Relationship of mitochondrial energy production with the rate and efficiency of whole body growth in young growing animals

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Trevor Russell Lutz

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Program of Study Committee:
Tim Stahly, Major Professor
Donald Beitz
Elisabeth Huff-Lonergan
James Reecy
Chad Stahl

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Graduate College
Iowa State University

This is to certify that the doctoral dissertation of

Trevor Russell Lutz

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

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ABSTRACT

Associations between mitochondria function and the mitochondrial protein adenine nucleotide translocator 1 (ANT1) content with rate and efficiency of growth in young growing rats and pigs were evaluated. Mitochondria were isolated from the gastrocnemius muscle in rats (n = 43) and the biceps femoris muscle in pigs (n = 44) to measure mitochondrial protein content and function among animals from a single strain and rearing environment. Lower rates of mitochondrial proton leak-dependent respiration (State 4; r = 0.42, $P < 0.01$) or improved mitochondrial metabolic efficiencies (RCR; r = 0.33, $P < 0.05$) in skeletal muscle were associated with improved gain/feed ratios in rats. In addition, rats with a lower muscle mitochondrial protein content exhibited improved efficiencies of feed utilization (r = 0.43, $P < 0.01$) and improved rates of growth (r = 0.31, $P < 0.05$). In pigs, higher rates of maximal mitochondrial respiration (State 3; r = .43, $P < .01$), an improved RCR (r = 0.34, $P < 0.05$) and a greater amount of ANT1 protein in the biceps femoris muscle (r = 0.44, $P < 0.05$) were each associated with improved daily body weight gains.

The ability of bilobalide and proanthocyanidins to positively modulate mitochondrial function also was evaluated. Weanling rats (16/trt) were allowed access to a diet containing either 0 or 78 ppm bilobalide for 22 days post-weaning. Gastrocnemius and liver mitochondria were isolated for mitochondrial function measurements. Dietary bilobalide addition resulted in increased liver weights ($P < 0.01$) and protein contents ($P < 0.01$), State 3 rates ($P < 0.05$) and RCR ($P = 0.08$) in liver mitochondria. However, mitochondrial function in the gastrocnemius muscle and daily BW gain, feed intake, and efficiency of feed utilization were not altered by bilobalide addition. In vitro incubation of pig skeletal muscle mitochondria with 0.36 μg/ml proanthocyanidins resulted in a 15% improvement ($P < 0.05$)

in State 3 respiration. These data establish that variation in mitochondrial function exists and is important to both the rate and efficiency of whole body growth in animals and bioactive compounds may have the potential to positively alter mitochondrial function.

CHAPTER 1. GENERAL INTRODUCTION

Mitochondria are often referred to as the "power houses" of a cell and in this capacity are responsible for producing the energy currency (ATP) utilized to sustain cell function and are responsible for 90% of cellular oxygen consumption (Rolfe and Brown, 1997). Because of the important role mitochondria play in cellular energy metabolism, we hypothesized that variation in mitochondrial content or rate and efficiency of mitochondrial energy production may be important to the rate and efficiency of whole body growth in animals. Previous research in adult rats has shown that inefficiency in mitochondrial energy production (proton leak) is responsible for 25% of whole body oxygen consumption during basal metabolic rate conditions, with skeletal muscle having a particularly high proportion of its oxygen consumption utilized to balance proton leak (Rolfe and Brand, 1996; Rolfe and Brown, 1997). In addition, two recent studies in poultry offer evidence indicating that mitochondrial function is associated with both the rate and efficiency of whole body growth (Opalka et al., 2000; Bottje et al., 2002). However, to date, there has been a scarcity of research conducted examining how variation in mitochondrial function alters whole body rate and efficiency of growth in young growing mammals. The aim of the current set of studies is to quantitate the degree of variation in mitochondrial energy production among young growing animals and determine whether or not this variation is associated with rate and efficiency of animal growth. If mitochondria prove to be of importance in regard to whole body growth in young growing mammals, future work should evaluate dietary compounds or genes/proteins that positively modulate mitochondrial function. To begin movement down this path, we have chosen to evaluate variation in the bioenergetic protein ANT1 and how this variation alters mitochondrial function and animal growth. In addition, bilobalide and proanthocyanidin

previously demonstrated to alter either the efficiency or rate of mitochondrial energy production, respectively, in rat liver mitochondria will be evaluated for their ability to alter muscle mitochondrial function.

Dissertation Organization

The dissertation is divided into a literature review, three papers, and a general conclusion. The papers were prepared in a style appropriate for submission to the *Journal of Animal Science.*

Literature Cited

Bottje, W., Z.X. Tang, M. Iqbal, D. Cawthon, R. Okimoto, T. Wing and M. Cooper. 2002 Association of mitochondrial function with feed efficiency within a single genetic line of male broilers. Poult. Sci. 81: 546-555.

Opalka, J.R., M. Wicke, F.N. Gellerich, S. Zierz and G. von Lengerken. 2000. Influence of mitochondrial function on growth and meat quality in turkey. Eur. J. Med. Res. 5 (Suppl I): 57.

Rolfe, D.F.S and M.D. Brand. 1996. Contribution of mitochondrial proton leak to skeletal muscle respiration and to standard metabolic rate. Am. J. Physiol. 271: C1380-C1389.

Rolfe, D.F.S. and G.C. Brown. 1997. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol. Rev. 77: 731-758.

CHAPTER 2. REVIEW OF LITERATURE

History of Mitochondrial Research

The developments leading to the description of biological energy production has a rich and interesting history and is fraught with sacrifices made by many of the investigators involved. Some of the researchers contributing to theories, which describe biological energy production, were considered mavericks in their time because they staunchly upheld their theories against strong popular scientific opinion that believed these theories to be ridiculous. Many of the advances in scientific knowledge in this area have proceeded in line with technological developments that have allowed for more precise and deeper probing of the underlying biological mechanisms.

In the early eighteenth century, the scientific community believed that biological energy or vital heat as it was described at the time was derived from a fire in the left ventricle of the heart and through respiration the lungs served to cool this internal fire (Blaxter, 1989). Today, we know this vital heat arises from both physical and chemical processes and the idea of an internal fire seems rather outlandish. Antoine Lavoisier was the first person to lead us toward our current understanding of biological energy production. He established that during respiration, oxygen in the air is diminished while carbon dioxide content is increased (Lavoisier, 1777). Later, utilizing the first animal calorimeter, Lavoisier successfully established the quantitative relationship between animal heat production, oxygen consumption, and carbon dioxide release (Lavoisier and Laplace, 1783).

Scientists of the eighteenth and nineteenth century revealed that after consuming and digesting food it is subsequently burned using oxygen that we breath in from the air. The next step for biochemists was to describe how the electrons get from the food we eat to

oxygen. From 1895 to 1920, two rival theories for tissue respiration existed. Wieland hypothesized that dehyrogenases removed hydrogen from food and this "activated hydrogen" somehow reacted with oxygen to form water (Tyler, 1992). Warburg strongly disagreed with this theory and believed an iron-containing oxidase utilized oxygen and removed electrons from food (Tyler, 1992). Warburg came to this conclusion after he observed tissue and cellular oxygen consumption was inhibited by the addition of cyanide. Both cyanide and oxygen were known to combine with iron, and Warburg believed that cyanide was binding to the iron containing oxidase, thus preventing oxygen binding and ultimately cellular respiration (Tyler, 1992). As it turned out, both hypotheses were correct; the two men were just looking at "opposite ends" of what we now know as the electron transport chain. At the front end of the chain are the dehydrogenases described by Wieland that remove electrons from substrates and at the back end of the chain is an oxidase that contains iron and moves electrons to oxygen. David Keilin next discovered the linkage between the front and backside of the electron transport chain. These linkages were named cytochromes, and they serve to take electrons from dehydrogenases and pass them on to the oxidase. The meaning of the word cytochrome is "cell color", and Keilin actually discovered the cytochromes using a light scattering prism applied to the working flight muscles of moths in the presence and absence of oxygen (Brown, 1999). Still an unanswered question was the mechanism by which foodstuffs are oxidized for entry into the electron transport chain. In 1937, Krebs described an enzymatic cycle, which today we call the citric acid or Krebs cycle.

Researchers next aspired to understand the energy currency of the cell and the mechanism by which the electron transport chain is coupled to the formation of this energy currency. Lipmann conducted much work in the area of cellular energetics, and originally

believing phosphocreatine to be the energy currency compound. However, in 1941, he came to the conclusion that it was not phosphocreatine that served as the major energy currency of the cell but rather ATP (Brown, 1999). It was originally hypothesized that the oxidization of food was linked to ATP formation via a chemical intermediate. However, Mitchell proposed that mitochondria pump protons generating proton electricity and this then is linked to the formation of ATP. Mitchell was involved in many bitter disputes with scientists believing that a chemical intermediate was involved in this coupling. He failed in convincing many of these scientists about the validity of proton electricity and his ideas really only gathered momentum as younger scientist, who supported Mitchell's theories, entered the field (Brown, 1999).

Previous discussion has focused on observations made in tissue homogenates or the whole animal, and no evidence has been presented indicating which cellular component may be responsible for energy production. With the establishment that biological energy was not produced in the heart, exploration was launched into which cell or cellular component was responsible for this energy production. This search began in earnest with the development of the light microscope and achromatic lenses in the early ninteenth century. Microscopists observed certain structures that had either a granule or threadlike appearance in various tissues beginning about 1850. The Greek work for thread (mitos) and granule (chondros) eventually led to the term mitochondria (Scheffler, 1999). The first recorded separation of mitochondria from tissue was conducted by Kolliker in 1888, and these granules were also observed to swell in water indicating the presence of a membrane (Tyler, 1992). Early studies on mitochondria structure were hampered by the use of acetic acid in tissue staining and fixative mixtures that dissolved mitochondria. Altmann, in 1890, conducted the first

systematic study of mitochondria by using a fixative with no acetic acid and was capable of preserving mitochondria. The staining procedure used by Altmann also stained secretory vesicles and fat droplets, and he referred to these structures as "bioplasts". Altmann hypothesized that these "bioplasts" were analogous to bacteria in that they were living particles capable of growth and division from preexisting bioplasts and could have the ability to exist in a system outside the cell. Fifty years later, the development of differential centrifugation and electron microscopy allowed mitochondria to be successfully isolated and their intactness to be verified. During the period from 1943 to 1947, Lehninger utilized differential centrifugation to isolate mitochondria and described the important role they play in respiration (Tyler, 1992).

Mitochondria Structure and Function

The number of mitochondria per cell varies by tissue, but ranges from a few hundred to a few thousand, with mitochondria typically exhibiting lengths and diameters of 3- 4 μm and 1 μm, respectively (Scheffler, 1999). Mitochondria are not randomly distributed in the cell, but rather are found in close proximity to energy requiring sites such as ATP-dependent, ion-translocating enzymes and sites involved in the motor activities of cells (e.g., actinomyosin ATPase) (Tyler, 1992). Additionally, in many cells and especially muscle, mitochondria have been shown to form networks that are maintained by the mitochondrial membrane protein, mitofusion-2. Repression of mitofusion-2 expression results in fragmentation of the mitochondrial network and is associated with reduced mitochondrial function (Bach et al., 2003). Interestingly, the expression of mitofusion-2 is decreased by 36% in obese humans, which may help explain deficiencies of muscle substrate oxidation observed in obese subjects (Bach et al., 2003). Within the cell, mitochondria are known to

translocate to energy requiring sites along microtubules and this movement can be disrupted by tumor necrosis factor α (De Vos et al., 2000). Mitochondria translocation also is an important factor involved in oocyte maturation and embryo development (Sun et al., 2001). These two factors raise some interesting questions regarding embryo survival and development in immune challenged pregnant females.

Two subpopulations of mitochondria that are located between the myofibrils (intermyofibrillar) and just under the cell membrane (subsarcolemmal) are present in both skeletal and cardiac muscle. Evidence indicates that intermyofibrillar mitochondria supply ATP for muscle contractile activity while subsarcolemmal mitochondria supply ATP for other cytoplasmic reactions (Cogswell et al., 1993; Bizeau et al., 1998). The proportion of these two different mitochondrial subpopulations varies according to muscle fiber type with glycolytic fibers having equal proportions of both subpopulations and oxidative fibers exhibiting a greater proportion of subsarcolemmal mitochondria (Philippi and Sillau, 1994). In addition, biochemical differences between the two populations also exist with the intermyofibrillar population exhibiting higher rates of oxygen consumption and proton leak along with a greater capacity to oxidize fatty acids than the subsarcolemmal population (Palmer et al., 1985; Cogswell et al., 1993; Iossa et al., 2001). Finally, these two populations are differently affected by muscle disuse and aging in that the respiration rate or number, respectively, of the intermyofibrillar population is reduced whereas the subsarcolemmal population is not altered (Farrar et al., 1981; Yajid et al., 1998).

Mitochondria divide from preexisting mitochondria to keep pace with cell growth and division in a process termed biogenesis (Tyler, 1992). Hormonal (i.e. thyroid hormone) or environmental stimuli (i.e. exercise, adaptive thermogenesis), which place an increased

energy demand on the cell, lead to increased biogenesis (Garesse and Vallejo, 2001). In addition, biogenesis is activated in order to replace mitochondria, whose turnover rate is estimated to be 3.8 days in the liver (Lipsky and Pedersen, 1981). Formation of new mitochondria requires replication of circular mitochondrial DNA (mtDNA) along with the incorporation of mitochondrial proteins encoded in the cell nucleus. The human mitochondrial genome consists of ~16,000 bp with 1-10 molecules of mtDNA per mitochondria (Marin-Garcia and Goldenthal, 2002). The vast majority of mitochondrial proteins are actually encoded within the cell's nucleus as human mitochondria contain ~1500 proteins of which only 37 are encoded by mtDNA (Anderson et al., 1981; Lopez et al., 2000). Mitochondrial DNA is thought to be strictly inherited from the mother because of selective destruction of sperm cell mitochondria during early embryogenesis (Birky, 1995; Cummins et al., 1997). However, a recent case of paternally inherited mtDNA has been observed in a human subject, but the authors stress this was a special case whereby the subject's mutated mitochondrial DNA had a selective proliferative advantage (Schwartz and Vissing, 2002). Recent efforts have focused on defining the mechanisms controlling mitochondrial biogenesis in hopes that a type of "exercise in a bottle" may someday be developed. Peroxisome-proliferator-activated receptor-γ co-activator-1 appears to play a central role in this process, because its expression parallels mitochondrial biogenesis, which is associated with exercise, and when over expressed in mouse muscle results in mitochondrial proliferation (Baar et al., 2002; Lin et al., 2002).

Mitochondria consist of an inner and outer membrane, matrix space, ribosomes and mtDNA. The enzymes involved in oxidative phosphorylation are bound to the inner mitochondrial membrane, while in contrast, Krebs cycle enzymes are soluble and are located

within the matrix space. Glucose, fatty acids, and amino acids are oxidized via glycolysis and the Krebs cycle subsequently transferring their electrons to NADH and $FADH_2$. These two molecules then carry electrons to the mitochondrial respiratory chain, which is composed of Complexes I-IV (Fig. 1). These electrons are subsequently transported along the

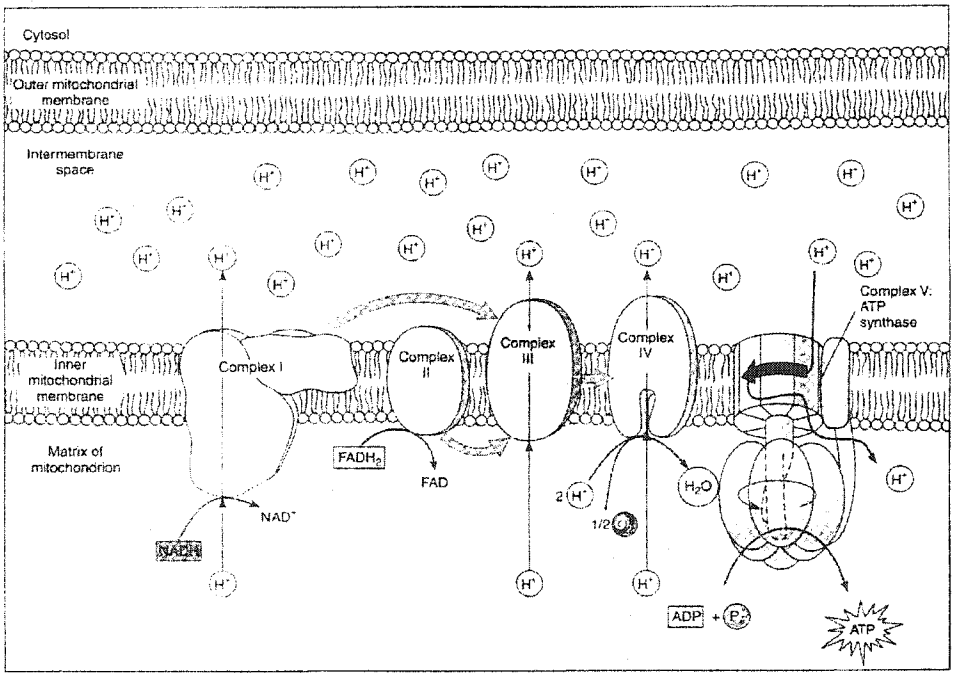

Figure 1. Mitochondrial electron transport chain. (adapted from Soloman et al., 2002)

mitochondrial respiratory chain to the final electron acceptor oxygen, thus supplying the energy to pump protons out of the mitochondrial matrix allowing for the formation of a proton motive force consisting of both a concentration and electric potential difference of protons across the inner mitochondrial membrane. This stored energy of the proton motive force is ultimately dissipated either through ATP formation via Complex V (Fig. 1) or proton leak.

As reviewed by Scheffler (1999), Complex I (NADH-ubiquinone oxidoreductase) is composed of 42 proteins and serves to accept electrons from NADH and transfer them to

ubiquinone via iron-sulphur-containing proteins while pumping protons from the matrix side of the mitochondria to the intermembrane space. Complex II (succinate:ubiquinone oxidoreductase) is composed of only four proteins. Two of these proteins are also components of the Krebs cycle and upon oxidation of succinate to fumarate electrons are channeled again to ubiquinone with $FADH_2$ serving as the electron carrier rather than NADH. Eleven proteins form Complex III (ubiquinone-cytochrome-c oxidoreductase), and in this step electrons are transferred from ubiquinone to cytochrome c via cytchrome b and c_1 allowing the transfer of additional protons across the innermembrane. Cytochrome c then moves to Complex IV (cytochrome c oxidase) that contains 13 proteins, and ultimately these electrons are transferred to the terminal electron acceptor oxygen via cytochrome a and a_3, and again there is further proton pumping. Protons, which were pumped out of the matrix in Complexes I, III and IV, then flow back into the matrix through the 16-protein Complex V (ATP synthase), which results in the formation of ATP. Adenine nucleotide translocator serves to transport ATP into the cellular cytosol while transporting ADP into the mitochondria to once again be phosphorylated.

The role that mitochondria play in cellular energy metabolism is well known and studied; however, in recent years a renewed interest in mitochondria has emerged because of the important role they play in cell death. Cellular death can occur either via necrosis or apoptosis with distinct differences observed between these two different death pathways. Necrosis results in cell rupture and eventual release of cytosolic components and is induced by rapid declines in ATP concentrations (Pedersen, 1999). In contrast, apoptosis is a programmed process that requires ATP and results in shrinking of the induced cell and eventual engulfment by neighboring healthy cells. Necrosis is characterized by the formation

of a mitochondrial permeability transition pore that allows for the movement of low molecular weight compounds into the mitochondria, which results in mitochondrial swelling and loss of the ability to maintain the proton gradient and ATP synthesis. Apoptosis is induced by various death factors (calcium, ceramide, caspases, reactive oxygen species (ROS), and Bcl-2 family members), which result once again in the formation of the mitochondrial permeability transition pore with subsequent release of cytochrome c and apoptosis-inducing factor from the mitochondria (Pedersen, 1999). ATP depletion is not a causative factor in apoptotic cell death, because it is believed that the mitochondrial permeability transition pore recloses during apoptosis, reestablishing the proton gradient and allowing for the maintenance of cellular ATP concentrations that are needed for subsequent apoptotic events (Pedersen, 1999).

Energy Use in the Whole Body

The amount of energy required to support life in animals is termed the basal metabolic rate and is measured on a fasted, mature, resting animal at thermo-neutrality. Basal metabolic rate does not measure the energetic cost of growing, feeding, processing food, or physical activity. Because oxygen is the terminal electron acceptor in the electron transport chain, it can be used to determine how much energy a particular organ is consuming when the oxygen consumption rate of that organ is known. Ultimately, the summation of oxygen consumption by individual organs contributes to the whole of basal metabolic rate. Muscle comprises the largest portion of body mass and total body oxygen use (Table 1).

Liver comprises only a small portion of total body mass, but it consumes a large portion of the body's oxygen (Table 1), thus illustrating the point that various body organs

have different metabolic rates per unit of mass and contribute differently to the body's basal metabolic rate.

Table 1. Contribution of the major oxygen-consuming organs to body mass and basal metabolic rate in adult rats[a]

Organ	Body mass, %	Body oxygen use, %
Muscle	42–46	20–33
Skin	18.5	7–11
Adipose tissue	5–15	1–12
Skeleton	6.7–10	1-4
Liver	3.4–5.5	10–20
Gastrointestinal tract	4–5.3	4–8
Brain	1.5	3–14
Kidneys	<1	4–14
Heart	<1	1–10
Lungs	<1	<1

[a]Adapted from Ramsey et al., 2000

Also of interest, is which cellular processes are the largest consumers of oxygen on an organ-by-organ basis during basal metabolism. Listed in Table 2 are the contributions that protein synthesis, Na^+/K^+ ATPase, and proton leak contribute to the total oxygen consumption/energy demand of some individual organs. These are the major consumers of cellular energy, but other reactions such as Ca^+-ATPase, substrate cycling, actinomyosin ATPase, protein degradation among others also contribute to varying degrees (Rolfe and Brown, 1997). As a proportion of basal metabolic rate, proton leak, protein synthesis, and Na^+/K^+-ATPase are estimated to consume 25, 12-25, and 14% of the whole body's oxygen (Rolfe and Brand, 1996; Rolfe and Brown, 1997). Proton leak is a source of energetic inefficiency, because the energy used to pump protons across the mitochondrial membrane is

not used to form the energy-storing molecule ATP, but rather is lost as heat to the animal's surroundings. The greatest opportunity to reduce endogenous rates of mitochondrial proton leak appears to reside in skeletal muscle, because of its proportionally large consumption of oxygen (50%) stemming from this process.

Table 2. Contribution (%) of major oxygen-consuming processes to oxygen consumption rate of rat tissues at standard state[a]

Tissue	Protein synthesis	Na^+/K^+-ATPase	Proton leak
Liver	24	5-10	26
Gastrointestinal tract	74	60	not determined
Kidney	6	40-70	not determined
Heart	3	1-5	15
Brain	5	50-60	not determined
Skeletal muscle	17	5-10	50

[a]Adapted from Rolfe and Brown, 1997

The partitioning of cellular oxygen use as an animal's energy demands increases beyond basal metabolic rate (i.e., young growing animal) are less clearly quantified (Martin Brand, personal communication). Certainly, we can predict the average amount of energy required to deposit protein and fat in pigs and also can assign mean energetic efficiencies to these two processes (Ewan, 2001). Energy not retained in the animal is dissipated as heat resulting from animal movement, intermediary metabolic reactions required in the synthesis of fat and protein, ion gradients and proton leak. The energetic cost of protein synthesis is estimated to be four ATP per peptide bond (Rolfe and Brown, 1997). Research also has indicated that as protein synthesis rate increases so to does Na^+/K^+-ATPase activity and associated ATP expenditure (Vandenburgh and Kaufman, 1981). However, as energy demand increases, the rate of proton leak decreases. Previously, it was mentioned that proton

leak accounts for 50% of muscle tissue oxygen consumption in perfused muscle tissue at rest. The contribution of proton leak to whole muscle oxygen consumption falls to a still significant but lower value (34%) when increasing energy demand of the tissue two-fold by simply inducing muscle contraction (Rolfe et al., 1999). This phenomena occurs because as mitochondrial membrane potential decreases via increased energy demand so to does the rate of proton leak (Nicholls, 2002). At the present time, the contribution of proton leak to whole body energetic efficiency in animals not at rest is unknown because the in vivo mitochondrial membrane potential has yet to be determined.

Inefficiencies in Mitochondrial Energy Production

In the past, it was thought that mitochondrial energy production (oxygen consumption) was fully coupled to ATP production. However, we now understand that a portion of the cellular oxygen consumption is nonmitochondrial and a significant portion of mitochondrial oxygen consumption is not coupled to ATP synthesis. Certain metabolic pathways outside the mitochondria are known to contain oxidases, with one example being the oxidation of fatty acids in the peroxisome. Nonmitochondrial oxygen consumption is estimated to be 10% of whole body oxygen consumption (Rolfe and Brown, 1997).

Inefficiencies in mitochondrial energy production have been shown to occur by two different means. First, a small proportion of mitochondrial oxygen consumption is not coupled to proton pumping. This inefficiency occurs when electrons leak from the electron transport chain and reduce oxygen to superoxide. This sort of inefficiency has been shown to account for only a maximum of 1-2% of the total oxygen consumption in isolated mitochondria (Boveris et al., 1972; Turrens et al., 1985). However, as discussed later, these free radicals cause significant damage to mitochondrial proteins, which may result in poor

efficiencies of whole body energy utilization in domestic animals (Pumford et al., 2002). The larger contributor to inefficiencies in mitochondrial energy production would be the leakage of protons through the mitochondrial inner membrane at a site other than ATP synthase. Electrical potential or the proton motive force is generated in mitochondria via differences of protons across the membrane (pH) and by differences in electrical potential across the membrane (Nicholls, 2002). Proton leak is most apparent at a high proton motive force (low rate of electron transport) and is not linearly related to proton motive force at high membrane potentials (Nicholls, 2002). It has been known for over a century that dinitrophenol can increase whole body oxygen consumption and heat production in animals due to respiratory chain uncoupling (Tyler, 1992). Nicholls was the first person to show that this respiratory chain uncoupling naturally occurs in brown adipose tissue of animals and functions to increase core body temperature by means of uncoupling protein-1 (UCP1) (Nicholls and Locke, 1984). Proton leakage also occurs in other tissues besides brown adipose tissue, but, at this time, it is not known if this uncoupling occurs via delocalised diffusion of protons through the inner mitochondrial membrane or by means of specific transport proteins (i.e. UCP2 or UCP3) (Porter, 2001).

At this time, a discussion of the method utilized to measure mitochondrial oxygen consumption is warranted. A common method used by researchers to measure the efficiency and rate of mitochondrial energy production is the polarographic measurement of oxygen consumption as described by Estabrook (1967). Mitochondria are isolated from tissue and incubated in a stirred reaction chamber and, through the use of an oxygen sensitive electrode, mitochondrial oxygen consumption over time is measured. In the presence of appropriate energy substrates that produce NADH or $FADH_2$, ADP administration causes a rapid

consumption of oxygen. This is called State 3 (maximal) respiration. Upon conversion of the administered ADP to ATP, the mitochondria return to a much slower rate of respiration termed State 4, during which time the consumption of oxygen is used to balance the protons leaking back through the mitochondrial inner membrane. Finally, the respiratory control ratio is calculated as State3/State4 and is a measure of mitochondrial metabolic efficiency or coupling.

Today, many researchers working with mitochondria refer to State 4 respiration as proton leak-dependent respiration. This type of terminology has been widely utilized, because in the absence of proton leak, State 4 oxygen consumption is speculated to be zero (Brand et al., 1994). However, this statement deserves further qualification because no absolute inhibitors of proton leak are currently known to exist. First, both free fatty acids and high calcium concentrations have been shown to uncouple isolated mitochondria, which result in increased State 4 respiration rates (Wojtczak and Schonfeld, 1993; Lemasters et al., 1997). To eliminate the effects of these two compounds in the estimation of State 4 (proton leak-dependent respiration), both calcium chelators (EDTA) and fatty acid binding proteins (e.g., bovine serum albumin) are commonly included in the mitochondrial incubation medium. Second, the classical attainment of State 4 respiration occurs when all ADP added to stimulate State 3 is converted to ATP (Estabrook, 1967). During State 4 respiration, there is the possibility that a portion of mitochondrial oxygen consumption may stem from hydrolysis of ATP via mitochondrial ATP utilizing reactions, resulting in the formation of ADP and further oxygen consumption via oxidative phosphorylation rather than proton leak. However, when oligomycin, an inhibitor of ATP synthase, is added to isolated muscle mitochondria, there is no reduction in State 4 respiration, indicating the lack of conversion

from ATP to ADP during this state (Tonkonogi and Sahlin, 1999; Iossa et al., 2002). On the other hand, hydrolysis of ATP via mitochondrial ATP utilizing reactions seems to play a larger role in liver mitochondria because oligomycin addition can reduce State 4 rates of respiration in these mitochondria (Masini et al., 1983).

To further describe the efficiency of mitochodrial energy production, researchers calculate the phosphorus to oxygen ratio (P/O), which is the ratio of ATP synthesized to oxygen consumed and is described as mechanistic or effective. The mechanistic P/O is the maximal theoretical yield of ATP per oxygen in the absence of proton leak, other uncoupling reactions, and oxygen consumption at a site other than cytochrome c oxidase. While the effective ratio is a more accurate measurement of the true P/O occurring in vivo after accounting for the proportion of oxygen consumption that takes place outside the mitochondria and that due to proton leak. Mechanistically, the P/O was thought to be 3 for mitochondria oxidizing NADH (Rolfe and Brown, 1997). However, after accounting for proton leak and non-mitochondrial oxygen consumption, the effective P/O is less and estimated to be 1.8 for mitochondria oxidizing NADH (Hinkle et al., 1991; Brand, 1995).

The real question is whether or not the above phenomena affects whole body energetic efficiency. Mitochondria are responsible for 90% of cellular energy production, and therefore it is plausible to hypothesize that inefficiencies in this process may relate to whole body energetic efficiency (Rolfe and Brown, 1997). Indeed, variations in mitochondrial oxygen use have been observed between different breeds of chickens, sheep and swine (Mukherjee et al., 1970; Wolanis et al., 1980; Dzapo and Wassmuth, 1983). In addition, mitochondria from major oxygen-consuming tissues (liver, skeletal muscle, kidney and brain) each exhibit significant proton leak (Rolfe et al., 1994). Of these different tissues,

skeletal muscle has the largest degree of proton leak estimated to account for 50% of the oxygen consumption at steady state conditions in skeletal muscle (Rolfe and Brand, 1996). By using proton leak rates of the various tissues in the rat body, it has been estimated that 25% of the whole body oxygen consumption at standard state conditions is utilized to balance proton leak (Rolfe and Brand, 1996).

The most widely studied example of protein-catalyzed proton leakage is the process involving UCP1 found in mitochondria of brown adipose tissue (Stuart et al., 2001). This uncoupling protein produces a futile cycling of protons in the mitochondria, resulting in heat production. Besides UCP1, two other proteins have been identified, named UCP2 and UCP3, which have 50 to 60% amino acid identity to UCP1 and therefore may also have uncoupling ability (Stuart et al., 2001). UCP2 is the most widely expressed protein of the three in kidney, brain, heart, skeletal muscle and liver, whereas UCP3 is found predominantly in skeletal muscle (Porter, 2001). Recent work from two laboratories has indicated that changes in the efficiency of mitochondrial energy utilization may affect whole body energy metabolism in animals. Clapham et al. (2000) overexpressed human UCP3 (expressed predominantly in muscle) in mice and found that body weight gain was decreased while food intake was dramatically increased in the mice with overexpression of UCP3. In addition, whole body resting oxygen consumption was increased in mice in which UCP3 was overexpressed. Mitochondrial uncoupling was evident in the mice that overexpressed UCP3 as measured by a decrease in mitochondrial metabolic efficiency due to increased proton leak and decreased maximal rate of mitochondrial respiration. In addition, Bottje et al. (2002) observed in chicks from a single genetic strain that low mitochondrial metabolic efficiencies in breast and leg muscle mitochondria were associated with low efficiency of dietary energy

utilization for body growth. Low efficiency of dietary energy utilization also was associated with higher rates of mitochondrial free radical production due to electron leak at Complex I and III of the respiratory chain in the chick (Bottje et al., 2002). This is important because increased electron leak has been implicated as one causative factor of proton leak (Liu, 1999; Shi et al., 1999). Approximately 95% of superoxide radicals ($O_2\cdot$) generated in the body are produced within the mitochondria during oxidative phosphorylation and then converted to other free radicals such as $HO_2\cdot$, H_2O_2, and $OH\cdot$ (Schapira, 1997). These free radicals within the mitochondria then are able to damage lipids, proteins, and DNA in the relatively unprotected mitochondria, resulting in further loss of mitochondrial function (Kristal et al., 1994; Kristal et al., 1997; Tonkonogi et al., 2000; Callahan et al., 2001).

Proton leak has gained much interest from researchers attempting to decrease the prevalence of human obesity. For example, a recent study in humans has suggested that weight reduction on a calorie-restricted diet is related to the amount of skeletal muscle proton leak (Harper et al., 2002). For this reason, there is currently a large effort to identify compounds that may increase proton leak with the eventual outcome being a reduction in the prevalence of human obesity. However, the goal in meat animal production should be the identification of compounds that decrease mitochondrial proton leak leading to the development of a "super-efficient" animal. Caution is warranted, though, in trying to decrease proton leak excessively, because it has been speculated that one physiological function of proton leak is to reduce reactive oxygen specie formation (Nedergaard and Cannon, 2003). Various bioactive compounds have been shown in vitro to increase metabolic efficiency in liver mitochondria incubations (Janssens et al., 2000). These increases in mitochondrial metabolic efficiency result from a lower proton leak-dependent

respiration (aescine, Ginkor Fort, naftidrofuryl and naphthoquinone) or greater maximal rates of respiration (hydroxyethylrutosides, proanthocyanidin and Cylo 3) (Janssens et al., 2000). For in vivo manipulations of proton leak in animals, a nontoxic, potentially economical regulator is desired. Furthermore, for manipulation of proton leak in existing, highly productive strains of meat-producing animals, an orally active compound that allows continuous availability of the regulator to tissues and is approved for animal and human consumption is desired. Oral ingestion of bilobalide, a component of Ginko biloba, by rats has been shown to improve mitochondrial metabolic efficiency by causing a 60% reduction in liver mitochondrial proton leak (Jannsens et al., 1999). The exact mechanism whereby bilobalide decreases proton leakage is unknown. It is known, though, that bilobalide must be fed to rats for at least 14 days before there is a decrease in mitochondrial proton leakage in liver (Janssens et al., 1995). These authors hypothesized that bilobalide may need to accumulate or insert itself into the inner mitochondrial membrane to elicit its effect. However, it is not known if bilobalide would be effective in skeletal muscle mitochondria, which has one of the larger degrees of proton leak.

Regulating mitochondrial energy production by reducing proton leak also may have implications in improving the quality and longevity of life in humans. Support for this hypothesis comes from two lines of evidence. First, Porter and Brand (1993) have described an inverse relationship between proton leak and longevity. Specifically, small mammals, such as rodents, have comparably higher rates of proton leak and shorter life spans than larger mammals. Second, proton leak is greater in old than in young rodents and energy restriction in aged rats reduces proton leak (Lal et al., 2001; Harper et al., 1998). This is interesting because caloric restriction in rodents has been demonstrated to increase the

longevity and quality of the aged rodent's life including a delay in the onset of skeletal muscle loss, hypertension and cancer (Weindruch and Walford, 1988; Weindruch and Sohall, 1997). Increased rates of proton leak in the aged rat results in increased rates of resting oxygen consumption that could potentially lead to increased concentrations of ROS (Harper et al., 1998; Brookes et al., 1998). These ROS may result in increased peroxidation of mitochondrial membrane lipids, leading to further proton leak and resulting in a cycle that damages the cell and mitochondria (Ramsey et al., 2000).

Control of Mitochondrial Energy Production

Shown in figure 2 are the different energy-producing and -utilizing reactions in the animal cell. Glucose, fatty acids, and amino acids are utilized as substrates for the respiratory chain following their oxidation and subsequent formation of NADH. NADH is

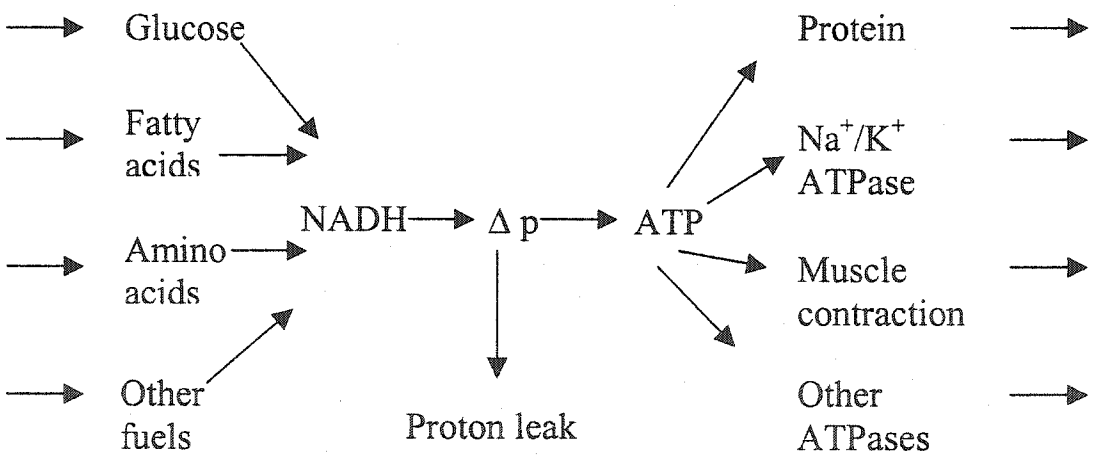

Figure 2. Production and dissipation of mitochondria membrane potential

subsequently oxidized by oxygen via the mitochondrial respiratory chain, forming a proton motive force (Δ p) that is dissipated either through ATP formation or proton leak. Potential

energy stored in the form of ATP is then utilized to drive various cellular process such as protein synthesis, muscle contraction and ATPase-dependent pumps.

An ever evolving question has been the description of mechanisms controlling mitochondrial energy production and utilization. These descriptions have ranged from very broad definitions of control acting through either pull generated via ATP-utilizing reactions or through substrate push to the more intricate description of individual enzymes or groups of enzymes that may be controlling mitochondrial energy production. Adding further complexity to this question is the fact that control may be different depending on the type and energy status of the tissue being examined.

Initially, it was thought that increased ATP usage could increase mitochondrial ATP synthesis by supplying an increased concentration of the substrate, ADP, for oxidative phosphorylation (Lardy and Wellman, 1952). Also, it is known that an increase in the supply of respiratory substrate (NADH) to mitochondria stimulates ATP synthesis (Brown, 1992). Thus, ATP synthesis responds to both the pull of ATP consumption and the push of increased electron supply at opposite ends of the electron transport chain. These observations developed the foundation for our understanding of the control of mitochondrial energy production, but a more comprehensive view of individual enzymes and enzyme systems was desired. To meet this need, metabolic control analysis was developed to quantitate the amount of control various mitochondrial parameters had over the flux through the system. Central to the idea of metabolic control analysis is that changes in the activity of one enzyme within a given metabolic system also brings about alterations in related metabolite pools and enzyme activities, culminating in the achievement of a new steady state. Mathematically these changes are quantified by using flux control coefficients calculated as the percentage

change in the steady state rate of the pathway divided by the percentage change in the enzyme activity causing the flux change (Brown, 1992). From these data, one can also construct threshold curves that depict the percentage of enzyme inhibition necessary to bring about a decline in flux through the system. Metabolic control analysis assumes that each component within a metabolic pathway contributes to the control of the pathway and these flux control coefficients sum to 1. This idea is a major shift from the concept that one rate-limiting enzyme controls flux through metabolic pathways that is taught in many biochemistry textbooks. If the one rate-limiting enzyme per metabolic pathway concept were true, the control coefficient for that particular enzyme would sum to one and very rarely in nature is this ever observed (Fell, 1998).

Recently, metabolic control analysis was utilized to quantitate the contribution made by substrate oxidation, the phosphorylating system and proton leak to mitochondrial respiration in pig muscle at various energy states (Lombardi et al., 2000). The authors found that, for mitochondria respiring in State 4, 87.3% of the metabolic control stemmed from proton leak and 12.7% from substrate oxidation. When mitochondrial energy production was increased to its maximal rate (State 3), a shift in the flux control was observed. Specifically, during State 3 respiration, 33.7% of the control over mitochondrial respiration could be attributed to substrate oxidation and 54.1% to phosphorylation, and a comparatively lower degree of control for proton leak at 8.3%. As measured in the mitochondria of other animals, it is evident that control over mitochondrial respiration is distributed among the various respiratory proteins and the distribution of this control depends on the energy demands of the mitochondria. In order to predict the control of mitochondrial respiration in vivo, one would need to know where along the continuum from State 3 to State 4 mitochondria respire. The

rate of mitochondrial oxygen consumption in vivo is not well known, but it is thought to be intermediate between State 3 and 4 and defined as State 3.5 (Korzeniewski, 2002). Evidence for this is that the ATP/ADP during State 3.5 is much closer to that found in intact tissues than during State 3 or 4 (Korzeniewski, 2002). However, very little work has been done to describe control over mitochondrial respiration in this intermediate, more physiologically relevant state.

Metabolic control analysis can also be useful in describing how much extra capacity exists for different enzymes involved in mitochondrial energy production. For instance, would a 20% decline in the activity of a particular enzyme/complex have any consequence on pathway flux or must a decline of 80% be observed to result in any appreciable change in pathway flux. The answer to this question depends on many different factors such as the flux control coefficient of the particular enzyme/complex, tissue type, energy demand, and oxygen concentration (Rossignol et al., 1999; Korzeniewski and Mazat, 1996). Researchers have identified two different types of threshold curves (Fig. 3) that different components involved in mitochondrial energy production may exhibit (Mazat et al., 2001). Processes displaying a Type I threshold curve exhibit a plateau response to inhibition and a large threshold value usually occurring at greater than 70% inhibition. While processes displaying a Type II threshold curve, have no discernable threshold value and each incremental level of enzyme/complex inhibition results in an inhibition of system flux. An example of different threshold values in different tissues can be observed for the respiratory complex cytochrome c oxidase. Specifically, an 80% inhibition in cytochrome c oxidase activity induces only a small decrease in liver mitochondrial respiration, but this same cytochrome c oxidase inhibition in heart mitochondria reduces respiration to 40% (Rossignol et al., 1999).

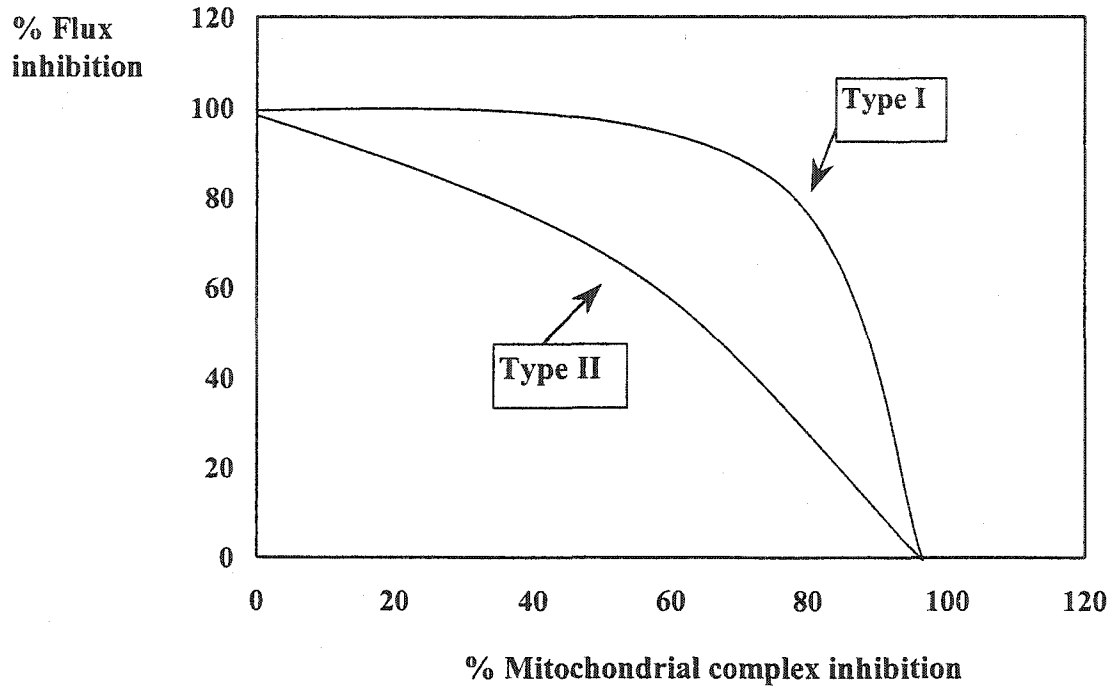

Figure 3. Conceptual relationship between mitochondrial complex inhibition and respiratory flux inhibition. Processes displaying a Type I threshold curve exhibit a plateau response to inhibition and a large threshold value usually occurring at greater than 70% inhibition. While in the Type II threshold curve a threshold value is not observable and each incremental level of enzyme/complex inhibition results in an inhibition of system flux.

The tissue specificity of these different thresholds also helps one to predict how a particular mitochondrial deficiency might effect phenotypic expression in the various body tissues. For example, skeletal and heart muscle mitochondria are controlled mostly at the level of the respiratory chain (Complex I-IV) and are therefore more sensitive to alterations in its function while the liver, kidney, and brain are more sensitive to defects in phosphorylation (ATP synthase, phosphate carrier) (Rossignol et al., 2000). Oxygen concentration has also been shown to affect mitochondrial threshold estimates (Korzeniewski and Mazat, 1996). Studies on isolated mitochondria are typically carried out using air-saturated media that contains approximately 240 μM O_2. At this oxygen concentration, the

threshold value for cytochrome c oxidase is close to 90%. However, tissue oxygen concentrations range from < 1 to 90 μM, with an average of 35 μM (Brown, 1992). At these oxygen concentrations, cytochrome c oxidase in some cases exerts greater control with a lower threshold value (Korzeniewski and Mazat, 1996). These data indicate that the threshold value for various enzymes/complexes involved in mitochondrial energy production in vivo may be different than those measured in isolated mitochondria.

The significance of the expression level of the bioenergetic gene for ANT1 on mitochondria energy production and growth in animals has not been elucidated. However, ANT1 has been estimated, based on metabolic control analysis, to elicit the greatest degree of control over mitochondrial energy production in skeletal muscle during State 3.5 respiration (Malgat et. al., 2000). ANT1 is a protein expressed predominantly in skeletal and heart muscle, is located in the inner mitochondrial membrane and serves to exchange ADP and ATP between mitochondrial matrix and cytosol (Li et. al., 1989). ANT1 function is very important to mitochondrial energy production (ATP formation), because the inner mitochondrial membrane is not permeable to adenine nucleotides (Nichols, 1992). In ANT1 knockout mice, state 3 respiration and mitochondrial metabolic efficiency are decreased as would be expected, because these mitochondria lack the ability to exchange ADP and ATP (Graham et. al., 1997). There is also a shift towards anaerobic metabolism as observed by a four-fold increase in blood lactate (Graham et. al., 1997). Blood lactate concentrations have also been shown to increase in humans exhibiting a four-fold decrease in the concentration of ANT1 (Bakker et al., 1993).

Recently, low rates of mitochondrial energy production have been associated with slow body growth rates of turkeys and intrauterine growth retarded rats (Opalka et al., 2000;

Selak et al., 2003). In addition, low expression of the ANT1 gene is implicated in both reduced in vitro cellular energy production and slow postnatal growth of intrauterine growth retarded rats (Nogueira et al., 2001; Lane et. al., 1998). Intrauterine growth retardation in rats and pigs has been shown to result in less efficient energy utilization and growth postnatally (Powell and Aberle, 1980; Ogata et. al., 1985). By using the degree of ANT1 inhibition reported by Lane et al. (1998) in growth retarded rats and the ANT1 threshold curves generated by Malgat et al. (2000) in their metabolic control analyses, mitochondrial energy production would be estimated to differ by 40% among animals. ANT1 function-expression also may be influenced by animal health in that the immune modulators nitric oxide (NO) and ROS alter ANT1 function as well as lactate production in animals (Olszanecki and Chlopicki, 1999; Vieira et al., 2001). Decreased expression of ANT1 may lead to decreased electron flow through the respiratory chain potentially contributing to decreased ATP production and reduced growth rates.

Proanthocyanidins derived from grape seed may help to normalize growth rates in animals with decreased expression levels of ANT1. Proanthocyanidins have been shown to alter the activity of ANT1 by increasing the velocity of ADP exchange in rat liver mitochondria (Janssens et al., 2000). Since ANT1 has been shown to have the highest control coefficient of all other enzymes and complexes involved in mitochondrial energy production, increasing its activity may increase State 3 respiration by increasing the rate of mitochondrial energy production.

General Summary

This review has served to summarize the importance of mitochondria in biological energy production. Because mitochondria play such an important role in this process, variation in mitochondrial function may have important consequences in relation to both the rate and efficiency of animal whole body growth. A limited number of reports have indicated that both the rate and efficiency of mitochondrial energy production has implications on whole body energy metabolism. However, in these reports experimental conditions were different than those commonly observed for food animals raised under modern production practices. Specifically, mitochondrial differences were studied in animals of perturbed physiological status or in older, non-growing animals consuming quantities of feed sufficient to meet only maintenance energy needs. Very little research has been conducted that examines the relationships between mitochondrial function and whole body growth in young, growing animals raised under normal production settings. If a significant degree of variation in mitochondrial function is observed among animals, then the ability to alter this variation in a beneficial direction is more likely to exist. Bilobalide and proanthocyanidins are two compounds previously shown to positively alter rat liver mitochondrial function when administered either in vivo or in vitro, respectively. However, the effects of these two compounds on muscle mitochondria that have the greatest rates of proton leak and are responsible for the largest percentage of whole body oxygen is not known. Finally, an important question that requires exploration is which protein or proteins of the approximately 1500 proteins in mitochondria account for the greatest degree of variability in mitochondrial function. Previous modeling efforts have indicated that ANT1 may be one important protein that regulates mitochondrial energy production. Therefore,

associations between variation in tissue concentrations of this protein and mitochondrial function and whole body growth should be examined.

Literature Cited

Anderson S., A.T. Bankier, B.G. Barrell, M.H.L. De Bruijn, A.R. Coulson, J. Drouin. 1981. Sequence and organization of human mitochondrial genome. Nature 290: 457-465.

Baar, K., A.R. Wende, T.E. Jones, M. Marison, L.A. Nolte, M. Chen, D.P. Kelly and J.O. Holloszy. 2002. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB J. 16: 1879-1886.

Bach, D., S. Pich, F.X. Soriano, N. Vega, B. Baumgartner, J. Oriola, J.R. Daugaard, J. Lloberas, M. Camps, J.r. Zierath, R. Rabasa-Lhoret, H. Wallberg-Henriksson, M. Laville, M. Palacin, H. Vidal, F. Rivera, M. Brand, and A. Zorzano. 2003. Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism a novel regulatory mechanism altered in obesity. J. Biol. Chem. 278: 17190-17197.

Bakker, H.D., H.R. Scholte, C. Van den Bogert, W. Ruitenbeek, J.A. Jeneson, R.J. Wanders, N.G. Abeling, B. Dorland, R.C. Sengers, A.H. Van Gennip. 1993. Deficiency of the adenine nucleotide translocator in muscle of a patient with myopathy and lactic acidosis: a new mitochondrial defect. Pediatr. Res. 33: 412-417.

Birky, C.W. Jr. 1995. Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. Proc. Natl. Acad. Sci. 92: 11331-11338.

Bizeau, M.E., W.T. Willis and J.R. Hazel. 1998. Differential responses to endurance training in subsarcolemmal and intermyofibrillar mitochondria. J. Appl. Physiol. 85: 1279-1284.

Blaxter K. 1989. Energy metabolism in animals and man. Cambridge University Press, Great Britain.

Bottje, W., Z.X. Tang, M. Iqbal, D. Cawthon, R. Okimoto, T. Wing and M. Cooper. 2002 Association of mitochondrial function with feed efficiency within a single genetic line of male broilers. Poult. Sci. 81: 546-555.

Boveris, A., N. Oshino and B. Chance. 1972. The cellular production of hydrogen peroxide. Biochem. J. 128: 617-630.

Brand, M.D. 1995. The stoichiometry of proton pumping and ATP synthesis in mitochondria. Biochemist 16: 20-24.

Brand, M.D., L. Chien, E.K. Ainscow, D.F.S. Rolfe and R.K. Porter. 1994. The causes and functions of mitochondrial proton leak. Biochim. Biophys. Acta 1187: 132-139.

Brookes, P.S., J. Lands, J. Clark, S. Heales. 1998. Peroxynitrite and brain mitochondria: evidence for increased proton leak. J. Neurochem. 70: 2195-2202.

Brown, G.C. 1992. Control of respiration and ATP synthesis in mammalian mitochondria and cells. Biochem. J. 284: 1-13.

Brown, G.C. 1999. *The Energy of Life*. Harper-Collins, London.

Callahan, L.A., D.A. Stofan, L.I. Szweda, D.E. Nethery, and G.S. Supinski. 2001. Free radicals alter maximal diaphragmatic mitochondrial oxygen consumption in endotoxin-induced sepsis. Free Radic. Biol. Med. 30: 129-138.

Clapham J.C., J.R. Arch, H. Chapman, A. Haynes, C. Lister, G.G. Moore, V. Piercy, S.A. Carter, I. Lehner, S.A. Smith, L.J. Beeley, R.J. Godden, N. Gerrity, M. Skehel, K.K. Changani, P.D. Hockings, D.G. Reid, S.M. Squires, J. Hatcher, B. Trail, J. Latcham, S. Rastan, A.J. Harper, S. Cadenas, J.A. Buckingham, M.D. Brand and A. Abuin. 2000. Mice over expressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. Nature 406: 415-418.

Cogswell, A.M., R.J. Stevens, D.A. Hood. 1993. Properties of skeletal muscle mitochondria isolated from subsarcolemmal and intermyofibrillar regions. Am. J. Physiol. 264: 383-389.

Cortopassi, G.A., D. Shibata, N.W. Soong, N. Arnheim. 1992. A pattern of accumulation of a somatic deletion of mitochondrial DNA in aging human tissues. Proc. Natl. Acad. Sci. 89: 7370–7374.

Cummins, J.M., T. Wakayama and R. Yanagimachi. 1997. Fate of microinjected sperm components in the mouse oocyte and embryo. Zygote 5: 301-308.

De Vos, K., F. Severin, F. Van Herreweghe, K. Vancompernolle, V. Goossens, A. Hyman and J. Grooten. 2000. Tumer necrosis factor induces hyperphosphorylation of kinesin light chain and inhibits kinesin-mediated transport of mitochondria. J. Cell Biol. 149: 1207-1214.

Dzapo, V.V. and R. Wassmuth. 1983. Mitochondrial metabolism and heterotic effects in pig. Results of a reciprocal crossbreeding experiment. II. Activity of oxygen uptake of cells and oxidative phosphorylation in heart, liver and scrotal mitochondria. Zeitschr. Tierz. Zuchtungsbiol. 100: 280-295.

Estabrook, 1967. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Meth. Enzymol. 10:41-47.

Ewan, R.C. 2001. Energy utilization in swine nutrition. In: A.J. Lewis and L.L. Southern (Ed.) *Swine Nutrition*. CRC Press, Boca Raton, Florida.

Farrar, R.P., T.P. Martin and C.M. Ardies. 1981. The interaction of aging and endurance exercise upon the mitochondrial function of skeletal muscle. J. Gerontol. 36: 642-647.

Fell, D.A. 1998. Increasing the flux in metabolic pathways: A metabolic control analysis perspective. Biotechnol. Bioeng. 58: 121-124.

Garesse, R. and C.G. Vallejo. 2001. Animal mitochondrial biogenesis and function: a regulatory cross-talk between two genomes. Gene 263: 1-16.

Graham, B.H., K.G. Way mire, B. Cottrell, I.A. Trounce, G.R. Macgregor, D.C. Wallace. 1997. A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoform of the adenine nucleotide translocator. Nat. Gen. 16: 226-234.

Harper, M-E., R. Dent, S. Monemdjou, V. Bezaire, L. Van Wyck, G. Wells, G.N. Kavaslar, A. Gauthier, F. Tesson and R. McPherson. 2002. Decreased mitochondrial proton leak and reduced expression of uncoupling protein 3 in skeletal muscle of obese diet-resistant women. Diabetes. 51: 2459-2466.

Harper, M-E., S. Monemdjou, J.J. Ransey, and R. Weindruch. 1998. Age-related increase in mitochondrial proton leak and decrease in ATP turnover reactions in mouse hepatocytes. Am. J. Physiol. 275: E197-E206.

Hinkle, P.C., M.A. Kumar, A. Resetar and D.L. Harris. 1991. Mechanistic stoichiometry of mitochondrial oxidative-phosphorylation. Biochemistry 30: 3576-3582.

Iossa, S., L. Lionetti, M.P. Mollica, R. Crescenzo, M. Botta, S. Samec, A.G. Dulloo and G. Liverini. 2001. Differences in proton leak kinetics, but not in UCP3 protein content, in subsarcolemmal and intermyofibrillar skeletal muscle mitochondria from fed and fasted rats. FEBS Lett. 505: 53-56.

Iossa, S., M.P. Mollica, L. Lionetti, R. Crescenzo, M. Botta, G. Liverini. 2002. Skeletal muscle oxidative capacity in rats fed high-fat diet. Int. J. Obes. Relat. Metab. Disord. 26: 65-72.

Janssens D., E. Delaive, A. Houbion, F. Eliaers, J. Remacle and C. Michiels. 2000. Effect of venotropic drugs on the respiratory activity of isolated mitochondria and in endothelial cells. Br. J. Pharmacol. 130: 1513-1524.

Janssens D., Michiels C., Delaive E., Eliaers F., Drieu K., and Remacle J. 1995. Protection of hypoxia-induced ATP decrease in endothelial cells by Ginkgo biloba extract and bilobalide. Biochem. Pharmacol. 50: 991-999.

Janssens D., J. Remacle, K. Drieu, and C. Michiels. 1999. Protection of mitochondrial respiration activity by bilobalide. Biochem. Pharmacol. 58: 109-119.

Korzeniewski, B. 2002. Effect of enzyme deficiencies on oxidative phosphorylation: from isolated mitochondria to intact tissues. Theoretical studies. Mol. Biol. Rep. 29: 197-202.

Korzeniewski, B. and J.P. Mazat. 1996. Theoretical studies on the control of oxidative phosphorylation in muscle mitochondria: application to mitochondrial deficiencies. Biochem. J. 319: 143-148.

Kristal, B., S. Koopmans, C.T. Jackson, Y. Ikeno, B. Par, and B.P. Yu. 1997. Oxidant-mediated repression of mitochondrial transcription in diabetic rats. Free Rad. Biol. Med. 22: 813-822.

Kristal, B., B. Park, and B.P. Yu. 1994. Antioxidants reduce peroxyl-mediated inhibition of mitochondrial transcription. Free Rad. Biol. Med. 16: 653-660.

Lal, S.B., J.J. Ramsey, S. Monemdjou, R. Weindruch , and M-E Harper. 2001. Effects of caloric restriction on skeletal muscle mitochondrial proton leak in aging rats. J. Gerontol. 56: B116-B122.

Lane, R.H., A.K. Chandorkar, A.S. Flozak, and R.A. Simmons. 1998. Intrauterine growth retardation alters mitochondrial gene expression and function in fetal and juvenile rat skeletal muscle. Pediatr. Res. 43: 563-570.

Lardy H.A. and H. Wellman H. 1952 Oxidative phosphorylations: Role of inorganic phosphate as an acceptor system in control of metabolic rates. J. Biol. Chem. 195: 215–224.

Lavoisier, A.L. 1777. Experiences sur la decomposition de l'air dans le poulmon, et sur un des principaux usages de la respiration dans l'economie animale. Archives de l'Academie des Sciences.

Lavoisier, A.L. and P.S. Laplace. 1783. Memoire sur la chaleur. Memoires de l'Academie des Sciences.

Lemasters, J.J., A.L. Nieminen, T. Qian, L.C. Trost and B. Herman. 1997. The mitochondiral permeability transition in toxic, hypoxic, and reperfusion injury. Mol. Cell. Biochem. 174: 159-165.

Li, K., C.K. Warner, J.A. Hodge, S. Minoshima, J. Kudoh, R. Fukuyama, M. Maekawa, Y. Shimizu, N. Shimizu, D.C. Wallace. 1989. A human muscle nucleotide translocator gene has four exons, is located on chromosome 4, and is differentially expressed. J. Biol. Chem. 264: 13998-14004.

Lin, J., H. Wu, P.T. Tarr, C. Zhang, Z. Wu, O. Boss, L.F. Michael, P. Pulgserver, E. Isotani, E. Olson, B.B. Lowell, R. Bassei-Duby and B.M. Spiegelman. 2002. Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibres. Nature 418: 797-801.

Lipsky, N.G. and P.L. Pedersen. 1981. Mitochondrial turnover in animal cells. Half-lives of mitochondria and mitochondrial subfractions of rat liver based on [14C]bicarbonate incorporation. J. Biol. Chem. 256: 8652-8657.

Liu, S.S. 1999. Cooperation of a "reactive oxygen cycle" with the Q cycle and the proton cycle in the respiratory chain- superoxide generating and cycling mechanisms in mitochondria. J. Bioenerg. Biomembr. 31: 367-376.

Lombardi A, M. Damon, A. Vincent, F. Goglia, P. Herpin. 2000. Characterisation of oxidative phosphorylation in skeletal muscle mitochondria subpopulations in pig: a study using top-down elasticity analysis. FEBS Lett. 475: 84-88.

Lopez, M.F., B.S. Kristal, E. Chernokalskaya, A. Lazarev, A.I. Shestopalov, A. Bogdanova, M. Robinson. 2000. High-throughput profiling of the mitochondrial proteome using affinity fractionation and automation. Electrophoresis 21: 3427-3440.

Malgat, M., T. Letellier, R. Rossignol and J.P. Mazat. 2000. Metabolic control analysis and threshold effect in oxidative phosphorylation: study in an intermediate state of respiration. Conference Programme of the 9th International BioTheroKinetics Meeting, Stellenbosch, South Africa pp.125-134.

Marin-Garcia, J., and M.J. Goldenthal. 2002. The mitochondrial organelle and the heart. Rev. Esp. Cardiol. 55: 1293-1310.

Masini, A., D. Ceccarelli-Stanzani and U. Muscatello. 1983. The effect of oligomycin on rat liver mitochondria respiring in state 4. FEBS Lett. 160: 137-140.

Mazat, J.P., R. Rossignol, M. Malgat, C. Rocher, B. Faustin, T. Letellier. 2001. What do mitochondrial diseases teach us about normal mitochondrial functions...that we already knew: threshold expression of mitochondrial defects. Biochim. Biophys. Acta 1504: 20-30.

Mukherjee, T.K., R.W.C. Stevens and M.P. Hoogendoorn. 1970. Oxygen uptake of mitochondrial isolates from two breeds of chickens and their F1 cross. Poult. Sci. 49: 1130-1131.

Nedergaard, J. and B. Cannon. 2003. The 'novel' 'uncoupling' proteins UCP2 and UCP3: what do they really do? Pros and cons for suggested functions. Exp. Physiol. 88: 65-84.

Nicholls, D.G. and R.M. Locke. 1984. Thermogenic mechanisms in brown fat. Physiol. Rev. 64: 1-64.

Nichols, D. 1992. *Bioenergetics 2.* Academic Press Limited, San Diego.

Nichols, D. 2002. *Bioenergetics 3.* Academic Press Limited, San Diego.

Ning, X.H., J. Zhang, J. Liu Y. Ye, S.H. Chen, A.H.L. From, R.J. Bache, M.A. Portman. 2000. Signaling and expression for mitochondrial membrane proteins during left ventricular remodeling and contractile failure after myocardial infarction. J. Am. Coll. Cardiol. 36: 282-287.

Nogueira, V., M. Rigoulet, M-A Piquet, A. Devin, E. Fontaine and X.M. Leverve. 2001. Mitochondrial respiratory chain adjustment to cellular energy demand. J. Biol. Chem. 276: 46104-46110.

Ogata, E.S., M.E. Bussey, A. LaBarbera, S. Finley. 1985. Altered growth, hypoglycemia hypoalaninemia, and ketonemia in the young rat: postnatal consequences of intrauterine growth retardation. Pediatr. Res. 19: 32-37.

Olszanecki, R. and S. Chlopicki. 1999. Endotoxaemia in rats: role of NO, PAF, and TXA2 in pulmonary neutrophil sequestration and hyperlactataemia. J. Physiol. Pharmacol. 50: 443-454.

Opalka, J.R., M. Wicke, F.N. Gellerich, S. Zierz and G. von Lengerken. 2000. Influence of mitochondrial function on growth and meat quality in turkey. Eur. J. Med. Res. 5 (Suppl I): 57.

Palmer, J.W., B. Tandler, C. Hoppell. 1985. Biochemical differences between subsarcolemmal and interfibrillar mitochondria from rat cardiac muscle: effects of procedural manipulations. Arch. Biochem. Biophys. 236: 691-702.

Pedersen, P.L. 1999. Mitochondrial events in the life and death of animal cells: A brief overview. J. Bioenerg. Biomembr. 31: 291-304.

Philippi, M. and A.H. Sillau. 1994. Oxidative capacity distribution in skeletal muscle fibers of the rat. J. Exp. Biol. 189: 1-11.

Porter, R.K. 2001. Mitochondrial proton leak: a role for uncoupling proteins 2 and 3? Biochim. Biophys. Acta 1504: 120-127.

Porter, R.K. and M.D. Brand. 1993. Body mass dependence of H^+ leak in mitochondria and its relevance to metabolic rate. Nature 362: 628-630.

Powell, S.E. and E.D. Aberle. 1980. Effects of birth weight on growth and carcass composition of swine. J. Anim. Sci. 50: 860-868.

Pumford N.R., M. Iqbal , D. Cawthon, W. Bottje. 2002. Increased protein oxidation and differences in protein expression in muscle mitochondria in animals with lower feed efficiency. Free Rad. Biol. Med. 33 (Suppl 1): 89.

Ramsey, J.J., M-E Harper, and R. Weindruch. 2000. Restriction of energy intake, energy expenditure and aging. Free Rad. Biol. Med. 29: 946-968.

Ramsey, J.J., D.E. Johnson, K.L. Hossner, K.A. Johnson. 1996. Metabolic rate, organ mass, and mitochondrial proton leak variations in lean and obese rats. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 113: 461-466.

Rolfe, D.F.S and M.D. Brand. 1996. Contribution of mitochondrial proton leak to skeletal muscle respiration and to standard metabolic rate. Am. J. Physiol. 271: C1380-C1389.

Rolfe, D.F.S. and G.C. Brown. 1997. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol. Rev. 77: 731-758.

Rolfe, D.F., A.J. Hulbert, M.D. Brand. 1994. Characteristics of mitochondrial proton leak and control of oxidative phosphorylation in the major oxygen-consuming tissues of the rat. Biochim. Biophys. Acta. 1188: 405-416.

Rolfe D.F., J.M. Newman, J.A. Buckingham, M.G. Clark, M.D. Brand MD. 1999. Contribution of mitochondrial proton leak to respiration rate in working skeletal muscle and liver and to SMR. Am. J. Physiol. 276: 692-699.

Rossignol, R., T. Letellier, M. Malgat, C. Rocher and J.P. Mazat. 2000. Tissue variation in the control of oxidative phosphorylation: implication for mitochondrial diseases. Biochem. J. 347: 45-53.

Rossignol, R. J. Malgat, J.P. Mazat and T. Letellier. 1999. Threshold effect and tissue specificity. Implication for mitochondrial cytopathies. J. Biol. Chem. 274: 33426-33432.

Schapira A.H. 1997. Mitochondrial disorders: an overview. J. Bioenerg. Biomembr. 29:105-107.

Scheffler, I.E. 1999. *Mitochondria*. J. Wiley and Sons, New York.

Schwartz, M. and J. Vissing. 2002. Paternal inheritance of mitochondrial DNA. N. Engl. J Med. 347: 576-580.

Selak, M.A., B.T. Storey, I. Peterside, and R.A. Simmons. 2003. Impaired oxidative phosphorylation in skeletal muscle of intrauterine growth retarded rats. Am. J. Physiol. Endocrinol. Metab. 285: 130-137.

Shi, Q.D., Y. Zhang, J.Q. Chen, S.S. Liu. 1999. Electron leak causes proton leak in skeletal muscle mitochondria in exercise-induced fatigue. Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao. 31: 97-100.

Soloman, E.P., L.R. Berg, D.W. Martin. 2002. Biology. 6th ed. Harcourt College Publishers.

Stacpoole, P.W. 1997. Lactic acidosis and other mitochondrial disorders. Metabolism 46: 306-321.

Stuart, J.A., S. Cadenas, M.B. Jekabsons, D. Roussel, M.D. Brand. 2001. Mitochondrial proton leak and the uncoupling protein 1 homologues. Biochim. Biophys. Acta 1504: 144-158.

Sun Q.Y., G.M. Wu, L. Lai, K.W. Park, R. Cabot, H.T. Cheong, B.N. Day, R.S. Prather and H. Schatten. 2001. Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development in vitro. Reproduction 122: 155-163.

Tonkonogi, M. and K. Sahlin. 1999. Actively phosphorylating mitochondria are more resistant to lactic acidosis than inactive mitochondria. Am. J. Physiol. 277: 288-293.

Tonkonogi, M., B. Walsh, M. Svensson, and K. Sahlin. 2000. Mitochondrial function and antioxidative defense in human muscle: effects of endurance training and oxidative stress. J. Physiol. 528: 379-388.

Turrens, J.F., A. Alexandre and A.L. Lehninger. 1985. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch. Biochem. Biophys. 237: 408-414.

Tyler, D.D. 1992. *The mitochondrion in health and disease.* VCH Publisher, Inc., New York.

Vandenburgh, H.H. and S. Kaufman. 1981. Stretch-induced growth of skeletal myotubes correlates with activation of the sodium pump. J. Cell. Physiol. 109: 205-214.

Vieira, H.L., A.S. Belzacq, D. haouzi, F. Bernassola, I. Cohen, E. Jocotot, K.F. Ferri, C. El Hamel, L.M. Bartle, G. Melino, C. Brenner, V. Goldmacher, G. Kroemer. 2001. The adenine nucleotide translocator: a target of nitric oxide, peroxynitrite, and 4-hydroxynonenal. Oncogene 20: 4305-4316.

Weindruch, R. and R.L. Walford. 1988. The retardation of aging and disease by dietary restriction. Springfield, IL: CC Thomas.

Weindruch, R., R.S. Sohal.. 1997. Caloric intake and aging. N. Engl. J. Med. 337: 986-994.

Wojtczak, L. and P. Schonfeld. 1993. Effect of fatty acids on energy coupling processes in mitochondria. Biochim. Biophys. Acta 1183: 41-57.

Wolanis, M., V. Dzapo and R. Wassmuth. 1980. The determination of biochemical parameters of energy metabolism and their relationships with vitality, fattening performance and carcass quality in sheep. 2. Respiration activity and oxidative phosphorylation of isolated diaphragm mitochondria. Z. Teriz. Zuechtungsbiol. 97: 28-36.

Yajid, F., J.G. Mercier, B.M. Mercier, H. Dubouchaud and C. Prefaut. 1998. Effects of 4 wk of hind limb suspension on skeletal muscle mitochondrial respiration in rats. J. Appl. Physiol. 84: 479-485.

CHAPTER 3. QUANTITATIVE RELATIONSHIP BETWEEN MITOCHONDRIAL FUNCTION AND EFFICIENCY OF ANIMAL GROWTH

A paper to be submitted to *Journal of Animal Science*
T.R. Lutz and T.S. Stahly

Abstract

Variation in muscle and liver mitochondrial energy production among animals from a single strain, gender and rearing environment was quantified, and the relationship of these differences to the efficiency and rate of body growth were evaluated. Male Sprague Dawley rats, initially weighing 54 grams, were individually penned and allowed to consume a nutritionally adequate diet ad libitum for 20 ± 2 days. Body weight gains and feed intakes of each rat (n = 43) were quantified, and mitochondria from the gastrocnemius muscle (n = 43) and liver (n = 16) were isolated and their mitochondrial protein content and State 4 (proton leak-dependent respiration) and State 3 (maximal respiration rate) oxygen consumption rates per unit of mitochondrial protein were determined. The mitochondrial RCR (ratio State3/State4), a measure of mitochondrial metabolic efficiency, also was calculated. Measures of mitochondrial energy production in the gastrocnemius muscle were correlated with the efficiency of feed utilization for BW gain. Specifically, lower rates of mitochondrial proton leak-dependent respiration in the muscle ($r = 0.42$, $P < 0.01$) or improved mitochondrial metabolic efficiencies in muscle ($r = 0.33$, $P < 0.05$) were associated with improved BW gain/feed ratios. In addition, rats with a lower muscle mitochondrial protein content exhibited improved efficiencies of feed utilization ($r = 0.43$, $P < 0.01$) and improved rates of growth ($r = 0.31$, $P < 0.05$). Based on multiple regression analysis, differences among animals in muscle mitochondrial protein content and proton leak-dependent respiration were additive and inversely associated with gain/feed and accounted for a significant portion of the

variation in this parameter ($R^2 = 0.33$, $P < 0.01$). These data establish that the efficiency of mitochondrial energy production and protein content in skeletal muscle influence the efficiency of feed utilization for animal growth.

Introduction

Mitochondria are the major energy producing organelles in mammalian cells and in this capacity are responsible for 90% of cellular oxygen consumption (Rolfe and Brown, 1997). Because mitochondria are so important to cellular energy production, variation in the rate and efficiency of energy production in the mitochondria and/or the number of mitochondria may be quantitatively important to both the rate and efficiency of whole body growth.

Proton leak is a process that leads to inefficiency in mitochondrial energy production and occurs when there is transfer of hydrogen ions between the mitochondrial membranes at a site other than ATP synthase. It has been estimated in adult rats that 25% of whole body oxygen consumption is utilized to balance mitochondrial proton leak during resting metabolic rate conditions with skeletal muscle being the largest contributor to this process (Rolfe and Brand, 1996). However, in growing animals, the relative importance of this mitochondrial inefficiency to whole body energetic efficiency has not been characterized. Furthermore, the importance of differences in mitochondria number among animals and efficiency of whole body growth has not been evaluated. With the recent identification of some of the genetic mechanisms controlling mitochondiral biogenesis as well as biological processes altering proton leak, future advantageous regulation of these processes may be possible.

The objectives of this research were to quantify the variation in muscle and liver mitochondrial protein content and energy production among growing rats from a single strain, gender and rearing environment and to determine the impact of these differences on whole body energetic efficiency and growth.

Materials and Methods

Animal management

Male Sprague Dawley weanling rats (20 d of age) were penned individually in cages on wire mesh floors. Rats were maintained on a 12 h light-dark cycle in rooms with an average temperature and relative humidity of 23 ± 0.5°C and 70 ± 7%, respectively. Rats (n = 43) weighing 54 ± 6 g at the initiation of the test were allowed ad libitum access to a diet (Table 1) formulated to meet or exceed all nutrient requirements of the rat (NRC, 1995). Body weight, feed consumption and feed wastage data were collected every two days. After 20 ± 2 days, rats weighing on average 195 ± 15 g were decapitated at 0900. The liver (n = 16) and gastrocnemius muscle (n = 43) were immediately removed, weighed and placed in cooled isolation media (IM) that contained (in mM) 100 sucrose, 10 EDTA, 100 Tris-HCl and 46 KCl, pH=7.4. The experimental protocol was approved by the Institutional Committee on Animal Care at Iowa State University.

Isolation of gastrocnemius muscle and liver mitochondria

The gastrocnemius muscle mitochondria were isolated as described by Bhattacharya et al. (1991). First, the muscle sample was minced on a cooled glass plate and resuspended in IM at a concentration of 10% (wt/vol). The IM contained 0.5% (wt/vol) fatty acid-free bovine serum albumin, which was used to bind free fatty acids liberated during tissue mincing and homogenization. The mince was then incubated with Nagarse (0.2 mg/ml;

Sigma, St. Louis, MO) at room temperature for 5 minutes while stirring to liberate the intermyofibrillar mitochondria. The mince was then homogenized using three, five-second bursts of a Polytron tissue homogenizer (Brinkmann, Westbury, NY) to liberate the subsarcolemmal mitochondria that was followed by a further five-minute incubation on ice. Liver mitochondria were isolated by first mincing the tissue on a cooled glass plate and then placing the mince in IM containing 0.5% fatty acid-free bovine serum albumin. The mixture was then homogenized utilizing five strokes of a Potter-Elvehjm tissue homogenizer. Both the gastrocnemius and liver homogenates were centrifuged at 750 x g for 10 min at 4°C to pellet the cellular debris. The supernatant was removed and centrifuged at 10,000 x g at 4°C for 10 min to pellet the mitchondrial fraction. The mitochondrial pellets were then resuspended in IM and again centrifuged at 10,000 x g at 4°C for 10 min. Finally, the mitochondrial pellet was resuspended in reaction media (0.2 µl/mg muscle or liver weight) that contained (in mM) 15 KCl, 30 K_2HPO_4, 25 Tris base, 45 sucrose, 12 mannitol, 5 $MgCl_2$ and 7 EDTA, pH=7.4.

Mitochondrial oxygen consumption

Mitochondrial oxygen consumption measurements were determined in triplicate using a Clark-type oxygen electrode and a thermostatically controlled chamber equipped with magnetic stirring (Yellow Springs Instrument Co., Inc., Yellow Springs, OH). Mitochondria were incubated in the respiration chamber at 30°C that contained 1.1 ml of reaction media with 0.2% (wt/vol) fatty acid-free bovine serum albumin and glutamate:malate (10:5 mM), which donates electrons to the respiratory chain at Complex I. Prior to initiation of this study, oxygen concentration of the reaction media was determined (410 nmol O/ml at 760 mm Hg and 30°C) according to a previously published method to calculate mitochondrial

oxygen consumption (Robinson and Cooper, 1970). Approximately, 0.5 mg of gastrocnemius or 2 mg of liver mitochondrial protein and either 165 nmol (gastrocnemius) or 110 nmol (liver) ADP was infused into the incubation chamber by using a Hamilton syringe. The final concentration of ADP in the reaction chamber that contained muscle or liver mitochondria was 146 and 95 μM, respectively. The administration of ADP stimulates State 3 (maximum) respiration and upon conversion of ADP to ATP the mitochondria return to a resting rate of respiration termed State 4 (Chance and Williams, 1956; Estabrook, 1967). Oxygen consumption during State 4 is principally utilized to balance proton leak, and it has been argued that in the absence of proton leak State 4 respiration would be zero, hence the term proton leak-dependent respiration (Brand et al., 1994). The respiratory control ratio (RCR), which is an index of mitochondrial metabolic efficiency or coupling, was calculated as State 3 divided by State 4 respiration (Estabrook, 1967). Protein concentration of each mitochondrial suspension was determined in duplicate with the Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA) utilizing a bovine gamma globulin standard. Oxygen consumption was expressed as nmol $O \cdot min^{-1} \cdot$mg mitochondrial $protein^{-1}$. In a preliminary assay, intra-assay coefficients of variation for State 3 and 4 of 6 and 9%, respectively, were obtained.

Statistical analysis

Correlations between of body weight gain, feed intake and efficiency of feed utilization and the various measures of mitochondrial function and protein content were analyzed by using the PROC CORR procedure of SAS (SAS Inst. Inc., Cary, NC). In addition, multiple regression analysis using backward-stepwise regression was utilized to further quantitate relationships between mitochondrial State 3 and 4 respiration and

mitochondrial protein content with measures of body weight gain, feed intake and efficiency of feed utilization. Two- and three- way interactions and quadratic terms for the various mitochondrial measurements were evaluated in the model. Variables that were not significant ($P > 0.10$) were removed from the regression model. Differences in mitochondrial measurements between the gastrocnemius muscle and liver were analyzed by analysis of variance techniques using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC).

Results and Discussion

Rats grew well throughout the duration of the experiment, exhibiting a body weight 20 g heavier at the termination of the experiment (20 ± 2 d of age) than that estimated from growth curve data provided by the supplier (Harlan, Indianapolis, IN). Daily body weight gains and efficiency of feed utilization (BW gain/feed) averaged 6.9 g and 465 g/kg, respectively, over the duration of the 20-d experiment (Table 2). As expected for rats from a single genetic strain, gender and rearing environment, variation in daily gains and gain/feed ratios were low with coefficient of variations of 7 and 6%, respectively. Body weight gains exhibited a quadratic pattern as the rats matured (Fig. 1). Specifically, daily body weight gains increased during the first 12 days of the experiment, plateaued on days 12 through 16 and then declined for the remainder of the experiment. Efficiency of feed utilization measured as g of body weight gain per kg of feed intake declined throughout the course of the experiment as the animals matured (Figure 1). These patterns of body weight gain and feed utilization reflect the shift toward greater deposition of body fat (an energetically costly tissue) and the lesser deposition of muscle as the rat matures from weaning to 195 g BW.

Mitochondrial rates of proton leak-dependent respiration (State 4) and the rates of maximal mitochondrial respiration (State 3) as well as metabolic efficiency of the

mitochondria (RCR) were higher in the gastrocnemius muscle than liver (Table 2). However, mitochondrial protein content (indicator of mitochondria number) was only about one-third in the gastrocnemius muscle versus liver. Based on the proton leak-dependent rate of oxygen consumption (nmol $O \cdot min^{-1} \cdot mg$ mitochondrial $protein^{-1}$) and the mitochondrial protein content (mg/g of tissue) in the gastrocnemius muscle and liver, it is estimated that the oxygen consumed to balance proton leak in the whole body muscle mass of the rat would be 11 times greater than that of the liver. The calculations are based on the assumptions that muscle represents 44% of the rats' body weight (Even et al., 2001), the gastrocnemius, a mixed fiber muscle, is representative of the body muscle mass (Armstrong and Phelps, 1984) and a liver weight of 11 g (current study). Based on these data, shifts in proton leak-dependent respiration in skeletal muscle would be expected to have the greatest effect on efficiency of feed utilization for body growth in rapidly growing animals.

Previous research in rats and mice has indicated that increased rates of proton leak-dependent respiration in skeletal muscle mitochondria result in increased metabolic rates or reductions in food utilization efficiency (Clapham et al., 2000; De Lange et al., 2001). However, these observations were made under highly perturbed physiological situations with proton leak induced either by thyroid hormone administration or via a 66-fold overexpression of uncoupling protein-3. Furthermore, the above observations were made in adult animals with low ATP tissue demands for tissue growth, which would exacerbate the proportion of energy utilized to balance proton leak because of an increased mitochondrial membrane potential (Nicholls, 2002). In resting muscle and liver with a low demand for ATP, proton leak accounts for 50 and 26% of muscle and liver oxygen consumption, respectively (Brand et al., 1994; Rolfe and Brand, 1996). However, the contribution of proton leak to muscle

and liver oxygen consumption falls to 34 and 22%, respectively, when doubling ATP tissue demand by either introducing a contraction to the muscle or inducing gluconeogenesis and ureagenesis in the liver (Rolfe et al., 1999). Growing animals are fueling the synthesis of large amounts of proteinaceous tissue (e.g., muscle) and other cellular processes, resulting in a high rate of ATP demand and production. Increased ATP production lowers the mitochondrial membrane potential; so, in theory, proton leak should account for a lower proportion of the total energy budget in young, rapidly growing animals, particularly in muscle. Furthermore, proton leak-dependent respiration in liver mitochondria has been shown to increase as an animal ages potentially due to increases in free radical damage to the mitochondria (Goodell and Cortopassi, 1998; Harper et al., 1998). To our knowledge, the quantitative relationship between proton leak-dependent respiration in muscle mitochondria and efficiency of feed utilization in growing mammals consuming feed ad libitum has not been previously evaluated.

Mitochondrial rates of proton leak-dependent respiration and other measures of mitochondrial function and protein content were demonstrated to vary among growing animals of the same genetic strain and gender and reared under similar environmental conditions (Table 2). The magnitude of variation in mitochondrial proton leak-dependent respiration (CV of 15%) was similar or less than that previously reported in muscle of growing or aged rats (Wardlaw and Kaplan, 1984; Wardlaw et al., 1986; Lal et al., 2001).

In the current study, mitochondrial proton leak-dependent respiration rates and protein content were determined to be quantitatively associated with the efficiency of feed utilization for body growth. Specifically, lower rates of mitochondrial proton leak-dependent respiration (State 4) and improved mitochondrial metabolic efficiencies (RCR) in muscle

were associated with improved BW gain/feed ratios in these growing animals (Table 3). Rates of proton leak-dependent respiration in muscle mitochondria tended ($P = 0.07$) to be associated with voluntary feed intakes but not daily body weight gains. Maximal rates of mitochondrial respiration (State 3) in gastrocnemius muscle were not associated with efficiency of feed utilization, feed intake, or body weight gain. The lack of relationship between maximal rates of mitochondrial respiration and body growth may be due in part to the stage of growth monitored. As animals mature, body weight gains become less responsive to changes in amounts of energy available due to shifts in hormone status as well as in the amounts of water accreted in proteinaceous tissues (Iossa et al., 1999).

An association between the mitochondrial protein content of muscle and efficiency of feed utilization for body growth was evident (Table 3). Lower mitochondrial protein content in muscle was associated with improved efficiencies of feed utilization as well as rates of body weight gain (Table 3). This inverse relationship between gastrocnemius muscle mitochondrial protein content, an estimate of mitochondria number, and efficiency of feed utilization is an interesting finding and could potentially be important for developing technologies that improve the energetic efficiency of animal growth. In addition, mitochondria from the gastrocnemius muscle of rats with reduced mitochondrial contents also had a higher rate of State 3 respiration (Table 4). Thus, it seems that muscle with fewer, but more metabolically active, mitochondria may be advantageous in promoting improvements in the energetic efficiency of whole body growth.

Differences in mitochondrial protein content may have occurred due to variations in muscle fiber type, exposure to, and/or ability to squelch reactive oxygen species or to differences among animals in the expression of key genes regulating mitochondrial

biogenesis. The gastrocnemius muscle was utilized in our studies because it is a mixed fiber type muscle and suitably represents the composition of all skeletal muscle fiber types in the rat (Armstrong and Phelps, 1984). The muscle fiber composition of gastrocnemius muscle is 78% fast-twitch glycolytic, 17% fast-twitch oxidative glycolytic and 5% slow-twitch oxidative and the mitochondrial content of these different types of fibers is known to vary with fast-twitch glycolytic fibers having the lowest number of mitochondria (Armstrong and Phelps, 1984; Jackman and Willis, 1996). So, rats with improved efficiencies of feed utilization may simply have had a greater proportion of glycolytic fibers along with the associated reduction in mitochondrial content of this fiber type because selection pressure for greater muscle mass and improved efficiencies of feed utilization has been shown to result in a greater glycolytic fiber content of skeletal muscle (Karlsson et al., 1993).

Mitochondrial biogenesis also is induced by reactive oxygen species (ROS) exposure (Miranda et al., 1999; Lee et al., 2002). Thus, rats with poor efficiencies of feed utilization and high muscle mitochondrial protein contents may have experienced greater exposure to ROS or may have had lower endogenous antioxidant capability. In the current study, rats were reared in a single environment, fed diets supplemented with three times the estimated requirement for the antioxidant vitamins A and E, and exhibited no visually apparent health problems which would be expected to minimize mitochondrial production of ROS.

Finally, differences in the inherent expression of genes regulating mitochondrial biogenesis may have altered muscle mitochondria concentration among animals even though all animals were from a single strain. This is especially relevant with the recent discovery of the peroxisome proliferator-activated receptor γ coactivator 1α protein, a master regulator of

mitochondrial biogenesis, and a potential target for genetic manipulation or bioactive compounds in food-producing animals (Lin et al., 2002, Nisoli et al., 2003).

Mitochondrial proton leak-dependent respiration was not related to mitochondrial maximum respiration rate or protein content (Table 4). Thus, improvements in the efficiency of body growth associated with lower proton leak-dependent respiration are not due to differences in the inherent capacity of the mitochondrial respiratory chain to produce energy but rather to the efficiency of this energy production. Previous research has indicated that as mitochondrial biogenesis increases the rate of proton leak per unit of mitochondrial protein also increases (St-Pierre et al., 2003). However, in this study, even though mitochondrial protein content was related to the efficiency of body growth, changes in the amount mitochondrial protein were not associated with proton leak-dependent respiration rates per unit of mitochondrial protein.

The amount of potential energy lost per gram of whole muscle as proton leak (accounts for variation in both proton leak per unit of mitochondrial protein and mitochondrial protein content) was negatively correlated ($R = 0.57$, $P < 0.01$) with the efficiency of utilization of feed for growth (g body weight gain/g feed) over the duration of the 20-d experiment (Figure 2). Based on multiple regression analysis, variation among animals in muscle mitochondrial protein content and proton leak-dependent respiration were additively associated with body weight gain/feed (g/kg) and accounted for a significant portion of variation observed for this criteria ($R^2 = 0.33$, $P < 0.01$; Table 5). Variation among animals in mitochondrial protein content (SD = 0.6) and proton leak-dependent respiration (SD = 4) equivalent to one SD from the population mean were associated with additive changes in body weight gain/feed ratios of +22 and +24 g/kg, respectively. Based on the

additivity of the responses, animals exhibiting mitochondrial traits one and two SD from the population mean would be estimated to differ in their BW gain/feed ratios by 46 and 92 g/kg, respectively. These values are equivalent, respectively, to a 10 and 20% difference in efficiency of feed utilization among the two mitochondrial based animal subpopulations.

Implications

Improvements in the efficiency of meat animal production during the past 40 years, largely have been achieved by increasing the proportion of high value, low energy content tissues (e.g., muscle) in the body. However, the muscle content of animal foods acceptable to processors and consumers is rapidly being approached. Therefore, future improvements in energetic efficiency likely need to be achieved by enhancing the efficiency of cellular processes within a tissue rather than solely by means of increasing muscle cell number and size.

Our data establish that variation in muscle mitochondrial protein content and the efficiency of mitochondrial energy production does exist and that deficiencies in mitochondrial function are quantitatively important to efficiency of animal growth. Therefore, manipulating muscle mitochondrial function seems to represent a viable target for improving the energetic efficiency of body growth in animals.

Literature Cited

Armstrong, R.B. and R.O. Phelps. 1984. Muscle fiber type composition of the rat hindlimb. Am. J. Anat. 171: 259-272.

Bhattacharya, S.K., J.H. Thakar, P.L. Johnson, and D.R. Shanklin. 1991. Isolation of skeletal muscle mitochondria from hamsters using an ionic medium containing EDTA and Nagarse. Anal. Biochem. 192: 344-349.

Brand, M.D., L. Chien, E.K. Ainscow, D.F.S. Rolfe and R.K. Porter. 1994. The causes and functions of mitochondrial proton leak. Biochim. Biophys. Acta 1187: 132-139.

Chance, B. and G.R. Williams. 1956. The respiratory chain and oxidative phosphorylation. Adv. Enzymol. 17: 65-134.

Clapham J.C., J.R. Arch, H. Chapman, A. Haynes, C. Lister, G.G. Moore, V. Piercy, S.A. Carter, I. Lehner, S.A. Smith, L.J. Beeley, R.J. Godden, N. Gerrity, M. Skehel, K.K. Changani, P.D. Hockings, D.G. Reid, S.M. Squires, J. Hatcher, B. Trail, J. Latcham, S. Rastan, A.J. Harper, S. Cadenas, J.A. Buckingham, M.D. Brand, and A. Abuin. 2000. Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. Nature. 406: 415-418.

De Lange, P., A. Lanni, L. Beneduce, M. Moreno, A. Lombardi, E. Silvestri, and F. Goglia. 2001. Uncoupling protein-3 is a molecular determinant for the regulation of resting metabolic rate by thyroid hormone. Endocrinology 142: 3414-3420.

Estabrook, R.W. 1967. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Meth. Enzymol. 10:41-47.

Even, P.C., V. Rolland, S. Roseau, J.C. Bouthegourd, and D. Tome. 2001. Prediction of basal metabolism from organ size in the rat: relationship to stain, feeding, age, and obesity. Am. J. Physiol. Regul. Integr. Comp. Physiol. 280: 1887-1896.

Goodell, S. and G. Cortopassi. 1998. Analysis of oxygen consumption and mitochondrial permeability with age in mice. Mech. Ageing Dev. 101: 245-256.

Harper, M-E, S. Monemdjou, J.J. Ramsey and R. Weindruch. 1998. Age-related increase in mitochondrial proton leak and decrease in ATP turnover reactions in mouse hepatocytes. Am. J. Physiol. 275: 197-206.

Iossa, S., L. Lionetti, M.P. Mollica, A. Barletta and G. Liverini. 1999. Energy intake and utilization vary during development in rats. J. Nutr. 129: 1593-1596.

Jackman, M.R. and W.T. Willis. 1996. Characteristics of mitochondria isolated from type I and type IIb skeletal muscle. Am. J. Physiol. 270: 673-678.

Karlsson, A., A.C. Enfalt, B. Essen-Gustavsson, K. Lundstrom, L. Rydhmer and S. Stern. 1993. Muscle histochemical and biochemical properties in relation to meat quality during selection for increased lean tissue growth rate in pigs. J. Anim. Sci. 71: 930-938.

Lal, S.B., J.J. Ramsey, S. Monemdjou, R. Weindruch and M-E Harper. 2001. Effects of caloric restriction on skeletal muscle mitochondrial proton leak in aging rats. J. Gerontol. Biol. Sci. 56: 116-122.

Lee, H.C., P.H. Yin, C.W. Chi, and Y.H. Wei. 2002. Increase in mitochondrial mass in human fibroblasts under oxidative stress and during replicative cell senescence. J. Biomed. Sci. 9: 517-526.

Lin, J., H. Wu, P.T. Tarr, C. Zhang, Z. Wu, O. Boss, L.F. Michael, P. Puigserver, E. Isotani, E.N. Olson, B.B. Lowell, R. Bassel-Duby, and B.M. Spiegelman. 2002. Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibers. Nature 418: 797-801.

Lutz, T.R. and T.S. Stahly. 2003. Relationship of mitochondrial function and ant1 protein content in muscle and efficiency and rate of body weight gain of pigs. J. Anim. Sci. (In Preparation).

Miranda, S., R. Foncea, J. Guerrero, and F. Leighton. 1999. Oxidative stress and up regulation of mitochondrial biogenesis genes in mitochondrial DNA-depeleted HeLa cells. Biochem. Biophys. Res. Commun. 258: 44-49.

National Research Council. 1995. Nutrient Requirements of Laboratory Animals, 4th ed. National Academy Press, Washington, DC.

Nichols, D. 2002. *Bioenergetics 3*. Academic Press Limited, San Diego.

Nisoli, E., E. Clementi, C. Paolucci, V. Cozzi, C. Tonello, C. Sciorati, R. Bracale, A. Valerio, M. Francolini, S. Moncada, and M.O. Carruba. 2003. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science 299: 896-899.

Porter, R.K. 2001. Mitochondrial proton leak: a role for uncoupling proteins 2 and 3? Biochim. Biophys. Acta 1504: 120-127.

Robinson J. and J.M. Cooper. 1970. Method of determining oxygen concentration in biological media, suitable for calibration of the oxygen electrode. Anal. Biochem. 33: 390-399.

Rolfe, D.F.S and M.D. Brand. 1996. Contribution of mitochondrial proton leak to skeletal muscle respiration and to standard metabolic rate. Am. J. Physiol. 271: C1380-C1389.

Rolfe, D.F.S. and G.C. Brown. 1997. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol. Rev. 77: 731-758.

Rolfe D.F., J.M. Newman, J.A. Buckingham, M.G. Clark, M.D. Brand MD. 1999. Contribution of mitochondrial proton leak to respiration rate in working skeletal muscle and liver and to SMR. Am. J. Physiol. 276:692-699.

St-Pierre, J., J. Lin, S. Krauss, P.T. Tarr, R. Yang, C.B. Newgard, B.M. Spiegelman. 2003. Bioenergetic analysis of peroxisome proliferator-activated receptor gamma coactivators 1alpha and 1beta (PGC-1alpha and PGC-1beta) in muscle cells. J. Biol. Chem. 278: 26597-26603.

Wardlaw, G.M. and M.L. Kaplan. 1984. Oxygen consumption and oxidative capacity of muscles from young obese and nonobese Zucker rats. Am. J. Physiol. 247: 911-917.

Wardlaw, G.M., M.L. Kaplan and S. Lanza-Jacoby. 1986. Effect of treadmill training on muscle oxidative capacity and accretion in young male obese and nonobese zucker rats. J. Nutr. 116: 1841-1852.

Table 1. Basal diet composition

Ingredient	%
Corn	62.30
Soy concentrate	28.95
D,L-Methionine	0.68
L-Threonine	0.03
L-Tryptophan	0.04
L-Valine	0.05
Corn oil	3.00
Dicalcium phosphate	1.61
Limestone	0.55
Sodium chloride	0.20
Starch	2.00
Vitamin premix[a]	0.49
Trace mineral premix[b]	0.10

[a]Contributed the following per kg of diet: biotin, 0.60 mg; riboflavin, 9 mg; pantothenic acid, 30 mg; niacin, 45 mg; folic acid, 3 mg; pyridoxine, 18 mg; thiamin, 10.8 mg; vitamin B_{12}, 0.15 mg; vitamin A, 6900 IU; vitamin D_3, 3000 IU; vitamin E, 81 IU; vitamin K, 3 mg; choline, 1509 mg.

[b] Provided per kg of diet: 88 mg Fe; 75 mg Zn; 30 mg Mn; 8.8 mg Cu; 1 mg I; 0.3 mg Se.

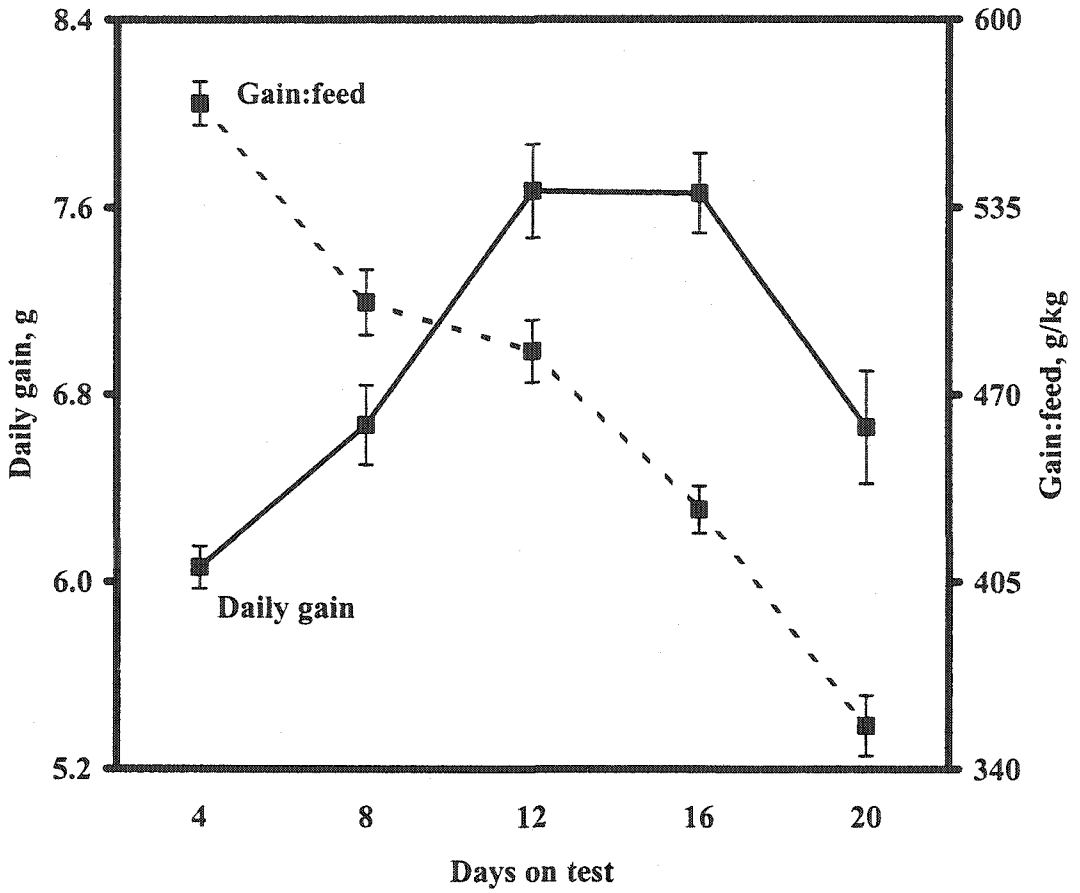

Figure 1. Rat daily body weight gain (g) and efficiency of feed utilization (gain:feed, g/kg) during each of five, 4-d periods from day 0 to 4, 4 to 8, 8 to 12, 12 to 16 and 16 to 20 of test, respectively. Values are means ± SEM for each 4-d period.

Table 2. Means and standard deviations of various measures of gastrocnemius muscle and liver mitochondrial function measured on day 20 of the test and body growth and feed utilization in rats averaged over the entire 20-day growth period

Criteria	Tissue	Mean	SD	CV
Mitochondrial function (Day 20)				
Respiration rate, nmol O·min^{-1}·mg mitochondrial protein^{-1}				
State 3 (maximal)[a]	Gastrocnemius	174	32	18
	Liver	31	6	19
State 4 (leak-dependent)[a]	Gastrocnemius	26	4	15
	Liver	6	1	17
Metabolic efficiency (RCR)[a]	Gastrocnemius	6.9	1.4	20
	Liver	5.0	0.9	18
Mitochondrial protein content, mg/g of liver or muscle (Day 20)[a]				
	Gastrocnemius	3.9	0.6	15
	Liver	10.7	1.5	14
Body growth and feed utilization (Day 0 to 20)				
Body weight (BW), g				
Initial (Day 0)		54	6	11
Final (Day 20)		195	15	8
BW gain, g/d		6.9	0.5	7
Feed, g/d		14.9	0.8	6
BW gain/feed, g/kg		465	27	6
Tissue mass, % of BW (Day 20)[a]				
	Gastrocnemius	0.6	0.04	7
	Liver	5.7	0.4	7

[a]Liver vs gastrocnemius muscle mitochondria ($P < 0.01$).

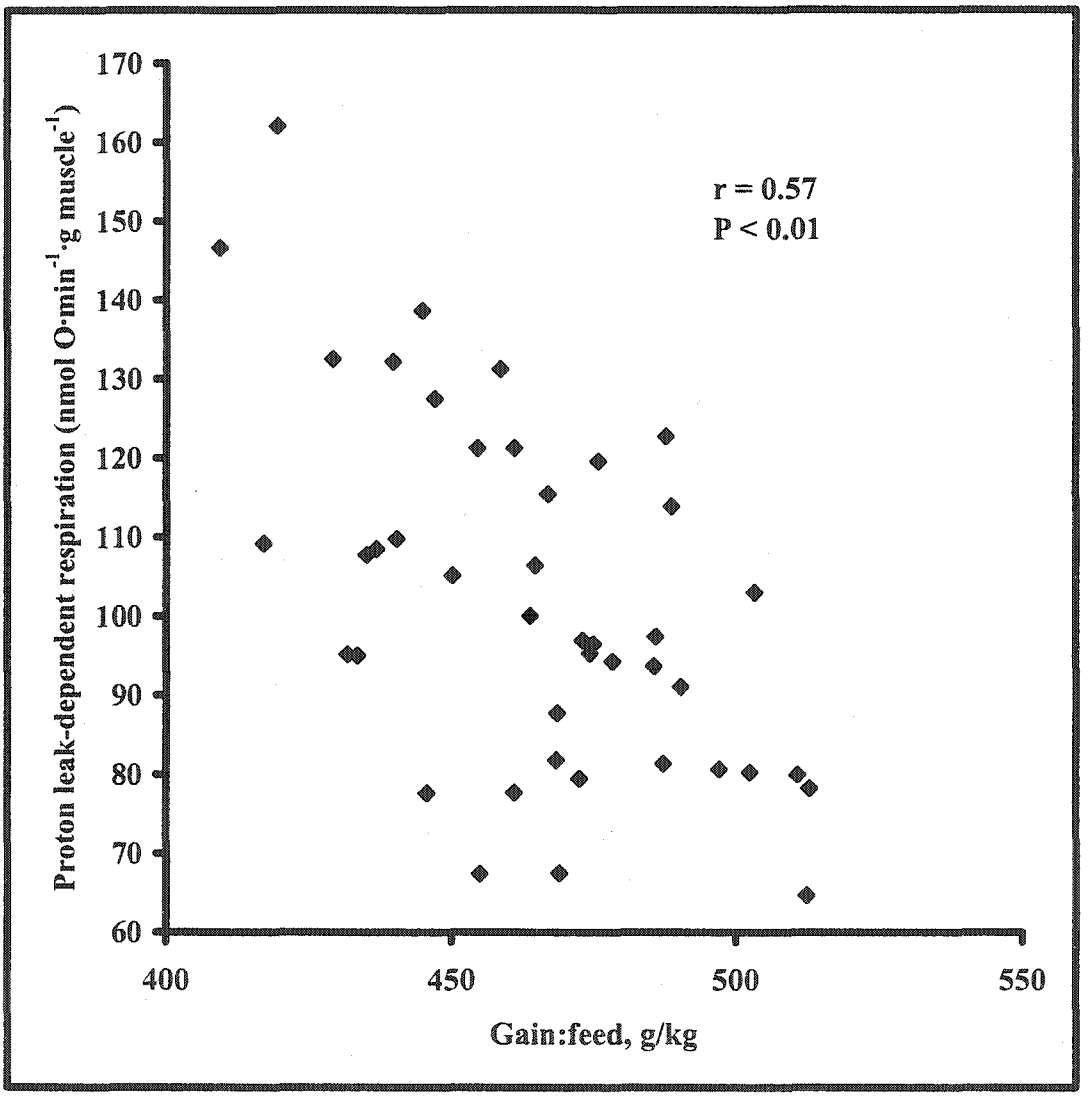

Figure 2. Association between mitochondrial proton leak-dependent respiration in the gastrocnemius muscle (nmol O·min^{-1}·g muscle^{-1}) measured on day 20 of test and efficiency of feed utilization for growth over the entire 20-d growth period.

Table 3. Correlation coefficients of measures of mitochondrial function and protein content in the gastrocnemius muscle with daily body weight gain, feed intake and efficiency of feed utilization of rats

	Mitochondrial function measurements[a]			Mitochondrial
Criteria[b]	State 3	State 4	RCR	protein content[c]
BW gain, g/d	-0.001[d]	-0.20	0.10	-0.31
	(0.99)	(0.21)	(0.54)	(0.04)
Feed, g/d	0.01	0.28	-0.22	0.03
	(0.96)	(0.07)	(0.16)	(0.87)
Gain:feed, g/kg	0.05	-0.42	0.33	-0.43
	(0.73)	(0.005)	(0.03)	(0.004)

[a]Maximal respiration rate (State 3) and proton leak-dependent respiration (State 4) on day 20 ± 2 of the growth period. Values reported as nmol $O \cdot min^{-1} \cdot mg$ mitochondrial $protein^{-1}$. Respiratory control ratio (RCR) is calculated as (State 3/State 4) and is a measure of mitochondrial metabolic efficiency.

[b]Daily body weight gain, daily feed intake and gain/feed ratios of rats over the 20 ± 2 day growth period.

[c]mg mitochondrial protein/g gastrocnemius muscle

[d]Correlation coefficient in upper row, P-values for difference from zero in parentheses in lower row.

Table 4. Relationships among the measures of mitochondrial function and protein content in the gastrocnemius muscle

	Mitochondrial function		
Criteria	State 3	State 4	RCR
Mitochondrial function			
Respiration rate, nmol O·min^{-1}·mg mitochondrial protein^{-1}			
State 3 (maximal)		0.24[a]	0.72
		(0.13)	(0.001)
State 4 (leak-dependent)			-0.49
			(0.001)
Mitochondrial protein, mg/g muscle			
Protein	-0.52	0.09	-0.50
	(0.001)	(0.56)	(0.001)

[a]Correlation coefficient in upper row, P-values for difference from zero in parentheses in lower row.

Table 5. Quantitative relationship between mitochondrial protein content and proton leak-dependent respiration in the gastrocnemius muscle and efficiency of feed utilization for body growth[a]

Criteria	Estimate	SE	P=
Intercept	606	32	0.001
Mitochondrial protein	-18	6	0.004
Mitochondrial proton leak-dependent respiration	-3	0.9	0.005

[a]Multiple regression analysis was used to estimate the relationship of mitochondrial protein content (mg/g of muscle) and proton leak-dependent respiration (nmol O·min^{-1}·mg mitochondrial protein^{-1}) in gastrocnemius muscle at the end of the 20-d test period with efficiency of feed utilization for body growth (g body weight gain/kg food intake) over the duration of the 20 ± 2-d test period. R^2 for the model = 0.33.

CHAPTER 4. EFFECT OF DIETARY BILOBALIDE ON MITOCHONDRIAL FUNCTION AND BODY GROWTH IN RATS

A paper to be submitted to *Journal of Animal Science*
T.R. Lutz and T.S. Stahly

Abstract

The objective of this study was to determine the effects of dietary addition of bilobalide, a potential inhibitor of mitochondrial proton leak, on muscle and liver mitochondrial function and body growth in rats. Weanling rats (16/trt; initial BW of 52 g) were individually penned and allowed ad libitum access to a diet that contained either 0 or 78 ppm bilobalide for 22 days post-weaning. Gastrocnemius (16/trt) and liver mitochondria (8/trt) were isolated and State 3 (maximum) or State 4 (proton leak-dependent) respiration rates and the respiratory control ratio (RCR=State3/State4), an index of respiratory chain coupling, were measured. Dietary bilobalide addition resulted in increased liver weights (12.0 vs 10.8 g; $P < 0.01$) and mitochondrial protein contents (11.6 vs 9.7 mg/g of liver; $P < 0.01$). Bilobalide ingestion also increased liver mitochondrial State 3 rates (33 vs 28 nmol $O \cdot min^{-1} \cdot mg\ protein^{-1}$; $P < 0.05$) and RCR (5.3 vs 4.5; $P = 0.08$), but not State 4 rates. However, bilobalide ingestion did not ($P > 0.10$) alter gastrocnemius weight, mitochondrial protein content, State 3 (158 vs 147 nmol $O \cdot min^{-1} \cdot mg\ protein^{-1}$) or State 4 respiration rates or the RCR (6.1 vs 5.8). Dietary bilobalide did not alter daily body weight gain, feed intake, efficiency of feed utilization or retroperitoneal and epididymal fat pad weights. Based on these data, bilobalide, when ingested orally, minimizes the proportion of energy in liver mitochondria lost due to proton leak, but does not alter muscle mitochondrial function or rate and efficiency of whole body growth in growing animals consuming feed ad libitum.

Introduction

Understanding and positively regulating the mitochondrial energy production system represents a new arena for improving energetic efficiency of animal growth. For years, scientists have been working to improve efficiency of animal production by increasing the body content of high value, low energy content tissues (e.g., muscle). However, the muscle content of animal food products that is acceptable to processors and consumers is rapidly being approached in some industries (e.g., pork). Thus, future improvements in energetic efficiency by means of increasing muscle cell number and size seem limited. Understanding the role of proton leak in energetic efficiency and the feasibility and consequences of regulating this cellular process is critical for continued improvement in the energetic efficiency of animal growth.

Mitochondria are the major energy-producing organelles in mammalian cells, and in this capacity are responsible for 90% of cellular oxygen consumption (Rolfe and Brown, 1997). Recent work in our laboratory has indicated that there is a relationship between the efficiency of mitochondrial energy production and whole body energetic efficiency in rats due to differences in proton leak-dependent respiration among animals (Lutz and Stahly, 2003). Therefore, a goal for improving energetic efficiency in meat animal production could be the identification of bioactive compounds that reduce mitochondrial proton leak and improve mitochondrial metabolic efficiency. Such compounds would ideally be targeted to skeletal muscle because this tissue is the largest consumer of oxygen on a whole animal basis and a proportionally large percentage of skeletal muscle's total oxygen consumption is utilized to balance proton leak under resting metabolic rate conditions (Rolfe and Brand, 1996; Ramsey et al., 2000). Bilobalide, a sesquiterpene trilactone found in the Ginkgo biloba

leaf, may be one compound of interest because, after oral administration, it has been reported to reduce proton leak and improve mitochondrial metabolic efficiencies (RCR) in livers of rats (Janssens et al., 1999). However, its effects on skeletal muscle mitochondria are not known.

Materials and Methods

Animals and diets

Thirty-two male Sprague Dawley weanling rats (20 d of age) were penned individually in cages on wire mesh floors. Rats were maintained on a 12 h light-dark cycle in rooms with an average temperature of 23 ± 0.5°C and 70 ± 7% relative humidity. Rats were randomly allotted from outcome groups based on body weight to a basal diet containing either 0 or 78 ppm bilobalide, projected to provide a daily dosage of about 0 or 9 mg of bilobalide/kg of BW. This oral dosage of bilobalide has previously been shown to effectively reduce proton leak-dependent respiration in rat liver mitochondria (Janssens et al., 1999). Bilobalide (100% purity) was obtained from Sigma, St. Louis, MO. The basal diet was formulated to meet or exceed all nutrient requirements of the rat (NRC, 1995; Table 1). Rats were allowed to consume feed and water ad libitum. Body weight, feed consumption and feed wastage data were determined every two days. After 22 days on their respective treatments, rats in each replicate were decapitated, and the liver, soleus and gastrocnemius muscles, and epididymal and retroperitoneal fat pads were isolated, removed and weighed. The experimental protocol was approved by the Institutional Committee on Animal Care at Iowa State University.

Mitochondrial isolation and oxygen consumption measurements

Upon decapitation, mitochondria were immediately isolated from the gastrocnemius muscle (16/trt) and liver (8/trt) according to procedures outlined by Lutz and Stahly, (2003). Mitochondrial oxygen consumption measurements were then determined in triplicate using a Clark-type oxygen electrode and a thermostatically controlled chamber equipped with magnetic stirring (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) utilizing glutamate-malate (10:5 mM) as the respiratory substrate according to procedures outlined by Lutz and Stahly (2003). Gastrocnemius (0.5 mg) or liver (2 mg) mitochondrial protein was infused into the reaction chamber. Then ADP was infused into the reaction chamber via a Hamilton syringe at a final concentration of 146 μM (gastrocnemius) or 95 μM (liver). The administration of ADP stimulates State 3 (maximum) respiration and upon conversion of ADP to ATP the mitochondria return to a resting rate of respiration termed State 4 (Chance and Williams, 1956; Estabrook, 1967). Oxygen consumption during State 4 is principally utilized to balance proton leak, and it has been argued that in the absence of proton leak State 4 respiration would be zero; hence, the term proton leak-dependent respiration is defined (Brand et al., 1994). The respiratory control ratio (RCR), which is an index of mitochondrial metabolic efficiency or coupling, was calculated as State 3 divided by State 4 respiration (Estabrook, 1967). Protein concentration of each mitochondrial suspension was determined with the Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA) utilizing a bovine gamma globulin standard and oxygen consumption was expressed as nmol $O \cdot min^{-1} \cdot mg$ mitochondrial $protein^{-1}$.

Statistical analysis

Data were analyzed as a randomized complete block design by analysis of variance techniques using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC) with rat considered the experimental unit and blocks based on initial body weight. Least square means are reported.

Results and Discussion

In the current study, rats consumed an average of 9.2 mg/kg BW of bilobalide per day which was similar to the desired intake of 9 mg/kg BW per day. Bilobalide feeding increased mitochondrial protein content and State 3 oxygen consumption rates in liver mitochondria as well as liver weights (Tables 2 and 3). State 4 oxygen consumption rates were not altered; therefore, metabolic efficiency (RCR) in liver mitochondria was improved due to the increased State 3 oxygen consumption rates. An improved metabolic efficiency in liver mitochondria of rats following bilobalide ingestion also was observed by Janssens et al., (1999). An understanding of the mechanism whereby bilobalide alters mitochondrial function is not well understood. However, it has been reported that bilobalide must be fed to rats for at least 14 days before there is an improvement in liver mitochondrial metabolic efficiency, indicating that bilobalide potentially must accumulate within the mitochondria (Janssens et al., 1995). In addition, bilobalide addition to a neuronal cell culture line has been shown to upregulate subunit III of cytochrome c oxidase and subunit ND1 of NADH dehydrogenase, both of which are mitochondrial-encoded genes (Chandrasekaran et al., 19998; Tendi et al., 2002).

Improvements in hepatic mitochondrial RCR could potentially result in improved efficiencies of feed utilization because improved RCR in muscle mitochondria have been

associated with improved efficiencies of whole body feed utilization in broilers and rats (Bottje et al., 2002; Lutz and Stahly, 2003). However, the amount of mitochondrial oxygen consumed as well as the amount utilized to balance proton leak is substantially smaller in the liver versus the animal's muscle mass (Rolfe and Brand, 1996; Lutz and Stahly, 2003). Thus, an efficacious regulator of mitochondrial metabolic efficiency would likely need to alter muscle mitochondria RCR if whole body energetic efficiencies are to be altered. Dietary bilobalide additions did not alter mitochondrial protein content, State 3 or State 4 respiration rates or RCR measurements in gastrocnemius muscle after 22 days of feeding (Table 4). Reasons for changes in liver but not muscle mitochondrial function are unknown but may be related to different concentrations of bilobalide arriving at the individual tissues. In humans, digestibility of bilobalide is estimated to be 70% with 30% of the digested dose excreted unchanged in the urine (Kleijnen and Knipschild, 1992). These data indicate that the remaining ingested bilobalide was either retained in body tissues or a portion of the ingested bilobalide may have been metabolized to an unidentified intermediate, raising the possibility that non-metabolized bilobalide concentrations bathing the liver versus skeletal muscle may have been different. However, studies utilizing radioactively labeled bilobalide to define tissue distribution and metabolism are currently not available in the literature.

Bilobalide additions did not alter daily body weight gain, feed intake or efficiency of feed utilization in rats fed their respective treatments for 22 days even though some alteration in liver mitochondrial function did occur as a result of bilobalide feeding (Table 5). Previously, it has been reported that bilobalide must be fed for at least 14 days before changes in liver mitochondrial function are observed. For this reason, rat body weight gain, feed intake, and efficiency of feed utilization also were examined during the last eight days

of the 22-day experiment, but no differences due to dietary treatment during this stage of growth were observed (Table 5). The similar muscle and fat depot weights in the bilobalide and control animals is in agreement with the absence of change in proton leak-dependent respiration in the major oxygen-consuming tissue, skeletal muscle (Table 3).

Implications

Dietary bilobalide additions enhanced metabolic efficiency of liver mitochondria but not in the major oxygen-consuming tissue, skeletal muscle. Therefore, future research describing the tissue distribution and metabolism of bilobalide after oral ingestion should be conducted. Finally, alterations in liver mitochondria induced by bilobalide feeding were not of sufficient nature to alter whole body energetic efficiency.

Literature Cited

Bottje, W., M. Iqbal, Z.X. Tang, D. Cawthon, R. Okimoto, T. Wing, and M. Cooper. 2002. Association of mitochondrial function with feed efficiency within a single genetic line of male broilers. Poult. Sci. 81: 546-555.

Brand, M.D., L. Chien, E.K. Ainscow, D.F.S. Rolfe and R.K. Porter. 1994. The causes and functions of mitochondrial proton leak. Biochim. Biophys. Acta 1187: 132-139.

Chance, B. and G.R. Williams. 1956. The respiratory chain and oxidative phosphorylation. Adv. Enzymol. 17: 65-134.

Chandrasekaran, K., L.I. Liu, K. Hatanpaa, K. Drieu, S.I. Rapoport. 1998. Stimulation of mitochondrial gene expression by bilobalide, a component of Ginkgo biloba extract (EGb : 761). Adv. Ginkgo. 7: 121-128.

Estabrook, R.W. 1967. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Meth. Enzymol. 10:41-47.

Janssens D., Michiels C., Delaive E., Eliaers F., Drieu K., and Remacle J. 1995. Protection of hypoxia-induced ATP decrease in endothelial cells by Ginkgo biloba extract and bilobalide. Biochem. Pharmacol. 50: 991-999.

Janssens D., J. Remacle, K. Drieu, and C. Michiels. 1999. Protection of mitochondrial respiration activity by bilobalide. Biochem. Pharmacol. 58: 109-119.

Kleijnen J. and P. Knipschild. 1992. Ginkgo biloba. Lancet 340: 1136-1139.

Lutz, T.R. and T.S. Stahly. 2003. Quantitative relationship between mitochondrial function and efficiency of animal growth. J. Anim. Sci. (In Preparation).

National Research Council. 1995. Nutrient Requirements of Laboratory Animals, 4th ed. National Academy Press, Washington, DC.

Ramsey, J.J., M-E Harper, and R. Weindruch. 2000. Restriction of energy intake, energy expenditure and aging. Free Rad. Biol. Med. 29: 946-968.

Rolfe, D.F.S and M.D. Brand. 1996. Contribution of mitochondrial proton leak to skeletal muscle respiration and to standard metabolic rate. Am. J. Physiol. 271: C1380-C1389.

Rolfe, D.F.S. and G.C. Brown. 1997. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol. Rev. 77: 731-758.

Tendi, E.A., F. Bosetti, S. F. DasGupta, A.M. Stella, K. Drieu, and S.I. Rapoport. 2002. Ginkgo biloba extracts EGb 761 and bilobalide increase NADH dehydrogenase mRNA level and mitochondrial respiratory control ratio in PC12 cells. Neurochem. Res. 27: 319-323.

Table 1. Basal diet composition

Ingredient	%
Corn	62.30
Soy concentrate	28.95
D,L-Methionine	0.68
L-Threonine	0.03
L-Tryptophan	0.04
L-Valine	0.05
Corn oil	3.00
Dicalcium phosphate	1.61
Limestone	0.55
Sodium chloride	0.20
Trace mineral premix[b]	0.10
Vitamin premix[a]	0.49
Bilobalide carrier (starch)	2.00

[a]Contributed the following per kg of diet: biotin, 0.60 mg; riboflavin, 9 mg; pantothenic acid, 30 mg; niacin, 45 mg; folic acid, 3 mg; pyridoxine, 18 mg; thiamin, 10.8 mg; vitamin B_{12}, 0.15 mg; vitamin A, 6900 IU; vitamin D_3, 3000 IU; vitamin E, 81 IU; vitamin K, 3 mg; choline, 1509 mg.

[b] Provided per kg of diet: 88 mg Fe; 75 mg Zn; 30 mg Mn; 8.8 mg Cu; 1 mg I; 0.3 mg Se.

Table 2. Effect of dietary bilobalide additions on liver mitochondrial respiration and protein content[a]

	Bilobalide, ppm			
Criteria	0	78	SEM	P=
Respiration rates, nmol O·min^{-1}·mg mitochondrial protein^{-1}				
State 3 (maximum)	28	33	1.5	0.04
State 4 (leak-dependant)	6	6	0.3	0.83
Metabolic efficiency				
RCR (State3/State4)	4.5	5.4	0.3	0.08
Protein content, mg/g of liver	9.7	11.6	0.3	0.01

[a]Means of 16 rats per treatment

Table 3. Effect of dietary bilobalide additions on different body tissue masses expressed as a percentage of body weight[a]

	Bilobalide, ppm			
Criteria	0	78	SEM	P=
Liver	5.35	5.92	0.07	0.01
Muscles				
Gastrocnemius	0.59	0.59	0.01	0.60
Soleus	0.037	0.036	0.001	0.48
Fat depots				
Epididymal	0.56	0.53	0.02	0.26
Retroperitoneal	0.36	0.36	0.01	0.84

[a]Means of 16 rats per treatment

Table 4. Effect of dietary bilobalide additions on gastrocnemius mitochondrial respiration and protein content[a]

	Bilobalide, ppm			
Criteria	0	78	SEM	P=
Respiration rates, nmol $O \cdot min^{-1} \cdot$mg mitochondrial $protein^{-1}$				
State 3 (maximum)	147	158	6	0.24
State 4 (leak-dependent)	26	26	0.8	0.67
Metabolic efficiency				
RCR (State3/State4)	5.8	6.1	0.2	0.21
Protein content, mg/g of muscle	4.0	4.1	0.1	0.20

[a]Means of 16 rats per treatment

Table 5. Effect of dietary bilobalide on feed intake, body weight gain, and efficiency of feed utilization during either the entire 22 days or last eight days of the experiment[a]

Criteria	Days on test	Bilobalide, ppm 0	78	SEM	P=
Body weight, g					
Initial		52.3	52.3	0.1	0.68
Final		203.8	201.6	2.7	0.56
Body weight gain, g/d					
	0–22	6.89	6.79	0.12	0.57
	14–22	7.23	6.96	0.18	0.29
Feed intake, g/d					
	0–22	15.05	15.00	0.18	0.82
	14–22	18.54	18.40	0.28	0.74
Gain/feed ratio, g/kg					
	0–22	457	453	5	0.48
	14–22	389	377	6	0.16

[a]Means of 16 rats per treatment

CHAPTER 5. RELATIONSHIP OF MITOCHONDRIAL FUNCTION AND ANT1 PROTEIN CONTENT IN MUSCLE AND EFFICIENCY AND RATE OF BODY WEIGHT GAIN OF PIGS

A paper to be submitted to *Journal of Animal Science*
T.R. Lutz, T.S. Stahly and E. Huff-Lonergan

Abstract

Variation in muscle mitochondrial energy production and the mitochondrial protein adenine nucleotide translocator 1 (ANT1) among pigs from a single strain and rearing environment was quantified, and the effects of these differences on the efficiency and rate of body growth were evaluated. In addition, the effects of proanthocyanidins, previously shown to positively alter hepatic ANT1 activity, on skeletal muscle mitochondrial function in vitro were evaluated. Pigs (n = 44) were weaned, individually penned and allowed ad libitum access to a nutritionally adequate diet from 8 to 28 kg BW. Body weight gain and gain per unit of feed were quantified. At 28 kg BW, mitochondria from the biceps femoris muscle were isolated and mitochondrial protein content, State 3 and State 4 respiration rates and RCR were determined. In addition, ANT1 protein content in the biceps femoris was measured. Muscle mitochondrial energy production and ANT1 content were correlated with daily body weight gains. Specifically, lower rates of State 3 respiration per mg of mitochondrial protein ($r = 0.43$, $P < 0.01$) and a lower RCR ($r = 0.34$, $P < 0.05$) were associated with reduced daily body weight gains in pigs growing from 18 to 28 kg BW. In addition, lower amounts of ANT1 protein in the biceps femoris muscle were associated with reduced daily body gains ($r = 0.44$, $P < 0.01$) and State 3 respiration rates per g of muscle ($r = 0.38$, $P < 0.02$). Finally, proanthocyanidins (0.36 μg/ml) in vitro induced a 15% increase ($P < .05$) in muscle mitochondria State 3 rates. These data establish that the rate of

mitochondrial energy production in muscle influences the rate of body growth in pigs and may potentially be modified by proanthocyanidins. Lastly, ANT1 may be an important bioenergetic protein influencing mitochondria function and body growth in pigs.

Introduction

Mitochondria are often referred to as the "power houses" of a cell and in this capacity are responsible for producing the energy currency (ATP) utilized to support cellular reactions and are responsible for 90% of cellular oxygen consumption (Rolfe and Brown, 1997). Previous research has indicated that variation in both the rate and efficiency of mitochondrial energy production is associated with the rate and efficiency of whole body growth in animals (Opalka et al., 2000; Lutz and Stahly, 2003). However, these relationships have not been well examined in pigs. The rate of mitochondrial energy production appears to be critically dependent on the mitochondrial protein ANT1 (Malgat et al., 2000). ANT1 is a heart-skeletal muscle specific inner mitochondrial membrane protein that serves to exchange ADP and ATP between the mitochondrial matrix and cytosol (Li et. al., 1989). Low tissue concentrations of ANT1 have been implicated in both reduced in vitro cellular energy production and reduced rates of mitochondrial energy production in vivo (Graham et al., 1997; Nogueira et al., 2001). The variation in skeletal muscle ANT1 content among pigs and the associated effects on energy production and utilization is unknown. Finally, proanthocyanidins, a group of bioactive compounds derived from grape seed, have been shown to alter ANT1 function by increasing the velocity of ADP exchange in hepatic mitochondria, thus leading to increased State 3 rates of respiration (Janssens et al., 2000). If proanthocyanidins increase the velocity of ADP exchange in skeletal muscle mitochondria

and increase the maximal rate of mitochondrial energy production, growth rates in pigs could potentially be improved upon proanthocyanidin feeding.

Our goals were to define variation in muscle mitochondrial function and ANT1 protein content among pigs, to determine if the observed variation is associated with the rate and efficiency of whole body growth, and to evaluate in vitro the effect of proanthocyanidins on State 3 respiration (maximal rate of energy production) in skeletal muscle mitochondria of pigs.

Materials and Methods

Animal management

Forty-four pigs from a high lean genetic strain were weaned (18 d of age) and moved to a facility physically isolated from other pigs. Pigs were individually penned on slotted floors in an environmentally regulated facility maintained at 26 ± 1.8°C and started on test as they individually reached 8 kg BW. From weaning to 18 kg BW, pigs were given ad libitum access to a diet (Table 1) formulated to meet or exceed all of their nutrient requirements (NRC, 1998). As pigs individually reached 18 kg BW, diets were altered to more closely match the nutrient needs of the heavier weight pigs (Table 1). Body weight, feed consumption and feed wastage data were collected every four days. As pigs individually reached 28 kg, they were euthanized via captive bolt gun at 0900. The biceps femoris muscle was rapidly isolated and sampled (about 3 g). The sample was placed in ice cold isolation media (IM) containing (in mM) 100 sucrose, 10 EDTA, 100 Tris-HCl and 46 KCl, pH=7.4 for subsequent mitochondrial isolation, and a second 50-g sample was flash frozen in liquid nitrogen and stored at -80°C for subsequent western blot analysis of ANT1 protein. Mitochondrial isolation was initiated within 30 min of muscle removal. The experimental

protocol was approved by the Institutional Committee on Animal Care at Iowa State University.

Isolation of biceps femoris muscle mitochondria

Biceps femoris muscle mitochondria were isolated according to published procedures (Bhattacharya et al., 1991). Briefly, the muscle sample was first minced on a cooled glass plate and resuspended in IM at a concentration of 10% (wt/vol). The IM contained 0.5% (wt/vol) fatty acid-free bovine serum albumin, which was used to bind free fatty acids liberated during tissue mincing and homogenization. This homogenate was then incubated with Nagarse (0.2 mg/ml; Sigma, St. Louis, MO) at room temperature for 5 minutes while stirring to liberate the intermyofibrillar mitochondria. A Polytron tissue homogenizer (Brinkmann, Westbury, NY) was then used to liberate the subsarcolemmal mitochondria by using three five-second bursts followed by a five-minute incubation on ice. The homogenate was then centrifuged at 750 x g for 10 min at 4°C to pellet cellular debris. Next, the supernatant was removed and centrifuged at 10,000 x g at 4°C for 10 min to pellet the mitchondrial fraction. The mitochondrial pellets were then resuspended in IM and again centrifuged at 10,000 x g at 4°C for 10 min. Finally, the mitochondrial pellet was resuspended to a final mitochondrial protein concentration of approximately 20 mg/ml in reaction media that contained (in mM) 15 KCl, 30 K_2HPO_4, 25 Tris base, 45 sucrose, 12 mannitol, 5 $MgCl_2$ and 7 EDTA, pH=7.4.

Mitochondrial incubation with proanthocyanidins

Mitochondrial preparations from 18 pigs were incubated with 0 or 0.36 μg/ml of IH636 grape seed proanthocyanidin extract (Dry Creek Nutrition, Inc., Modesto, CA) for at least one hour at 4°C before mitochondrial function measurements were made with the

untreated mitochondrial preparation from the same pig serving as its own control. In a preliminary experiment, pig muscle mitochondria were incubated with a proanthocyanidin dosage per unit of mitochondrial protein previously found to be most efficacious at improving State 3 respiration in rat liver mitochondria (Janssens et al., 2000). However, this dosage was excessive and resulted in a decline in State 3 respiration rather than an increase; therefore, half the dosage per unit of mitochondrial protein (0.36 μg/ml) of that utilized by Janssens et al. (2000) was used in the current experiment. A one-hour incubation time was used because this was the length of incubation previously utilized to demonstrate the positive effects of proanthocyanidins on ADP exchange rate, State 3 and RCR in rat liver mitochondria (Janssens et al., 2000). Proanthocyanidins are oligomeric units composed of monomeric flavan-3-ol units, with the three major identified monomeric units being catechin, epicatechin, and epicatechin-3-O-gallate (Santos-Buelga et al., 1995; Waterhouse and Walzem, 1998). Previous gas chromatography-mass spectrometry analysis of the product used in this experiment indicated the composition to be 54% dimeric proanthocyanidins, 13% trimeric proanthocyanidins, 7% tetrameric proanthocyanidins, and less than 5% each of monomeric and high-molecular weight oligomeric proanthocyanidins and flavonoids (Pataki et al., 2002).

Mitochondrial oxygen consumption

Mitochondrial oxygen consumption measurements were determined in triplicate using a Clark-type oxygen electrode and a thermostatically controlled chamber equipped with magnetic stirring (Yellow Springs Instrument Co., Inc., Yellow Springs, OH). Mitochondrial oxygen consumption measurements were conducted according to procedures previously outlined by Lutz and Stahly (2003) utilizing glutamate:malate (10:5 mM) as the respiratory

substrate and 0.5 mg of biceps femoris mitochondrial protein. State 3 respiration or maximal respiration was induced by injection of ADP into the reaction chamber sufficient to achieve a final ADP concentration of 146 μM. Upon conversion of this ADP to ATP, the mitochondria return to a resting rate of respiration termed State 4 (Chance and Williams, 1956; Estabrook, 1967). Oxygen consumption during State 4 is principally utilized to balance proton leak, and it has been argued that in the absence of proton leak State 4 respiration would be zero; hence, the term proton leak-dependent respiration is defined (Brand et al., 1994). The respiratory control ratio (RCR), which is an index of mitochondrial metabolic efficiency or coupling, was calculated as State 3 divided by State 4 respiration (Estabrook, 1967). Protein concentration of each mitochondrial suspension was determined with the Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA) utilizing a bovine gamma globulin standard and oxygen consumption was expressed as nmol $O \cdot min^{-1} \cdot mg$ mitochondrial $protein^{-1}$.

Western blot analysis of ANT1 protein

Frozen biceps femoris muscle (350 mg) from each pig was homogenized in solubilizing buffer (2% SDS, 10 mM phosphate, pH 7.0) by using 30 strokes of a Potter-Elvehjm tissue homogenizer. The homogenates were centrifuged at 1,500 g for 30 min at 25°C to precipitate insoluble particles. Muscle supernatant protein concentration was then determined using the DC (detergent compatible) Protein Assay (Bio-Rad, Hercules, CA), and all aliquots were diluted to the same protein concentration with solubilizing buffer. One-half volume of tracking dye and buffer (3 mM EDTA, 3% SDS, 30% glycerol, 0.001% pyronin Y, 30 mM Tris-HCl, pH 8.0) was then combined with the muscle supernatant fraction along with a 0.1 volume of 2-mercaptoethanol and heated at 50°C for 20 min. These samples were subsequently frozen at -80°C until further analysis.

Aliquots of the muscle protein supernatant (100 μg) were separated on a 5% stacking and 12% separating SDS-PAGE gel using a triple wide system, which ran for 3 h at a constant voltage of 90 V at 4°C. Proteins in the gels were then transferred by electroelution to polyvinylidene difluoride (PVDF) membranes (Perkin Elmer Life Sciences, Inc., Boston, MA) for 24 h using a constant amperage of 160 mA at 4°C. Solute concentrations of the gel running buffer and membrane transfer buffer were as previously described (Wiegand et al., 2001). PVDF membranes were incubated in a blocking solution (80 mM disodium hydrogen orthophosphate, anhydrous, 20 m*M* sodium dihydrogen orthophosphate, 100 mM sodium chloride, 0.1% [vol/vol] Tween-20) and 5% nonfat dry milk (NFDM) for 1 h at room temperature. The membrane was then washed three times (10 min/wash) with the blocking buffer excluding the nonfat dry milk. The PVDF membrane was then incubated for 2 h at room temperature with a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) raised against human ANT (1:8000 dilution in the above described blocking buffer with 1% NFDM). An initial database search using the BLAST server from the National Center for Biotechnology Information, indicated the nucleotide sequence of human and porcine ANT to be 93% similar. After washing as described above, the PVDF membrane was incubated for 2 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000 dilution in blocking buffer with 1% NFDM) purchased from Amersham Biosciences, Arlington Heights, IL. The membrane was again washed, and the immunoreactive protein was visualized with the ECL Advance chemiluminescence system (Amersham Biosciences, Arlington Heights, IL). Next, each membrane was scanned and densitometry analysis was conducted using the computer program ImageJ (National Institutes of Health, Bethesda, MD). All samples were run in

duplicate and results were analyzed as a ratio of sample band densitometry relative to that of a pig muscle preparation utilized as an internal control standard. Regression analysis indicated a linear increase in band densitometry ($R^2 = 0.97$) as the amount of biceps femoris muscle protein loaded on the gel was increased from 50 to 200 μg (Fig. 1).

Statistical analysis

Correlations between measures of body weight gain, feed intake and efficiency of feed utilization and the various measures of mitochondrial function and protein content and ANT1 protein were analyzed using the PROC CORR procedure of SAS (SAS Inst. Inc., Cary, NC). To determine the in vitro effects of proanthocyanidins on muscle mitochondrial function, data were analyzed as a completely randomized design by analysis of variance techniques by using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC).

Results and Discussion

In the current study, utilizing young, growing pigs derived from the same genetic strain and reared under similar environmental conditions, a significant amount of variation was observed in mitochondrial function measurements (State 3, State 4, RCR), protein content and ANT1 protein (Table 2). Previously, we also have conducted mitochondrial function and protein measurements in a group of growing rats from a single genetic strain, gender and rearing environment and observed similar variation to that of the pigs (Lutz and Stahly, 2003). Specifically, the coefficient of variation in pigs tended to be greater than rats for State 3 (24 vs 18%), State 4 (21 vs 15%) and mitochondrial protein content (25 vs 15%).

Various factors including genetic differences and environmental factors (i.e., climate, in utero conditions, health status or oxidative stress) may have contributed to this variation in skeletal muscle mitochondrial function. Variation in mitochondrial function has been

observed among various breeds of chickens, sheep and swine; however, in the current study, genetic differences were minimized because all pigs were derived from the same genetic strain (Mukherjee et al., 1970; Wolanis et al., 1980; Dzapo and Wassmuth, 1983). In pigs, cold exposure induces mitochondrial proliferation and proton leak-dependent respiration resulting in increased variation in mitochondrial measurements (Berthon et al., 1996; Herpin et al., 2002), but in the current experiment pigs were maintained in a warm environment with minimal environmental temperature variation. Inducing uteroplacental insufficiency in pregnant rats has been shown to result in reduced postnatal growth rates and has been associated with reduced mitochondrial State 3 respiration and ANT1 gene expression (Lane et al., 1998; Selak et al., 2003). The effects of uteroplacental insufficiency on mitochondrial function has not been evaluated in pigs, but it has been observed that intrauterine location alters fetal weight in pigs potentially due to differences in nutrient supply (McPherson et al., 2003). Differences in health status among the pigs housed in a common environment also may have contributed to variation in mitochondrial function as lipopolysaccharide administration has been shown to negatively impact mitochondrial function (Trumbeckaite et al., 2001). Finally, differences in exposure and/or ability to eliminate free radicals may contribute to variation in mitochondrial function. Recently, it has been demonstrated that chickens with reduced mitochondrial metabolic efficiencies and poor efficiencies of feed utilization also have increased concentrations of mitochondrial oxidized proteins with ANT1 appearing to be particularly sensitive to oxidative damage in a housefly model of oxidative stress (Yan and Sohal, 1998; Pumford et al., 2002).

In the current experiment, the rate of mitochondrial energy production was associated with the rate of daily body weight gain in pigs. Specifically, lower rates of State 3

respiration (maximal rate of mitochondrial energy production) expressed either per milligram of mitochondrial protein or per gram of biceps femoris muscle was associated with reduced daily body weight gains in pigs fed from 18 to 28 kg BW (Table 3 and 4). A similar association also has been recently observed in turkeys and intrauterine growth retarded rats where low rates of mitochondrial energy production were associated with slow body growth rates (Opalka et al., 2000; Selak et al., 2003). No association between mitochondrial energy production at 28 kg BW and the pigs' initial growth stage from 8 to 18 kg BW was detected (Table 3). Declines in mitochondrial metabolic efficiency (RCR) also were associated with reductions in daily body weight gain in pigs growing from 18 to 28 kg BW primarily due to reduced State 3 rates of respiration (Table 3). Declines in proton leak-dependent respiration per mg of mitochondrial protein (State 4) in pigs growing from 8 to 18 kg BW were associated with improvements in the efficiency of feed utilization. We have previously observed this same association in rats, but why this relationship did not persist in pigs growing from 18 to 28 kg BW is not known (Lutz and Stahly, 2003).

Because of the significant association observed in this study between the maximal rate of mitochondrial energy production and pig growth rate, we hypothesized that variation among pigs in the expression of a key bioenergetic protein ANT1 may influence mitochondrial energy production and ultimately growth especially in the young pig in an energy-dependent phase of growth. ANT1 has been estimated based on metabolic control analysis to elicit the greatest degree of control over mitochondrial energy production (Malgat et. al., 2000). ANT1 knockout mice have been shown to have lower rates of state 3 respiration as would be expected for mitochondria that lack the ability to exchange ADP and ATP (Graham et. al., 1997). In these mice, there also is a shift towards anaerobic

metabolism as observed by a four-fold increase in blood lactate concentration (Graham et. al., 1997). Low expression of the ANT1 gene also has been associated with low in vitro cellular energy production and slow postnatal growth of intrauterine growth-retarded rats (Nogueira et al., 2001; Lane et. al., 1998). However, the significance of the expression level of ANT1 on growth and mitochondrial function in pigs has not been elucidated. In the current study, we observed that there was an association between ANT1 protein expression levels measured in biceps femoris muscle and daily weight gain in pigs growing from 8 to 18 and 18 to 28 kg BW (Table 4). In addition, low expression of the ANT1 protein in the biceps femoris was associated with reduced rates of State 3 respiration per gram of biceps femoris muscle (Table 5). This was as expected because ANT1 has been reported to have the largest degree of control over mitochondrial energy production so declines in ANT1 should result in declines in State 3 respiration (Malgat et al., 2000).

Additional associations also were observed among the various measures of mitochondrial function. First, as the rate of State 3 respiration increased, either per milligram of mitochondrial protein or per gram of biceps femoris muscle, State 4 respiration rates also increased (Table 5). Nogueira et al. (2001), also have observed in rat liver mitochondria that increases in respiration rates are associated with declines in the efficiency of the respiratory chain. The authors contributed this relationship primarily to increases in mitochondrial cytochrome oxidase content because its concentration was positively related to both State 3 and 4 respiration rates and the authors have previously shown that increases in cytochrome oxidase are associated with declines in the efficiency of oxidative phosphorylation (Piquet et al., 2000). Next, as muscle mitochondrial protein concentration increased, both State 3 and 4 rates of respiration per unit of mitochondrial protein also increased (Table 5). A similar

association between muscle mitochondrial protein content and proton leak-dependent respiration also has been observed in transgenic mice with an increased number of mitochondria induced by overexpression of the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (St-Pierre et al., 2003).

Proanthocyanidins have been shown to alter in vitro ANT1 function by increasing the velocity of ADP exchange in rat liver mitochondria, thus leading to increased State 3 rates (Janssens et al., 2000). Therefore, we hypothesized that proanthocyanidins also may increase the velocity of ADP exchange in skeletal muscle mitochondria and increase the maximal rate of mitochondrial energy production. In the current experiment, proanthocyanidins induced ($P < 0.05$) a 15% increase in State 3 respiration in muscle mitochondria (Table 6). Since a significant association between State 3 and body weight gain was observed in the current study, proanthocyanidin feeding may serve to improve growth rates in pigs. Utilizing regression analysis, it was estimated that for each one unit change in the rate of State 3 respiration daily gain increases by 1.73 g ($R^2 = 0.18$; $P = 0.005$). Therefore, the 16 unit increase in State 3 respiration induced by proanthocyanidins (Table 6), could be hypothesized to increase daily body weight gain by 28 g. However, it has been recently determined in a rat model that oligomeric proanthocyanidins are not absorbed intact nor cleaved into their bioavailable monomers (Donovan et al., 2002). The proanthocyanidins monomer units (catechin and epicatechin) are absorbed by the rat, but the efficacy of these monomeric units relative to their oligomeric form on mitochondrial energy production is not known at the current time (Donovan et al., 2002).

Implications

Our data establish that the maximal rate of mitochondrial energy production and ANT1 protein content in skeletal muscle is quantitatively important to whole body growth in pigs. In addition, using an in vitro system we have shown that proanthocyanidins positively alter the rate of mitochondrial energy production. Future work is needed to identify compounds that will effectively improve mitochondrial function and ultimately growth in pigs.

Literature Cited

Berthon, D., P. Herpin, R. Bertin, F. De Marco, J. le Dividich. 1996. Metabolic changes associated with sustained 48-hr shivering thermogenesis in the newborn pig. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 114: 327-335.

Bhattacharya, S.K., J.H. Thakar, P.L. Johnson, and D.R. Shanklin. 1991. Isolation of skeletal muscle mitochondria from hamsters using an ionic medium containing EDTA and Nagarse. Anal. Biochem. 192: 344-349.

Brand, M.D., L. Chien, E.K. Ainscow, D.F.S. Rolfe and R.K. Porter. 1994. The causes and functions of mitochondrial proton leak. Biochim. Biophys. Acta 1187: 132-139.

Chance, B. and G.R. Williams. 1956. The respiratory chain and oxidative phosphorylation. Adv. Enzymol. 17: 65-134.

Donovan, J.L., C. Manach, L. Rios, C. Morand, A. Scalbert, and C. Remesy. 2002. Procyanidins are not bioavailable in rats fed a single meal containing a grape seed extract or the procyanidin dimer B_3. Brit. J. Nutr. 87: 299-306.

Dzapo, V.V. and R. Wassmuth. 1983. Mitochondrial metabolism and heterotic effects in pig. Results of a reciprocal crossbreeding experiment. II. Activity of oxygen uptake of cells and oxidative phosphorylation in heart, liver and scrotal mitochondria. Zeitschr. Tierz. Zuchtungsbiol. 100: 280-295.

Estabrook, 1967. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Meth. Enzymol. 10:41-47.

Graham, B.H., K.G. Way mire, B. Cottrell, I.A. Trounce, G.R. Macgregor, D.C. Wallace. 1997. A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoform of the adenine nucleotide translocator. Nat. Gen. 16: 226-234.

Herpin, P., G. Lossec, I. Schmidt, F. Cohen-Adad, C. Duchamp, L. Lefaucheur, F. Goglia and A. Lanni. 2002. Effect of age and cold exposure on morphofunctional characteristics of skeletal muscle in neonatal pigs. Pflugers Arch. 444: 610-618.

Janssens D., E. Delaive, A. Houbion, F. Eliaers, J. Remacle and C. Michiels. 2000. Effect of venotropic drugs on the respiratory activity of isolated mitochondria and in endothelial cells. Br. J. Pharmacol. 130: 1513-1524.

Lane, R.H., A.K. Chandorkar, A.S. Flozak, and R.A. Simmons. 1998. Intrauterine growth retardation alters mitochondrial gene expression and function in fetal and juvenile rat skeletal muscle. Pediatr. Res. 43: 563-570.

Li, K., C.K. Warner, J.A. Hodge, S. Minoshima, J. Kudoh, R. Fukuyama, M. Maekawa, Y. Shimizu, N. Shimizu, D.C. Wallace. 1989. A human muscle nucleotide translocator gene has four exons, is located on chromosome 4, and is differentially expressed. J. Biol. Chem. 264: 13998-14004.

Lutz, T.R. and T.S. Stahly. 2003. Quantitative relationship between mitochondrial function and efficiency of animal growth. J. Anim. Sci. (In Preparation).

Malgat, M., T. Letellier, R. Rossignol and J.P. Mazat. 2000. Metabolic control analysis and threshold effect in oxidative phosphorylation: study in an intermediate state of respiration. Conference Programme of the 9th International BioTheroKinetics Meeting, Stellenbosch, South Africa pp.125-134.

McPherson, R.L., F. Ji, G. Wu and S.W. Kim. 2003. Effects of intrauterine location on fetal growth and development in the pig: nutritional implications. Abstract presented at the Midwestern Section ASAS and Midwest Branch ADSA 2003 Meeting, Des Moines, IA (Abstract# 126).

Mukherjee, T.K., R.W.C. Stevens and M.P. Hoogendoorn. 1970. Oxygen uptake of mitochondrial isolates from two breeds of chickens and their F1 cross. Poult. Sci. 49: 1130-1131.

Nogueira, V., M. Rigoulet, M-A Piquet, A. Devin, E. Fontaine and X.M. Leverve. 2001. Mitochondrial respiratory chain adjustment to cellular energy demand. J. Biol. Chem. 276: 46104-46110.

NRC. 1998. Nutrient Requirements of Swine (10th Ed.). National Academy Press. Washington, DC.

Opalka, J.R., M. Wicke, F.N. Gellerich, S. Zierz and G. von Lengerken. 2000. Influence of mitochondrial function on growth and meat quality in turkey. Eur. J. Med. Res. 5 (Suppl I): 57

Pataki, T., I. Bak, P. Kovacs, D. Bagchi, D.K. Das, and A. Tosaki. 2002. Grape seed proanthocyanidins improved cardiac recovery during reperfusion after ischemia in isolated rat hearts. Am. J. Clin. Nutr. 75: 894-899.

Piquet, M.A., V. Nogueira, A. Devin, B. Sibille, C. Filippi, E. Fontaine, M. Roulet, M. Rigoulet and X.M. Leverve. 2000. Chronic ethanol ingestion increases efficiency of oxidative phosphorylation in rat liver mitochondria. FEBS Lett. 468: 239-242.

Pumford N.R., M. Iqbal , D. Cawthon, W. Bottje. 2002. Increased protein oxidation and differences in protein expression in muscle mitochondria in animals with lower feed efficiency. Free Rad. Biol. Med. 33: 89 Suppl. 1.

Rolfe, D.F.S. and G.C. Brown. 1997. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol. Rev. 77: 731-758.

Santos-Buelga, C., E.M. Francia-Aricha, M.T. Escribano-Bailon. 1995. Comparative flavan-3-ol composition of seeds from different grape varieties. Food Chem. 53: 197-201.

Selak, M.A., B.T. Storey, I. Peterside, and R.A. Simmons. 2003. Impaired oxidative phosphorylation in skeletal muscle of intrauterine growth retarded rats. Am. J. Physiol. Endocrinol. Metab. 285: 130-137.

St-Pierre, J., J. Lin, S. Krauss, P.T. Tarr, R. Yang, C.B. Newgard, B.M. Spiegelman. 2003. Bioenergetic analysis of peroxisome proliferator-activated receptor gamma coactivators 1alpha and 1beta (PGC-1alpha and PGC-1beta) in muscle cells. J. Biol. Chem. 278: 26597-26603.

Trumbeckaite, S., J.R. Opalka, C. Neuhof, S. Zierz, F.N. Gellerich. 2001. Different sensitivity of rabbit heart and skeletal muscle to endotoxin-induced impairment of mitochondrial function. Eur. J. Biochem. 268: 1422-1429.

Waterhouse, A.L. and R.L. Walzem. 1998. Nutrition of Grape Phenolics. In *Flavonoids in Health and Disease*. New York: Marcel Dekker.

Wiegand, B.R., F.C. Parrish Jr., D.G. Morrical, E. Huff-Lonergan. 2001. Feeding high levels of vitamin D3 does not improve tenderness of callipyge lamb loin chops. J. Anim. Sci. 79: 2086-2091.

Wolanis, M., V. Dzapo and R. Wassmuth. 1980. The determination of biochemical parameters of energy metabolism and their relationships with vitality, fattening performance and carcass quality in sheep. 2. Respiration activity and oxidative phosphorylation of isolated diaphragm mitochondria. Z. Teriz. Zuechtungsbiol. 97: 28-36.

Yan L-J and R.S. Sohal. 1998. Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. Proc. Natl. Acad. Sci. 95: 12896-12901.

Table1. Composition of diets

Ingredient	Stage of Pig Growth, kg	
	8 – 18	18 – 28
Corn	31.97	42.99
Soybean meal	45.51	39.95
Whey, dried	15.00	10.00
Choice white grease	3.00	3.00
L-Lysine·HCl	0.20	0.18
L-Threonine	0.14	0.12
D,L Methionine	0.20	0.14
Dicalcium phosphate	1.74	1.39
Limestone	0.78	0.62
Sodium chloride	0.25	0.40
Vitamin premix[a]	0.54	0.54
Trace mineral premix[b]	0.17	0.17
Antimicrobial agent[c]	0.50	0.50

[a]Contributed the following per kg of diet: biotin, 0.30 mg; riboflavin, 18 mg; pantothenic acid, 54 mg; niacin, 75 mg; folic acid, 1.8 mg; pyridoxine, 9 mg; thiamin, 6 mg; vitamin B_{12}, 0.90 mg; vitamin A, 10,500 IU; vitamin D_3, 1,200 IU; vitamin E, 66 IU; vitamin K, 3 mg; choline, 1197 mg.

[b]Provided per kg of diet: 210 mg Fe; 180 mg Zn; 72 mg Mn; 21 mg Cu; 2.4 mg I; 0.3 mg Se.

[c]Contributed the following per kg of diet: chlortetracycline, 110 mg; sulfathiazole, 110 mg; penicillin, 50 mg.

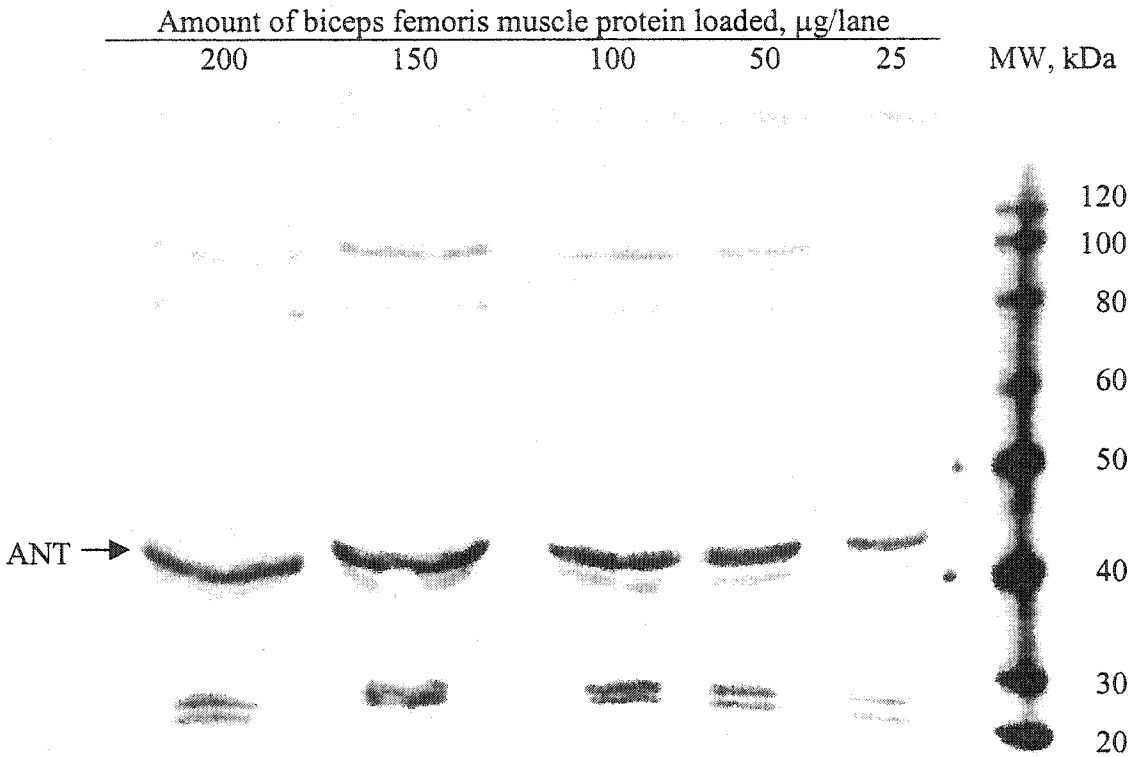

Fig. 1. Western blot analysis of ANT protein in pig biceps femoris muscle. Incremental levels of pig biceps femoris muscle protein (200, 150, 100, 50, 25 µg/lane) were loaded to examine linearity of band intensity. Regression analysis of the lanes that contained 200 to 50 µg of biceps femoris muscle protein indicated a linear increase in densitometry (R^2 = 0.97) as protein concentration increased.

Table 2. Means and standard deviations of various measures of biceps femoris muscle mitochondrial function measured at 28 kg BW and body growth and feed utilization of pigs fed from 8 to 18 kg BW and 18 to 28 kg BW

Criteria	Mean	SD	CV
Mitochondrial measures			
Respiration rates, nmol $O \cdot min^{-1} \cdot$mg mitochondrial $protein^{-1}$			
State 3	94	23	24
State 4	14	3	21
RCR (State 3/State 4)	6.9	1.1	16
Protein, mg/g muscle	1.6	0.4	25
ANT1, ratio to internal std[a]	1.17	0.13	11
Body growth and feed utilization			
Daily gain, g			
8 to 18 kg BW	595	92	15
18 to 28 kg BW	798	87	11
Daily feed, g			
8 to 18 kg BW	767	132	17
18 to 28 kg BW	1155	157	14
Gain:feed, g/kg			
8 to 18 kg BW	785	100	13
18 to 28 kg BW	696	60	9

[a]Ratio of muscle sample band densitometry area relative to that of a pig muscle sample utilized as an internal control standard.

Table 3. Correlation coefficients of measures of mitochondrial protein and function per mg of mitochondrial protein in the biceps femoris muscle at 28 kg BW with daily body weight gain, feed intake and efficiency of feed utilization in pigs during stages of growth from 8 to 18 and 18 to 28 kg BW

Criteria	Mitochondrial protein[a]	Per mg mitochondrial protein[b]		
		State 3	State 4	RCR
Daily gain, g				
8 to 18 kg BW	0.11[c]	0.13	-0.02	0.27
	(0.47)	(0.40)	(0.91)	(0.08)
18 to 28 kg BW	0.10	0.43	0.22	0.34
	(0.52)	(.005)	(0.16)	(0.03)
Daily feed, g				
8 to 18 kg BW	0.21	0.22	0.11	0.20
	(0.18)	(0.17)	(0.50)	(0.20)
18 to 28 kg BW	0.01	0.25	0.12	0.25
	(0.97)	(0.11)	(0.46)	(0.11)
Gain:feed, g/kg				
8 to 18 kg BW	-0.19	-0.27	-0.32	0.06
	(0.23)	(0.08)	(0.04)	(0.73)
18 to 28 kg BW	0.16	0.12	0.10	0.05
	(0.31)	(0.44)	(0.51)	(0.74)

[a]Milligrams mitochondrial protein/g of biceps femoris muscle.

[b]State 3 indicates the maximal rate of mitochondrial respiration while oxygen consumed during State 4 in principally utilized to balance proton leak. The units for these two functional measurements are nmol $O \cdot min^{-1} \cdot$mg mitochondrial $protein^{-1}$. RCR represents the respiratory control ratio (State 3/State 4) and is a measure of mitochondrial metabolic efficiency.

[c]Upper row = correlation coefficient, P-values for difference from zero in parentheses.

Table 4. Correlation coefficients of measures of ANT1 protein and mitochondrial function per g of biceps femoris muscle at 28 kg BW with daily body weight gain, feed intake and efficiency of feed utilization in pigs during stages of growth from 8 to 18 and 18 to 28 kg BW

Criteria	ANT1 protein[a]	Per g of biceps femoris muscle[b]	
		State 3	State 4
Daily gain, g			
8 to 18 kg BW	0.36[c]	0.16	0.09
	(0.02)	(0.31)	(0.58)
18 to 28 kg BW	0.44	0.32	0.17
	(0.004)	(0.04)	(0.27)
Daily feed, g			
8 to 18 kg BW	0.40	0.28	0.22
	(0.01)	(0.08)	(0.18)
18 to 28 kg BW	0.26	0.19	0.07
	(0.11)	(0.23)	(0.64)
Gain:feed, g/kg			
8 to 18 kg BW	-0.07	-0.27	-0.28
	(0.68)	(0.08)	(0.07)
18 to 28 kg BW	0.15	0.14	0.13
	(0.35)	(0.38)	(0.41)

[a]Ratio of muscle sample densitometric area relative to that of a pig muscle sample utilized as an internal control standard.

[b]State 3 indicates the maximal rate of mitochondrial respiration while oxygen consumed during State 4 in principally utilized to balance proton leak. The units for these two functional measurements are nmol $O \cdot min^{-1} \cdot g$ of biceps femoris $muscle^{-1}$.

[c]Upper row = correlation coefficient, P-values for difference from zero in parentheses.

Table 5. Relationships among measures of mitochondrial function and ANT1 protein content in the biceps femoris muscle

	Mitochondria measure				
Criteria	State 3	State 4	RCR	Protein	ANT1
Mitochondria measure/mg mitochondrial protein					
State 3		0.80[a]	0.35	0.34	
		(0.001)	(0.02)	(0.03)	
State 4			-0.25	0.49	
			(0.10)	(0.001)	
RCR (State 3/State 4)				-0.19	0.21
				(0.24)	(0.20)
Mitochondrial protein[b]					0.32
					(0.05)
Mitochondria measure/g of biceps femoris muscle					
State 3		0.92			0.38
		(0.001)			(0.02)
State 4					0.28
					(0.08)

[a]Upper row = correlation coefficient, P-values for difference from zero in parentheses.

[b]mg mitochondrial protein/g muscle

Table 6. Effect of proanthocyanidin incubation on biceps femoris muscle mitochondrial function in vitro.

	Proanthocyanidin concentration, μg/ml			
Criteria	0	0.36	SEM	P=
Respiration rates, nmol $O \cdot min^{-1} \cdot$mg mitochondrial $protein^{-1}$				
State 3	105	121	4.6	0.03
State 4	15	17	0.65	0.06
RCR (State 3/State 4)	7.0	7.2	0.21	0.64

CHAPTER 6. GENERAL CONCLUSIONS

Because of the important role mitochondria play in cellular energy metabolism, we hypothesized that variation in mitochondrial content or rate and efficiency of mitochondrial energy production may be important to the rate and efficiency of whole body growth in animals. To evaluate this hypothesis, a series of experiments were conducted to evaluate associations between mitochondria function and the mitochondrial protein adenine nucleotide translocator 1 (ANT1) content (pigs) with rate and efficiency of growth in young growing rats and pigs from a single strain and rearing environment. Measures of mitochondrial function were determined to be correlated with the efficiency of whole body feed utilization in rats and daily body weight gain in pigs. Specifically, lower rates of mitochondrial proton leak-dependent respiration ($r = 0.42$, $P < 0.01$) or improved mitochondrial metabolic efficiencies ($r = 0.33$, $P < 0.05$) were associated with improved gain/feed ratios in rats. In addition, rats with a lower muscle mitochondrial protein content exhibited improved efficiencies of feed utilization ($r = 0.43$, $P < 0.01$) and improved rates of growth ($r = 0.31$, $P < 0.05$). In pigs, higher rates of State 3 respiration ($r = 0.43$, $P < 0.01$), improved mitochondrial metabolic efficiency ($r = 0.34$, $P < 0.05$) and an increased amount of ANT1 protein in the biceps femoris muscle ($r = 0.44$, $P < 0.01$) were associated with improved daily body weight gains.

The ability of bilobalide and proanthocyanidins to positively modulate mitochondrial function also was evaluated. These compounds have previously been shown to positively modulate mitochondrial function in rat liver, but we were specifically interested in evaluating the efficacy of these compounds in muscle mitochondria which are responsible for the largest percentage of whole body oxygen consumption. In the rat, dietary bilobalide addition

resulted in increased liver weights ($P < 0.01$) and liver mitochondrial protein contents ($P < 0.01$). Bilobalide addition also increased liver mitochondrial State 3 rates ($P < 0.05$) and RCR ($P = 0.08$), but State 4 rates were unaltered. Dietary bilobalide did not alter muscle mitochodrial function, daily body weight gain, feed intake or efficiency of feed utilization. In vitro incubation of pig biceps femoris mitochondria with 0.36 μg/ml proanthocyanidins resulted in a 15% increase ($P < 0.05$) in State 3 respiration. These data establish that mitochondrial energy production and number are important to both the rate and efficiency of whole body growth in animals and certain bioactive compounds may have the potential to positively alter mitochondrial function.

The new information developed from this research improves our understanding of the role that muscle mitochondrial energy production plays in energetic efficiency of animal growth and establishes new cellular mechanisms as targets for favorable modifications by genes and/or bioactive molecules. Furthermore, the knowledge generated relative to muscle mitochondria has implications on potential cellular mechanisms/opportunities to alter meat quality (shifts in mitochondrial proliferation), animal susceptibility to free radical damage (ROS signaling, oxidized proteins), and metabolic diseases ('downer pigs'- associated with excess muscle lactate), and productive longevity of mature breeding animals (mitochondrial damage with aging).