

ANTIOXIDANT GENE EXPRESSION AND MITOCHONDRIAL FUNCTION DURING  
 $\beta$ -OXIDATION IN BEEF CATTLE

By

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To the Faculty of Washington State University,

The members of the Committee appointed to examine the dissertation of Kristen M. Brennan find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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Elevated  $\beta$ -oxidation leads to increased reactive oxygen species (ROS) production and oxidative stress. Many genes can help protect from damage sustained during this physiological state. To investigate the effect of fat mobilization on oxidative stress and gene expression in skeletal muscle, Angus cows were examined during weight loss and weight maintenance. At both physiological states, body weight, serum non-esterified fatty acids (NEFA), erythrocyte superoxide dismutase (SOD) and glutathione peroxidase (Gpx) activity, and mRNA levels of target genes in the *Biceps femoris* were measured. When cows were losing weight, serum NEFA levels were higher but SOD and Gpx activity did not change compared to weight maintenance. Expression of the NEFA responsive signaling proteins peroxisome proliferator-activated receptors (PPAR)  $\alpha$ ,  $\delta$  and  $\gamma$  increased during weight loss. Expression of the  $\beta$ -oxidation genes, carnitine palmitoyltransferase 1, fatty acid-binding protein 3, and acyl-coenzyme A oxidase 1 was highest during weight loss. Though antioxidant enzyme activity did not change, mitochondrial superoxide dismutase, glutathione peroxidase 4, selenoprotein W and thioredoxin reductase

expression increased during weight loss. During weight loss cows also had higher expression of the novel antioxidant genes: uncoupling proteins 2 and 3, tumor protein 53, nuclear respiratory factor 1, PPAR-gamma coactivator 1  $\alpha$  and estrogen-related receptor  $\alpha$ . During increased  $\beta$ -oxidation in cattle, erythrocyte antioxidant enzyme activity does not change, but expression of genes that play a key role in protecting skeletal muscle against oxidative stress increase.

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## **Dedication**

This dissertation is dedicated to the many people who have unconditionally supported my nine years as a “professional student”, especially my mom and dad.

CHAPTER 1:  
INTRODUCTION

Oxidative stress occurs when there is an imbalance between free radical production and antioxidant defense (Halliwell and Gutteridge, 1999). An imbalance occurs when there is increased production of reactive oxygen species (ROS) or when there are deficiencies in exogenous or endogenous antioxidants (Halliwell and Chirico, 1993). When free radical levels increase cellular damage can occur if adequate protective systems are not available to the cell or organism to terminate the radical reactions. A lack of adequate protective antioxidant systems can occur with deficiencies in dietary antioxidants, changes in antioxidant requirements, or disease. Prolonged exposure to elevated oxidative stress can impact on animal health by increasing susceptibility to disease and decreasing production and growth rates (reviewed in Miller and Brzezinska-Slebodzinska, 1993).

On a cellular level, free radicals can damage cellular components such as DNA and proteins and cellular structures such as membranes. Low levels of ROS are normal cellular by-products, so cells have developed several defense mechanisms to scavenge free radicals and repair radical-induced damage. These defense mechanisms can be loosely grouped in two types, exogenous antioxidants and endogenous antioxidants. Exogenous antioxidants include vitamins and minerals, such as selenium and vitamin E, obtained from the diet. Endogenous antioxidants include genes and proteins that act as the first line of defense by directly scavenging or decreasing the production of free radicals. However, endogenous and exogenous antioxidants are not mutually exclusive-some dietary antioxidants are components of endogenous antioxidant enzymes.

The body weight of cattle changes throughout the year as production states change. For example, cows lose weight shortly after calving when energy demands are high, and then gain weight during late lactation as energy requirements decrease. In order to meet the high energy

requirements during early lactation, cows mobilize and oxidize body fat. In vitro and in vivo studies indicate that when cells utilize fatty acids for ATP production, ROS production is elevated (Inoguchi et al., 2000; Li and Shah, 2004; Chinen et al., 2007). In dairy cattle, weight loss can result in metabolic stresses related to the increase in ROS production (Miller and Brzezinska-Slebodzinska, 1993). However, little information is available about how ruminants regulate antioxidant defenses during weight loss and increased  $\beta$ -oxidation. Cows in early lactation have elevated circulating non-esterified fatty acids (NEFA), higher levels of reactive oxygen metabolites and elevated erythrocyte SOD activity (Bernabucci et al., 2005) indicating an increase in ROS levels during body fat mobilization.

There is an intricate network of antioxidant genes and signaling molecules that regulate the body's response to oxidative stress. These molecules include, but are not limited to, scavenging molecules that detoxify ROS, transcription factors that induce the expression of antioxidant genes, and repair proteins and enzymes that fix damage by radicals to cellular components. However, these pathways have not been characterized in ruminants, leaving many questions as to how cattle deal with elevated ROS. By identifying changes in antioxidant gene expression patterns, we can better understand how ruminants deal with mild oxidative stress during weight cycling.

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CHAPTER 2:  
REVIEW OF THE LITERATURE

## **Oxidative Stress**

Free radicals are molecules with one or more unpaired electron in outer atomic (or molecular) orbit (Roberford and Calderon, 1995). These molecules are highly reactive and propagate a chain of reactions typically consisting of three parts: initiation, propagation and termination. Initiation begins with bond homolysis and redox reactions. During bond homolysis, a bond is broken in such a way that the two resulting molecules obtain a single electron and become radicals. However, bond homolysis occurs at a very slow rate in an uncatalyzed biological system (Roberford and Calderon, 1995). Propagation maintains the number of free radicals in the system through transfer of electrons from a non-radical to a radical, creating a new radical molecule. Termination of radical chain reactions occurs through scavenging to a less reactive production by antioxidant enzymes, or electron transfer.

Redox reactions can also generate free radicals as intermediates during their cycle. Both one-electron oxidation and one electron-reduction, such as in the electron transport chain, can result in free radical formation. Superoxide anion, oxygen with a single electron in its outer orbit, is the most common radical formed from redox reactions (Roberford and Calderon, 1995).

### *Beta-oxidation*

The mobilization of body fat for use as an energy source in other areas of the body begins in adipose tissue with the cleavage of triglycerides into free fatty acids (FFA) and glycerol (*Figure 1*). Hormone-sensitive lipase and adipose triglyceride lipase (Zimmerman et al., 2004; Schwieger et al., 2006) catalyzes the cleavage of a triacylglycerol to a FFA and a diacylglycerol

(reviewed in Vernon, 1981). Diacylglycerol is then further cleaved into free fatty acids and glycerol (Nelson and Cox, 2005). Prolonged fasting in sheep results in a consistent release of FFA into the blood stream. Most of the FFA in the blood stream of fed animals comes from lipolysis, with only about 20% of FFA being absorbed directly from the gut (reviewed in Vernon, 1981). There is a basal level of fat mobilization in animals, but this level increases in response to stimulants such as increased energy demands or catabolic hormones.

In the blood stream FFA are transported bound to albumin. After reaching the target tissue, such as skeletal muscle, fatty acids are transported into the cytosol where they are bound by fatty acid binding proteins (FABP). Beta-oxidation in the mitochondria begins in the mitochondrial matrix after three enzymatic reactions of the carnitine shuttle. First, fatty acids are activated by conversion to fatty acyl-CoA on the cytosolic side of the outer mitochondrial membrane. Fatty acyl-CoA formed on the cytosolic side of the mitochondrial membrane can either be used to produce ATP in the mitochondrial matrix or in the cytosol for production of membrane lipids.

Fatty acyl-CoA fated for mitochondrial oxidation is converted to fatty acyl-carnitine either in the outer membrane of the mitochondria or in the inner membrane space. Fatty acyl-carnitine is then transported into the matrix through the acyl-carnitine/carnitine transporter. Inside the matrix, the fatty acyl is transferred from carnitine to coenzyme A by carnitine palmitoyltransferase (CPT). This enzyme regenerates fatty acyl-CoA, releasing it and free carnitine into the matrix. Carnitine is transported back out to the inner membrane space via the acyl-carnitine/carnitine transporter. Once inside, the fatty acyl-CoA enters the mitochondrial beta-oxidation pathway to generate ATP.

The beta-oxidation of saturated fatty acids is a four-step process (*Figure 2*). Each pass through the four-step reaction yields an acetyl-CoA from the carboxyl end of the fatty acyl chain. The four steps are repeated as the process continues until all carbons of the fatty acid are converted to acetyl-CoA to be used by the Krebs's cycle to produce ATP and electrons for the electron transport chain (*Figure 3*). In addition, the reducing equivalents NADH and FADH<sub>2</sub>, which feeds through the electron transfer flavoprotein (ETF), are produced and used by the electron transport chain to make ATP (Nelson and Cox, 2005).

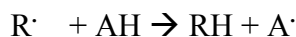
Fat mobilization as a fuel source occurs when energy demands are high, such as during early lactation. From parturition until peak lactation (around 8 weeks post calving in cattle), energy intake is less than energy requirements in high producing cows. This results in weight loss during lactation as body fat is mobilized to meet energy demands (Brockman, 1979). Fat is mobilized from adipose stores in the body, resulting in elevated circulating non-esterified fatty acid (NEFA) concentrations. In dairy cows, NEFA levels are significantly higher during early lactation (highest at 2-weeks after-calving) when energy requirements are higher than during late lactation (around the 7<sup>th</sup> month of lactation) (Castillo et al., 2005).

The mobilization of body stores for energy requirements can result in metabolic stresses related to the increase in reactive oxygen species production (Miller and Brzezinska-Slebodzinska, 1993). Elevated NEFA levels have been shown to increase reactive oxygen species (ROS) production by the mitochondria (Mataix et al, 1998, Koshkin et al., 2003, Srivastava and Chan, 2007). B-oxidation yields the reduced electron donors electron transfer flavoprotein (ETF) and electron transfer flavoprotein quinone oxidoreductase (ETF-QOR) (St. Pierre et al., 2002). It is thought that since both ETF and ETF-QOR occur in a their semiquinone

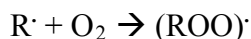
forms during  $\beta$ -oxidation, their semi-reduced states allow for the formation of ROS by-products (St-Pierre et al., 2002) during lipid oxidation.

### *Propagation of Free Radicals*

Propagation, or conservation of the number of free radicals in a system, can be the result of three mechanisms: atom or group transfer, electron transfer, or addition of radicals to a molecule (Roberford and Calderon, 1995). Atom or group transfer is the most frequent reaction of radical propagation and occurs when the outer most atom (either a hydrogen or a halogen) of a molecule is attacked by a radical. The resulting reaction where  $R\cdot$  is a radical and A is a protonated molecule is:



Electron transfer is the movement of an electron from a radical to a non-radical substrate. Electron transfer has an important role in lipid peroxidation, resulting in the formation of lipid hydroperoxide radicals. The final form of propagation is through the addition of radicals to a molecular oxygen or aromatic ring. This is a frequently observed reaction in physiological systems and also plays a role in lipid peroxidation (Roberford and Calderon, 1995). For example, the reaction with molecular oxygen would look similar to:



Termination of the radical chain reactions can occur by homolinking or crosslinking of radicals, radical scavenging or electron transfer (Roberford and Calderon, 1995). Homolinking and crosslinking of radicals occurs when two similar (homolinking) or different (crosslinking) radicals bond to form a molecule with a shared electron pair. Radical scavenging is the natural

or artificial termination of radical reactions. Both endogenous and exogenous antioxidants are the active scavengers in this method of radical termination (Roberford and Calderon, 1995). The final method of termination is electron transfer between identical radicals, such as the dismutation of superoxide to yield molecular oxygen and hydrogen peroxide (Roberford and Calderon, 1995).

The most frequent and most dangerous radicals are oxygen-derived radicals, or reactive oxygen species (ROS), such as superoxide ( $O_2^-$ ), hydroxide anion ( $OH^-$ ), and hydrogen peroxide ( $H_2O_2$ ) (Roberford and Calderon, 1995). Since they are strong producers of ROS, mitochondria are susceptible to oxidative damage to their DNA, lipids and proteins (reviewed in Genova, 2004). Damage to mitochondrial DNA (mtDNA) and proteins encoded by mtDNA decrease electron transfer and oxidative phosphorylation (reviewed in Genova et al., 2004). An excess of ROS leads to further increased production of radicals, creating a cycle of increased oxidative stress and decreased energy metabolism (reviewed in Genova et al., 2004). Under normal physiological conditions, about 4-5% of oxygen in the mitochondria does not get converted to water, but instead is reduced to a superoxide radical. The main sites for ROS production in the electron transport chain are located within complexes I and III (reviewed in Genova, 2004).

#### *Pathophysiological effects of oxidative stress in livestock*

When free radicals are not adequately scavenged, oxidative stress may directly and indirectly affect animal health, resulting in decreased production and herd health problems (reviewed in Miller and Brzezinska-Slebodzinska, 1993). Changes in oxidant/antioxidant balance may be the result of inadequate dietary antioxidants, body condition, disease, stress or the environment. For instance, bovine Tropical Theileriosis (a disease caused by the tick-born

*Theileria annulata* protozoa) causes decreased production and high death rates in infected cattle in endemic areas. Theileriosis results in increased levels of lipid peroxidation, indicating increased oxidative stress in diseased animals (Grewal et al., 2005). Peroxidation decreases membrane fluidity and increases cell lysis, contributing to the disease state (Grewal et al., 2005). In response to this increase in oxidative stress, levels of plasma glutathione peroxidase are significantly increased as part of the antioxidant defense system to protect cells from further oxidative injury from lipid peroxides (Grewal et al., 2005).

Oxidative stress can also occur in livestock as the result of body condition and metabolic status. Bernabucci et al. (2005) showed dairy cows with higher body condition scores (HBCS, BCS > 3.0) had greater losses in body condition from late pregnancy to 30 days in milk (DIM) when compared to medium (MBCS, BCS from 2.6 to 3.0) and lower (LBCS, BCS < 2.5) body condition score cows. High body condition score cows also had higher NEFA levels in their blood pre- and post-calving, indicating increased lipid mobilization.

Higher body condition score cows also had increased blood markers of oxidative stress. Reactive oxygen metabolites in plasma were higher post-calving than pre-calving (142.2 U, 129.3 U and 155.4 U versus 137.4 U, 128.9 U, and 142.2 U, for LBCS, MBCS, and HBCS respectively) in all three groups of cows. In red blood cells, SOD activity expressed as units per packed cell volume was lower during the postpartum period (1828 U, 1587 U, and 1528 U, for LBCS, MBCS, and HBCS) than the prepartum period (1830, 1560, and 1737, for LBCS, MBCS, and HBCS) (Bernabucci et al., 2005).

Thiobarbituric acid-reactive substances (TBARS) in plasma were maximal in all cows at 25 DIM (1.77, 1.69 and 2.1 nmols/mL, for LBCS, MBCS, and HBCS respectively) and increases were the highest in high body condition score cows. The transition from late pregnancy to early

lactation was characterized by a depletion of antioxidants in blood, resulting in oxidative stress in these cattle (Bernabucci et al., 2005). The considerable loss of body weight in high body condition score cattle resulted in a greater increase in antioxidant depletion and oxidant levels (Bernabucci et al., 2005). Thioredoxin reductase (TrxR) activity in white blood cells decreases during the first 21 DIM while glutathione peroxidase (GPx) activity and lipid peroxide levels remained high, indicating that TrxR is compromised during the periparturient period in dairy cows (Sordillo et al., 2007).

High milk production can be associated with increased oxidative stress in cattle (Lohrke et al., 2005). Lohrke et al. (2005) found that high producing dairy cows (51.7kg of milk/day) have increased levels of plasma lipid peroxides when compared to average producing dairy cows (34.2kg of milk/day). Therefore, highly productive dairy cows have insufficient detoxification of lipid peroxides and may be more susceptible to oxidative damage (Lohrke et al., 2005).

Minerals such as magnesium, iron, copper, manganese and selenium are essential components of the endogenous antioxidant defense system. Thus, deficiencies in these minerals can result in a misbalance of oxidants to antioxidants, and result in oxidative stress. Copper deficient cattle have decreased levels of the antioxidant enzyme, copper/zinc superoxide dismutase (CuZnSOD) and increased numbers of chromosomal abnormalities (Abba et al., 2000) which can be induced by radicals like superoxide.

Some diseases result in increased susceptibility to oxidative stress (Fernaes et al., 2005). Fernaes et al. (2005) found that scrapie infected neural cell lines had increased oxidative toxicity measured by a MTT cell viability assay. Scrapie is a fatal ovine neurodegenerative disease and part of the family of transmissible spongiform encephalopathy (TSE) family of diseases. Scrapie infected cells were significantly more sensitive to hydrogen peroxide than



normal cells. This study suggests that cells infected with scrapie have a weakened antioxidant defense. This increased susceptibility may play a role in the progression of disease in infected animals (Fernaesus et al., 2005).

### **Cellular Consequences of Oxidative Stress**

Reactive oxygen species are by-products of normal mitochondrial and metabolic processes, and when kept under control, are not always harmful. For example, superoxide is involved in the functioning of several enzymes and is used by macrophages to kill bacteria (Miller and Brzezinska-Slebodzinska, 1993). Damage to macromolecules and tissues occurs when ROS are not properly removed or when levels increase above normal, resulting in oxidative stress to the animal. Effects of ROS include direct peroxidative damage to lipids and nucleic acids and indirect damage induced by ROS to membranes and cellular pathways (Miller and Brzezinska-Slebodzinska, 1993). This damage results in dysfunctional regulation of essential cellular processes and in many cases, disease and pathology. At a cellular level, ROS damage lipids, DNA, polysaccharides, and proteins. Since ROS are oxidized molecules they can abstract electrons from other “normal” molecules. This results in a chain reaction that can cause tissue damage and affect normal physiology (Miller and Brzezinska-Slebodzinska, 1993).

#### *Lipid Peroxidation*

Free radicals can induce damage to membranes through lipid peroxidation (reviewed in Kowaltowski and Vercesi, 1999). Lipid peroxidation is the abstraction of a hydrogen atom from the methylene group of a lipid by a reactive species (Halliwell and Gutteridge, 1999) resulting in

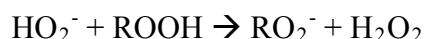
the formation of lipid peroxides. Peroxidized lipids are cleaved from the membrane to create a free lipid radical. Lipid radicals then combine with molecular oxygen and initiate a chain of lipid peroxidation. Lipid peroxidation is the interaction of ROS with free unsaturated fatty acids, lipoproteins and the acyl residues of membrane lipids (Evstigneeva et al., 1998). Under normal physiological conditions antioxidant enzymes, such as glutathione peroxidase 4 (GPx4) scavenge these peroxides.

Polyunsaturated fatty acids (PUFA) are the main targets for oxidative damage because of the availability of hydrogen atoms on the fatty acid chain. The adjacent double bond weakens the bond of the hydrogen on the next carbon, resulting in easy abstraction of the hydrogen by radicals. Saturated (no double bonds) and monounsaturated (one double bond) fatty acids are less susceptible to lipid peroxidation than PUFA.

Like other radicals, the process of lipid peroxidation begins with initiation. Initiation is usually the result of attack by a molecule that is reactive enough to abstract hydrogen from the methylene group. Hydroxyl radicals ( $\text{HO}^\cdot$ ) can easily initiate peroxidation by the reaction:



Superoxide radicals are not reactive enough to abstract hydrogen from lipids, but are in the protonated form ( $\text{HO}_2^-$ ). These hydroperoxyl radicals are the major contributors to lipid peroxidation in cells. Hydroperoxyl radicals initiate the reaction by attacking a lipid molecule, forming a lipid radical. Propagation of the reaction, whereby a lipid radical reacts with molecular oxygen, then attacks another lipid molecule, results in the formation of lipid peroxides. The propagation reaction results in the generation of hydrogen peroxide and a reactive lipid peroxide (Halliwell and Gutteridge, 1999):



In any case, peroxidation of a methylene group results in an unpaired electron on the carbon. This carbon radical can undergo various reactions including reacting with another carbon radical in the membrane to cross-link fatty acid side chains (Halliwell and Gutteridge, 1999). However, in the presence of oxygen, the most likely fate of carbon radicals is to combine with molecular oxygen to form peroxy radicals ( $\text{RO}_2^\cdot$ ). Peroxy radicals can abstract hydrogen from other lipid molecules or adjacent fatty-acid side chains, to continue the chain react of lipid peroxidation (Halliwell and Gutteridge, 1999). Combining of a peroxy radical with a hydrogen results in the formation of a lipid hydroperoxide (LOOH). Under very low concentrations of oxygen, carbon radicals can react with other molecules such the thiol group on some proteins (Halliwell and Gutteridge, 1999). Peroxidation is damaging because a single initiation event can result in formation of many peroxide molecules resulting from the chain reaction. Initial hydrogen abstraction from a PUFA on a membrane can occur at different points and thus produce varying amounts of hydroperoxides (Halliwell and Gutteridge, 1999). For example, peroxidation of linoleic acid gives two hydroperoxides while the peroxidation of arachidonic acid gives six hydroperoxides (Halliwell and Gutteridge, 1999).

Lipid peroxidation of cell membranes leads to an effect on normal function (Evstigneeva et al., 1998). Peroxidation of mitochondrial membrane lipids results in irreversible damage and loss of function to the respiratory chain and other components of the mitochondria (reviewed in Kowaltowski and Vercesi, 1999).

Lipid peroxidation can be terminated by antioxidants, or in some cases, by the reaction of two lipid radicals (Halliwell and Gutteridge, 1999). Avanzo et al. (2001) found that the rate of lipid peroxidation was higher in chicks fed a diet deficient in vitamin E than chicks fed a normal diet. When vitamin E is inadequate,  $\text{H}_2\text{O}_2$  can be converted to a hydroxyl radical.

## *DNA Damage*

Free radicals can damage DNA by direct attack or indirectly by interfering with replication and repair enzymes (Halliwell and Gutteridge, 1999). Oxidative stress greatly increases DNA damage such as strand breakage. Cells exposed to hydrogen peroxide or elevated oxygen concentrations, both inductors of oxidative stress, increases levels of DNA strand breakage (Halliwell and Gutteridge, 1999).

At normal physiological levels, most ROS do not react significantly with DNA or RNA bases. But like all other aspects of oxidative stress, problems appear when ROS levels are significantly higher than normal. Addition of hydrogen peroxide to animal cells results in an increase in DNA strand breakage and an increase in DNA base modification products (Halliwell and Gutteridge, 1999).

Hydroxyl radicals (OH<sup>•</sup>) can add to bases or abstract hydrogen from carbons on the rings and attack the C-4, 5 or 6 position of guanine or adenine. These radicals can also abstract hydrogen from thymine to create thymine peroxide radicals (Halliwell and Gutteridge, 1999). Radicals formed during lipid peroxidation can also damage DNA, mainly attacking guanine, but not to the extent of hydroxyl radicals.

Oxidative DNA damage has been detected at levels several-fold higher in mitochondrial DNA than nuclear DNA (Halliwell and Gutteridge, 1999). This is thought to be the result of the proximity of mitochondria DNA to ROS generated by the electron transport chain and because mitochondria DNA is not protected by histone proteins (Halliwell and Gutteridge, 1999). Radicals formed from lipid peroxidation of the inner mitochondrial membrane also target mitochondrial DNA because of proximity.

### *Protein Damage*

Protein damage by free radicals can affect the function of enzymes, receptors and signal-transduction. Damage can also indirectly affect other aspects of cell function, such as damage to DNA repair enzymes (Halliwell and Gutteridge, 1999). In addition, oxidized proteins may be recognized as foreign materials, thus initiating an autoimmune response (Halliwell and Gutteridge, 1999).

Reactive oxygen species can damage protein in several ways, such as the oxidation of –SH groups and the creation of protein peroxides. ROS preferentially attack certain amino acid residues (Halliwell and Gutteridge, 1999). Three targets of ROS are histidine, cysteine and methionine residues.

The oxidation of histidine residues on proteins can alter protein function. On enzymes, this can lead to the inactivation, thus resulting in indirect oxidative damage to cell functions such as DNA repair. Oxidation of histidine bases can also target proteins for degradation (Halliwell and Gutteridge, 1999). Histidine seems to have high reaction rates with superoxide molecules. Reactive oxygen species can also attack the thiol (-SH) groups on cysteine and methionine to form thiol radicals.

### **Endogenous Antioxidant Defenses**

As a natural protection, the body has several endogenous and exogenous antioxidants that function to scavenge free radicals. Endogenous antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (reviewed in Urso and Clarkson, 2003).

Exogenous antioxidants include water-soluble antioxidants, such as ascorbic acid (vitamin C) and lipid-soluble antioxidants, such as  $\alpha$ -tocopherol (vitamin E) and  $\beta$ -carotene (Miller and Brzezinska-Slebodzinska., 1993). Damage to tissues occurs when these antioxidants are not available at levels high enough to scavenge ROS. Several of these endogenous antioxidants require essential dietary trace elements such as Se, Mn, Cu and Fe (Miller and Brzezinska-Slebodzinska, 1993). There are several possible mechanisms by which antioxidants can work. They can: prevent free radical formation; scavenge free radicals and convert them into less reactive molecules; repair the damage caused by free radicals; induce expression of genes that code for antioxidant proteins; or provide an environment for the functioning of other antioxidants (Sen et al., 1995).

The body's naturally occurring antioxidant defense mechanisms can be thought of as a multi-step system. Each step requires both endogenous and exogenous antioxidants. The first step consists of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase that scavenge free radicals and break down scavenging products before they can react with macromolecules (reviewed in Evstigneeva et al., 1998). The second consists of fat and water-soluble antioxidants that interrupt radical reactions. These molecules include vitamin A, C, and E and several trace minerals such as selenium, copper, zinc and manganese. The third step involves the elimination and replacement of radical-damaged membrane lipids (reviewed in Evstigneeva et al., 1998) by enzymes such as phospholipase A2. All three steps are utilized together to stop the progression of free radicals and repair damage to cellular components.

Though there are many enzymes and nutritional components that make up the body's antioxidant defense systems, I have chosen to discuss a select number of key endogenous and exogenous antioxidants in this section.

### *Catalase*

Catalase is an antioxidant enzyme that functions to break down cellular H<sub>2</sub>O<sub>2</sub> into oxygen and water. Superoxide is converted by SOD into hydrogen peroxide, which is then broken down into water by GPx or catalase. Catalase and Gpx equally detoxify H<sub>2</sub>O<sub>2</sub> in erythrocytes (Gaetani et al., 1989). Overexpression of catalase in the cytosol of hepatocytes decreased intracellular H<sub>2</sub>O<sub>2</sub> levels compared to wild type cells (Bai et al., 1999). We did not measure catalase activity, so the rest of this section will focus on the two enzymes we did measure, SOD and GPx.

### *Superoxide dismutase (SOD)*

Superoxide is a highly reactive, unstable oxygen free radical. Superoxide can be formed in several ways, including as a byproduct of oxidative phosphorylation. During oxidative phosphorylation, occasionally (estimated less than 1% of the time) a single electron is transferred to molecular oxygen at complexes I and III of the electron transport chain, creating an extremely reactive oxygen molecule with a single electron in the outer orbit.

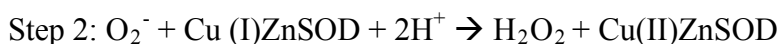
Superoxide can be scavenged by superoxide dismutases (SOD), a family of metalloenzymes that can catalyze the reaction to convert two superoxide molecules into hydrogen peroxide and oxygen (McCord and Edeas, 2005):



Superoxide dismutase is found as three main isoforms classified by the metal ion cofactor with which they are associated (reviewed in Frealle et al., 2005). All three forms, manganese (MnSOD), Iron (FeSOD) and copper/zinc (CuZnSOD), catalyze the conversion of superoxide to hydrogen peroxide (Halliwell and Gutteridge, 1999). The Manganese and copper/zinc SOD are found in mammals while FeSOD is found only in bacteria and some plants (Halliwell and Gutteridge, 1999). MnSOD is encoded for by the SOD2 gene and is localized to the mitochondrial matrix, while CuZnSOD is encoded by the SOD1 gene and originally found only in the cytosol (reviewed in Frealle et al., 2005).

### ***CuZnSOD***

Recent studies indicate that CuZnSOD (SOD1) is located in the cytosol, nucleus, peroxisomes and the mitochondrial inner membrane space (reviewed in Valentine et al., 2005). CuZnSOD is a highly conserved, homodimeric protein that has one copper binding site and one zinc binding site (Hart et al., 1999). CuZnSOD scavenges two superoxide anions in a two-step reaction (Hart et al., 1999):



CuZnSOD is the predominant SOD in most tissues, as it is responsible for approximately 70-80% of the total tissue SOD activity (Halliwell and Gutteridge, 1999). Mutations in SOD1 can result in increased levels of free radicals that can damage neurons and have been indicated in several other neurodegenerative diseases (reviewed in Rosen et al., 1993).

Mutations in the SOD1 gene are associated with familial amyotrophic lateral sclerosis (fALS), a neurodegenerative disease that affects motor neurons of the brainstem, spinal column



and cortex (Rosen, 1993). Mice with the targeted deletion of SOD1 have a reduced lifespan and increased incidences of liver tumors (Elchuri et al., 2005). SOD1 knockout mice showed a slight increase in MnSOD activity, but this declined as the mouse aged. In addition, SOD1 knockout mice had increased oxidative damage to the mitochondria and nucleus of cells and increased susceptibility of free radical damage to DNA, protein and lipids tumors (Elchuri et al., 2005). Since mice can live with a targeted deletion of SOD1, as opposed to the severely shortened lifespan of a SOD2 knockout mouse, this suggests that although CuZnSOD is the predominate SOD, it is not the most vital.

### *MnSOD*

MnSOD (SOD2) is also a homodimer and contributes approximately 10-20% of the total SOD activity in tissues (Halliwell and Gutteridge, 1999). The localization of MnSOD to the inner mitochondrial membrane (matrix side) contributes to its critical role in radical scavenging (Raha et al., 2000). Because of its position, MnSOD can readily destroy superoxide anions produced in the electron transport chain. Since superoxide is a negatively charged molecule and cannot readily cross the mitochondrial membrane (Gus'kova et al., 1984), it remains inside the matrix causing damage to macromolecules inside the mitochondria. Thus the location of MnSOD molecules allows for immediate scavenging of superoxide ions produced as by-products of oxidative phosphorylation.

The essential role of MnSOD was demonstrated by the generation of SOD2 knockout mice (Lebovitz et al., 1996). Mice with the targeted deletion of the SOD2 gene had no MnSOD activity and exhibited physiological abnormalities until their early death at approximately 21 days old (Lebovitz et al., 1996). Levels of CuZnSOD were increased by approximately 25% in

SOD2 knockout mice suggesting that the isoforms of SOD can partially compensate for each other. SOD2 knockout mice had reduced growth rates, reduced adipose and skeletal muscle mass and hypocellular bone marrow resulting in anemia, when compared to their wild type littermates. SOD2 knockout mice also had neurodegeneration in large central nervous system neurons. Degeneration was characterized by extensive damage to the mitochondria (Lebovitz et al., 1996). Ten-percent of the SOD2 knockout mice had damaged cardiac myocardium including swelling and fragmentation of the mitochondria and in some cases, lipid peroxidation of mitochondrial membranes (Lebovitz et al., 1996). SOD2 knockout mice indicate the vital role of MnSOD in the protection of mitochondria and the whole cell from oxidative damage.

Additional studies have supported the critical role of MnSOD in free radical scavenging. Yeast cells with the targeted deletion of the SOD2 gene (encoding MnSOD) have a significant increase in intracellular ROS levels, supporting the theory that SOD works to scavenge ROS in cells (Doudican et al., 2005). An increase in ROS levels in these mutant strains resulted in a direct increase in oxidative lesions, such as single-strand breaks and point mutagenesis, to mtDNA (Doudican et al., 2005). The livers of SOD2 heterozygous mice (containing only one normal allele for SOD2) have increased levels of mitochondrial superoxide anions, mtDNA oxidation and increased uncoupling of oxygen consumption to ATP synthesis (Williams et al., 1998). Both models indicate that MnSOD is important for suppression of superoxide in the mitochondria and to protect mitochondrial function during exposure to oxidative stress.

### *Glutathione*

Glutathione (GSH) is an endogenous antioxidant that randomly scavenges a variety of free radicals (reviewed in Sen, 1995) and plays a major role in the coordination of antioxidant

defenses in the body (Sen et al., 1994). In coordination with SOD, glutathione scavenges highly reactive  $H_2O_2$  and converts it to water (Halliwell and Chirico, 1993). During a scavenging reaction, two molecules of GSH are reduced to the oxidized form of glutathione, glutathione disulfide (GSSG), by the catalytic enzyme glutathione peroxidase (GPx) in the reaction:  $2GSH + H_2O_2 \rightarrow GS-SG + 2H_2O$ . GSH is resynthesized from GSSG by glutathione reductase (reviewed in Avanzo et al., 2001) in the reaction:  $GS-SG + NADPH + H^+ \rightarrow 2GSH + NADP^+$ . The ratio of GSH to GSSG is an important indicator of oxidative stress status in the body.

Glutathione is suspected to be able to regenerate  $\alpha$ -tocopherol from tocopherol-radical byproducts (reviewed in Sen et al., 1994). Mitochondria contain high levels of glutathione and thus scavenging of mitochondrial reactive oxygen species is largely glutathione dependent. Glutathione can come from exogenous sources, but it is not efficiently transported across cell membranes. In mice, glutathione treatment (1g/kg body weight) increased swimming endurance by 140% more than control animals. (Cazzulani et al., 1991).

In a study by Sen et al. (1994), total glutathione (tGSH) decreased significantly after exhaustive exercise in skeletal muscle, while serum GSH was elevated compared to nonexercised rats. Exhaustive exercise also resulted in a significant increase in lipid peroxides in skeletal muscle (Sen et al., 1994). The reduction in glutathione is likely the result of it being oxidized to its reduced form, GSSG, as the result of increased oxidative stress. Total GSH levels would decrease in muscle when oxidized GSH is transported out of the cells.

Skeletal muscle cells from rats treated with BSO (buthionine sulfoximine), an inhibitor of glutathione synthesis, decreased GSH 80-90% (Sen et al., 1994). When exercised, endurance decreased 50% potentially because of a decreased ability to scavenge exercise-induced ROS production (Sen et al., 1994). In addition, levels of lipid peroxides were higher in GSH deficient

rats compared to control animals (Sen et al., 1994). This study suggests an important role for glutathione in the control of exercise induced-oxidative stress. However, it is not clear what role glutathione plays in the regulation of oxidative stress induced by other means.

### *Glutathione Peroxidase*

Glutathione peroxidase (GPx) was first identified in 1973 as a selenoenzyme (reviewed in Behne and Kyriakoulos, 2001) and is the key enzyme involved in the reduction of glutathione to glutathione disulfide. GPx is composed of four protein subunits each of which contains selenium at the active site in the form of selenocysteine (Halliwell and Gutteridge, 1999).

Though GPx is specific for GSH as its H<sup>+</sup> donor (Halliwell and Gutteridge, 1999), different homologues can act on peroxides other than H<sub>2</sub>O<sub>2</sub>. Though some forms of GPx can reduce fatty acid hydroperoxides, they cannot act upon membrane-bound forms. Instead, hydroperoxides must first be released by other lipase enzymes (Halliwell and Gutteridge, 1999).

In mammals, there are five identified GPx homologues, GPx1-4 and GPx6 (Kryukov et al., 2003). GPx1, also called cellular glutathione peroxidase (cGPx), was the first glutathione peroxidase discovered and is the most ubiquitously expressed (Ran et al., 2004). Other glutathione peroxidases are tissue specific: GPx2 is expressed primarily in the digestive tract and GPx3 is found in blood plasma. GPx4, also called phospholipid-hydroperoxide glutathione peroxidase (PHGPx), associates closely with lipids in cellular membranes (Ran et al., 2004). The most recently discovered glutathione peroxidase, GPx6, has not been characterized (Ran et al., 2004).

## ***GPx1***

GPx1 was the first identified mammalian selenoprotein (reviewed in Behne and Kyriakoulos, 2001). It is present in almost all tissues and consists of four identical selenocystine containing subunits (reviewed in Behne and Kyriakoulos, 2001). GPx1 is the most abundant glutathione peroxidase, which would lead to the assumption that it is the most active and essential GPx. However a study Ho and colleagues (1997) showed that GPx1 knockout mice were healthy and fertile, with a completely normal phenotype. GPx1 knockout mice had no difference in lipid peroxidation in brain, heart, kidneys, liver or lung tissues from wild type mice. Knockout mice did not have increased in sensitivity to hyperoxia (exposure to high levels of oxygen) when compared to wild type mice (Ho et al., 1997). This normal phenotype suggested that GPx1 has a limited role in the oxidative damage defense mechanisms. However, de Haan and colleagues published a study in 1998 that conflicted with the results of Ho's study. Like Ho's study, de Haan's study showed the GPx1 knockout mice had a normal phenotype under normal phenotypic conditions. However, when GPx1 knockout mice were treated with 30mg/kgBW of paraquat, a potent generator of free radicals, all GPx1 knockout mice died within five hours post-injection (de Haan et al., 1998). All wild type mice survived when treated with the same dose, and GPx1 activity increased two-fold in their tissues when compared to untreated mice (de Haan et al., 1998). The same study also used neuronal cell cultures from knockout and wild type mice to look at susceptibility to H<sub>2</sub>O<sub>2</sub>, another oxidant. Cells were exposed to hydrogen peroxide and cell death was assessed. Cells from knockout mice had increased cell death, compared to wild type cells, as a result of increased susceptibility to the oxidant (de Haan et al., 1998). These two somewhat conflicting studies suggest that under normal conditions, low

GPx1 activity can be compensated for by other endogenous antioxidants. However, during certain forms of oxidative stress, this compensation may not occur or may not be adequate.

### ***GPx2***

GPx2, or gastrointestinal glutathione peroxidase, is a tissue specific selenoenzyme expressed primarily in the gastrointestinal tract of rodents and in the gastrointestinal tract and liver of humans (reviewed in Behne and Kyriakoulos, 2001). Like GPx1, it consists of four selenocystine containing subunits. The localization of GPx2 suggests that it may play a role in the body's defense against ingested lipid peroxides as well as products of lipid peroxidation occurring in the gastrointestinal tract (Halliwell and Gutteridge, 1999).

### ***GPx3***

GPx3, or plasma glutathione peroxidase, is another selenoenzyme expressed in various tissues then excreted into extracellular fluids (reviewed in Behne and Kyriakoulos, 2001). Yamamoto and Takahashi (1993) examined the reactivity of purified GPx3 to phosphatidylcholine hydroperoxide (PCOOH), a typical lipid peroxide. Phosphatidylcholine hydroperoxide was treated with purified GPx3 and produced two peaks (Yamamoto and Takahashi, 1993). Hydroxyl derivatives of phosphatidylcholine were identified using HPLC. The metabolites were the same as these changed when was treated with PCOOH with NaBH<sub>4</sub>, a compound known to produce hydroxyl derivatives from PCOOH (Yamamoto and Takahashi, 1993). In addition, Yamamoto and Takahashi found incubation of phosphatidylcholine hydroperoxides with GPx3 resulted in the almost complete disappearance of phospholipid-

derived hydroperoxides (Yamamoto and Takahashi, 1993). Phospholipid hydroperoxides are reactive with GPx3 indicating GPx3 is a scavenger for membrane oxidation products.

### ***GPx4***

Glutathione peroxidase 4 was the second mammalian selenoprotein to be identified (reviewed in Behne and Kyriakoulos, 2001). It is found in tissues in both cytosolic and membrane-bound forms and is imported into the mitochondria (reviewed in Behne and Kyriakoulos, 2001). GPx4 has the unique ability to directly reduce hydroperoxide groups on lipids and lipoproteins, making it part of the protection system against membrane oxidative damage (Ran et al., 2004). Since GPx4 is much smaller than the other glutathione peroxidases, it interacts with membrane lipids and reduces esterified fatty acid hydroperoxides, thus scavenging membrane lipid peroxidation products (reviewed in Ran et al., 2004). GPx4 also reduces thiamine hydroperoxide, a product of a free radical attack on a DNA molecule (Halliwell and Gutteridge, 1999).

Glutathione peroxidase 4 is considered to be the primary cellular defense against oxidative damage to cell membranes (Ran et al., 2004). Mice overexpressing the human GPx4 have increased levels of GPx4 mRNA and protein in heart, brain, kidney, liver and skeletal muscle. Interestingly, these mice have reduced oxidative injury after an oxidative stressor (Ran et al., 2004), solidifying GPx4's role in the endogenous antioxidant defense system. When normal mice are treated with diquat, a potent pro-oxidant that generates superoxide, liver damage that is associated with lipid peroxidation is evident. When mice overexpressing GPx4 were treated with diquat, liver damage and lipid peroxidation markers were reduced when

compared to normal mice (Ran et al., 2004). Therefore levels of diquat-induced lipid peroxidation were reduced in mice overexpressing GPx4.

Mice with the target deletion of GPx4 (GPx<sup>-/-</sup>) die during the first week of embryonic development, a much more severe phenotype than any other glutathione peroxidase mutant mouse (Yant et al., 2003). Mice null for other glutathione peroxidases show no abnormal phenotype or at the most, a mild increase in sensitivity to oxidative stress (Yant et al., 2003). Mouse embryonic fibroblasts (MEF) obtained from mice heterozygous for GPx4 (GPx4 <sup>+/-</sup>) showed increased sensitivity to oxidative stress induced by radiation, t-BOOH (a metabolic stressor), paraquat and H<sub>2</sub>O<sub>2</sub> concentration (Yant et al., 2003). The embryonic lethality and increase oxidative stress susceptibility in heterozygous mice indicates that GPx4 has a vital role in antioxidant defense that cannot be restored by other glutathione peroxidase homologs.

In the mitochondria, GPx4 helps to maintain electron transport chain function during oxidative stress. ATP production in liver mitochondria from mice overexpressing Gpx4 is not different from liver mitochondria from wild type mice. However, after mice were treated with diquat, an herbicide that generates superoxide, significantly decreased ATP production in wild type mitochondria but had no effect on GPx4 mitochondria (Liang et al., 2007b). Mitochondrial membrane potential also significantly decreased after wild type mice were treated with diquat, but remained unchanged in diquat treated GPx4-overexpressing mice. Therefore, GPx4 overexpression protected liver mitochondrial ATP production and maintained mitochondrial membrane potential during oxidative stress (Liang et al., 2007b).



## Exogenous Antioxidant Defenses

### *Vitamin E*

Alpha-tocopherol (vitamin E) is an essential lipid-soluble vitamin that functions as a multipurpose antioxidant (Evstigneeva et al., 1998). Alpha-tocopherol can quench superoxide anion and can protect polyunsaturated fatty acids in membranes from oxidation by reactive oxygen species (Mazor et al., 1997) by stopping the chain of lipid peroxy formation (*Figure 4*). Vitamin E acts as an antioxidant by donating hydrogen from its hydroxyl group to a lipid peroxide radical, resulting in a stable lipid species and a relatively unreactive tocopherol radical. Ascorbate (vitamin C) then aids in the regeneration of  $\alpha$ -tocopherol from the tocopherol radical (Rimach et al., 2002).

In a study by Brzezinska-Slebodzinska (1994) periparturient dairy cows supplemented with vitamin E had increased fast acting antioxidants in plasma. Plasma from cows treated with vitamin E demonstrated increased protection from ROS, resulting from an increase in fast acting antioxidants, than untreated cows. Cows supplemented daily with vitamin E tended to have lower instances of retained fetal membranes than unsupplemented cows (Brzezinska-Slebodzinska, 1994).

Vitamin E also prevented exercise induced oxidative damage in skeletal muscle of rats (Goldfarb et al., 1994). Control rats and rats supplemented with vitamin E were exercised on treadmills prior to euthanasia. Exercise increased the lipid peroxide marker TBARS, but this increase was prevented with vitamin E supplementation (Goldfarb et al., 1994). A decrease in lipid peroxide markers with vitamin E treatment indicates a decrease in oxidative stress in these cells. This study further supports the role for vitamin E in free radical scavenging.

In rats, hypercholesterolemia increases the production of free radicals. Vitamin E treatment during hypercholesterolemia increased tissue levels of glutathione and glutathione peroxidase and decreased markers of oxidative stress (Gokkusu and Mostafazadeh, 2003). Vitamin E treatment also reduced oxidative stress-induced cell death in chicken muscle cell cultures (Nunes et al., 2005). In these cells, oxidative stress was induced using low doses of menadione (vitamin K) and resulted in a 70% decrease in cell viability. When cells were treated with vitamin E following menadione, cell death was prevented (Nunes et al., 2005). Vitamin E may also play a role in stabilizing lipids from oxidative stress induced peroxidation and thus leads to decreased damage and cell death. Electron microscopy showed that membranes of liver cells from vitamin E deficient ducklings had decreases in polyunsaturated fatty acids in the membranes of liver cell organelles (Molenaar et al., 1972). In isolated bovine heart mitochondria lipid peroxidation and protein carbonyl formation increased exponentially with time. When single inner membrane vesicles, called submitochondrial particles (SMP), isolated from bovine heart mitochondria were incubated with  $\alpha$ -tocopherol, as little as 1 nmol/ mg SMP protein of  $\alpha$ -tocopherol prevented TBARS formation (Lass and Sohal, 1998).

Chicks fed a diet deficient in vitamin E develop a condition called exudative diathesis, resulting in swelling, bleeding and poor feathering (Avanzo et al., 2001). Markers of lipid peroxidation were greatly enhanced in the skeletal muscle of vitamin E deficient chicks when compared to chicks receiving adequate vitamin E. Supplementation also significantly increased levels of glutathione peroxidase in muscle mitochondria (Avanzo et al., 2001). The ratio of GSH/GSSG, a marker of antioxidant status, was significantly different between the two groups and indicated that the oxidative stress was induced by a vitamin E deficiency (Avanzo et al., 2001).

A study by Rafique and colleagues (2001) supported these findings using rats fed a vitamin E-deficient diet for 48 weeks. The depleted rats had undetectable levels of vitamin E in their skeletal muscle and liver and an 82% and 41% increase in malonaldehyde, a marker of lipid peroxidation, in the gastrocnemius muscle and liver, respectively. Mitochondria from the gastrocnemius muscle of the vitamin E depleted rats also had a decrease in membrane fluidity and decreased respiratory chain activity (Rafique et al., 2001). Supplementation of isolated heart mitochondria with vitamin E inhibits lipid peroxidation (Lass and Sohal, 1998).

Recent studies have examined the role of  $\alpha$ -tocopherol as a signaling molecule in the regulation of gene expression. A vitamin E responsive transcription factor (tocopherol-associated protein, TAP) was identified to interact with vitamin E sensitive genes (Zimmer et al, 2000). Few antioxidant genes have been identified as having TAP-dependent transcriptional regulation to date. However, studies have shown that vitamin E treatment and deficiency results in changes in antioxidant gene expression such as GPx1 and metallothionein (Rimbach et al., 2002).

### *Selenium*

Selenium is an antioxidant that works primarily in biochemical partnerships with other antioxidants, such as vitamin E and glutathione. Selenium is an essential component of the endogenous antioxidant, glutathione peroxidase (GPx) (reviewed in Burk, 1983). Nutritional deficiency of Se results in a decline in GPx activity and causes several diseases such as White Muscle Disease (horse, sheep and cattle) and Mulberry Heart Disease in pigs (Halliwell and Gutteridge, 1999). In the absence of GPx or when levels of Se are low,  $H_2O_2$  is converted to the highly reactive hydroxyl radical ( $OH^\cdot$ ). In rats, GPx activity decreases to undetectable levels after four weeks of feeding a Se deficient diet (reviewed in Burk, 1983). Selenium deficiency

significantly affects GPx activity and metabolism and results in a rise in the levels of cellular hydrogen peroxide levels (reviewed in Burk, 1983).

In cardiac tissue, Se deficiency resulted in a significant decrease in GPx and glutathione reductase enzymes (Venardos et al., 2005). Treatment with Se resulted in a dose-dependent increase in glutathione peroxidase expression. Not only is Se essential for the production of GPx, but a deficiency can result in reduced endogenous antioxidant defenses.

Exercise results in increased oxygen consumption and increases free radical production. One possible method of counteracting this increase of free radicals is through supplementation of antioxidants like Se. In a study by Palazzetti et al. (2004), control-trained and non-trained subjects were supplemented with Se during endurance training and markers of oxidative stress were measured. Selenium supplementation resulted in an increase in GPx activity in trained subjects suggesting that supplementation could provide protection against exercise induced oxidative damage to muscle cells.

Chicks fed a Se-deficient diet had elevated TBARS levels in muscle mitochondria compared to control chicks (0.73 and 0.27 nmols/mg of protein, respectively), indicating an increase in oxidative stress and lipid peroxidation (Avanzo et al., 2001). In muscle mitochondria from Se-deficient chicks, total GPx activity decreased compared to control chicks (75.1 versus 29.3 nmol NADPH oxidized/min/mg of protein, respectively). Selenium-dependent GPx activity, measured using  $H_2O_2$  as a substrate, also decreased from 23.8 nmol NADPH oxidized/min/mg of protein to 13.7 nmol NADPH oxidized/min/mg of protein (Avanzo et al., 2001). However the GSH/GSSG ratio remained unchanged between treatment and control animals.

## **The Mitochondria**

### *Membrane Composition*

Biological membranes are lipid bilayers that surround cells and organelles and contain large amounts of polyunsaturated fatty acid (PUFA) side chains. Membrane lipids are amphipathic, meaning that they have both hydrophobic tails and polar, hydrophilic heads. Proportions of fatty acids in animal membranes vary among species, tissue type and membrane type and function (Guidotti, 1972). In addition, animal membranes also contain small amounts of carbohydrates, in the form of glycolipids, and up to 25% cholesterol (Tanford, 1973). The relative ratio of protein to lipid varies among membrane type and the amount of protein in a membrane increases with the diversity of the particular membrane's function. Plasma membranes of animal cells tend to contain approximately 50% protein, 40-50% lipid and small amounts of carbohydrate. The outer mitochondrial membrane contains approximately 52% protein, 48% lipid and little (2-4%) carbohydrate. In comparison the inner mitochondrial membrane, where the enzymes of the electron transport chain reside, contains 76% protein, 24% lipid and very little (less than 2%) carbohydrate (Guidotti, 1972).

Membrane fluidity is associated with the presence of unsaturated and poly-unsaturated fatty acid side chains. These fatty acids lower the melting point of the membrane and thus increase viscosity. Fluidity is essential to the proper function of biological membranes and damage to membrane fatty acids results in decreased membrane function (Halliwell and Gutteridge, 1999).

The structural integrity of mitochondrial membranes is directly linked to mitochondrial function. The fatty acid composition of the inner mitochondrial membrane is suggested to play a

role in both aging and protection from oxidative damage (reviewed in Pamplona et al., 2002). Polyunsaturated fatty acids (PUFA) have the highest sensitivity to oxidative damage. Sensitivity increases as a function of the number of double bonds per a fatty acid molecule (reviewed in Pamplona et al., 2002).

The degree of fatty acid saturation in membranes may be linked to the membrane's susceptibility to oxidation. Sensitivity to oxidation by oxygen radicals increases as the number of double bonds in a fatty acid increase (Bielski et al., 1983). An in vitro study showed that oxygen radicals reacted strongest to fatty acids with multiple double bonds compared to those with only a single double bond (Bielski et al., 1983). The rate constant of the reaction between oxygen radicals and an unsaturated fatty acid, were highest in arachidonic acid (4 carbon-carbon double bonds) and negligible with oleic acid (one carbon-carbon double bond). These data support the theory that oxygen radicals account for some of the damage that occurs to cellular membranes (rich in PUFA) during times when free radical production is elevated. Therefore a higher degree of fatty acid saturation may be advantageous by decreasing susceptibility of membranes to free radical attack and lipid peroxidation (Pamplona et al., 2002).

The free radical theory of aging suggests that animals with the lowest rate of mitochondrial ROS production have the greatest maximal longevity (Maximum Lifespan Potential, or MLSP). Pamplona et al. (1998) found that the double-bond index (DBI) of mitochondrial phospholipids was negatively correlated with MLSP. A similar relationship was found between MLSP and in vivo lipid peroxidation levels. Therefore, animals with the longest MLSP have the lowest degree of membrane phospholipid unsaturation and the lowest level of lipid peroxidation (Pamplona et al., 1998). The actual physiological significance of a low DBI is unclear. However, the low DBI of membranes, especially mitochondrial membranes, in animals

with high MLSP could result in protection from lipid peroxidation and therefore, reduce the overall production of ROS (Pamplona et al., 2002). Overall, these aging studies have helped elucidate the role of membrane composition in protection from oxidative damage.

Little is known about the mechanisms by which the body regulates membrane fatty acid composition. To an extent, membrane remodeling can occur in response to and to cope with different physiological states (Hulbert et al., 2005). The structures of the fatty acid chains that make up membrane lipids can not only affect membrane fluidity, but also affect the function of membrane proteins (Hulbert et al., 2005). Dairy cows fed a diet to meet 80% of their energy requirements showed moderate changes in the fatty acid profiles of liver phospholipids (Douglas et al., 2007). However, regardless of the pre-partum diet, cows had a change in abundance of several fatty acids in liver phospholipids post-calving (Douglas et al., 2007). Long-chain PUFA (20:3, 20:5, 22:5, and 22:6) decreased at calving compared to pre-calving, then increased post-calving. In addition, the unsaturation index of liver phospholipids was lower at calving than at day 45 pre-calving or day 65 post calving (Douglas et al., 2007). There was no difference in unsaturation index between control and restriction-fed cows. Dietary manipulation and physiological state changes can alter phospholipid profiles of certain tissues including liver (Douglas et al., 2007). In rats, caloric restrictions of 8.5% and 25% both lead to a moderate decrease in the degree of liver mitochondrial membrane lipid unsaturation (Gomez et al., 2007).

Membrane lipid composition also varies among tissue types and membrane function. In addition, the membrane composition of organelles can be very different from that of cell membranes. In rats skeletal muscle cell and mitochondrial membranes have very different fatty acid composition (Tsalouhidou et al., 2006). Skeletal muscle mitochondrial membranes had higher monounsaturated fatty acids (MUFA) and lower PUFA than cell membranes. The

unsaturation index of mitochondrial membranes was lower than cell membranes in rats (Tsalouhidou et al., 2006).

### *Electron Transport Chain*

The electron transport chain (ETC) sits in the inner mitochondrial membrane and allows electrons from reduced substrates (such as NADH, FADH<sub>2</sub>, etc) to be eventually passed to oxygen to create water and ATP (*Figure 5*). Protons are pumped from the matrix to the inner membrane space at Complexes I, III, and IV. Proton pumping establishes a proton gradient used to drive ATP synthesis at Complex V (Voet and Voet, 1990).

Several different molecules act as electron carriers in the electron transport chain including ubiquinone (also known as coenzyme Q), iron-containing proteins such as cytochromes, iron-sulfur proteins, and flavin nucleotides. Ubiquinone can accept one or two electrons, becoming semi-quinone radical or ubiquinol, respectively. Flavins are also two-electron carriers with stable one-electron intermediates, while iron-sulfur centers and cytochromes are one-electron carriers.

In the overall electron transport chain reaction, electrons are moved from a reduced electron donor through a series of electron transport proteins to molecular oxygen. Complexes I and II catalyze the transfer of electrons from two different electron donors, NADH (complex I) and succinate (complex II), to ubiquinone. Complex III then takes electrons from reduced ubiquinone to cytochrome c, where Complex IV transfers the electrons to molecular oxygen (Nelson and Cox, 2005).

Complex I, or NADH dehydrogenase, is a large protein made up of several iron-sulfur centers. Complex I is thought to be the enzyme most affected by reactive oxygen species



because it consists of seven of the 13 protein subunits encoded for by mitochondrial DNA (Genova et al., 2004). Complex I catalyzes two reactions simultaneously: the transfer of four protons from the matrix to the inner membrane space and the transfer of a hydride ion from NADH and a proton from the matrix to ubiquinone. This makes Complex I a proton pump driven by electron transfer, specifically moving protons from the matrix to the inner membrane space. The now reduced ubiquinone, called ubiquinol, diffuses to Complex III where it is oxidized to ubiquinone once again (Nelson and Cox, 2005).

Complex II, or succinate dehydrogenase, is smaller and simpler than Complex I. Complex II is made up of four subunits: two membrane proteins (C and D) and two that extend into the matrix (A and B). C and D contain heme groups that are binding sites for ubiquinone. Subunits A and B bind the enzyme substrate which is succinate. Electrons in this complex move from succinate to FAD then through the iron-sulfur centers to ubiquinone (Nelson and Cox, 2005).

The next complex, Complex III or cytochrome bc<sub>1</sub> complex (or ubiquinone: cytochrome c oxidoreductase), couples the transfer of electrons from ubiquinol to cytochrome c to the pumping of protons from the matrix to the inner membrane space. Cytochrome c then moves to Complex IV, pushing electrons to this complex (Nelson and Cox, 2005).

Complex IV, or cytochrome oxidase, is the final step in the electron transport chain before ATP synthesis. Here, electrons are carried from cytochrome c to molecular oxygen, resulting in its reduction to water. At this complex, for every four electrons used to reduce oxygen, four protons are pumped into the intermembrane space. However, since the electron carriers at this complex can only carry one electron at a time, incompletely reduced intermediates (such as hydroxyl free radicals) can be released (Nelson and Cox, 2005).

Oxidative phosphorylation is the process by which most ATP is synthesized from metabolic intermediates in animals. This process is used to convert sugars and lipids to ATP, a form of energy that can be stored. Oxidative phosphorylation occurs in Complex V, or ATP Synthase, of the electron transport chain. Complex V has two main subunits,  $F_o$  and  $F_1$ . The  $F_o$  subunit translocates protons while the  $F_1$  subunit synthesizes ATP from ADP and  $P_i$ . Electron transport and oxidative phosphorylation are tightly coupled under normal physiological conditions (Voet and Voet, 1990).

During times when the demand for ATP is low, these two processes can become uncoupled allowing electron transport to continue without oxidative phosphorylation. Several compounds and proteins can also induce this uncoupling effect. When this occurs, the proton gradient across the membrane is reduced and energy is dissipated (Voet and Voet, 1990).

### *Production of ROS*

Complex I and III of the electron transport chain and the ETF and ETF-QOR of the  $\beta$ -oxidation pathway all have the potential to produce ROS because of the presence of partially-reduced electron donors. Production of ROS by Complex I is currently under debate because of the inability to measure ROS production by an isolated complex (Andreyev et al., 2005). In Complex I, two electrons are transferred from a reduced substrate such as NADH to a series of electron carriers in the complex. Because the distance between the substrate and the final electron carrier (Ubiquinone, Q) in Complex I is too great to allow direct electron transfer, the electrons are transferred through a series of four carriers in this complex. Two electrons are donated from NADH to a flavin nucleotide (FMN). FMN is a two electron shuttle with a stable one electron intermediate. From here, one electron at a time is passed on to a two iron-two sulfur

cluster then to a four iron-four sulfur cluster. Each of these iron-sulfur clusters are one electron carriers, hence the need for an initial carrier that has stable one and two electron states. From here, each electron is passed to a ubiquinone carrier. Ubiquinone has stable one (Ubisemiquinone, QH) and two electron states (Ubiquinol, QH<sub>2</sub>). However, the one electron intermediate results in a radical form of the ubiquinone, which under some circumstances may react with nearby oxygen, pass an electron and generate superoxide. Superoxide produced at this complex is released on the matrix side of the membrane (Nelson and Cox, 2005).

The second primary site for superoxide production in the ETC is Complex III. Like Complex I, Complex III involves ubiquinone as a one and two electron carrier. In Complex III, fully reduced ubiquinol (QH<sub>2</sub>) carries two electrons but needs to ultimately pass them to a one electron carrier (Cytochrome C). In order to accomplish this, Complex III uses the “Q cycle”: two electrons from ubiquinol are diverted down two separate pathways to ultimately reduce two cytochrome C molecules. The Q cycle accommodates the conversion from two electron carriers to a series of one electron carriers (in this case cytochromes b<sub>562</sub>, b<sub>566</sub> and c<sub>1</sub> and a two iron-two sulfur cluster) to the final one electron carrier. Complex III has two ubiquinone binding sites located between cytochrome b<sub>562</sub> and the two iron-two sulfur proteins (Q<sub>o</sub>) and between the matrix membrane and Cytochrome b<sub>566</sub> (Q<sub>i</sub>). In the Q cycle, a first molecule of ubiquinol (QH<sub>2</sub>) is oxidized to ubiquinone, releasing two protons into the inner membrane space and donating one electron to the iron-sulfur protein then cytochrome c<sub>1</sub>. The other electron is sent to a second ubiquinone molecule via cytochrome b<sub>566</sub> to cytochrome b<sub>562</sub>. A second molecule of ubiquinol is oxidized, again sending an electron through the iron-sulfur protein and a second electron to an ubisemiquinone molecule. Two protons are taken from the matrix to form the fully reduced ubiquinol (QH<sub>2</sub>). The passage of electrons from ubiquinol to cytochrome

b566 through Complex III again involves the ubisemiquinone radical intermediate, which could lead to the side reaction of superoxide production. Superoxide produced at this complex is released at the cytoplasmic side of the membrane.

### *Proton Leak and Uncoupling*

Under normal physiological conditions, not all of the mitochondria's oxygen consumption is coupled to the synthesis of ATP (reviewed in Rolfe and Brand, 1997). The result of this uncoupling of oxidative phosphorylation is proton leak (*Figure 6*). Proton leak reduces the efficiency of ATP synthesis by dissipating energy from metabolic intermediates as heat. Proton leak creates a futile cycle in which protons are pumped across the inner mitochondrial membrane and then leak back across into the inner membrane, bypassing ATP production. Studies using isolated cells, perfused muscle and an intact heart have shown that proton leak is not an artifact of mitochondrial isolation (reviewed in Rolfe and Brand, 1997). This leak is thought to make up approximately 25% of an individual's basal metabolic rate (reviewed in Brookes, 2005). The mechanism of proton leak and uncoupling is not well understood, but uncoupling proteins (UCP) are thought to play a role, possibly by transporting protons across the membrane. Proton leak rate increases with aging (Harper et al., 1998) and in response to certain hormones such as thyroid hormones (Lanni et al., 1999). Proton leak decreases during times where energy efficiency is reduced, such as hibernation (Barger et al., 2003) and in obesity (Harper et al., 2002).

Though all functions of proton leak have yet to be determined, several theories exist. Proton leak is thought to be a means of heat production in animals that must maintain a constant

body temperature (Rolfe and Brand, 1997). A second proposed function for proton leak is to reduce the production of free radicals as a cellular antioxidant defense.

It is believed that uncoupling and proton leak may help to reduce the generation of free radicals such as reactive oxygen species (ROS), since the generation of free radicals is dependent on coupling of oxidative phosphorylation (reviewed in Brookes, 2005). Under normal conditions, free radicals or ROS are produced as a natural product of the electron transport chain. The majority of ROS produced are superoxide molecules (molecular oxygen with one unshared electron in its outer orbit). In the electron transport chain, molecular oxygen is reduced to water and the energy from this reaction is used to create a proton pump. Approximately 2-5% of the time a single electron is passed to molecular oxygen creating a superoxide radical (reviewed in Brookes, 2005). Superoxide is highly unstable and is scavenged to hydrogen peroxide by superoxide dismutase. Uncoupling of mitochondria, resulting in an increase in proton leak, decreases ROS generation in both yeast and liver cells (reviewed in Brookes, 2005). At the same time, increased ROS can increase proton leak. This suggests that proton leak plays a beneficial physiological role in the reduction of oxidative damage in cells by both decreasing ROS production and by responding to increased ROS levels.

The rate of ROS production by the mitochondria is influenced by many factors including membrane potential, overall redox state, and concentration of ROS-generating compounds. The mitochondrial membrane potential ( $\Delta\Psi$ ) is the electrical potential between the difference between the two sides of the inner mitochondrial membrane (matrix and inner membrane space) and drives the production of ATP. Changes in the membrane potential caused by alterations in the proton gradient can alter ROS production. For example, a negative membrane potential favors the formation of superoxide. A slight reduction in the membrane potential leads to tighter

coupling between electrons and the ETC complexes, reducing the chance for the production of superoxide (Korshunov and Skulachev, 1997). A pH gradient ( $\Delta$  pH) is also necessary for maximal superoxide production in the mitochondria (Lambert and Brand, 2004a). The rate of superoxide production by Complex I is highly dependent on the pH gradient across the inner mitochondrial membrane (Lambert and Brand, 2004b)

One of the main regulators of ROS generation is the redox state of the mitochondrial ETC complexes. Each ETC complex has its own redox potential and responds differently to changes in membrane potential and the membrane proton gradient (Brookes, 2005). More reduced superoxide-generating sites on the ETC complexes can lead to increased superoxide production during electron transport (Lambert and Brand, 2004a). Lambert and Brand (2004a) found that though a relatively overall reduced complex I is required for superoxide production, an increase does not occur without the presence of an inhibitor at the Q site.

The inhibition of the ROS generating sites on the ETC complexes can also result in an increase in ROS production (Brookes, 2005). The presence of antimycin (complex III inhibitor) results in elevated ROS levels in isolate mitochondria (Cadenas and Boveris, 1980). While each of these factors influence the rate of ROS production, they alone are not sufficient for high production. Many of these factors work through altering  $\Delta\Psi$  or  $\Delta$ pH resulting in elevated ROS levels.

### **Uncoupling Proteins**

The family of mammalian uncoupling proteins consists of five known proteins: UCP1, UCP2, UCP3, UCP4 and brain mitochondrial carrier protein 1 (BMCP1), or UCP5. Of these,

UCP1-3 have high sequence homology and UCP4 and UCP5 have low sequence homology to UCP1. UCP1-3 are also known to regulate mitochondrial proton leak (reviewed in Krauss, 2005). UCP proteins have six transmembrane domains and (with the exception of BMCP1) are located in the inner mitochondrial membrane. UCP1 maps to human chromosome 4, rat chromosome 19, mouse chromosome 8, and bovine chromosome 17. UCP2 and UCP3 have been mapped together on human chromosome 11, rat chromosome 1, mouse chromosome 7 and bovine chromosome BTA15 (Stone et al., 1999). UCP4 mapped to human chromosome 6 (Mao et al., 1999), rat chromosome 9, mouse chromosome 17 and bovine chromosome 23. BMCP1 is located on chromosome X in humans and mice (Sanchis et al., 1998). The uncoupling protein family has a potential role in the regulation of energy expenditure and in regulation of oxidative damage in cattle.

Two main theories on the mechanisms behind the uncoupling protein dependent decrease in ROS production have been proposed. First, UCP alter the membrane potential by mild uncoupling. Mild uncoupling slightly limits the proton gradient which in turn slightly increases oxygen consumption but allows continuing of ATP synthesis. Since the generation of ROS in the ETC is very sensitive to the proton gradient established by the pumping of protons into the inner membrane space, mild uncoupling could decrease the gradient very slightly to decrease ROS production.

The second theory proposes that UCP aid in the export fatty acids and lipid peroxides from the matrix. When fatty acid supply exceeds oxidation rates, fatty acids can enter the matrix through a transmembrane transporter instead of CPT. These fatty acids are susceptible to peroxidation and thus exporting by UCP would aid in the protection against the accumulation of lipid peroxides and oxidative damage (Schrauwen et al., 2006).

## *UCP1*

Uncoupling protein 1 (UCP1) was the first member of the uncoupling protein family to be discovered (Gimeno et al., 1997). The thermogenic function of brown adipose tissue (BAT) was discovered in the early 1960's, but it wasn't until 1982 that BAT uncoupling protein (renamed UCP1 in 1997) was purified (Ricquier and Bouillaud, 2000).

UCP1 is expressed only in brown adipose tissue and plays an important role in adaptive thermogenesis (reviewed in Ricquier and Bouillaud, 2000). UCP1 mediates proton leak in BAT and is controlled by the sympathetic nervous system (reviewed in Krauss, 2005). UCP1 null mice do not maintain body temperature when exposed to cold at 4°C, indicating a defect in their thermoregulation capabilities (Enerback et al., 1997). In addition, UCP1 null mice were not obese when fed a normal or high fat diet, but did show increased adiposity in the interscapular brown fat stores supporting the theory that the tissue is not able to utilize lipids for thermogenesis. UCP1 null mice showed a five-fold increase in UCP2 mRNA in brown adipose tissue stores suggesting that the members of the UCP family can function for each other. These data, along with the normal phenotype of UCP1 heterozygous mice support the theory that UCP homologues can compensate for each other (Enerback et al., 1997). Though UCP2 may have some functions similar to UCP1, studies suggest that UCP2 has other important roles in animals.

## *UCP2*

Uncoupling protein 2 is an uncoupling protein homologue discovered in 1997 by Fleury and colleagues. UCP2 has 55% amino acid identity to the previous identified uncoupling protein



1 (UCP1) (Boss, 1997a). Unlike UCP1, UCP2 seems to be expressed ubiquitously, including brown adipose tissue (BAT), white adipose tissue (WAT), skeletal muscle, lung, heart, placenta, kidney, pancreatic tissue and the hypothalamus. In addition, high levels of UCP2 expression are found in macrophages, spleen, thymus, bone marrow and leukocytes (Fleury et al., 1997).

Though all of the functions of UCP2 are not known, there is an association between UCP2 and energy expenditure, though conclusions are conflicting. Yeast strains transfected with UCP2 expression vectors showed a strong decrease in mitochondrial membrane potential and partially uncoupled respiration when compared to control strains (Fleury et al., 1997). However, changes in membrane potential may be due to improper insertion of the overexpressed UCP2 protein in the mitochondrial membrane. Thymocytes isolated from UCP2 knockout (KO) mice have levels of mitochondrial uncoupling at least 50% lower than thymocytes from normal mice (Krauss et al, 2005). Lower levels of uncoupling were also supported by a reduction in oxygen consumption (a driving force in proton leak). In support of UCP2's role in energy balance regulation, levels of UCP2 mRNA are increased in ob/ob (leptin deficient) mice (Chavin et al., 1999). The high level of UCP2 expression in white blood cells and organs of the immune system suggests a possible role of UCP2 in disease resistance and response to infection.

Studies using UCP2 KO mice have also suggested a stronger role for UCP2 in the prevention of reactive oxygen species (ROS) formation. A study published in 2000 demonstrated that UCP2 deficient mice are resistant to an infection by *Toxoplasma gondii*, a fatal murine brain parasite (Arsenijevic et al., 2000). UCP2 deficient mice produce 80% more ROS than wild type mice, suggesting that UCP2 is essential for the suppression ROS generation (Arsenijevic et al., 2000). In a healthy animal, the suppression of ROS is essential but in an infected animal an increase might aid the host immune defense. The same study also found that

when isolated macrophages from wild type mice were challenged with lipopolysaccharide (LPS), the potent endotoxin found in gram-negative bacteria, UCP2 expression decreased and ROS production increased. When UCP2 KO macrophages were challenged with LPS, expression of MnSOD increased. Decreased UCP2 expression was associated with increased ROS production, increased cytokine production and increased SOD2 production (Arsenijevic et al., 2000). These data support the hypothesis that UCP2 aids in the suppression of ROS production most likely through an increase in uncoupling of the mitochondria. Another study using UCP2 knockout mice further supported the role of UCP2 in the suppression of ROS. UCP2 knockout mice had increased levels of malonaldehyde (MDA), a marker of lipid peroxidation, in their livers (Horimoto et al., 2004).

UCP2 expression also increases during increased  $\beta$ -oxidation rates in liver cells (Grav et al., 2002). In a study where rats were fed with the modified fatty acid tetradecylthioacetic acid (TTA), production of acid-soluble products from labeled fatty acid substrates increased, indicating that  $\beta$ -oxidation rates increased in liver cells. UCP2 mRNA expression was elevated in liver cells concurrent with the increase in  $\beta$ -oxidation markers (Grav et al., 2002).

### *UCP 3*

Uncoupling protein 3 (UCP3) is another uncoupling protein homologue first discovered in 1997 (Boss, 1997a). UCP1 and UCP3 share a 56% amino acid identity. The UCP3 gene encodes two transcripts, UCP3 long (UCP3<sub>L</sub>) and UCP3 short (UCP3<sub>S</sub>), which are transcribed into the long and short form proteins. These two proteins differ only by 37 amino acids in the C-terminus of the proteins (Boss, 1997a).

Originally studies suggested that UCP3 was expressed at low levels in white adipose tissue (WAT), cardiac tissue and other tissues. However UCP3 expression is almost exclusively found in skeletal muscle tissue and brown adipose tissue (BAT) suggesting that previous papers were looking at other uncoupling proteins besides UCP3 (Boss, 1997a).

Though all of UCP3's functions are not known, UCP3 may play a role in regulating energy metabolism and expenditure by increasing resting metabolic rate (Clapham et al., 2000). However, studies using UCP3 knockout mice showed that UCP3 is not required for energy balance maintenance. UCP3 KO mice have normal body weight, resting metabolic rate, and thermoregulation and can adapt normally to cold temperatures. Unlike other uncoupling protein KO mouse models, UCP3 KO do not have increased UCP1 or UCP2 mRNA levels (Vidal-Puig et al., 2000).

Although UCP3 KO mice are healthy and have normal body weights and resting energy expenditure, they have abnormal mitochondrial respiration in skeletal muscle. Skeletal muscle mitochondria are more coupled suggesting that UCP3 functions as a mitochondrial uncoupling protein. In addition, skeletal muscle mitochondria in UCP3 KO mice have increased ROS levels (Vidal-Puig, 2000) suggesting a possible role for UCP3 in the regulation of ROS generation and control of oxidative stress in this tissue.

To further complicate things, a study was published in Nature (Clapham et al., 2000) shortly after the UCP3 KO studies were published using transgenic mice overexpressing UCP3. Mice overexpressing UCP3 had a reduced body weight despite being hyperphagic. These mice also had reduced adiposity and increased insulin sensitivity (Clapham et al., 2000). In April 2005, a study was published using mice that overexpressed UCP3 at two-fold the physiological level. This study found no difference in total energy expenditure among UCP3 null mice, mice

overexpressing UCP3, or WT mice (Bezaire et al., 2005). Mice overexpressing UCP3 had decreased intramuscular triglyceride levels, indicating a shift towards fat oxidation in skeletal muscle of these transgenic mice (Bezaire et al., 2005). These mice also have increased levels of plasma membrane fatty acid binding protein (FABP<sub>pm</sub>) in muscle cells, indicating that the skeletal muscle of UCP3 overexpressing mice is better able to handle increased fatty acid entry into the cells. In addition, both UCP3 null mice and UCP3 overexpressing mice had increased levels of ATP and ATP and ADP, respectively, arguing against the proposed role of UCP3 in mitochondrial uncoupling.

Skeletal muscle is a highly metabolic tissue, comprising approximately 30% of basal metabolic rate (Zurlo et al., 1990). UCP3 expression levels in skeletal muscle change in accordance to changes in energy balance. UCP3 expression is increased in the skeletal muscle of rats during long-term (6-12 months) caloric restriction (Bevilacqua et al., 2004). Food-restricted animals also maintained normal capacity for ATP production despite caloric restriction, suggesting that a decrease in this uncoupling protein allows ATP levels to remain normal despite negative energy balance. Several studies have shown that UCP3 expression and proteins levels increase in skeletal muscle during times where fat oxidation and circulating free fatty acid (FFA) levels are increased, such as starvation and high fat diets (Chou et al., 2001). These reports suggest that during calorie restriction without malnutrition, UCP3 is decreased because of lower levels of free radicals. However, when animals are starved (complete food restriction), the mobilization of body fat and lipid oxidation results in increased UCP3 expression.

During periods of acute exercise in humans, both plasma FFA and UCP3 levels increase in skeletal muscle (reviewed in Schrauwen et al., 2001). UCP3 levels increase in response to

treatment with thyroid hormone, a condition also known to increase fat oxidation and circulating FFA levels (reviewed in Schrauwen et al., 2001).

UCP3 decreases during times where circulating FFA levels are decreased in mice (Brun et al., 1999). During lactation, where mammary uptake of circulating FFA is increased (therefore circulating FFA levels are low), UCP3 expression in skeletal muscle is decreased in mice (Xiao et al., 2004). When nursing pups were removed from the lactating animal, circulating FFA levels return to normal and UCP3 levels showed a full recovery within 48 hours of pup removal (Xiao et al., 2004). In addition Brun et al. (1999) found that UCP3 expression in skeletal muscle of neonatal mice responded to alterations in their diet. Mice fed a high-carbohydrate diet post-weaning had lower UCP3 expression levels when compared to mice weaned onto a high-fat diet. When 15 and 21-day old pups were fasted, UCP3 expression increased in skeletal muscle, as did non-esterified fatty acids (Brun et al., 1999).

Recently more evidence has accumulated suggesting that UCP3 is not a major factor in the regulation of mitochondrial ATP production and energy expenditure (Vidal-Puig, 2000). Since UCP3 knockout mice have normal phenotypes and body weight but increased ROS levels, focus has shifted to other potential functions of UCP3. UCP3 is now thought to play a role in both oxidative stress and preventing the formation of ROS formation and in the regulation of FFA uptake in skeletal muscle. In rats that have increased UCP3 levels in skeletal muscle in response to a high fat diet, ATP production and 24-hour energy expenditure are normal (Chou et al., 2001).

One hypothesis is that the physiological function of UCP3 is to protect the mitochondria from the accumulation of fatty acids in the inner mitochondrial matrix. This is especially important in situations where fatty acid delivery exceeds oxidation (Schrauwen et al., 2001).

UCP3 is expressed at higher levels in glycolytic (type 2) muscle tissue than oxidative (type 1) muscle tissue. In human skeletal muscle, UCP3 expression is highest in type 2b (fast glycolytic) muscle fibers, followed by type 2a (fast oxidative) and type 1 (slow oxidative) (Hesselink et al., 2001). Oxidative stress is highest in type 2b and lowest in type 1 muscle fibers, so the high level of expression in type 2 fibers supports the role of UCP3 in reducing oxidative stress (Hesselink et al., 2001). Increased UCP3 may minimize the damage to mitochondria and cells in these high oxidative muscle fiber types.

#### *UCP4 and BMCP1*

Human UCP4 was cloned in 1999 using the protein sequence of UCP3 (Mao et al., 1999). Sequence analysis revealed that UCP4 possesses a 19%, 33%, and 34% amino acid homology to human UCP1, UCP2 and UCP3, respectively. Unlike other UCPs, UCP4 expression is brain-specific. UCP4 is expressed in most brain tissues and at low levels in the spinal cord, and is localized in the mitochondria of neural cells (Mao et al., 1999). Like the other UCPs, UCP4 decreases mitochondrial membrane potential.

BMCP1 was discovered in 1998 and shares a 34%, 38% and 39% sequence homology to UCP1-3, respectively (Sanchis et al., 1998). Like UCP4 it is expressed predominately in brain tissue, however BMCP1 has slight expression levels in the heart, skeletal muscle, gut, lung, kidneys, reproductive organs and adipose tissue (Sanchis et al., 1998). BMCP1 has six transmembrane domains like other members of the UCP family and BMCP1 decreases mitochondrial membrane potential and increases mitochondrial uncoupling (Kim-Han et al., 2001). Neuronal cell lines that overexpress BMCP1 had a 25% decrease in superoxide production and an increase in mitochondrial uncoupling (Kim-Han et al., 2001). Reactive

oxygen species are an important aspect of many neurodegenerative diseases, so BMCP1 may be a part of the body's endogenous defense system to these diseases.

### **Activation and Regulation of Uncoupling Proteins**

#### *Superoxide*

An increase in superoxide levels increases proton leak through all three UCP homologues in isolated mitochondria (Echtay et al., 2002). Echtay et al. (2002) demonstrated that superoxide could activate UCP3 resulting in increased proton conductance in vitro (*Figure 7*). Incubating isolated mouse skeletal muscle mitochondria with xanthine plus xanthine oxidase to generate superoxide increased proton conductance. When superoxide dismutase was added, the increased proton conductance was abolished, indicating that the increased proton conduction was dependent on the presence of superoxide. The effect was also abolished by incubating cells with the purine nucleotide GDP, a known inhibitor of UCPs, or with bovine serum albumin (BSA), suggesting that the increase in proton leak was dependent on fatty acid-activation of UCP3 (Echtay et al., 2002).

In order to confirm that the increase in proton conductance was dependent on UCP3, Echtay et al. (2002) used mitochondria isolated from UCP3 knockout mice. Superoxide produced by incubating with xanthine and xanthine oxidase had no effect on the proton leak rate in mitochondria from UCP3 knockout mice. Further confirmation came from measuring proton leak in mitochondria isolated from starved wild type rats. A two-fold increase in proton leak correlated with a two-fold increase in UCP3 protein levels in skeletal muscle (Echtay et al., 2002). Mitochondria isolated from tissue expressing only UCP2 (rat kidney, spleen and

pancreatic beta cells) responded to superoxide with increased proton leak. This increase required fatty acids and was inhibited by GDP.

Avian uncoupling protein (avUCP) increases during fasting in chicken skeletal muscle. Fasting for 24 hours resulted in elevated serum NEFA levels and increased superoxide production by skeletal muscle mitochondria compared to control (fed) chickens. Further analysis of avUCP expression after the 24-hour fast showed a 7.7-fold increase in skeletal muscle compared to control chickens (Abe et al., 2006).

Electrostimulation of isolated rat muscle cells increased ROS levels and resulted in a significant increase in UCP3 expression. In the same study, in vitro incubation of rat skeletal muscle cells with xanthine/xanthine oxidase for 10 days resulted in a 115% increase in UCP3 expression compared to control cells (Silveira et al., 2006).

### *Lipid peroxides*

Evidence for the control of UCP2 and UCP3 by lipid peroxides is very circumstantial. Goglia and Skulachev (2003) proposed a theory that uncoupling proteins act as carriers of fatty acid peroxides in the mitochondria, protecting the matrix and its components from oxidative damage. They suggest that UCPs are activated by, and aid in, the translocation of lipid peroxides through the inner membrane to protect mtDNA and other parts of the matrix.

Lipid peroxidation by-products, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are cytotoxic. HNE specifically targets mitochondrial DNA and proteins and can induce mitochondrial uncoupling through UCP2 and UCP3 (Echtay et al., 2003). HNE may also aid in the protection against oxidative damage by activating UCPs, resulting in mild uncoupling and diminished superoxide production (Echtay et al., 2005).



Because the measures used to determine lipid peroxidation are indirect, many data show merely correlations. For example, Khalfallah et al. (2000) used lipid infusion to induce fatty acid oxidation in skeletal muscle cells of human subjects. In response to increased lipid oxidation rates, both plasma FFA levels and UCP3 mRNA expression increased. Levels of UCP3 mRNA positively correlated with plasma FFA levels,  $\beta$ -hydroxybutrate and lipid oxidation rates indicating that lipid oxidation may be an important factor in UCP3 transcriptional regulation. However, this evidence suggesting that lipid peroxides regulate UCPs can be explained by either ROS activation of UCPs or by fatty acid activation.

### *Fatty acids*

There is a high correlation between the pattern of UCP expression and conditions in which fatty acids are the primary fuel source such as fasting (de Lange et al., 2006), high fat diets (Chou et al., 2001), lipid infusion (Weigle et al., 1998) or hibernation (Barger et al., 2006). Both UCP2 and UCP3 expression have been shown to increase in response to free fatty acid levels in serum or plasma.

Upregulation of UCP3 in response to fasting has been reported in rats (de Lange et al., 2006), collared lemmings (Blaylock et al., 2004), marsupials (Jastroch et al., 2004), and arctic ground squirrels (Barger et al., 2006), while an increase in UCP2 during fasting has been reported in rats (Boss et al., 1997b). Food deprivation in rats for 24 or 48 hours results in elevated serum NEFA levels and this increase coincides with elevated fatty acid oxidation in isolated mitochondria and UCP3 protein in skeletal muscle cells (de Lange et al., 2006). Treatment with nicotinic acid during food deprivation reduced serum NEFA levels and inhibited this increase in UCP3 expression, supporting the hypothesis that UCP3 transcription is

upregulated by fatty acids. UCP2 expression in skeletal muscle increases after a 72-hour fast in rats (Weigle et al., 1998). Studies using collared lemmings also report a significant increase in UCP3 expression, correlated to plasma free fatty acid levels, in skeletal muscle after a 6, 12 or 18 hour fast (Blaylock et al, 2004). Additional reports have shown that a 46-hour fast in rats results in increased expression of UCP2 and UCP3, but that the degree of the response is dependent on muscle fiber type (Samec et al., 2002). The highest increases in both genes were found in fast-twitch type fibers and a moderate increase was found in slow-twitch fibers.

UCP3 expression increases in the skeletal muscle of rats fed a high fat diet compared to muscle of rats on a standard diet (Matsuda et al., 1997). Chou et al. (2001) showed that UCP3 protein levels increased two-fold in skeletal muscle and were highly correlated to serum NEFA levels in rats fed a high fat diet (60% of energy from fat) compared to rats on a standard diet (12% of energy from fat).

UCP3 expression in skeletal muscle of arctic ground squirrels increases 10-fold and 3-fold during non-hibernating fasting and hibernation, respectively, but is not effected by environmental temperature (Barger et al., 2006). Serum NEFA levels were significantly higher in non-hibernating fasting and hibernating squirrels compared to awake, fed squirrels. UCP3 protein levels in skeletal muscle were higher after a 48-hour non-hibernating fast than in samples from fed squirrels, but this change in UCP3 did not correspond to a change in proton leak rates.

Lipid infusion also increases UCP3 expression in skeletal muscle of rats compared to saline infusion (Weigle et al., 1998). A 24-hour lipid infusion (0.0023 grams/min) resulted in a significant increase in UCP2 expression and UCP3 expression in rat skeletal muscle (Vettor et al., 2002).

During hibernation, animals switch to oxidizing lipid stores as a fuel source and thus circulating free fatty acid levels are higher than during aroused states (Nizielski et al., 1989). Boyer et al. (1998) showed a 3-fold increase in UCP3 mRNA in the skeletal muscle of hibernating ground squirrels.

Data support the hypothesis that UCP levels increase when lipids are utilized as a fuel source, and this increase correlates to increased circulating NEFA levels. However, it is unclear if this is a direct regulation or if the by-products of  $\beta$ -oxidation control these genes (*Figure 8*).

### *Cold exposure*

Unlike UCP1, it is unlikely that UCP2 and UCP3 expression in skeletal muscle is involved in thermoregulation. Though all three homologues have been implicated in playing a role in thermogenesis in BAT, evidence supporting a role in skeletal muscle is lacking. Of the homologues, only UCP1 in BAT can mediate a non-shivering thermogenesis (NST) response to cold exposure (Golozoubova et al., 2001).

Boss et al. (1997b) reported a 2.6-fold increase in UCP2 expression in skeletal muscle in rats following a 48-hour exposure to 6°C. However UCP2 knockout mice have a normal response to cold exposure (Arsenijevic et al., 2000) and there have been no other studies that have confirmed this increase.

Studies using Artic ground squirrels indicate that UCP3 expression in skeletal muscle is unaffected by degree of cold exposure (Barger et al., 2006). Cold temperatures also failed to increase UCP3 expression in skeletal muscle of rats (Larkin et al., 1997). Data collected from collared lemmings further dispute the hypothesis that UCP3 is activated by cold temperatures because UCP3 expression in skeletal muscle significantly decreased after mild (10°C) cold

exposure (Blaylock et al., 2004). Similar reports indicate this is also true in human subjects where UCP3 expression and protein was decreased following mild cold exposure (Schrauwen et al., 2002).

### **Other Target Genes**

The antioxidant defense system in the body is a complex network of genes, proteins and enzymes that function to scavenge oxidants and help repair damage sustained to cells. We have selected a few target genes that are involved in the regulation of endogenous antioxidant defenses to study in cattle.

#### *ERR $\alpha$*

The estrogen-related receptors (ERR) are a family of orphan nuclear hormone receptors that have ligand-independent transcriptional activity (Rangwala et al., 2007). Two of the family members, ERR $\alpha$  and ERR $\gamma$ , are expressed in tissues with high metabolic activity, including the heart, skeletal muscle and brain. The third member, ERR $\beta$ , is highly expressed during development in the embryo.

Estrogen related receptors are coactivated by the PPAR gamma coactivator (PGC) family of transcription coactivators. ERR are thought to work with PGC1 $\alpha$  to regulate mitochondrial gene expression, implicating a role for both genes in metabolism and proper mitochondrial function. PGC1 $\alpha$  regulates the expression of ERR $\alpha$  (Schreiber et al., 2003). Cells infected with adenoviral vectors expressing PGC1 $\alpha$  showed strong induction of ERR $\alpha$  mRNA and an increase

in ERR $\alpha$  protein levels that appeared shortly after the appearance of PGC1 $\alpha$  protein (Schreiber et al., 2003).

As a coactivator, ERR $\alpha$  is required for the PGC1 $\alpha$ -dependent induction of mitochondrial ROS detoxification genes such as superoxide dismutase 2, thioredoxin 2 and peroxiredoxin 3 and 5 (Rangwala et al., 2007). ERR $\alpha$ -null fibroblasts have lower levels of oxidative phosphorylation (cytochrome C and ubiquinol c reductase b) and fatty acid oxidation gene (carnitine palmitoyl transferase 1b) expression compared to wild type fibroblasts, despite equivalent PGC1 $\alpha$  expression (Rangwala et al., 2007). Microarray analysis also found gene clusters describing mitochondrial pathways such as oxidative phosphorylation, fatty acid oxidation and electron transport were down-regulated in ERR $\alpha$ -null fibroblasts compared to wild type cells (Rangwala et al., 2007).

Inhibition of basal ERR $\alpha$  expression by small interfering RNA (siRNA) in cells infected with PGC1 $\alpha$  expressing adenoviral vectors had significantly lower expression of mitochondrial biogenesis genes (Schreiber et al., 2004). These genes included genes involved in mitochondrial DNA replication and transcription (mtTFA), protein import (Tim22), fatty acid oxidation (carnitine/acylcarnitine translocase, and oxidative phosphorylation (cytochrome C and ATPsyntase  $\beta$ ). Overexpression of ERR $\alpha$  (7.3 fold higher than wild type cells) in cardiac myocytes leads to increased expression of genes involved in fatty acid oxidation and mitochondrial respiration. Fatty acid oxidation genes included lipoprotein lipase, fatty acid binding protein 3, acyl-CoA oxidase, and fatty acyl coenzyme A synthetase were induced during ERR $\alpha$  overexpression (Huss et al., 2004). Mitochondrial respiration genes included cytochrome c oxidase and NADH dehydrogenase. In addition, ERR $\alpha$  overexpression increased mitochondrial fatty acid utilization in transfected cells. Oxidation of labeled palmitic acid

increased 49.3% in ERR $\alpha$ -overexpressing cardiac myocytes (Huss et al., 2004). Oxidation rates were decreased 85% when etomoxir, a CPT1 inhibitor, was added verifying that mitochondrial fatty acid oxidation was specifically measured.

ERR $\alpha$  may play a role in the control of  $\beta$ -oxidation in brown adipose tissue (BAT) cells. In developing BAT and cardiac cells, the appearance of ERR $\alpha$  protein parallels appearance of the  $\beta$ -oxidation gene medium-chain acyl coenzyme A dehydrogenase (MCAD) protein (Vega and Kelly, 1997). In addition, ERR $\alpha$  protein levels are greatest in tissues with the highest capacity for  $\beta$ -oxidation and abundant MCAD protein levels, including BAT and heart tissue. Tissues with low  $\beta$ -oxidation rates, such as white adipose, brain and lung tissues have low expression of MCAD and ERR $\alpha$  (Vega and Kelly, 1997). ERR $\alpha$  also binds to a key regulatory element on the MCAD promoter, regulating the tissue-specific expression of MCAD and possibly play a role in cellular energy balance (Sladek et al., 1997).

PGC1 $\alpha$  regulates both the expression and the transcriptional activity of ERR $\alpha$  (Schreiber et al., 2003). Using cell lines infected with adenoviral vectors expressing PGC1 $\alpha$ , Schreiber et al. (2003) showed that PGC1 $\alpha$  can induce ERR $\alpha$  expression at both the mRNA and protein levels. Schreiber et al. (2003) also showed that PGC1 $\alpha$  enhances the activity of ERR $\alpha$  regulated promoters by physically interacting with ERR $\alpha$ . PGC1 $\alpha$  induces the expression of the ERR $\alpha$  target gene, MCAD. In the presense of PGC1 $\alpha$ , the suppression of ERR $\alpha$  by small interfering RNA (siRNA) resulted in a decrease in ERR $\alpha$  mRNA and a decrease in MCAD. Therefore ERR $\alpha$  is required for the PGC1 $\alpha$ -dependent activation of MCAD (Schreiber et al., 2003). These studies demonstrate that both ERR $\alpha$  and PGC1 $\alpha$  are required for the activation of genes involved in mitochondrial function,  $\beta$ -oxidation, and energy metabolism, and that PGC1 $\alpha$  may in fact be a ligand to regulate ERR $\alpha$ -dependent transcriptional activation.

## *P53*

P53 is a member of mitochondrial transcription factors and acts as a mediator between cellular stress signals and coping mechanisms. P53 was originally identified as an oncogene, but has since been found to regulate a large family of target genes to mediate many different responses. Research has focused on p53 as a tumor suppressor that promotes apoptosis in abnormal cells. However, recent data suggest that p53 has additional functions addressing physiological stressors. Induction of p53 gene expression results in a number of different responses, including antioxidant responses and most recently, the regulation of mitochondrial respiration (Matoba et al., 2006). In addition, intracellular ROS levels are increased in p53 deficient mice (Sablina et al., 2005).

P53 plays an antioxidant role by increasing the expression of antioxidant target genes to reduce ROS production in cells. P53 is normally thought of as a pro-oxidant gene, because it promotes apoptosis. However, under mild oxidative stress, p53 actually regulates antioxidant genes to control ROS levels (Sablina et al, 2005). P53 activates the transcription of glutathione peroxidase in vitro (Tan et al., 1999). Gel shift assays showed that human p53 binds to a p53-binding site on the promoter of the GPx gene. Tan et al., (1999) also transfected cells containing GPx promoter-luciferase constructs (with or without the p53 binding site) with p53. Cells expressing Gpx with the p53-binding site had a 4-fold increase in GPx activity compared to cells expressing Gpx without the p53-binding site, indicating that p53 is necessary for strong Gpx promoter activity.

In vitro p53 expression is responsive to hydrogen peroxide levels and triggers an increase in p53-responsive genes (Sablina et al., 2005). One p53 inducible gene, TIGAR (TP53-induced

glycolysis and apoptosis regulator), decreases glycolysis by lowering fructose-2, 6-bisphosphate levels in cells resulting in lowered intracellular ROS levels (Bensaad et al., 2006). TIGAR expression also results in increased NADPH and increased the GSH/GSSG ratio in transfected cells. In vitro, TIGAR overexpression decreased ROS levels following oxidant treatment, while mutant TIGAR (non-functional) overexpression resulted in no change in ROS levels (Bensaad et al., 2006).

### *NRF1 and NRF2*

Nuclear respiratory factors (NRF) 1 and 2 are members of the CNC family of bZIP (basic leucine zipper) transcription factors critical for cell function. NRF bind to the antioxidant response element (ARE) in response to elevated intracellular levels of ROS (Scarpulla, 2006 and Kwong et al., 1999). The antioxidant response element drives expression and coordinates the induction of many free radical-detoxifying genes, including enzymes required for glutathione synthesis (Venugopal and Jaiswal, 1998). NRF regulation of gene expression is redox responsive and is thought to foster communication between the mitochondria and nucleus after free radical induced mitochondrial damage.

NRF1 is widely expressed, with the highest expression in muscle, liver, lung, kidney and heart (Kwong et al., 1999). NRF1 knockout fibroblasts show an increased sensitivity to the toxic effects of oxidant-producing chemicals such as paraquat, cadmium chloride and diamide compared to wild type cells. The concentration of paraquat required to kill half of the NRF1 knockout fibroblasts was three times lower than the concentration required to kill half of the wild type cells (0.6mM and 0.2mM, respectively). This suggests that NRF1 knockout cells are more susceptible to oxidative stress-inducing compounds than wild type cells. In addition, NRF1



knockout fibroblasts have decreased glutathione levels and expression of glutathione synthesis genes compared to wild type cells (Kwong et al., 1999). Overexpression of NRF1 in transfected fibroblasts resulted in a 24% increase in cellular glutathione compared to cells transfected with an empty plasmid (Myhrstad et al., 2001). In fact, NRF1 specifically activates the antioxidant response element (ARE) on the promoter of  $\gamma$ -glutamylcysteine synthetase (GCS), one of the two enzymes that catalyze the synthesis of glutathione from amino acids (Myhrstad et al., 2001). Further confirming NRF1's role in antioxidant defenses, when NRF1 knockout fibroblasts were cultured in 20% oxygen, intracellular levels of ROS increased two-fold higher than wild type cells (Leung et al., 2003).

Fibroblasts from NRF2-null mice exposed to diquat for 24 hours exhibited increased lipid peroxidation compared to fibroblasts from wild type mice (Osburn et al., 2006). While there was no overall difference in total antioxidant gene expression between the two, genes involved in lipid peroxidation and repair of oxidized proteins tended to decrease in NRF2-null fibroblasts. The ratio of GSH/GSSG and total glutathione was lower in NRF2 null cells compared to wild type after diquat treatment indicating a decreased adaptive response to oxidative stress in NRF2-null mice. In addition, diquat treatment resulted in a 2.5-fold increase in transcription of an antioxidant response element (ARE)-luciferase reporter gene in wild type fibroblasts. ARE-luciferase reporter gene transcription remained unchanged after diquat treatment in NRF2-null fibroblasts, indicating that NRF2 is required for activation of the ARE on antioxidant genes (Osburn et al., 2006).

Some studies suggest that both NRF proteins work together in protecting cells from elevated ROS. Double knock-out cells (NRF1  $-/-$  NRF2  $-/-$ ) had a four-fold increase in ROS levels compared to wild type cells when subjected to high levels of oxygen (Leung et al., 2003).

Leung et al. (2003) also found that the expression of two NRF-target genes involved in glutathione synthesis (the catalytic and the regulatory subunits of  $\gamma$ -glutamylcysteine ligase) was abolished and glutathione levels were significantly reduced in double NRF-knockout cells. Transfection of the double knock-outs with plasmids containing NRF1 and NRF2 cDNA restored glutathione levels back to normal, confirming that the loss of NRF1 and NRF2 account for change in glutathione levels (Leung et al., 2003).

### *PGC1 $\alpha$*

PGC1 $\alpha$  is a transcriptional co-activator abundant in tissues with high metabolic activity that has been shown to activate several genes, including UCP1 in BAT. Together, PGC1 $\alpha$  and NRF1 $\alpha$  regulate the transcription of genes involved in lipid catabolism and mitochondrial function (Valle et al., 2005). PGC1 $\alpha$  expression is induced during cold exposure in cardiac and skeletal muscle (Puigserver et al., 1998) and in response to ROS (St-Pierre et al., 2006), suggesting PGC1 $\alpha$  has a role in energy metabolism. PGC1 $\alpha$  mRNA expression is elevated in response to fasting and is associated with increased mitochondrial energy production (Lin et al., 2005). PGC1 $\alpha$  (and PGC1 $\beta$ ) have been shown to stimulate UCP2 (Oberkofler et al., 2006) and UCP3 expression, potential as a way to modulate ROS production in mitochondria (reviewed in Scarpulla, 2006).

A recent study by Silveria et al. (2006) showed that rat skeletal muscle cells incubated with xanthine oxidase for 10 days had elevated UCP3 (58%) and PGC1 $\alpha$  (175%) compared to control cells on day 10. In vascular cells, PGC1 $\alpha$  overexpression reduces accumulation of ROS damage. In the same cells, PGC1 $\alpha$  induces the expression of genes involved in the mitochondrial electron transport chain, lipid metabolism and antioxidant defenses, including a

two-fold increase in UCP2 expression. Small interfering RNA suppression of PGC1 $\alpha$  in vitro results in a significant decrease in antioxidant genes including superoxide dismutase (MnSOD), thioredoxin 2 and thioredoxin reductase 2 (Valle et al, 2005). PGC1 $\alpha$  is important in the induction of certain genes involved in the regulation of the cellular response to oxidative stress.

Overexpression of PGC1 $\alpha$  (approximately a two-fold increase in mRNA) in fibroblasts increased levels of mitochondrial biogenesis protein markers such as mitochondrial transcription factor A and cytochrome C (Liang et al., 2007a). PGC1 $\alpha$  overexpression resulted in cellular ATP levels approximately 72% higher than control fibroblast cells. Control fibroblasts treated with the oxidative stress inducing agent, t-butyl hydroperoxide (t-BOOH), had a 60% decrease in mitochondrial membrane potential. However, cells overexpressing PGC1 $\alpha$  had unchanged membrane potentials after t-BOOH treatment (Liang et al., 2007). This study suggests that PGC1 $\alpha$  overexpression not only stimulates mitochondrial biogenesis but also makes cells more resistant to oxidative stress (Liang et al., 2007a).

During food deprivation in rats, PGC1 $\alpha$  and UCP3 are upregulated in skeletal muscle along with increased levels of serum free fatty acids and mitochondrial fatty acid oxidation (de Lange et al, 2006). PGC1 $\alpha$  can also induce expression of genes involved in fatty acid oxidation (Scarpulla, 2006). In pre-adipocytes, overexpression of PGC1 $\alpha$  increased palmitate oxidation in vitro (Vega et al., 2000). After a 6-hour incubation with labeled palmitate, the production of labeled CO<sub>2</sub> was approximately 2-fold higher in cells transfected with PGC1 $\alpha$  than control cells transfected with LacZ (Vega et al., 2000).

The increase in fatty acid oxidation was even greater (three-fold higher than control) in cells cotransfected with PGC1 $\alpha$  and PPAR $\alpha$ . GST pull down assays indicate that PPAR $\alpha$  binds PGC1 $\alpha$ . Expression of fatty acid oxidation genes, such as MCAD, LCAD and CPT1 were

highest in cells expressing both PGC1 $\alpha$  and PPAR $\alpha$ . This study identified PGC1 $\alpha$  as a coactivator for PPAR $\alpha$  control of the transcription of genes involved in fatty acid metabolism (Vega et al., 2000).

### *Peroxisome Proliferation Activation Receptors*

The first genetic sensor for fats in the body was discovered in the early 1990s and named PPAR (Peroxisome Proliferation Activation Receptor), because of its ability to induce peroxisome proliferation (Issemann and Green, 1990). Studies identified three family members: PPAR $\alpha$ , PPAR $\beta$  (also known as PPAR $\delta$ ) and PPAR $\gamma$  (reviewed in Evans et al., 2004). These receptors are members of the nuclear receptor super family and work by controlling target genes involved in metabolism. PPARs can be activated by both dietary fatty acids and fatty acid derivatives in the body (Evans, 2004). PPAR $\alpha$  is expressed primarily in tissues with a high capacity for fatty acid oxidation, such as skeletal muscle, heart and liver tissue (reviewed in Gremlich et al., 2004). The PPAR $\gamma$  isoform is highly expressed in adipose tissue and expressed at low levels in the heart, liver and skeletal muscle (Vidal-Puig et al., 1997). The PPAR $\beta/\delta$  isoform is ubiquitously expressed.

PPAR $\alpha$  is expressed abundantly in tissues that exhibit high rates of fatty acid oxidation and PPAR $\alpha$  knockout mice have low rates of  $\beta$ -oxidation. PPAR $\alpha$  knockout mice have abnormal metabolic responses to starvation and exhaustive exercise. Fatty acid oxidation capacity is 28% lower in the skeletal muscle of PPAR $\alpha$  knockout mice compared to wild type mice (Muoio et al., 2002). The PPAR $\alpha$  agonist, ureido-fibrate-5 (UF-5), stimulates mitochondrial  $\beta$ -oxidation in skeletal muscle and liver. After three weeks of increasing doses of UF-5 in fat-fed hamsters, plasma triglycerides were decreased 70% and CPT1 expression increased 2.3-fold in muscle

compared to control fat-fed hamsters. In vitro, UF-5 stimulated mitochondrial palmitate oxidation up to 1.6-fold in skeletal muscle and 2.7-fold in liver, in a dose-dependent manner (Minnich et al., 2001).

Rats fed a high fat diet had elevated plasma NEFA levels, increased expression of fatty acid oxidation genes and elevated PPAR $\alpha$  expression in skeletal muscle compared to rats on a control diet (Garcia-Roves et al., 2007). Mitochondrial biogenesis, measured by an increase in mitochondrial DNA copy number, increased in skeletal muscle from rats fed a high fat diet.

When fed a high fat diet, PPAR $\beta$  null mice show decreased mitochondrial uncoupling (Wang et al., 2003). In skeletal muscle, PPAR $\beta$  upregulates fatty acid oxidation and energy expenditure (Barak et al., 2002) and in cultured myocytes, PPAR $\beta$  was found to stimulate fatty acid oxidation (Wang et al., 2003). Myocytes treated with a PPAR $\beta$  agonist demonstrated significant increases in fatty acid oxidation and had increased expression of genes required for fatty acid metabolism and mitochondrial uncoupling (Wang et al., 2003). Analysis of gene expression in cultured rat myotubes treated with the PPAR $\beta$  agonist, GW501516, showed an increase in expression of genes involved in  $\beta$ -oxidation (LCAD, catalase), fatty acid transport (hormone sensitive lipase, CPT1) and mitochondrial respiration (UCP2, UCP3) (Tanaka et al., 2003). Mice fed a normal diet and treated with GW501516 (10mg/kg bodyweight) had a 1.8-fold increase in  $\beta$ -oxidation in skeletal muscle compared to vehicle treated mice. This increase accompanied a decrease in plasma NEFA levels and an increase in  $\beta$ -oxidation gene expression in skeletal muscle (Tanaka et al., 2003). In fact, in rats with elevated plasma NEFA levels and increased PPAR $\beta$  binding to the promoter of CPT1 in skeletal muscle compared to control rats (Garcia-Roves et al., 2007).

PPAR $\gamma$  agonists increase UCP2 mRNA levels in muscle cells. In vitro, PPAR $\gamma$  increases UCP2 promoter activity and stimulates UCP2 expression. Serial deletion constructs were used to identify the regulatory sequences that were important for transcriptional control of the UCP2 gene. The region between -86 and -44 was found to be required for response to PPAR $\gamma$ . (Medvedev et al., 2001). However, electromobility shift assays (EMSA) showed that PPAR $\gamma$  does not bind to this region of the UCP2 promoter, suggesting that transcriptional activation involves other transcription factors. Point mutation analysis also showed that the E-box motif on the UCP2 promoter is required for PPAR $\gamma$  responsiveness. USF1 and USF2 (Upstream Stimulator Factor 1 and 2) bind to the E-box containing region of the promoter, making these transcription factors likely candidates for mediating the effect of PPAR $\gamma$  on UCP2 (Medvedev et al., 2001). Since several other response elements have been identified on the UCP2 promoter such as SRE, other transcription factors cannot be ruled out. Sterol regulatory element binding protein (SREBP) can interact with both E-boxes and sterol regulatory element (SRE) (Osborne, 2000). Coactivators, such as peroxisome proliferator-activated receptor gamma coactivator-1 (PGC1) also increase UCP2 gene expression and may also be responsible for the regulation of PPAR $\gamma$ 's effect on UCP2 transcription.

Sequencing of the human UCP3 promoter revealed three PPAR response elements (PPRE) in the proximal promoter region (Acin, 1999). High fat diets result in increased circulating free fatty acids, which in turn activate PPARs. High fat diets also increase UCP3 expression, possibly through PPAR binding to its response elements on the UCP3 promoter.

## **Conclusion**

Little information regarding the role of the uncoupling proteins and other novel antioxidant genes in ruminants is available. Oxidative stress in ruminants has not been well characterized and even less is known about the changes that occur during weight cycle changes in ruminants. Research into the mechanism behind genes with antioxidant function can help us better understand ruminant physiology and the importance of these cellular changes. By identifying important changes in antioxidant gene expression and enzyme activity, we can better understand the physiology of ruminants during these weight cycles.

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Figure 1: Free fatty acid mobilization (source: M.W. King, 1996):

### Hormone-Induced Fatty Acid Mobilization in Adipocytes

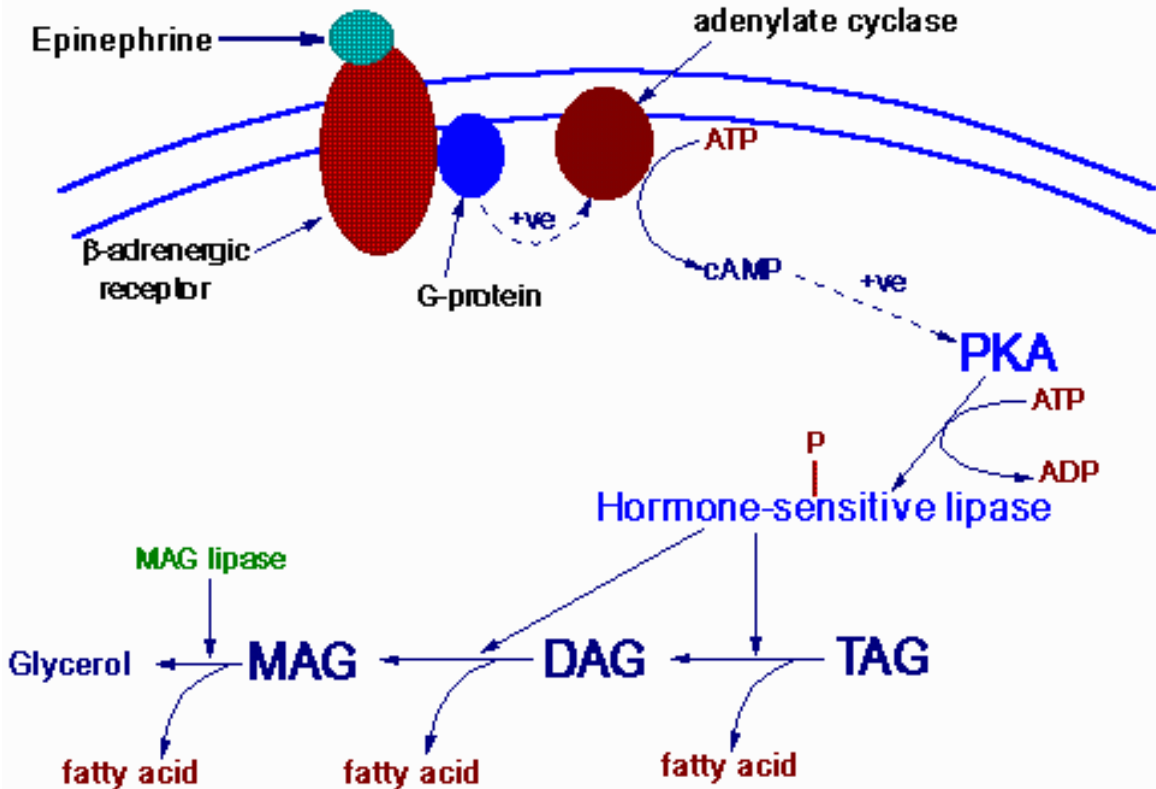


Figure 2: Enzymes and products of mitochondrial  $\beta$ -oxidation (Eaton et al., 1996):

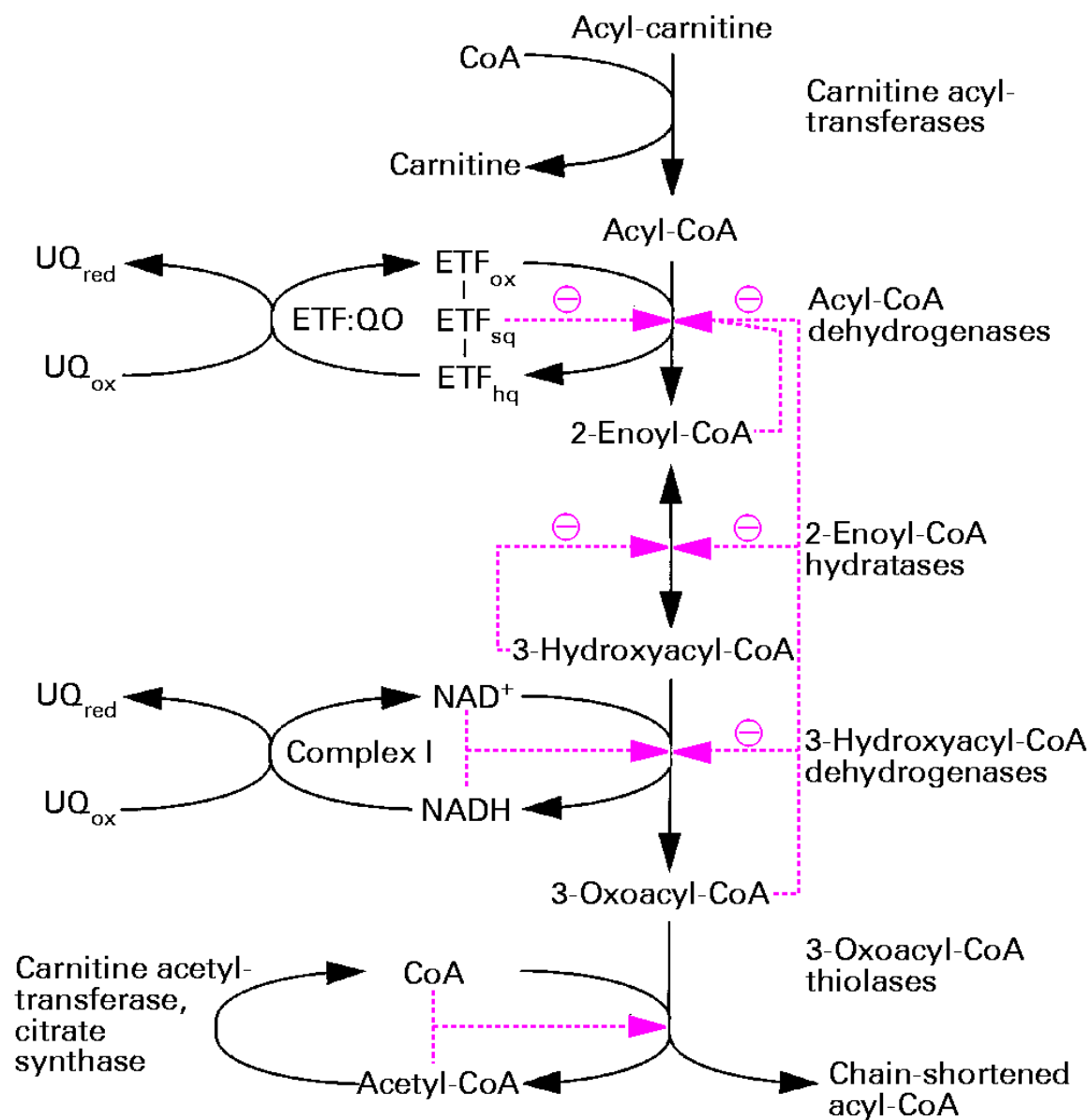


Figure 3: Mitochondrial  $\beta$ -oxidation pathway (Eaton et al., 1996):

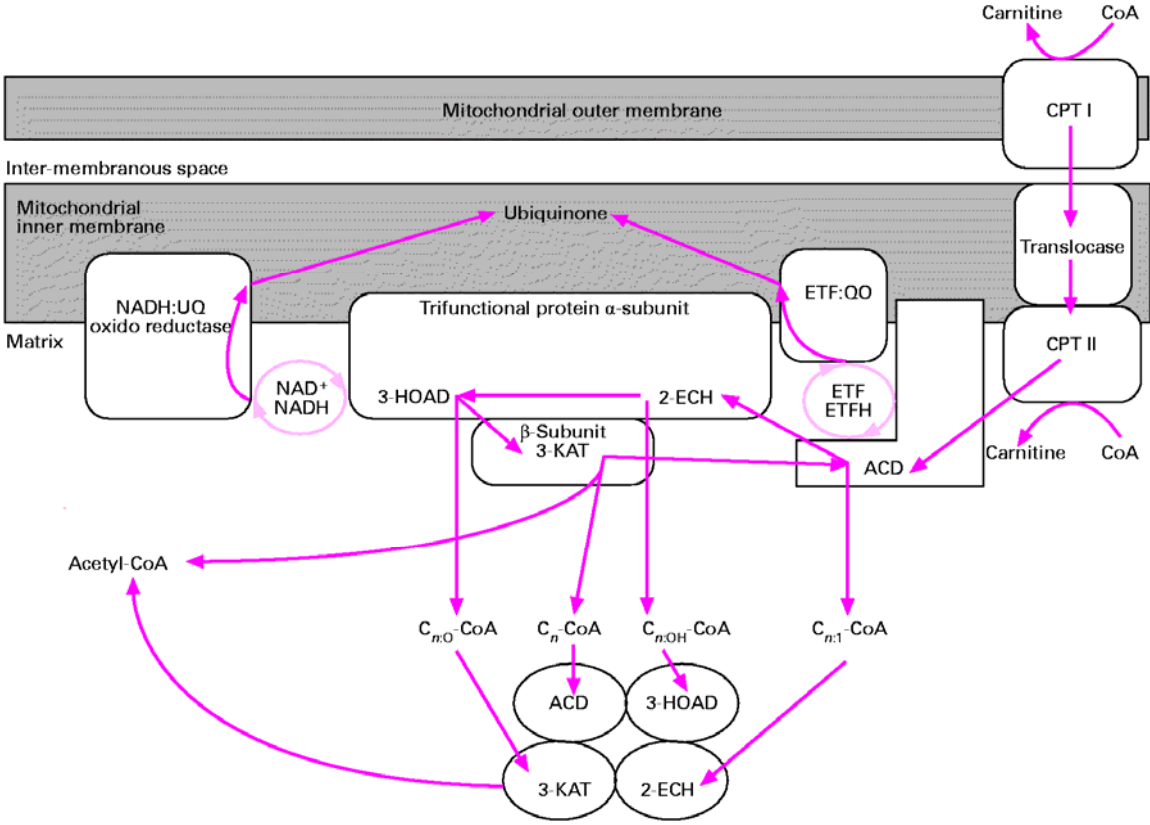


Figure 4: Vitamin E and membrane protection (van Meeteren et al., 2005):

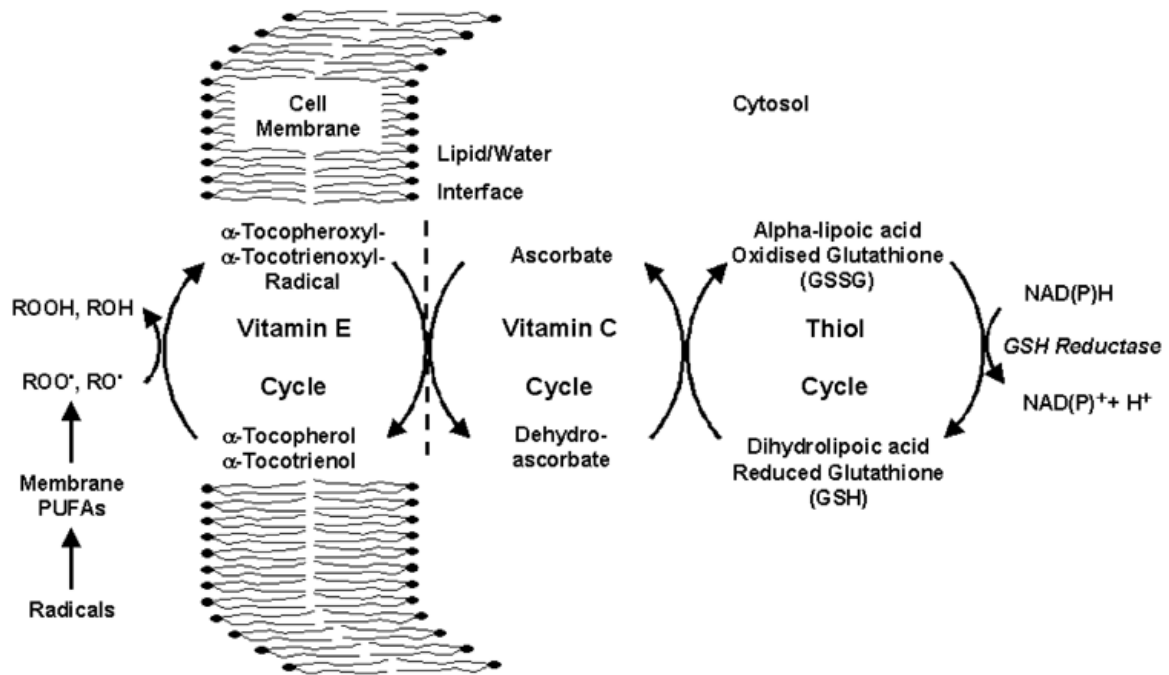




Figure 5: Electron transport chain (Saraste, 1999):

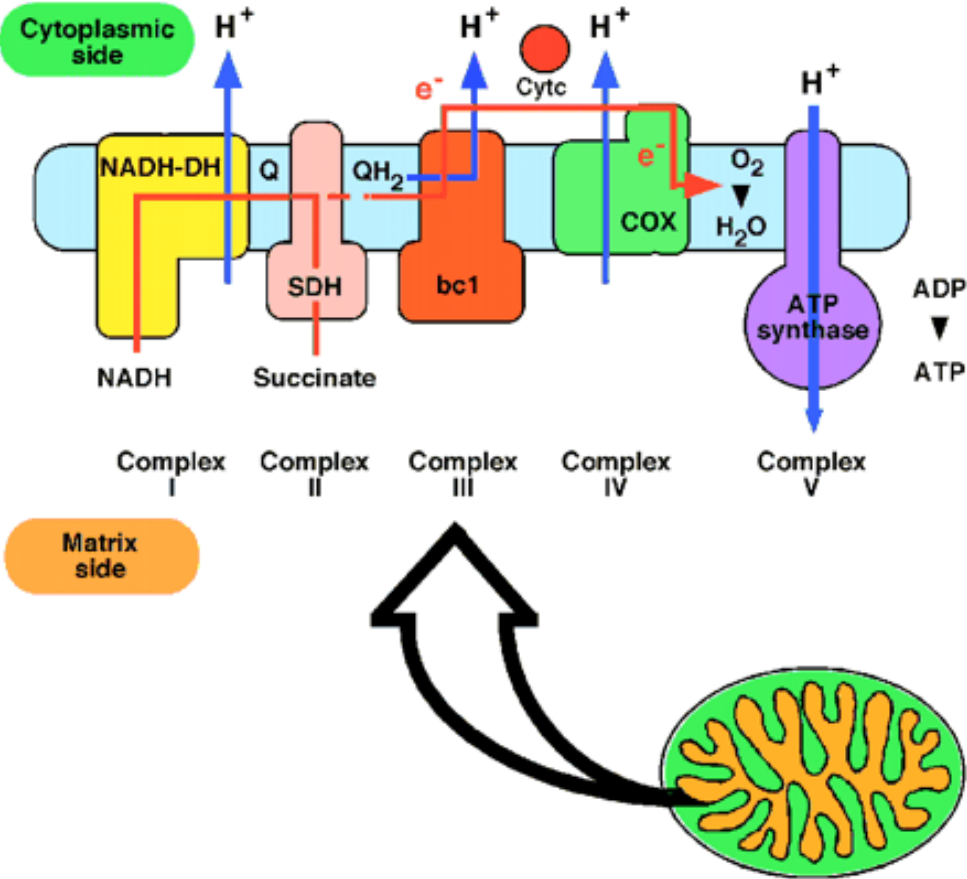


Figure 6: Uncoupling oxidative phosphorylation (Krauss et al., 2005):

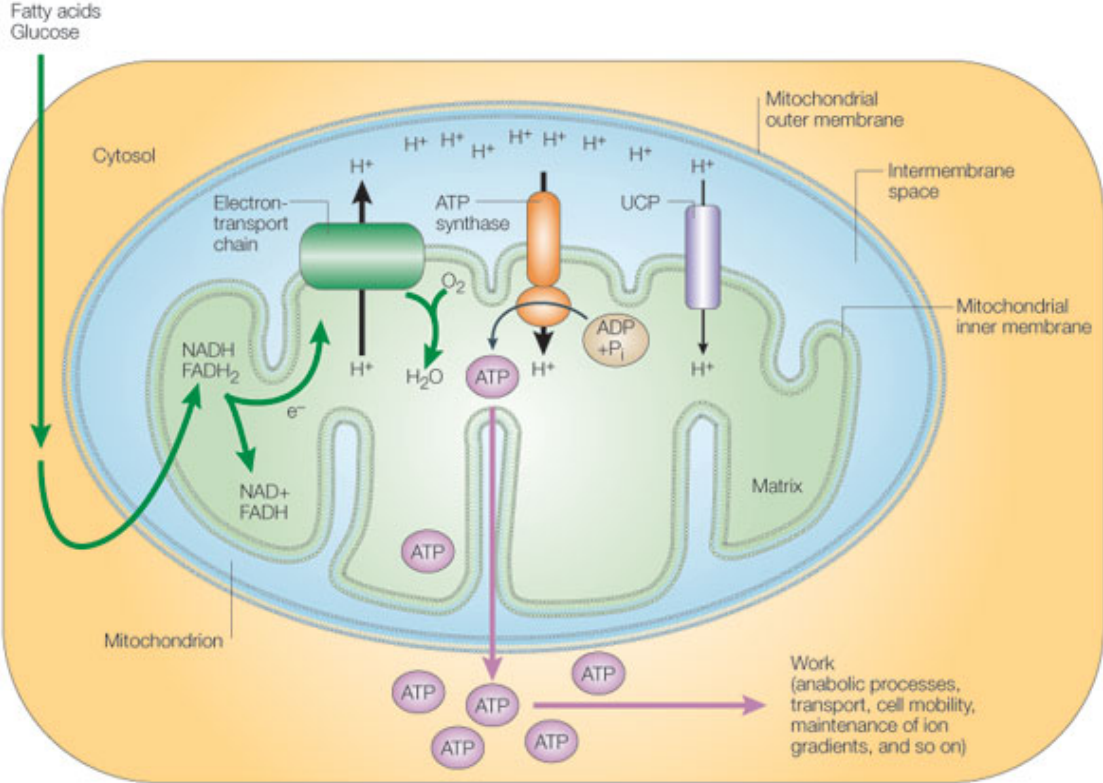
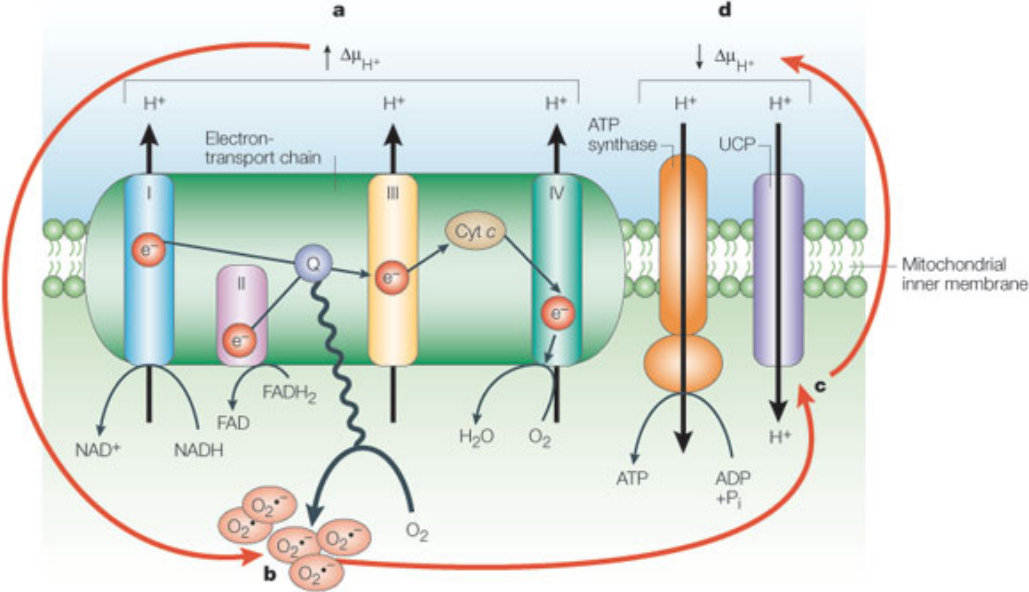
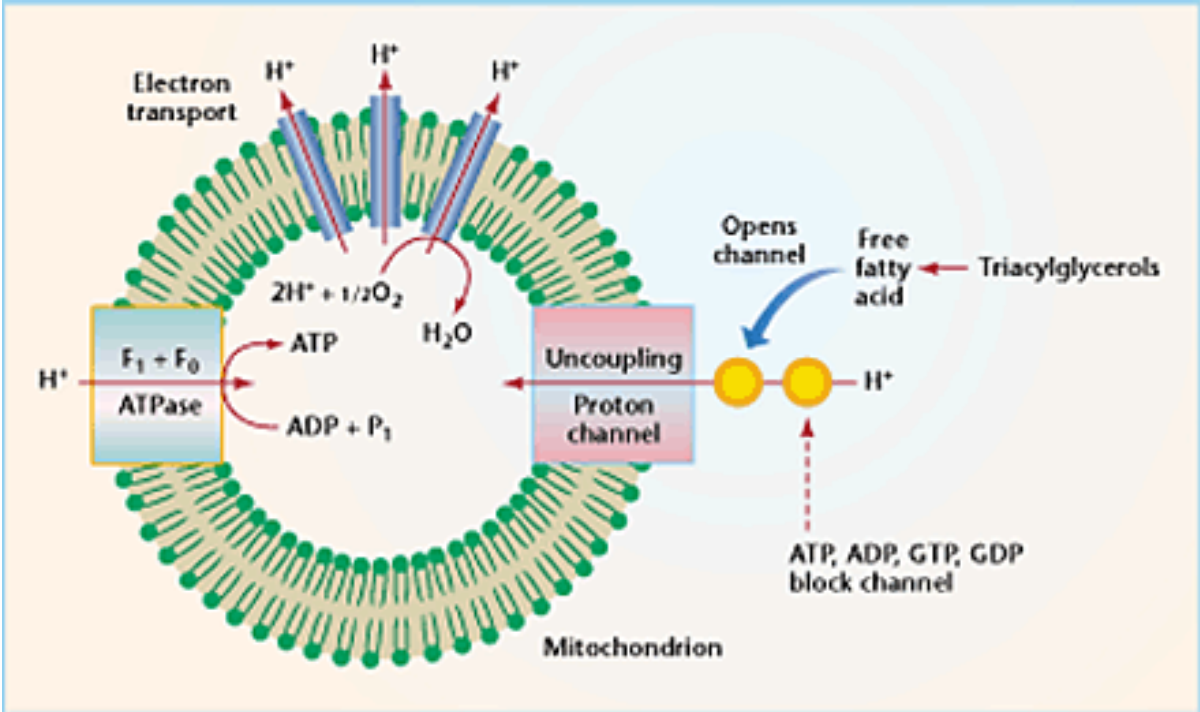


Figure 7: Activation of Uncoupling proteins by superoxide radicals (Krauss et al., 2005):



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Figure 8: Activation of UCP by free fatty acids (Kozak, 2000):



CHAPTER 3:  
EFFECT OF INCREASED  $\beta$ -OXIDATION ON mRNA LEVELS OF UNCOUPLING  
PROTEINS 2 AND 3 AND PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS IN  
THE SKELETAL MUSCLE OF BEEF COWS

## Abstract

Twenty-six Angus-cross cows were studied during weight loss (WL) and weight maintenance (WM) to examine the effects of elevated  $\beta$ -oxidation on mRNA levels in skeletal muscle. At the end of both the WL and WM sampling periods, muscle biopsies were removed from the *Biceps femoris* and mRNA levels were measured using real-time PCR. During WL, cows had elevated mRNA levels of  $\beta$ -oxidation markers, CPT1 (4.6 fold), FABP3 (2.0 fold) and ACOX (2.8 fold) compared to WM. mRNA levels of the non-esterified fatty acid (NEFA)-responsive signaling molecules, PPAR $\alpha$ , PPAR $\delta$  and PPAR $\gamma$  increased two fold, 2.2 fold and 1.84 fold, respectively, during WL. Uncoupling proteins 2 and 3 also had increased mRNA (3.0 fold and 6.0 fold, respectively) during WL, but western blot analysis found no changes in protein abundance of UCP3. Thus, an increase in the expression of genes involved in  $\beta$ -oxidation and in the signaling by fatty acids and fatty acid by-products occurs during weight loss in beef cattle.

**Key words:** beef cattle, uncoupling protein, peroxisome proliferator-activated receptors,  $\beta$ -oxidation, skeletal muscle

## Introduction

Net fat mobilization occurs when energy requirements exceed energy intake. Several genes are responsive to changes in circulating lipid and  $\beta$ -oxidation by-products. Uncoupling proteins (UCP) are a family of ion carrier proteins that reside in the inner mitochondrial membrane. Both UCP2 and UCP3 mRNA levels increase in skeletal muscle during short-term fasting in rats (Boss et al., 1997; Weigle et al., 1998; de Lange et al., 2006) while UCP3 also increases in arctic ground squirrels (Barger et al., 2006) and collared lemmings (Blaylock et al.,

2004). Short-term food deprivation results in elevated NEFA levels and increased UCP3 protein in rat skeletal muscle (de Lange et al., 2006). The increase in UCP3 coincides with elevated mitochondrial fatty acid oxidation rates and the increased UCP3 is completely abolished when cells are treated with the fatty acid oxidation inhibitor, nicotinic acid (de Lange et al., 2006). Peroxisome proliferator-activated receptors (PPAR) are members of the nuclear receptor super family and work by controlling target genes involved in metabolism. PPARs can be activated by both dietary fatty acids and fatty acid derivatives (Evans et al., 2004) and all three PPAR isoforms are expressed in tissues with high capacity for fatty acid oxidation, such as skeletal muscle (Gremlich et al., 2004). To date there have been few studies establishing the role of uncoupling proteins and PPAR in ruminants. Previous studies have largely focused on the role of UCP and PPAR in energy efficiency in cattle rather than during energy restriction. The objective of this study was to determine if mRNA levels for PPARs and UCPs, and protein expression of UCP3 are increased during weight loss in beef cattle.

## **Materials and Methods**

### *Animals*

Twenty-six Angus-cross cows were blocked by body weight and housed in pens of 6 to 10 hd. Cows were  $5 \pm 0.4$  yr old and weighed approximately  $700 \pm 10$  kg. Measurements were taken at two time points: weight loss (WL) and weight maintenance (WM). The WL period occurred for 60 d after calving. Cows were weighed prior to feeding approximately 5 d post-calving to determine initial weight and weighed weekly thereafter. Final weight, also measured prior to feeding, was recorded on d 60. Cows were turned out on pasture after the WL period. The WM portion of the experiment took place after weaning. Approximately 10 d after weaning

and prior to feeding, cows were weighed for initial body weight. Cows were weighed weekly thereafter. Final weight was measured prior to feeding on d 60. The diet fed during WL and WM consisted of bluegrass straw and alfalfa hay (Table 1) to meet maintenance requirements for cows in mid-gestation (NRC, 2000).

Muscle biopsy samples (approximately 0.5 g) were taken from the *Biceps femoris* of each animal on d 60 of each treatment. A 10 cm x 10 cm area was clipped over the *Biceps femoris*, followed by lidocaine infiltration. Once analgesia was established, a 1 cm incision was made longitudinally through the skin. The muscle biopsy instrument was inserted and the biopsy obtained. Two skin sutures closed the incision. Biopsies were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}$  C. All experimental protocols were approved by the Washington State University Institutional Animal Care and Use Committee.

#### *Total RNA isolation from skeletal muscle*

Total RNA was isolated from skeletal muscle using Trizol reagent (Invitrogen, Carlsbad, CA) with modifications as described by Sambrook and Russell (2001). Total RNA was quantified by absorbance at 260 nm and the integrity of total RNA was checked by agarose gel electrophoresis and ethidium bromide staining of the 28s and 18s bands. RNA (2 ug) was DNase treated using Turbo Free DNase (Ambion, Foster City, CA) and first-strand cDNA was synthesized using Superscript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand synthesis was checked using polymerase chain reaction (PCR) at 50 cycles using primers described below.

#### *Real time PCR*



Target gene mRNA levels were determined using Real-time polymerase chain reaction (quantitative PCR). Real-time PCR was performed using a Biorad thermocycler and iQ SYBR Green Master Mix (Biorad, Hercules, CA) according to the manufacturer's instructions. Genes examined were: fatty acid binding protein 3 (FABP3), carnitine palmitoyltransferase (CPT1), acyl-coenzyme A oxidase 1 (ACOX1), uncoupling proteins 2 and 3 (UCP2 and UCP3) and peroxisome proliferator-activated receptors alpha, delta and gamma (PPAR $\alpha$ ,  $\delta$ ,  $\gamma$ ). Primers were designed using *Bos taurus* sequences for target genes when available (Table 2).  $\beta$ -actin was used as a control to account for any variation in efficiency of reverse transcription and PCR.

PCR conditions were as follows: 95° C for 2.5 min, 50 cycles of 95° C for 30s, 58° C for 30 s and 72° C for 30 s, a melt curve of increasing temperature of 2° C every 10 s starting at 55° C followed by a hold at 4° C. For each individual animal, a relative quantification of mRNA expression was performed using standard curves of each primer set. The relative expression was expressed as a ratio of the target gene to control gene using the Pfaffl equation (Pfaffl, 2001). Relative expression was normalized within cow, where expression value was expressed as a ratio to expression during WM. Therefore relative expression of target genes during WM was 1.0 and during WL relative expression was expressed as a fold change from the WM value.

### *Western blotting*

Protein samples were isolated from skeletal muscle samples according to the protocol described by Harlow and Lane (1998). Protein quantity was determined using the Bradford method (Bradford, 1976). Twenty micrograms of protein (12  $\mu$ l) was mixed with 3x sample buffer, boiled for 3 min and loaded onto a 12% SDS-PAGE gel. After bands were sufficiently separated, the proteins were transferred to a nitrocellulose membrane. UCP3 proteins were

identified by incubation overnight with a rabbit polyclonal antibody recognizing UCP3 (#3477; Abcam, Cambridge, MA) diluted 1 to 5000 in 5% BSA. Blots were then incubated for 30 min with a horseradish peroxidase-conjugated goat-anti rabbit secondary antibody (#8277) diluted 1 to 5000 (Abcam, Cambridge, MA) and detected using ECL chemiluminescence substrate (Pierce, Rockford, IL). For a loading control blots were incubated at room temperature overnight and then reprobbed using a polyclonal antibody to  $\beta$ -actin diluted 1 to 10,000 according to Liao et al. (2000). Samples were quantified using Adobe Photoshop.

### *Statistics*

Cow body weight data were analyzed using the general linear model procedure (Proc GLM) from the SAS version 9.1 statistical package (SAS Institute Inc., 2002). The model was: Model:  $Y_{it} = u + t_i + E_{it}$ , where  $t_i$  was the effect of treatment. Real-time PCR and western blot data were analyzed using a student T-test to test the hypothesis that the fold-change was equal to 1.0. Statistical differences were determined with  $P < 0.05$ . Correlations were determined using SAS (Proc Corr).

## **Results and Discussion**

Cows lost more weight during WL ( $-41.5 \pm 5.0$  kg) than WM ( $+ 5.5$  kg  $\pm 5.0$ ;  $P < 0.0001$ ). During WL, serum NEFA increased 5 fold ( $P < 0.0001$ ; Brennan et al., 2007), indicating that body fat was being mobilized to meet energy requirements. As a result of increased body fat mobilization, the expression of genes involved in  $\beta$ -oxidation increased in skeletal muscle ( $P < 0.05$ ). Carnitine palmitoyltransferase 1 is the protein responsible for transporting fatty acids into the mitochondria and mRNA levels of this gene increased 4.6 fold

during WL. mRNA levels of ACOX, the first enzyme of  $\beta$ -oxidation, increased 2.8 fold during WL. Fatty acid binding protein 3 mRNA levels increased 2 fold ( $P < .05$ ). Although the function of FABP3 remains somewhat unclear, studies in vitro using hepatocytes found rates of fatty acid uptake and metabolism increased when FABP levels increased (Burczynski et al., 1999). Blaak et al. (2001) found skeletal muscle FABP protein levels are positively correlated to increased weight loss and increased fat oxidation in humans. ACOX mRNA levels were more highly correlated ( $P < 0.05$ ) with CPT1 mRNA levels ( $r = 0.91$ ) than with FABP3 mRNA levels ( $P < 0.05$ ;  $r = 0.67$ ) and CPT mRNA levels were correlated ( $P < 0.05$ ) to FABP3 mRNA levels ( $r = 0.65$ ).

Weight loss also resulted in an increase in mRNA levels of all three PPAR isoforms in skeletal muscle (Figure 2): mRNA levels for PPAR $\alpha$  and PPAR $\gamma$  increased 2 fold and 1.84 fold, respectively whereas PPAR $\delta$  mRNA levels increased 2.2-fold ( $P < 0.05$ ). PPAR $\alpha$  is considered the main isoform involved in the control of  $\beta$ -oxidation. PPAR $\alpha$ -null mice have reduced fatty acid oxidation during fasting, indicating the importance of this gene in utilizing fatty acids as a fuel source (Muio et al., 2002). Ren et al. (1997) reported that feeding fatty acids to wild type mice induced the expression of ACOX mRNA in hepatocytes but PPAR $\alpha$ -null mice fed the same fats did not have a change in ACOX mRNA levels. Further, Minnich et al. (2001) found that mice treated with the PPAR $\alpha$ -agonist UF-5 had a 2.3 fold increase in CPT1 mRNA levels and elevated fatty acid oxidation in skeletal muscle. In this study, PPAR $\alpha$  mRNA levels were positively related ( $P < 0.01$ ) to ACOX and CPT mRNA levels ( $r = 0.81$  and  $r = 0.80$ , respectively). Though the increase in PPAR $\alpha$  mRNA was modest, previous work (Patsouris et al., 2006) has shown that even a 25% increase in PPAR $\alpha$  mRNA can result in increased PPAR $\alpha$  target genes in mouse hepatic tissue.

PPAR $\delta$  mRNA levels were increased most of all three PPAR isoforms. PPAR $\delta$ -agonists increase skeletal muscle fatty acid oxidation (Brunmair et al., 2006) and the mRNA levels of  $\beta$ -oxidation genes in skeletal muscle cells (Luquet et al., 2005). PPAR $\delta$  mRNA levels were correlated ( $P < 0.05$ ) only to ACOX and CPT1 mRNA levels ( $r = 0.5$  and  $r = 0.44$ , respectively), but tended ( $P = 0.09$ ) to correlate to FABP3 mRNA levels ( $r = 0.35$ ). PPAR $\delta$ -agonists also increased fatty acid oxidation and CPT1 mRNA levels 2-fold in skeletal muscle (Dimopoulos et al., 2007). PPAR $\delta$  also interacts with FABP by enhancing its transcriptional activity in adipocytes and keranocytes (Tan et al., 2002). These correlations suggest that PPARs, activated by free fatty acids, are involved in the regulation of genes involved in the utilization of fatty acids to generate ATP.

Levels of mRNA for uncoupling proteins 2 and 3 increased ( $P < 0.0001$ ) during WL, by 3 fold and 6 fold, respectively (Figure 2). NEFAs may act as signaling molecules to activate UCP to protect the mitochondria from damage by lipid peroxides (Dulloo et al., 2001). Other hypotheses are that lipid peroxides, the oxidative by-products of  $\beta$ -oxidation, activate UCP (Brand and Esteves, 2005). In support of both hypotheses, UCP mRNA levels increase during food deprivation when there is a shift towards lipid oxidation (de Lange et al., 2006) and UCP mRNA levels in muscle are closely associated with the mRNA expression of key genes, such as CPT and medium-chain acyl-CoA dehydrogenase, involved in  $\beta$ -oxidation during fasting (Samec et al., 2002). UCP2 and UCP3 mRNA levels increase in response to both increased plasma NEFA levels and increased fatty acid oxidation in skeletal muscle in myotubes and in rats and mice (Weigle et al., 1998; Brun et al., 1999 Hwang and Lane, 1999; Samec et al., 2002). In the current study, UCP2 mRNA levels were positively correlated ( $P < 0.0001$ ) to ACOX, CPT1 and FABP3 mRNA levels ( $r = 0.78$ ,  $r = 0.75$ , and  $r = 0.81$ , respectively) supporting findings that

UCP expression increases with increased expression of CPT when plasma NEFA are elevated (Abe et al., 2006) indicating that these two genes are involved in fatty acid metabolism. UCP3 mRNA levels were not related to ACOX ( $r = 0.34$ ) and FABP3 ( $r = 0.37$ ) mRNA levels.

In our study PPAR $\alpha$  mRNA levels were positively related ( $P < 0.05$ ) to UCP2 mRNA levels ( $r = 0.63$ ) and moderately related to UCP3 ( $r = 0.4$ ) mRNA levels. PPAR $\alpha$  regulates the expression of UCP2 in the liver (Kelly et al., 1998) and UCP3 expression in skeletal muscle (Stavinoha et al., 2004). PPAR $\alpha$ -agonists induce the expression of UCP3 in a time and dose-dependent manner in mice (Brun et al., 1999). Both UCP2 and UCP3 expression correlated ( $P < 0.05$ ) to PPAR $\delta$  expression ( $r = 0.51$  and  $r = 0.45$ , respectively). PPAR $\delta$  regulates the expression of UCP2 in response to elevated fatty acids in vitro (Chevillotte et al., 2001) and the PPAR $\delta$ -activator, GW- 501516, induces a three-fold increase in UCP3 expression in skeletal muscle (Terada et al., 2006). The correlation between PPAR $\delta$  and PPAR $\alpha$  mRNA levels and UCP mRNA levels further supports the hypothesis that these genes work closely to regulate the body's response to increased fatty acid oxidation. Even though PPAR $\gamma$  can induce UCP expression (Kelly et al., 1998), there was no significant correlation between PPAR $\gamma$  and UCP2 or UCP3.

Because UCP3 gene mRNA levels increased 6 fold during WL, western blot analysis was conducted to measure protein expression levels (Figure 3). The mouse polyclonal antibody detected two distinct bands as reported previously with several different UCP antibodies (Sprague et al., 2007). UCP3 protein levels did not change during WL. The lack of change in UCP3 protein levels can be explained by the lack of a clear relationship between mRNA transcripts and protein levels. This can be attributed to post-translational modifications or a high protein turnover rate. In fact, previous studies have reported a decrease in UCP3 protein levels in muscle despite an increase in mRNA levels in rats (Kontani et al., 2002). A recent study also

shows that the half life of UCP2 is only 25 minutes (Rousset et al., 2007). Though the half life of UCP3 is unknown, the similarity in function between UCP2 and UCP3 suggests that UCP3 may also be very unstable explaining why we did not see a difference in UCP3 protein levels.

When energy demands exceed energy intake, body fat mobilization increases the expression of genes involved in fatty acid oxidation and utilization. In addition, expression of genes that use fatty acids as ligands, such as PPAR, increase in skeletal muscle. Finally, the expression of uncoupling proteins increases during WL in ruminants similar to the increases reported in monogastrics. However, the function of uncoupling proteins during  $\beta$ -oxidation is not clear. During WL, circulating NEFA's increase and act as ligands to PPAR expressed in the periphery. In skeletal muscle, PPAR regulate the expression and activation of genes involved in  $\beta$ -oxidation such as ACOX, CPT, and FABP as well as UCP. UCP expression can also be directly stimulated by elevated NEFA, potentially to protect cells from damage by lipid oxidation by products. These data support previous findings in non-ruminants and suggest that these genes play a role in the same physiological processes in ruminants.

### **Implications**

The body weight of beef cattle changes throughout the year as production states change. Although the exact function of PPARs and UCPs in ruminants is not clear, our data indicate PPARs and UCPs have a role in the regulation of the body's response to elevated circulating fatty acids. These data help further explain changes in expression of genes involved in  $\beta$ -oxidation during weigh loss in cattle production cycles.

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Table 1: Diet composition fed to cows during weight loss (WL) or weight maintenance (WM).

Item	WL	WM
Ingredient		
Bluegrass-alfalfa mix	100%	100%
Chemical composition		
DM, %	90.6	90.0
CP, %	9.8	9.8
Ash, %	7.7	7.9
NDF, %	85	74
ADF, %	48	45
TDN, %	47	47
TM Salt <sup>1</sup>	Ad lib	Ad lib

<sup>1</sup>TM salt provided the following: 0.03 % Ca, 42.7 % Na, 350 ppm Zn, 60 ppm Co, 350 ppm Cu, 1800 ppm Mn, 370 ppm Mg, 2000 ppm Fe, 100 ppm I, 90 ppm Se.

Table 2: Real-time PCR primers.

<b>Gene</b>	<b>Accession</b>	<b>Forward 5'-3'</b>	<b>Reverse 5'-3'</b>
$\beta$ -actin	AY141970	TGCTTCTAGGCGGACTGTTA	ATAAAGCCATGCCAATCTCA
UCP2	BC102839	GTGCTGAGCTGGTGACCTAC	CCCGAAGGCAGAAGTAAAGT
UCP3	NM174210	GGGAGGCAACAGAAAGTACA	ATGTTGGGCAGAATTCCTTT
FABP3	DQ026674	AGTGGACAGCAAGAATTTTCG	GATTGTGGTAGGCTTGGTCA
ACOX1	NM_001035289	TGCATTCATCCAAAGGACTC	CATAAGTGGCTGTGGTTTCC
CPT1	BC123511	ACGTATCGCCGTAAACTGG	TGGTGTGTAACATCCTCTCC
PPAR $\alpha$	NM_001034036	GTGCCAATACTGTCGTTTCC	CGTAAGGATTTCTGCCTTCA
PPAR $\delta$	NM_001083636	TACTGCCGCTTCCAGAAAT	CCACCAGCTTCCTCTTCTCT
PPAR $\gamma$	AY179866	CCGTGGACCTTTCTATGATG	TCATAGTGCGGAGTGGAAT

Figure 1: Relative expression of fatty acid binding protein 3 (FABP3), carnitine palmitoyltransferase 1 (CPT1), acyl-coenzyme A oxidase 1 (ACOX1) using real time PCR during WL (solid bars) and WM (striped bars). \* P < 0.05.

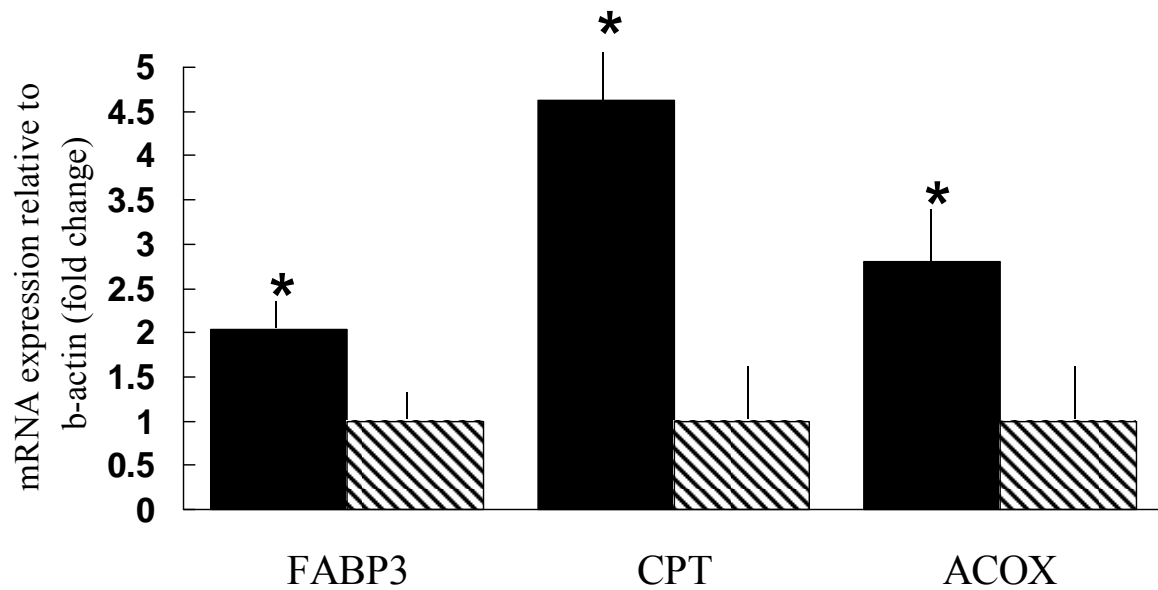




Figure 2: Relative expression of uncoupling proteins 2 and 3 (UCP2 and UCP3) and peroxisome proliferator-activated receptors alpha, delta and gamma (PPAR $\alpha$ ,  $\delta$ ,  $\gamma$ ) using real time PCR during WL (solid bars) and WM (striped bars). \* P < 0.05.

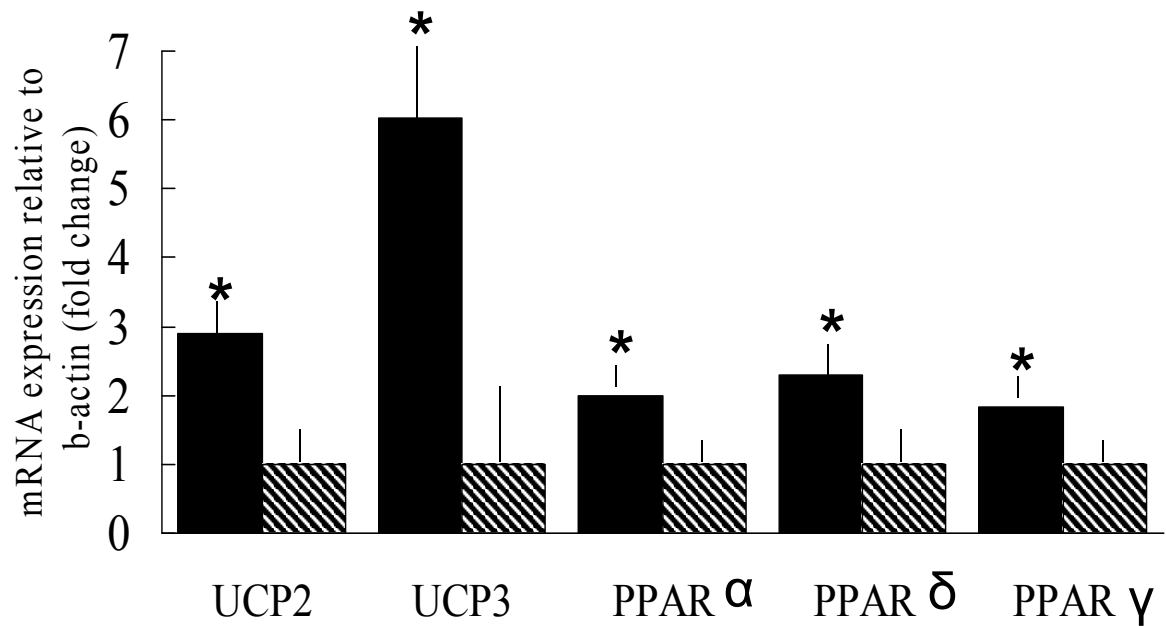
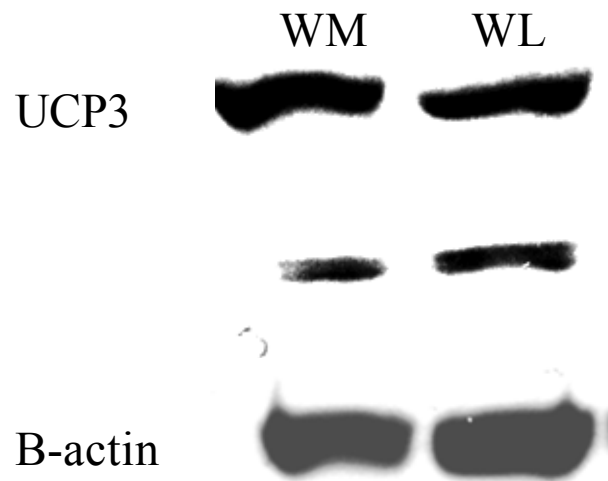


Figure 3: Sample Western blot image of UCP3 protein levels in skeletal muscle. Upper band represents the UCP3 protein (approximately 37 kDa). Both bands are from the same cow, sample on the left was taken during weight maintenance and sample on the right was taken during weight loss. Blots were reprobbed for  $\beta$ -actin (approximately 30 kDa) as a control (lower blot).



CHAPTER 4:

WEIGHT LOSS INCREASES ANTIOXIDANT GENE EXPRESSION IN SKELETAL  
MUSCLE BUT NOT ERYTHROCYTE ANTIOXIDANT ACTIVITY IN BEEF COWS

## **Abstract**

Twenty-six Angus-cross cows were used to determine the effect of body weight loss on skeletal muscle and erythrocyte markers of oxidative stress. Serum NEFA levels and erythrocyte superoxide dismutase (CuZnSOD) and glutathione peroxidase (GPx) activities were measured during weight loss (WL) and weight maintenance (WM). Erythrocyte enzyme activity was used to determine whole animal antioxidant status. Muscle biopsies were obtained at both WL and WM and mRNA levels of antioxidant genes were measured using real time PCR to determine response to reactive oxygen species in skeletal muscle. WL resulted in elevated serum NEFA levels but no change in erythrocyte CuZnSOD and GPx activity. During WL, mRNA levels of antioxidant genes GPx4, MnSOD, TxrR1 and SelW, and genes involved in antioxidant signaling, PGC1 $\alpha$ , NRF1 $\alpha$ , p53 and ERR $\alpha$  were also increased. During WL cows had no changes in antioxidant enzyme activity, but mRNA levels of genes involved in protecting the body from oxidative stress increased in skeletal muscle.

**Key words:** beef cattle, antioxidant, oxidative stress, glutathione peroxidase, superoxide dismutase, skeletal muscle

## **Introduction**

When energy requirements exceed energy intake, the body must shift to using adipose stores as a fuel source resulting in weight loss as body fat is mobilized (Brockman, 1979). The mobilization of body stores can result in metabolic stresses related to the increase in reactive oxygen species (ROS) production (Miller and Brzezinska-Slebodzinska, 1993). Elevated NEFA

levels increase ROS production by the mitochondria in vitro (Mataix et al., 1998, Koshkin et al., 2003, Srivastava and Chan, 2007).  $\beta$ -oxidation produces the reduced electron donors electron transfer flavoprotein (ETF) and electron transfer flavoprotein quinone oxidoreductase (ETF-QOR) (St. Pierre et al., 2002). Because both ETF and ETF-QOR occur in semiquinone forms during  $\beta$ -oxidation, their semi-reduced states may allow for the formation of ROS by-products (St-Pierre et al., 2002) during lipid oxidation. Elevated fat oxidation, such as when NEFAs are the primary energy source, results in elevated ROS levels. Chronic production of ROS can lead to cellular damage resulting in oxidative damage to lipids and other macromolecules.

The objective of this study was to determine the effect of body fat mobilization on markers of oxidative stress in beef cattle.

## **Materials and Methods**

### *Animals*

Twenty-six Angus-cross cows were blocked by body weight and housed in pens of 6 to 10 hd. Cows were  $5 \pm 0.4$  yr old and weighed approximately  $700 \pm 10$  kg. Measurements were taken at two time points: weight loss (WL) and weight maintenance (WM). The WL period occurred for 60 d after calving. Cows were weighed prior to feeding approximately 5 d post-calving to determine initial weight and weighed weekly thereafter. Final weight, also measured prior to feeding, was recorded on d 60. Cows were turned out on pasture after the WL period. The WM portion of the experiment took place after weaning. Approximately 10 d after weaning and prior to feeding, cows were weighed for initial body weight. Cows were weighed weekly thereafter. Final weight was measured prior to feeding on d 60. The diet fed during WL and

WM consisted of bluegrass straw and alfalfa hay to meet maintenance requirements for cows in mid-gestation (NRC, 2000).

Blood samples were collected from the tail vein on d 60 of the WL and WM periods. Packed cell volume (PCV) was determined by centrifugation for each sample from whole blood samples. Samples for plasma and red blood cells (RBC) were collected in vacutainer tubes with EDTA, processed and stored at  $-80^{\circ}$  C. RBC were thawed on ice, diluted 1:5 with ice-cold ultrapure water to lyse red blood cells and centrifuged at  $10,000 \times g$  for 15 minutes at  $4^{\circ}$  C. Lysate was stored on ice until analysis. Muscle biopsy samples (approximately 0.5 g) were taken from the *Biceps femoris* of each animal on d 60 of each treatment. A 10 cm x 10 cm area was clipped over the *Biceps femoris*, followed by lidocaine infiltration. Once analgesia was established, a 1 cm incision was made longitudinally through the skin. The muscle biopsy instrument was inserted and the biopsy obtained. Two skin sutures closed the incision. Biopsies were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}$  C. All experimental protocols were approved by the Washington State University Institutional Animal Care and Use Committee.

#### *Nonesterified Fatty Acid Concentrations*

Serum samples were collected and stored at  $-80^{\circ}$  C. Nonesterified fatty acid (NEFA) concentrations were measured spectrophotometrically using NEFA-C (HR) kit (Wako Chemicals, Richmond, VA). The procedure was modified according to manufacturer's instructions for microplate analysis. NEFA concentrations (mEq/L) were determined using a standard curve.

### *Superoxide Dismutase Activity*

Superoxide dismutase (CuZnSOD) activity in red blood cell (RBC) lysate was measured spectrophotometrically (Cayman Chemical, Ann Arbor, MI). All reagents and standards were prepared according to manufacturer's instructions. SOD activity (U/mL) of each sample was determined using the standard curve generated on the plate. SOD activity was corrected to U/mL of PCV.

### *Glutathione Peroxidase (GPx) Activity*

Glutathione peroxidase activity in RBC lysate was measured spectrophotometrically (Oxis Research, Foster City, CA). The total activity of GPx (mU/mL) in the samples was determined using the molar extinction coefficient for NADPH,  $6220 \text{ M}^{-1} \text{ cm}^{-1}$ . GPx activity was corrected to mU/mL of PCV.

### *Total RNA isolation from skeletal muscle*

Total RNA was isolated from skeletal muscle using Trizol reagent (Invitrogen, Carlsbad, CA) with modifications (Sambrook and Russell, 2001). Total RNA was then quantified by absorbance at 260 nm and the integrity of total RNA was checked by agarose gel electrophoresis and ethidium bromide staining of the 28s and 18s bands. RNA (2 ug) was DNase treated using Turbo Free DNase (Ambion, Foster City, CA) and first-strand cDNA was synthesized using Superscript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand synthesis was checked using polymerase chain reaction (PCR) at 50 cycles using primers described below.

### *Real time PCR*

Target gene mRNA levels were measured using Real-time polymerase chain reaction (quantitative PCR). Real-time PCR was performed using a Biorad thermocycler (Biorad, Hercules, CA) and iQ SYBR Green Master Mix (Biorad, Hercules, CA). Primers were designed for the following genes:  $\beta$ -actin, mitochondrial superoxide dismutase (MnSOD), glutathione peroxidase 1 (GPx1), glutathione peroxidase 4 (GPx4), thioredoxin reductase 1 (TrxR1), Selenoprotein W (SelW), peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC1 $\alpha$ ), nuclear respiratory factor 1 (NRF1), estrogen-related receptor alpha (ERR $\alpha$ ) and tumor protein 53 (p53). Primers were designed using *Bos taurus* sequences when available for target genes and a housekeeping gene (Table 1).  $\beta$ -actin was used as a control to account for any variation in efficiency of reverse transcription and PCR.

PCR conditions were as follows: 95° C for 2.5 min, 50 cycles of 95° C for 30 s, 58° C for 30 s and 72° C for 30 s, a melt curve of increasing temperature of 2° C every 10 s starting at 55° C followed by a hold at 4° C. For each individual animal, a relative quantification of mRNA levels was performed using standard curves of each primer set. The relative expression was expressed as a ratio of the target gene to control gene using the Pfaffl equation (Pfaffl, 2001). Relative expression was normalized within cow, where expression value was expressed as a ratio to expression during maintenance. Therefore relative expression of target genes was expressed as a fold change from the WM value.

### *Statistics*

NEFA, CuZnSOD and GPx data were analyzed using the general linear model procedure (Proc GLM) from the SAS version 9.1 statistical package (SAS Institute Inc., 2002). The model



was:  $Y_{it} = u + t_i + E_{it}$ , where  $t_i$  was the effect of treatment. Statistical differences were determined with  $P < 0.05$ . Real-time PCR data were analyzed using a student T-test to test the hypothesis that the fold-change was equal to 1.0. Statistical differences were determined with  $P < 0.05$ . Correlations were determined using the Proc Corr program on SAS.

## Results and Discussion

Cows lost more weight ( $- 41.5 \pm 5.0$  kg) during WL than ( $+ 5.5$  kg  $\pm 5.0$ ) WM ( $P < 0.0001$ ; Brennan et al., 2007) and serum NEFA concentrations were elevated ( $P < 0.01$ ) during WL. Cows averaged  $0.51 (\pm 0.02)$  mEq/L of NEFA during WL and  $0.10 (\pm 0.02)$  mEq/L of NEFA during WM. Circulating NEFA increase during lipolysis to provide fatty acids for  $\beta$ -oxidation (Brockman, 1979). Bernabucci et al. (2005) reported cows with elevated plasma NEFA levels also had higher plasma levels of reactive oxygen metabolites. NEFA can increase the production of ROS in vitro in cells (Inoguchi et al., 2000 and Li and Shah, 2004) and in isolated mitochondria (Mataix et al., 1998; Koshkin et al., 2003; Srivastava and Chan, 2007) as well as in vivo in rats (Chinen et al., 2007).

In this study, although NEFA levels were elevated, no differences were found the activities of in erythrocyte CuZnSOD or GPx during WL. CuZnSOD activity averaged 715 U/mL of PCV ( $\pm 28.7$ ) during WL and 673 U/mL of PCV ( $\pm 28.7$ ) during WM. SOD is an endogenous antioxidant enzyme that is the main component of the body's intracellular defense system against ROS. SOD converts superoxide ( $O_2^-$ ) to the less toxic ROS hydrogen peroxide ( $H_2O_2$ ), which is further scavenged by GPx to less toxic compounds (Halliwell and Chirico, 1993).

GPx activity was 261 mU/mL of PCV ( $\pm 9.0$ ) during WL and 241 mU/mL of PCV ( $\pm 9.0$ ) during WM. Bernabucci et al. (2005) found dairy cows with elevated NEFA levels had no change in red blood cell GPx activity compared to cows with lower NEFA level. Medium body condition score (BCS) cows had a decreased (BCS) after calving and elevated NEFA levels, but no change in SOD or GPx activity (Bernabucci et al., 2005). High BCS cows had higher NEFA than medium BCS and had an increase in reactive oxygen metabolites (ROM) and erythrocyte SOD activity indicating that link between NEFA levels and oxidative status. In this study, SOD and Gpx data indicate that any ROS production did not affect antioxidant capacity activity of erythrocyte enzymes.

Whereas SOD and GPx activity did not change, real time PCR quantification of mRNA levels revealed an upregulation of several antioxidant genes during WL (Figure 1). During WL, MnSOD mRNA levels were 1.8 fold higher. Although mRNA levels of GPx1 did not change, GPx4 was 1.4 fold higher ( $P < 0.05$ ), TrxR1 mRNA levels increased 10.3 fold ( $P = 0.05$ ), and SelW mRNA levels increased ( $P < 0.05$ ) 2.7 fold during WL. GPx4 mRNA levels were strongly correlated ( $P < 0.0001$ ) to the mRNA levels of MnSOD ( $r = 0.76$ ). MnSOD is localized the inner mitochondrial membrane and acts at the primary defense from superoxide produced in the electron transport chain. GPx4 is one of the main cellular defenses against oxidative damage to cell membranes (Ran et al., 2004) because GPx4 is the only GPx that can scavenge lipid hydroperoxides (Thomas et al., 1990). Increased GPx4 mRNA levels in these cows could protect them from the increased NEFA levels and thus an increase in lipid peroxide products, however there was no correlation between serum NEFA and antioxidant mRNA levels.

Thioredoxin reductase 1 and SelW were upregulated (Figure 1) during WL. Expression of TrxR1 mRNA is induced in a time-dependent manner in the liver and lung of mice following

treatment with the superoxide-producing chemical paraquat (Jurado et al., 2003). Treatment of human carcinoma cell lines with H<sub>2</sub>O<sub>2</sub> not only rapidly induced the mRNA expression of TrxR1, but also resulted in the oxidation of the selenocysteine residue on TrxR1, further supporting the role of TrxR1 as an antioxidant (Sun et al., 1999). TrxR1 is one of the main factors maintaining the overall redox state of the cell and also reduces the active site of GPx (Nordberg and Arner, 2001). SelW is a selenoprotein highly expressed in skeletal muscle (Yeh et al., 1995; Gu et al., 2000) and overexpression of SelW protects cells in vitro from oxidative damage and cell death following treatment with H<sub>2</sub>O<sub>2</sub> (Jeong et al., 2002).

In addition to antioxidant genes, we examined the effect of weight loss and elevated NEFA levels on four transcription factors or co-activators that play a role in the antioxidant response in non-ruminants (Figure 2). These genes include PGC1 $\alpha$  (peroxisome proliferator-activated receptor gamma coactivator-1 alpha), NRF1 (nuclear respiratory factor 1), ERR $\alpha$  (estrogen-related receptor alpha) and p53 (tumor protein 53). mRNA levels of PGC1 $\alpha$  increased 1.8 fold (P = 0.02), NRF1 $\alpha$  increased 2.5 fold (P = 0.002), p53 increased 1.9 fold (P < 0.0001) and ERR $\alpha$  increased 2.3 fold (P = 0.0005) during WL compared to WM. Though these genes seem to respond to elevated NEFA during WL, we did not find any correlations between NEFA levels or weight loss and mRNA levels.

PGC1 $\alpha$  mRNA levels are elevated in response to fasting and are associated with increased mitochondrial energy production (Lin et al., 2005). PGC1 $\alpha$  regulates the biological responses that drive a cell's ability to meet changing energy demands and can activate pathways that regulate the capacity for energy production (Finck and Kelly, 2006). PGC1 $\alpha$  mRNA levels may be increased due to increased energy demands resulting from lactation during the WL period. However, PGC1 $\alpha$  has also been implicated in the protection from oxidative stress. Rat

skeletal muscle cells subjected to xanthine oxidase (an oxidant) for 10 d had elevated PGC1 $\alpha$  mRNA (175%) compared to control cells on d 10 (Silveria et al., 2006). In vitro PGC1 $\alpha$  regulates the mRNA expression antioxidant genes including MnSOD, thioredoxin 2, TrxR2 (Valle et al., 2005), CuZnSOD and Gpx1 (St-Pierre et al., 2006). In this study, PGC1 $\alpha$  mRNA levels were strongly correlated ( $P < 0.001$ ) to both MnSOD ( $r = 0.64$ ) and GPx4 ( $r = 0.65$ ) mRNA levels indicating that PGC1 $\alpha$  may be involved inducing the transcription of MnSOD and GPx4 in vivo.

The increase in mRNA levels of ERR $\alpha$  (2.3 fold) was similar to that of PGC1 $\alpha$  (1.8 fold). ERR $\alpha$  is required as a coactivator for the PGC1 $\alpha$ -dependent induction of mitochondrial ROS detoxification genes such as SOD2, thioredoxin 2 and peroxiredoxin 3 and 5 (Rangwala et al., 2007), suggesting PGC1 $\alpha$  may also indirectly signal antioxidant genes through ERR $\alpha$ . Nuclear respiratory factor 1 (NRF1) binds the antioxidant response element (ARE) in response to elevated intracellular levels of ROS (Kwong et al., 1999; Scarpulla, 2006) driving the mRNA expression of many free radical-detoxifying genes, including enzymes required for glutathione synthesis (Venugopal and Jaiswal, 1998). NRF1 knockout fibroblasts have increased sensitivity to oxidant-producing chemicals and have decreased glutathione levels (Kwong et al., 1999). In adult mice the disruption of NRF1 in the liver results in elevated oxidative stress, increased fatty acid oxidation, and pathological conditions such as liver damage and spontaneous tumor development (Xu et al., 2005).

In the current study, p53 mRNA levels were correlated ( $P < 0.05$ ) to both MnSOD ( $r = 0.47$ ) and GPx4 ( $r = 0.63$ ) mRNA levels, suggesting that p53 is also involved in the induction of MnSOD and GPx4 transcription. In addition to these two genes, p53 was also correlated ( $P < 0.05$ ) to PGC1 $\alpha$  ( $r = 0.55$ ) and to NRF1 ( $r = 0.49$ ). These relationships have not been previously

described but may show some insight into the shared signaling pathway among these genes. There is an intricate network of antioxidant genes and signaling molecules that regulate the body's response to oxidative stress (Figure 3). During times when NEFA are used as a fuel source, signals such as mild ROS production or lipid by-products activate the transcription of nuclear signaling molecules. These genes work together to activate antioxidant genes to aid in the detoxification of ROS.

In conclusion, these data provide evidence that during mobilization of body fat in beef cows, serum NEFA levels increase and mRNA levels of target antioxidant genes increases in skeletal muscle. While ROS production did not affect peripheral antioxidant enzymes, antioxidant genes in skeletal muscle had increased expression in response. These genes act in a common pathway to regulate the transcription of well-established antioxidant genes (such as GPx, SOD or TrxR) that contribute to the animal's adaptation to mild oxidative stress. Finally, these data indicate that antioxidant genes are oxidative stress responsive and have important roles in cattle during body fat mobilization when fatty acid oxidation mildly elevates ROS production.

### **Implications**

When energy demands exceed energy intake, cattle mobilize body fat stores. This can cause oxidative stress as the result of ROS produced as by products of  $\beta$ -oxidation. As part of the protection from oxidative damage, several genes aid in the signaling of the body's antioxidant pathways. By identifying these key genes, we can help better understand how cattle protect themselves from oxidative damage sustained during elevated ROS production.

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Table 1: Real-time PCR primers.

<b>Gene</b>	<b>Accession</b>	<b>Forward 5'-3'</b>	<b>Reverse 5'-3'</b>
MnSOD	NM_201527	TGGACAAATCTGAGCCCTAA	TAAATTTGGCAAAGGAACCA
GPx1	X13684	GGAGATCCTGAATTGCCTCAAG	GCATGAAGTTGGGCTCGAA
GPx4	NM_174770	GATACGCCGAGTGTGGTTTA	GCGAACTCTTTGATCTCTGC
TrxR1	NM174625	AGGAGAAAGCTGTGGAGAAA	TTATCCCTTGATGGAATCGT
SelW	AF380118	ACTCCAAGAAGGGAGGTGAT	ACATTAGCCCTGAGCCAAA
PGC1 $\alpha$	AB106108	GTACCCAGAACCATGCAAAC	TGACAAATGCTCTTCGCTTT
NRF1	L22454	CATGCGTTGAGCTACTGACA	CAGCAGGGAGAGTTCTGTGT
ERR $\alpha$	NM_004451	ACTATGGTGTGGCATCCTGT	TGGTGATCTCACACTCGTTG
p53	U74486	TCTCCACAGCCAAAGAAGAA	ATCATTCAGCTCTCGGAACA

Figure 1: Relative quantification of mitochondrial superoxide dismutase (MnSOD), glutathione peroxidase 1 (GPx1), glutathione peroxidase 4 (GPx4), thioredoxin reductase 1 (TrxR1), Selenoprotein W (SelW) expression during WL (dark bars) and WM (striped bars). For GPx1, TrxR1, and SelW n=18. \* P < 0.05

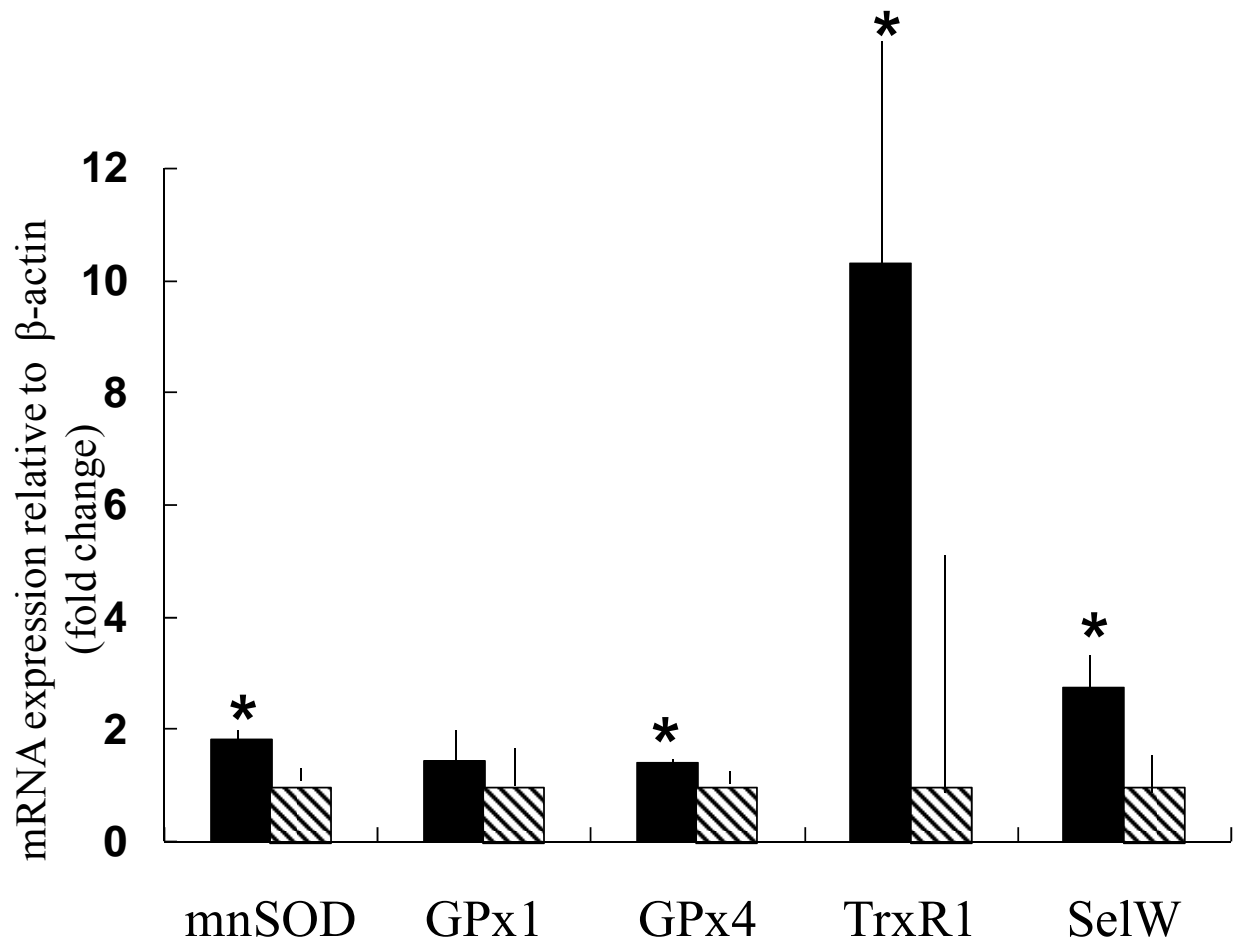


Figure 2: Relative quantification of estrogen-related receptor alpha (ERR $\alpha$ ), tumor protein 53 (p53), nuclear respiratory factor 1 (NRF1), and peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC1 $\alpha$ ) mRNA expression during WL (dark bars) and WM (striped bars). \* P < 0.05

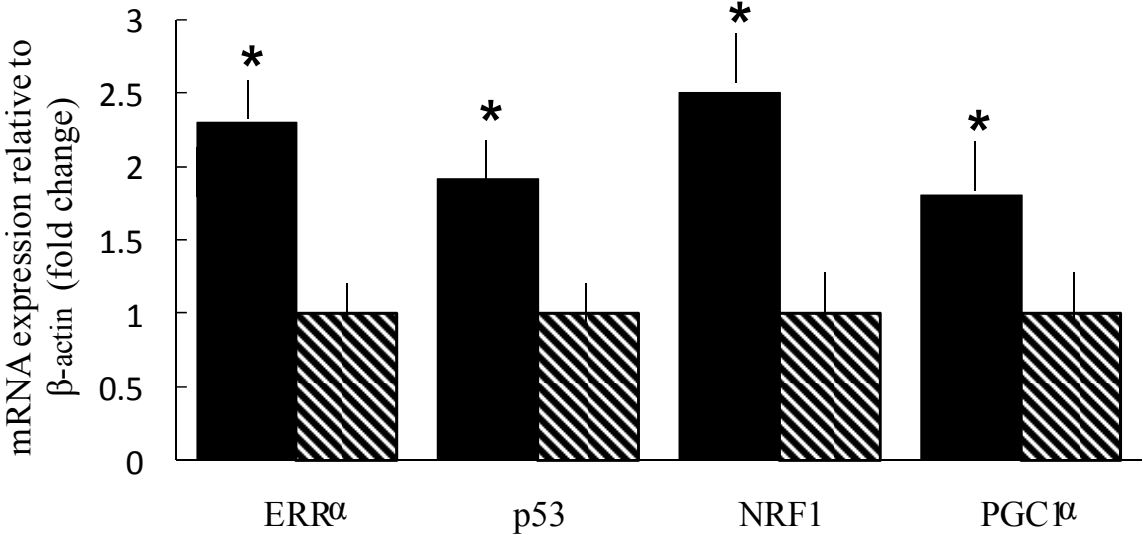
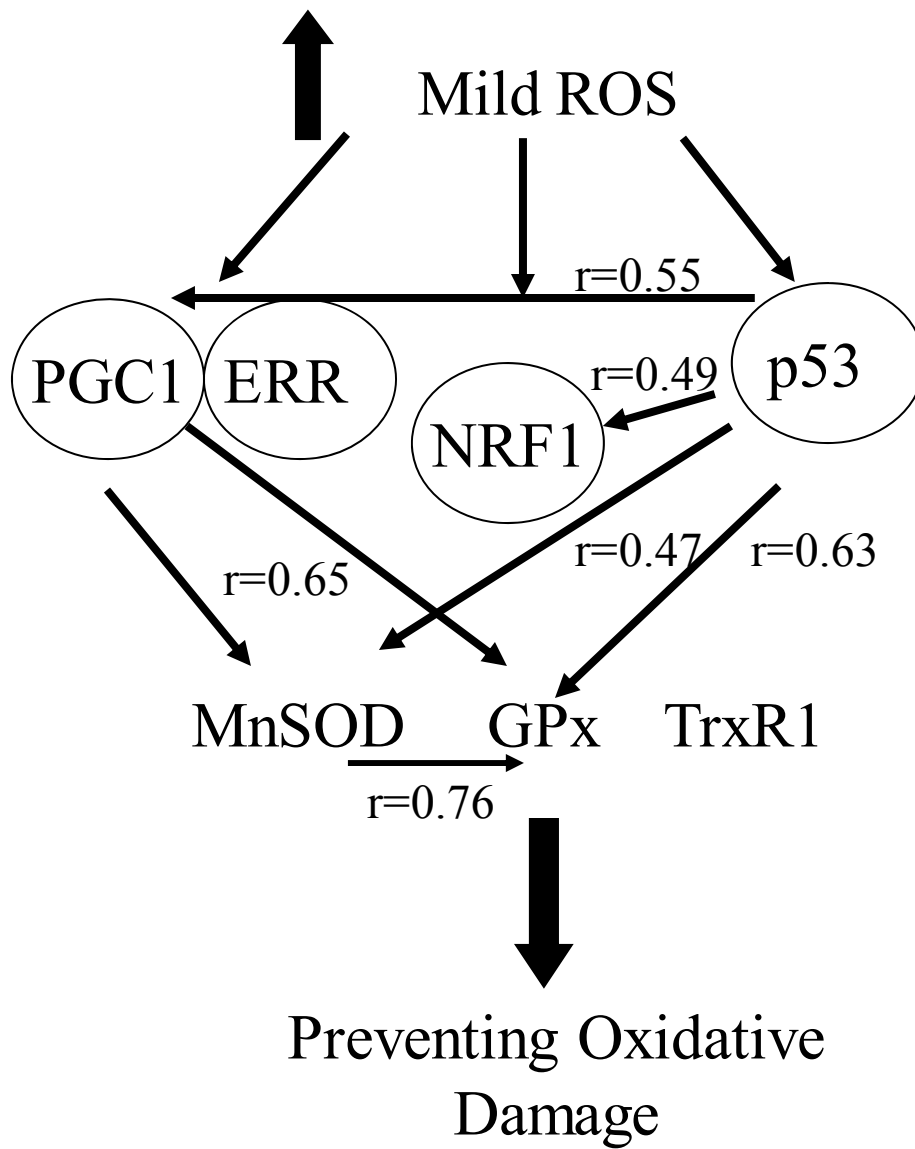


Figure 3: Proposed pathway for the regulation of antioxidant gene expression during mild oxidative stress.



CHAPTER 6:  
CONCLUSION



An elaborate network of antioxidant genes, enzymes and signaling molecules regulate the body's response to increased oxidative stress. The pathways involved in scavenging ROS, regulating the transcription of antioxidant genes and repairing damage sustained by radicals have not been elucidated in ruminants. During a normal year, dairy and beef cattle both undergo weight loss and weight gain as production states change. Previous studies indicate that during weight loss, when serum NEFA levels increase, there is an increase in markers of oxidative stress (Bernabucci et al., 2005). In vitro, oxidative stress markers increase and antioxidant enzyme activity decreases when fatty acids are used as a primary fuel source (Mataix et al., 1998; Inoguchi et al., 2000; Koshkin et al., 2003; Li and Shah, 2004; Srivastava and Chan, 2007). Previous data leads to the hypothesis that increased  $\beta$ -oxidation during weight loss elevates oxidative stress in beef cows.

During weight loss, serum NEFA levels were elevated but there was no change in erythrocyte antioxidant activity. This indicates that though  $\beta$ -oxidation was being used to for a fuel source, ROS production did not have a peripheral affect on antioxidant activity and these cows had an adequate erythrocyte antioxidant capacity. In skeletal muscle, mRNA levels of  $\beta$ -oxidation genes and NEFA-activated PPARs increased during weight loss. Though ROS did not have a peripheral effect in these animals, ROS production locally induced an increase in antioxidant gene expression in skeletal muscle. mRNA levels of genes involved in regulating the expression of well know antioxidant genes (SOD, GPx, etc.) also increased with increased  $\beta$ -oxidation. Finally, UCP2 and UCP3 mRNA levels increased with elevated  $\beta$ -oxidation. Figure 1 indicates the proposed pathway during weight loss. The increase in NEFA results in elevated PPAR expression. PPAR can not only regulate  $\beta$ -oxidation genes, but can also regulate the

expression of UCPs. Increased  $\beta$ -oxidation results in increased UCP2 and UCP3 mRNA levels through either an increase in ROS production and/or an increase in lipid peroxides. Increased ROS also increased mRNA levels of well known antioxidant genes, including GPx4, mitochondrial SOD and TrxR1. This increase corresponded to an increase in several transcription factors involved in the regulation of antioxidant gene transcription. The proposed pathway was supported by correlations between these genes (Figure 1).

This study indicates though cows did not have a change in erythrocyte antioxidant activity, increased  $\beta$ -oxidation results in increased levels of genes associated with the protection from oxidative damage in skeletal muscle. These data support previous findings in non-ruminants and suggests that these genes have similar functions in cattle. One of the most important findings in our study is that UCP2 and UCP3 aid in protecting the mitochondria during elevated  $\beta$ -oxidation since previous studies in ruminants have not found a role for UCP2 and UCP3 in cattle.

Understanding the changes that cattle undergo during a normal production year will help researchers better understand ruminant physiology and metabolism. By determining that cows do undergo oxidative stress during increased  $\beta$ -oxidation, we can design diets that provide optimal antioxidants during times when cows are losing weight. Finally, the goal of genetic and molecular studies in production animals is to eventually be able to identify genetically superior animals. By identifying genes involved in the antioxidant response, selection of animals that are best able to cope with oxidative damage may eventually be possible.

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Figure 1: Proposed pathway for the control of antioxidant and  $\beta$ -oxidation genes during weight loss in beef cows. Correlations between genes are indicated by  $r$ .

