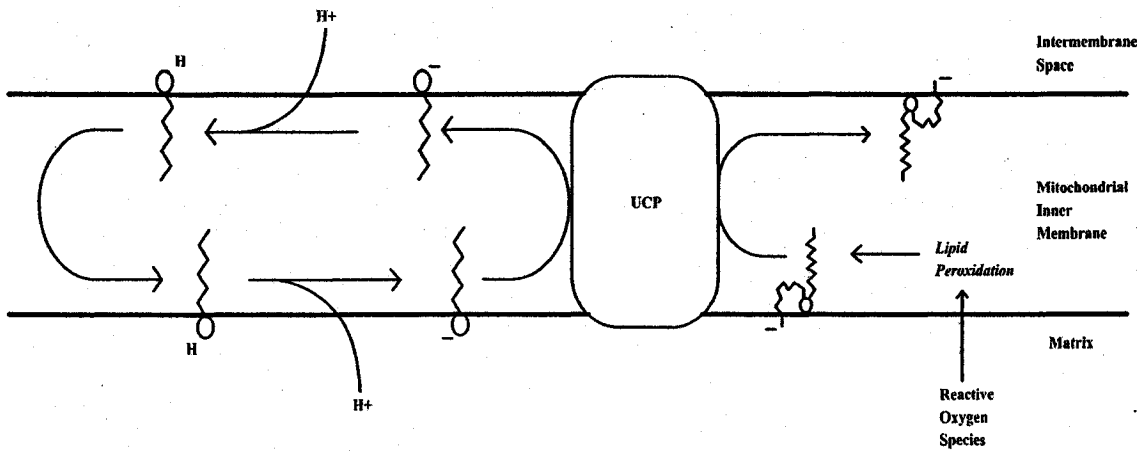

Figure 4.4 UCP3 Translocates Lipid Peroxides: Goglia and Skulachev Model

UCP3 acts as a carrier of fatty acid anions and fatty acid peroxides. Transfer of these molecules to the outer leaflet of the mitochondrial inner membrane is thought to reduce the potential for oxidative damage of matrix-localized macromolecules such as mitochondrial DNA. Fatty acid anions in the outer leaflet can be protonated allowing them to flip-flop back across the membrane bilayer. Whereas, lipid peroxides in the outer leaflet may be removed by antioxidant defence system such as glutathione peroxidase.

DNP is present in the presence and absence of ROS. In our assays of basal oxygen consumption and mitochondrial membrane potential, ROS production would have been much lower then during assays of the rates of ROS formation due to differences in glucose concentrations. This may explain the absence of a measurable UCP3-mediated uncoupling during assays of oxygen consumption and mitochondrial membrane potential.

ROS-induced mild uncoupling by UCP3 may explain the similarity in rates of ROS production between UCP3 overexpressing and DNP treated myotubes. However, it does not explain the disparity between UCP3 and DNP with respect to fuel oxidation measurements. If ROS stimulated a mild uncoupling we would expect to see similar levels of substrate oxidation between UCP3 overexpressing cells and DNP treated cells. Instead, the effects of UCP3 and DNP on glucose and fatty acid oxidation were opposite. Levels of ROS induced during oxidation experiments should have been sufficient to stimulate UCP3- mediated proton leak but this would require verification. It is also unclear if a mild uncoupling by UCP3 would be sufficient to cause a significant increase in fatty acid oxidation. Based on the inability of DNP to increase fatty acid oxidation, this would appear unlikely. Therefore, UCP3 may function in the translocation of multiple forms of fatty acid anions, including those derived from lipid peroxidation and acyl CoA hydrolysis. With the ability to translocate both these anions, UCP3 could improve both lipid oxidation rates and reduce the formation of deleterious ROS.

Interestingly, a recent study from Fink and colleagues (2005) failed to demonstrate a relationship between UCP1 or UCP2 overexpression in bovine aortic endothelial cell mitochondria with ROS formation. Brand and colleagues (2002) have

reported similar findings from the mitochondria of UCP3 overexpressing mice. These mice show no changes in oxidative damage compared to wild type mice. However, they also demonstrated that the mitochondria from UCP3-ablated mice produce significantly greater ROS-mediated damage then wild type mice. Brand and colleagues (2002) have proposed that the expression of UCP3 protein in wild type mice is sufficient to prevent the oxidative damage observed in UCP3-ablated mice and that the increased expression of UCP3 in overexpressing mice provides no further improvements in antioxidant defence. Our findings are in opposition to these as we demonstrate improved antioxidant defence with increased expression of UCP3 protein. However, because L6 myotubes express a relatively low level of endogenous UCP3, it may not be sufficient to defend against oxidative damage.

5. CONCLUSION

Physiological increases in UCP3 protein expression that do not cause artifactual uncoupling stimulate increased rates of palmitate oxidation and mitigate the formation of deleterious ROS but have no effect on glucose oxidation rates in L6 myotubes. These effects of UCP3 are distinct from those of the chronic treatment of myotubes with low levels of DNP and from myotubes transfected with UCP2. UCP3 therefore has an important role in maintaining mitochondrial function. An improved understanding of the regulation and function of UCP3 may have a significant impact in the prevention and treatment of major metabolic diseases such as obesity, insulin resistance, and T2DM. Future research is necessary to elucidate the mechanisms by which UCP3 regulates mitochondrial fatty acid metabolism and ROS formation.

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7. CURRICULUM VITAE

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Research Interests

- Mitochondrial bioenergetics
- Mitochondrial uncoupling protein
- Skeletal muscle and pancreatic beta cell fuel metabolism
- Reactive oxygen species formation and regulation

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Publications

1. McQuaid TS, Saleh MC, Joseph JW, Gyulkhandanyan A, Manning-fox JE, **MacLellan JD**, Wheeler MB, and Chan CB. cAMP-mediated signalling normalizes glucose-stimulated insulin secretion in uncoupling prtein-2 overexpressing beta-cells. J Endocrinol. 2006. 190:669-80

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3. Rusyniak DE, Tandy SL, Hekmatyar SK, Mills E, Smith DJ, Bansal N, **MacLellan D**, Harper ME, and Sprague JE. The role of mitochondrial uncoupling in 3,4-methylenedioxymethamphetamine-mediated skeletal muscle hyperthermia and rhabdomyolysis. J Pharmacol Exp. 2005. 313:629-39