REGULATION OF SELECTED SELENOPROTEINS IN PORCINE AND BOVINE SKELETAL MUSCLE

By

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To the faculty of Washington State University:

The members of the committee appointed to examine the thesis of EMILY NICOLE TERRY find it satisfactory and recommend that it be accepted.

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Chair

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REGULATION OF SELECTED SELENOPROTEINS IN PORCINE AND BOVINE SKELETAL MUSCLE

ABSTRACT

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Selenium (Se) is a nutritionally essential trace element needed to prevent myopathies in skeletal and cardiac muscle. Selenium acts via the 21st amino acid, selenocysteine (Sec), which is incorporated into the selenoprotein family at the UGA codon during translation. Because this codon also acts as a normal stop codon, special machinery i.e., the selenocysteine insertion sequence, during translation allows UGA to signal for Sec. There are 25 described selenoproteins, with only a few having well-defined functions. However, the entire family has redox capability because Sec is located in the active site. Selenoproteins considered in this paper are glutathione peroxidase-1 (GPx-1), thioredoxin reductase-1 (TrxR-1), selenoprotein W (Sel-W) and selenoprotein N (Sel-N). Glutathione peroxidase-1 and Sel-W are glutathione dependent antioxidants *in vivo* where GPx-1 helps reduce lipid peroxides. Low activity of Sel-W is implicated in white muscle disease, a calcification of skeletal and cardiac muscle. Thioredoxin reductase-1 reduces the disulfide bridge in thioredoxin and is highly responsive to changes in oxidant status in the cell. The role of Sel-N is still undefined with the exception of an involvement in muscle cell maturation and in muscular dystrophy when the gene encoding SepN is mutated. In the current studies mRNA expression of GPx-1, Sel-W and Sel-N was measured in fetal and neonatal skeletal muscle from swine with varied maternal Se intake (adequate or

deficient). Maternal intake of Se affected mRNA expression of Sel-W, but not GPx-1 or Sel-N in fetal skeletal muscle. Selenoprotein W expression in fetal skeletal muscle increased in late gestation, whereas Sel-N expression was affected by the interaction of diet by time. Additionally, Se concentration in fetal muscle was affected by maternal Se intake and decreased with gestational age. Thus, Sel-W is an important antioxidant for neonatal antioxidant protection. In another study, levels of mRNA for GPx-1, Sel-W and TrxR-1 were measured in bovine skeletal muscle during weight maintenance and weight loss, which induces oxidative stress. GPx-1 mRNA level was unaffected by weight loss whereas mRNA levels for Sel-W and TrxR-1 were upregulated during weight loss. In summary, Sel-W and TrxR-1 are important antioxidants during oxidative stress. These studies further the known action of selenoproteins in protecting cells from reactive oxygen species.

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CHAPTER 1

LITERATURE REVIEW

LITERATURE REVIEW

A Brief History of Selenium and Selenium-Containing Proteins

Selenium was first isolated and described in 1817 by Jons Jacob Berzelius after analysis of a red deposit on a lead wall in a sulfuric acid plant in Sweden (NRC, 1983; Barceloux, 1999). Berzelius named selenium (Se) after Selene, the Greek moon goddess because Se was similar in nature to tellurium, an element named for earth. The first recorded incident of Se toxicity probably occurred during Marco Polo's travels in 1295, where he observed "hoof rot" and hair loss in the mountains of Turkestan and Tibet, an area now known to be rich in Se. Throughout the ages, other occurrences of what is now recognized as Se toxicity have been noted; Father Pedro Simon recorded the loss of hair and nails in humans in Colombia in 1560 and Lt. Colonel Custer's defeat has been partially attributed to the late arrival of reinforcements, which were most likely affected by a form of Se toxicity resulting in lameness of the animals (NRC, 1983; Hintz and Thompson, 2000).

Following the description of Se by Berzelius, the study of Se did not progress until Se was determined to have toxic effects on animals in the early part of the 20th century. The toxicant was found in various types of corn, wheat and barley, and subsequently in animal proteins if animals had eaten these toxic grains (Franke and Painter, 1935). Selenium toxicity, i.e. selenosis, was found to be the cause of lameness and death for animals grazing on specific plants in Wyoming and the Dakotas (Franke, 1934). The disease in an acute or long term chronic phase was termed "blind staggers," and occurred in the Upper Great Plains of the United States, where the ingestion of Se

reached levels upwards of 10,000 ppm (Barceloux, 1999). Animals affected by blind staggers exhibited weight loss, blindness, respiratory issues and ataxia. This early accusation of Se in toxicosis prevented research into its benefits and Se was labeled as a poison for many years. The dangers of selenium were further supported when it was discovered that symptoms of selenosis were delayed by up to several months after ingestion of toxic levels of Se (Beath, 1935). This delayed onset prevented treatment from occurring in a timely manner, ultimately resulting in the death of the affected animal a few days after the onset of the acute symptoms. An intake of large amounts of Se affects tumor growth. Rats with liver cirrhosis caused by the continued feeding of a seleniferous diet produced hepatic adenomas and low grade carcinomas 18 months after onset of the diet (Nelson et al., 1943)

In 1957, Schwarz and Foltz produced the first major study to counter the prevailing negative opinion of Se. They showed that Se supplementation prevented liver necrosis induced by vitamin E deficiency in rats. The protective compound previously termed “Factor 3” was determined to be an organic selenium compound (Schwarz and Foltz, 1958). Factor 3 was found to be replaceable in the diet by inorganic Se, which would also protect against deficiency diseases in multiple animal species. The term “Factor 3” has since fallen out of use. The discovery that Se prevented some health disorders spurred further research into other possible benefits. Selenium was subsequently determined to prevent liver necrosis in pigs, exudative diathesis in chickens and nutritional muscular dystrophy in calves and lambs (Patterson et al., 1957; Eggert et al., 1957; Muth et al., 1958). These discoveries led to the description of Se as a nutritionally essential trace element. The essentiality of Se in the human diet was

determined after it was determined to be involved in Keshan's disease, a fatal cardiomyopathy that occurs in areas of China that have Se deficient soil (Coppinger and Diamond, 2001).

Selenium in Proteins

In 1973, Rotruck and colleagues determined that Se was present in purified glutathione peroxidase from rat erythrocytes, after injections of ^{75}Se as sodium selenite two to four weeks prior. This was the first solid evidence of Se incorporation into a protein, and this gave our first insight into the role that Se plays in the body. Glutathione peroxidase (GPx) has six isoenzymes in humans, the most recently discovered being GPx 6 in 2003 after mining the genetic sequence using selenocysteine insertion sequence (SECIS) search programs of the human genome (Kryukov et al., 2003). The first isoenzyme, GPx-1/cGPx, was described in 1973 by Flohe et al. GPx-1 is expressed in every cell within the mammal, making it the most abundant selenoprotein (Gladyshev, 2001) GPx is responsible for the detoxification of various peroxides, especially hydrogen peroxide (H_2O_2) (Mills, 1957; Shamberger, 1983; Gromer et al., 2005). These harmful peroxides are produced naturally by the cell through various cellular processes and GPx aids in catalyzing their reduction via glutathione (GSH), a mechanism that will be described in more detail later.

Nine years after the description of GPx, selenoprotein P was discovered and described as a selenium-transport protein in the plasma of rats (Motsenbocker and Tappel, 1982). The exact function of selenoprotein P has yet to be determined, however it is known to be essential for normal functioning in both brain and testis (Koga et al., 1998; Hill et al., 2003; Olson et al., 2005). A few years after the discovery of

selenoprotein P, evidence emerged of another Se-influenced protein. It was discovered that hepatic deiodination of thyroxine was inhibited by Se deficiency in rats, suggesting that Se influences both 5- and 5'-deiodination and that Se might have a role in the deiodinase complex (Becket et al., 1987). Three years later, two papers emerged within one month of each other, with the evidence that iodothyronine-5'-deiodinase (ID) was indeed a selenoenzyme (Behne et al., 1990; Arthur et al., 1990). Iodothyronine deiodinases are responsible for cleaving the 5'-iodine off thyroxine (T_4), to produce T_3 (Burk and Hill, 1993). There are three 5'-deiodinase isoenzymes, each reportedly containing their own SECIS element, each localized to different organ systems (Gromer et al., 2005). The selenoprotein status of one of these, ID Type II, was questioned since its substrate binding subunit differed from Type I and Type III and because it did not respond to increases in Se in cultured glial cells, where it is localized (Safran et al., 1991). In 1998, it was found that a SECIS element supposedly exists within the 3'-untranslated region of Type II ID, which allows for decoding of the UGA sequence to allow insertion of a selenocysteine (Sec) (Buettner, et al., 1998). This was later refuted by Leonard et al. (2000) after isolating an essential ID-II subunit that does not contain UGA.

Thioredoxin reductase (TrxR) as a Se containing enzyme was first described in 1996 after isolation from human lung adenocarcinomas (Tamura and Stadtman, 1996). TrxR had previously been isolated and purified from human placenta, but the presence of Sec was not detected (Oblong et al., 1993). There are two major forms of TrxRs: a large and a small (Holmgren, 2001; Gromer et al., 2005). Small TrxRs are not found in higher eukaryotes, however, they are present in bacteria, plants and unicellular eukaryotes.

Large TrxRs are found both in higher eukaryotes, unicellular eukaryotes and in the green algae *Chlamydomonas reinhardii,* suggesting a non-animal origin for TrxRs (Novoselov and Gladyshev, 2003). The difference between the two forms of TrxR comes in both their molecular weight, where small is approximately 35 kDa and large is approximately 55 kDa and in their structures and mechanisms of action (Gromer et al., 2005).

Large TrxR is a member of the NADPH dependent pyridine nucleotide disulfide oxidoreductase family and is responsible for catalyzing the reduction of its substrate thioredoxin (Trx), which plays a role in electron transport, thiol redox control of transcription factors and protects against oxidative stress (Holmgren, 2001). The Sec amino acid is found in the C-terminal of a redox motif (Gly-Cys-Sec-Gly), which is essential for enzyme activity, where Sec is the penultimate amino acid of the entire enzyme (Gladyshev et al., 1996). During catalysis, the redox center is reduced via the dithiol active center located in the N-terminus of the enzyme. This Sec reduction results in a conformational change, exposing the C-terminal motif and thereby allowing interaction of the enzyme with thioredoxin (Trx). There are three isoenzymes, the most abundant of which is TrxR1, described as a cytoplasmic housekeeping gene involved in cellular redox regulation (Gromer et al., 2005). A second TrxR is defined as a mitochondrial enzyme; and the third is a thioredoxin glutathione reductase, which contains the C-terminal TrxR domain and the N-terminal glutaredoxin-like domain, is capable of reducing both glutathione and thioredoxin, and functions only during puberty (Hatfield, 2001). The activity of TrxR is affected by Se status, where it is decreased during a deficiency (Hill et al., 1997).

The Biochemistry of Selenoproteins

A selenoprotein is a protein into which a Sec residue has been incorporated in a specific manner. There are three schools of thought with regards to the method of Sec incorporation: that it undergoes post-translational binding as a cofactor, that there is non-specific incorporation, and finally, that it is incorporated specifically during translation (Gromer et al., 2005). The most common method of Sec incorporation is the latter-most: specific incorporation during translation. That Sec undergoes post-translational binding as a cofactor has been determined to only occur in bacterial systems. The other major selenoamino acid, selenomethionine (Sem) is treated as methionine by the cell and undergoes mainly non-specific incorporation (Gromer et al., 2005). There is some cotranslational incorporation because Sem has an extremely close relationship with sulfur.

Selenocysteine as an amino acid was first discovered in 1976 by Cone et al., and was found to be primarily incorporated into proteins. Evidence that Sec is an amino acid in its own right is free Sec is not loaded onto cysteine (Cys) bearing tRNA ($tRNA^{Cys}$), because Sec is actually in competition with Cys (Gromer et al., 2005). If Sec is loaded onto $tRNA^{Cys}$, this results in improper functioning of the protein into which Sec is incorporated. The bacterial system of Sec synthesis is an expansion of the genetic code, because it undergoes cotranslational insertion into the polypeptide chain and that a specific tRNA exists for Sec (Böck et al., 1991). Sec incorporation into proteins is a multistep process that has been greatly studied in bacteria, and it is known that Sec is not directly loaded onto its own tRNA, but is synthesized after serine (Ser) is loaded (Gromer et al., 2005). The Sec tRNA is therefore labeled as: $tRNA^{[Ser]Sec}$. An L-Ser is first

loaded onto $tRNA^{Sec}$ in the presence adenosine triphosphate (ATP), resulting in L-seryl-$tRNA^{Sec}$ (Böck, 2001). In the presence of selenocysteine synthase, L-seryl-$tRNA^{Sec}$ is converted to dehydroalanyl-$tRNA^{Sec}$. The selenide that is required to convert the dehydroalanyl-$tRNA^{Sec}$ into a Sec bearing tRNA comes from selenomonophosphate, which will result in selenocysteyl-$tRNA^{Sec}$.

It was originally thought that phosphoseryl-tRNA was an intermediate in the process of Sec synthesis, and that it was converted to Sec via monoselenophosphate (Mizutani and Hitaka, 1988; Carlson et al., 2001). This was later found to be untrue, as evidence was uncovered pointing to an aminoacryl-$tRNA^{Sec}$ as the true intermediate during Sec synthesis and that phosphoseryl-tRNA is not involved in the process, resulting in the synthesis pathway described above (Forchhammer and Böck, 1991).

The codon that signals for Sec insertion serves a dual functionality, as it was first described as the universal stop codon, UGA (Caskey et al., 1968). UGA (TGA in genomic DNA) as a Sec insertion signal is found in-frame, indicating that it must not function as a stop codon in these proteins, and that there is some method by which mRNA is able to direct Sec insertion of the termination of translation (Chambers et al., 1986; Zinoni et al., 1986). $tRNA^{Sec}$ plays a role suppressing UGA as a termination sequence, by acting as a nonsense suppressor tRNA (Carlson, et al., 2001). Chambers et al. (1986) found the in-frame codon in mice, while almost simultaneously, Zinoni et al. (1986) found UGA present in *Escherichia coli* (E. coli). The proof that UGA signaled for Sec insertion was shown by Zinoni et al. (1986), after the analysis of the bacterial gene fdhF, which codes for the selenoprotein formate dehydrogenase H. As the protein sequence was unavailable, a series of deletion experiments in the coding gene were

carried out, to find if Sec insertion was affected. There were three series of deletions fdhF underwent. The first was an internal or 3' terminal deletion, which resulted in truncated polypeptides, and if TGA was deleted from the gene, Sec was not present. The second deletion experiment involved fusion of the 5' terminal portions of fdhF with LacZ, which, if the fusion joint was downstream of TGA, resulted in selenated fdhF-LacZ fusion proteins. If the fusion joint was upstream of TGA, no selenation occurred. Finally, a point mutation was inserted, changing the TGA codon into a TCA codon, which codes for serine, and this also resulted in no Sec incorporation. This provided proof that UGA encodes for the insertion of Sec during translation.

In addition to Sec synthesis during translation, free Sec can also be metabolized in the cell to selenocystathionine, which subsequently is metabolized to selenomethionine, a process that will be discussed later (Böck, 2001). Selenocysteine is also capable of acting as a substrate for cysteyl-tRNA ligase, which will form a selenocysteyl-$tRNA^{Cys}$, resulting in incorporation of Sec at Cys positions.

The tRNA for selenocysteine is unique, for more than just the synthesis of Sec. In mammals, there are two major isoforms of $tRNA^{Sec}$ which vary by just a single methylation group on the 2'-o-ribosyl moiety at position 34, the wobble position of the anticodon (Carlson et al., 2001). Both the mammalian and bacterial tRNA are the longest, at 90 and 95 nucleotides in length, respectively. In bacteria, this extra length is due to an aminoacyl acceptor arm that is 8 base pairs long and to a 22 nucleotide long extra arm (Böck, 2001). In mammals, the aminoacyl acceptor stem is 7 base pairs long, as it is in most other tRNAs, but evidence of a 9 base pair acceptor stem exists (Carlson

et al., 2001). Selenocysteine tRNA does not have a leader sequence for transcription, but it does, however, have a trailer sequence that requires post-transcriptional processing.

The cellular concentration of $tRNA^{Sec}$ is not a limiting factor in the incorporation of Sec into a polypeptide chain. Inserting additional gene copy numbers into transgenic mice does not proportionally increase the concentration of $tRNA^{Sec}$ within the cell; however the numbers vary among tissues (Moustafa et al., 2001). Even deletion of one allele still results in normal protein levels but deletion of both alleles (a double knock out) is embryonic lethal, which suggests that Sec-tRNA and therefore selenoproteins, are vital to life (Bösl et al., 1997).

As UGA signals for both Sec insertion and to halt translation, mRNA containing UGA in-frame must have a way of suppressing the stop codon in order to allow the insertion of Sec. This process is mediated by a unique secondary and tertiary structure and by the presence of a Selenoprotein Insertion Sequence (SECIS), which is located on the 3' side of the codon on mRNA (Böck, 2001). SECIS forms a hairpin structure that is able to complex with $tRNA^{Sec}$ and the translation factor SelB (Baron et al., 1993). However the hairpin is not entirely necessary for Sec insertion; the most critical element is the SECIS sequence (Shen et al., 1993). Deletions in the hairpin portion of GPx do not severely deplete Sec incorporation, but when 4-nucleotide sequences are deleted within SECIS, Sec incorporation is completely abolished. The translation factor SelB has two binding sites, allowing it to complex with both SECIS and the ribosome, which will stabilize the structure of the whole complex (Böck, 2001).

A quarternary complex is formed between SelB, selenocysteyl-tRNA and SECIS. The presence of selenocysteyl-tRNA stabilizes the bonds between SelB and SECIS

(Figure 1). While translation is occurring, the complex moves to the ribosome and the lower helical portion of SECIS melts, the UGA then arrives at the A site of the ribosome, allowing SelB to make contact and bind, which will induce guanosine triphosphate (GTP) hydrolysis. The charged tRNA is then released and the SelB-SECIS element will dissociate. The mRNA is freed to refold and begin the process again.

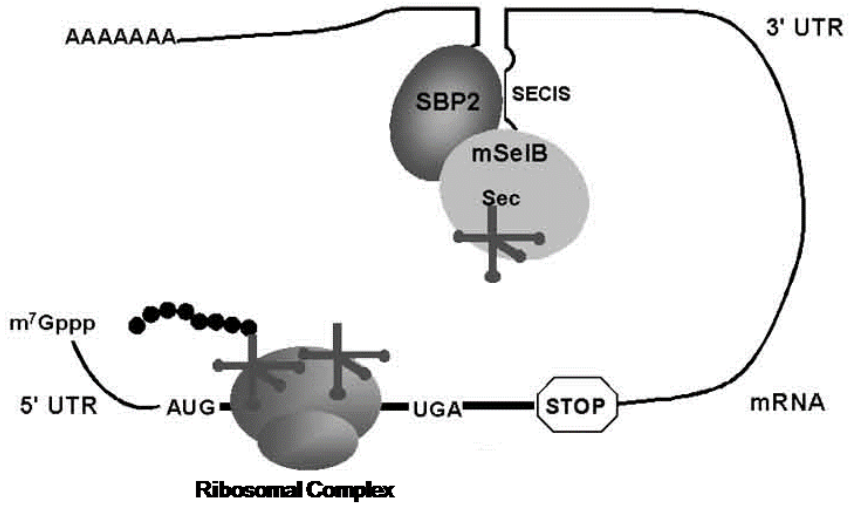


Figure 1- Schematic model of Sec incorporation proteins and SECIS binding element. Adapted from Lescure et al., 2002.

The Biochemistry of Selenium

Selenium is a non-metallic element belonging to Group VIa of the periodic table, located between sulfur and tellurium (ICPS, 1987). Selenium shares properties with both sulfur and tellurium, but is most closely related with sulfur (Foster and Sumar, 1997; Barceloux, 1999). Despite their similarities, sulfur and Se are not interchangeable within biological systems. Selenium exists in four natural oxidation states: 0, -2, +4, and +6. Elemental selenium or selenodiglutathione exist at 0; the selenides (Na_2Se or H_2Se) exist at -2; sodium selenite (Na_2SeO_3), selenium dioxide (SeO_2) and selenious acid (H_2SeO_3)

exist at +4; and sodium selenate (Na_2SeO_4) and selenic acid (H_2SeO_4) exist at +6. Hydrogen selenide is the commonly occurring ionized form of selenium in biological systems (Barceloux, 1999).

There are several dietary forms of Se, those that occur naturally in feedstuffs, and those that are added as nutritional supplements. Selenocystine, selenocysteine, selenomethionine and Se-methyl-selenomethionine are the organic forms of Se, whereas selenite is an inorganic salt that can be added to foods that are deficient in Se (Combs, Jr. and Combs, 1984). Selenocysteine and selenomethionine are both selenoamino acids and are provided in different manners to the mammal. Selenomethionine is unable to be synthesized by the mammalian body system, so it must be ingested in foods (Sunde, 1990). Selenocysteine is synthesized by the body utilizing L-serine in a manner that has already been discussed.

The various forms of Se are absorbed in different ways, and at different efficiencies, depending on the amount ingested, form of Se and species of animal (Shamberger, 1983). A schematic of the metabolism of Se is shown in Figure 2. In monogastrics, selenite is better absorbed than it is in ruminants, with a retention rate greater than 75% (Shamberger, 1983; Combs, Jr. and Combs, 1984). In ruminants, the absorption rate is much lower, only 30-40%. This dramatic decrease could result from a reduction of ingested Se to insoluble forms in the rumen. Selenite is taken up by the gastrointestinal mucosa in a manner similar to that of sulfate, which has been shown to be a competitive system and is mainly absorbed in lower small intestine, cecum and colon (Combs, Jr. and Combs, 1984; Boyd and Shennan, 1986). However, the retention of selenite is much lower than that of Sem, which can be up to 90% and is much more

variable. Selenomethionine and methionine are indiscriminately transported via a Na^+-dependent amino acid transport system, which follows that Sem follows the methionine transamination pathway for degradation (McConnell and Cho, 1965; Schrauzer, 2000). The absorption of Se is greater in feedstuffs of a plant origin (>60%) than from those of an animal origin (<25%) (Shamberger, 1983). Overall, the absorption and retention of Se is better for organic forms than inorganic, and that absorption of organic Se occurs more quickly, thereby raising plasma, erythrocyte and whole blood levels more quickly and to a greater degree (Daniels, 1996).

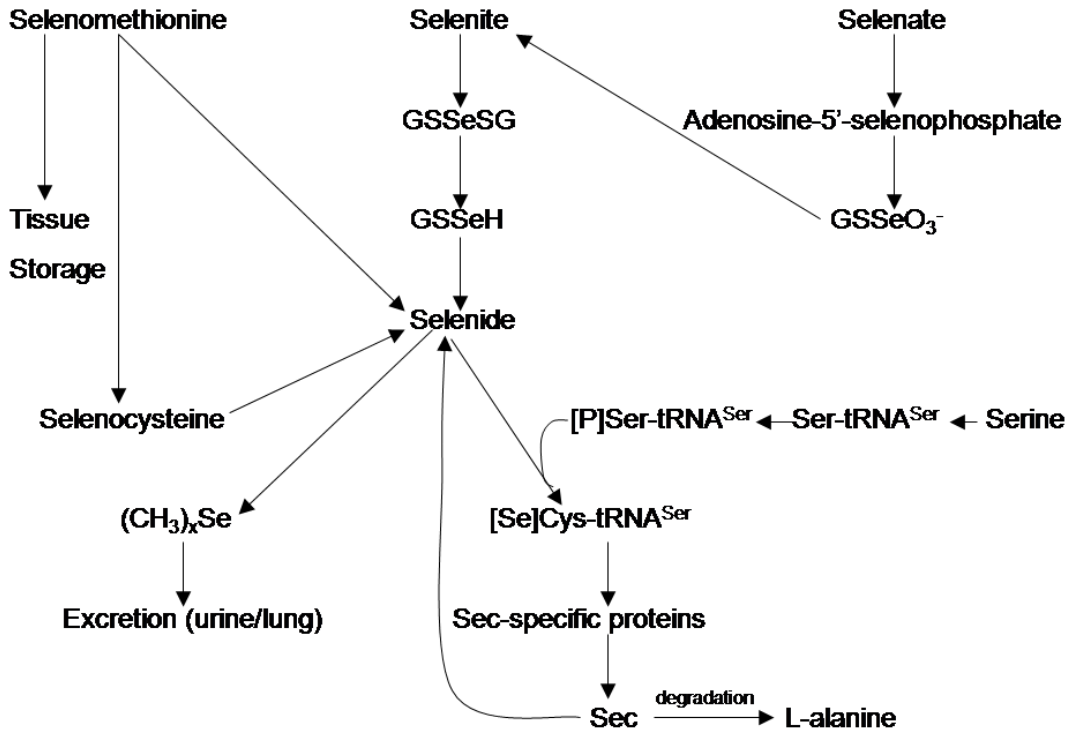


Figure 2- Schematic representation of selenium metabolism, adapted from Sunde, 1990.

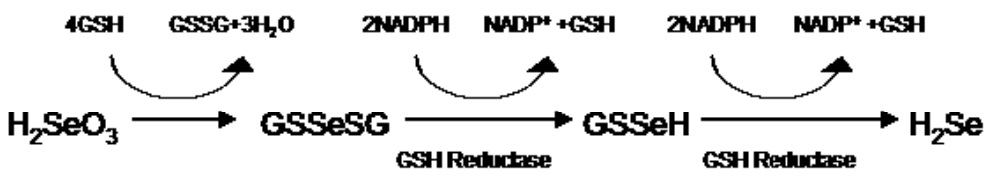
Following the initial absorption of the various Se forms, Se is transported in plasma, bound to plasma proteins like hemoglobin, selenoprotein P, and the glutathione

complex (Combs, Jr. and Combs, 1984). The initial uptake and metabolism occurs in the erythrocytes in order to reduce the Se to selenide. As Se is carried in the plasma, it will enter into the tissues, red blood cells and the leukocytes (Shamberger, 1983). The kidneys appear to have the richest stock of Se, followed by the liver and pancreas. The liver and the kidneys are the most sensitive indicators of selenium status.

Once Se has been absorbed, it needs to undergo reduction to a form that is useable by the body. Selenite first undergoes a four step non-enzymatic reaction by reduced glutathione (GSH), which forms a selenotrisulfide (GSH-S-Se-S-GSH/GSSeSG) (Combs, Jr. and Combs, 1984). Reduction can be carried out by any thiol group, the most common being GSH, which occurs in the cytosol (Ganther, 1968). Selenite reacts with the first thiol group to form an alkylthioselenic acid intermediate ($RSSeO_2H$, R referring to the thiol group), the second thiol reaction yields RSSe(O)SR, the third yields RSSeSR and sulfenic acid (RSSeH) and finally the fourth reaction yields RSO_2SR or RSSR. The final reaction of selenite and GSH is:

$$4GSH + H_2SeO_3 \rightarrow GSSeSG + GSSG + 3H_2O$$

resulting in selenodiglutathione (GSSeSG) and oxidized glutathione (GSSG). Following this initial reduction, selenodiglutathione undergoes a two-step enzymatic reduction by GSH reductase and NADPH to form H_2Se. The first step produces a selenopersulfide intermediate (GSSeH) and the second step results in the acid volatile selenide. The whole reduction pathway from selenite to selenide is as follows:



Erythrocytes then release selenide into the plasma where it can be readily transported to the tissues.

Dietary selenate (SeO_4^{2-}) is reduced to selenite through ATP, also in the cytosol, where the selenite is then reduced by GSH, glutathione reductase or NADPH (Combs, Jr. and Combs, 1984). The reaction is as follows:

where $GSSeO_3^-$ is thioselenic acid.

Thus, due to the capabilities of the cell to reduce inorganic Se forms, the cell should be capable of synthesizing amino acids from selenate (Combs, Jr and Combs, 1984). This is true for Sec, which is synthesized from condensation with serine after the reduction of inorganic Se to selenide. However, Sem must be ingested from the diet because there is no cellular machinery capable of the synthesis of Sem.

Catabolism of Sec, either following turnover of endogenous selenoproteins or from ingested selenoproteins, is carried out by Sec-β-lyase (Esaki et al., 1982). Selenocysteine lyase carries out the β-elimination of L-Sec to L-alanine and selenide, and for every one mole of Sec converted, equimolar amounts of alanine and selenide are produced. This catabolism of Sec might supply reduced Se for the SelD protein, which is an essential component for selenocysteine biosynthesis in eubacteria, its homolog in mammals is selenophosphate synthetase 2 (Xu et al., 2007). Additionally, Sec catabolism provides a means to detoxify free Sec, which is highly reactive (Burk, 1991).

Selenomethionine, which occurs naturally in plants, can replace methionine in the cell when methionine is limited, with minimal effect on structure (Schrauzer, 2000). If

replacement occurs near the active site there can be an effect on the enzymatic capability of proteins. Selenomethionine can be metabolized to yield Sec or degraded to release selenide via the methionine transamination pathway, which is less likely to occur. The metabolism of Sem to yield Sec occurs via adenosylation, demethylation and final conversion to Sec with selenohomocysteine and selenocystathionine as intermediates (Esaki et al., 1981). Some Sem is incorporated into muscle, erythrocytes, kidneys, liver, stomach and the gastrointestinal mucosa, and can also be retained in the brain (Schrauzer, 2000).

Following absorption and reduction, Se products must be excreted. The primary form of excreted Se is methylated selenide, of which there are multiple forms (Combs, Jr and Combs, 1984). The main pathway for excretion of Se metabolites is via the urine, in the form of a methylated selenosugar, 1β-methylseleno-*N*-acetyl-D-galactosamine (Kobayashi et al., 2002). The sugar component of the metabolite is *N*-acetyl galactosamine, but it is unclear how the body uses this component to form selenosugars. The formation of the final excreted selenosugar form occurs in the liver, following methylation and reduction of another selenosugar conjugated with GSH. The excretion of selenosugar occurs in the required to low toxic range of Se intake; at higher, more toxic levels, trimethylated and dimethylated Se metabolites are excreted via urine and expiration respectively. In rats, the major metabolite is the selenosugar, and in younger animals at excessive intakes of Se, trimethylated Se (TMSe) is excreted for a short period of time after which excretion decreases (Suzuki et al., 2004). This mode of action differs in adult rats, where TMSe is a minor metabolite in urine, even at extremely high intakes of Se, indicating that endogenous supply of the sugar component of the selenosugar is in

greater supply in adult rats than it is in young rats. Excretion via feces is mainly unabsorbed Se and is much greater in ruminants than monogastrics (Shamberger, 1983; Daniels, 1996). Fecal excretion is the main form of Se excretion in ruminants.

Tissue Distribution and the Assessment of Status

Selenium is found throughout the body, but the tissues with the highest concentrations in livestock are the kidneys and liver, followed by the lungs, heart, pancreas, spleen, skin and the brain (ICPS, 1987). Human kidneys contain on average 1.04 ppm Se, the liver 0.54 ppm Se and the heart 0.28 ppm Se (Schroeder et al., 1970). However, the skeletal muscle contains the largest overall amount of Se. (Gibson, 1989). Overall the human body contains between 3 and 15 mg of Se, depending upon Se intake. The blood also carries a large proportion of the body's Se content, and blood Se concentration is correlated with dietary intake, i.e. as more Se is ingested, the concentration of Se in the blood increases (ICPS, 1987).

There are several methods to assess Se status and to analyze Se content in biological samples. Common procedures for Se analysis include fluorometry, neutron activation analysis (NAA) and flameless atomic absorption spectrometry (AAS), all of which require skilled analysts (ICPS, 1987). There is not a truly quick and reliable field test to establish the Se status of an individual. In mammals, the most common medium for assessment is the blood, but urine can also be used for short term response studies. Other materials that can be used are hair, liver and nails.

Whole blood, serum or plasma can be indicators of both deficiencies and excess intake (ICPS, 1987). However, because Se interacts with several other elements, including mercury and cadmium, Se concentration in blood can lead to wrong diagnosis.

Serum Se measurements are the most sensitive to status change, therefore are best to indicate short term status (Gibson, 1989; Waldner et al., 1998; Thomson, 2003). Serum Se concentrations are usually 0.119 to 0.134 μg/ml in humans in the United States; however, as with total Se in the body, serum Se varies according to Se intake. Plasma Se concentration is similar to serum, in that it indicates short term Se status. In both serum and plasma, Se is found bound to albumin, selenoprotein P, plasma GPx and to other organic compounds, and following separation of the components are best measured by AAS or fluorometry (Gibson, 1989; Foster and Sumar, 1997). Whole blood Se has been reported to be a better indicator of status because it is less likely to fluctuate with daily intake (Waldner et al., 1998). A complete response to a change in Se intake will take the average life span of a red blood cell, which is approximately 90 to 120 days. A normal range of blood Se in humans in the United States is 0.19 to 0.25 ug/dL (Gibson, 1989). Low concentrations of Se in whole blood indicate chronic Se deficiency, because of the longer time period for adjustment.

With the understanding of the function of GPx came another fairly reliable method of determining Se status; blood GPx activity. Blood GPx activity is best used to determine if a deficiency is present as Se intake and GPx activity levels are highly correlated until GPx activity threshold is reached (ICPS, 1988; Gibson, 1989; Thomson, 2003). In the laboratory, it takes less time to analyze GPx activity than it does the Se content of blood. Glutathione peroxidase activity also indicates long term Se status, as a status change is only detectable 3 to 4 months after it has occurred, the equivalent of the average erythrocyte lifespan (Waldner et al., 1998). A drawback to using GPx as a measure of Se status is that the test is based upon maximal activity of GPx; however, it is

uncertain as to whether or not maximal activity is required to prevent chronic diseases. Also, the effect of different life stages on the expression of GPx needs to be considered. In Se adequate rats that are pregnant or lactating, GPx mRNA levels decline 40% and activity declines 50% from the nonpregnant Se adequate levels, indicating that life stage affects an important status marker (Sunde et al., 2005). Additionally, GPx is extremely unstable, so measurements must be made soon after samples are collected (ICPS, 1988; Waldner et al., 1998).

As mentioned, there are other measurements to indicate Se status, in the short term and long term. Urinary loss of Se can be used to assess Se status and recent exposure because it is closely correlated to Se intake and to plasma Se (ICPS, 1988; Gibson, 1989; Foster and Sumar, 1997; Thomson, 2003). Urinary Se output is a good indicator of toxic intake or environmental overexposure, where a maximal allowable urine concentration is approximately 100 μg/L in humans. Hair and nails can be used to indicate long term exposure to Se. Nails provide the best long term indicator of status, as they can track up to 6 months of status history, depending upon the length of the nail and the rate of growth (Gibson, 1989). Hair, on the other hand, is much more susceptible to outside chemicals, especially with the use of Se in anti-dandruff shampoos (LeBlanc et al., 1999).

Soil Distribution in the United States and Bioaccumulation in Plants

The soil distribution of Se is highly varied throughout the United States; some areas are extremely deficient, whereas others provide Se levels that can cause severe toxicosis via specific plant species. Selenium is present in the soil because of leeching that occurs of the parent bedrock materials in an area (Barceloux, 1999). Because the

bedrock materials are extremely variable in Se content, this leads to the high variation in soils throughout the United States (Figure 3). A distribution of sedimentary rocks from the Permian, Triassic, Cretaceous and Tertiary eras are found in areas with the highest concentrations of Se in soil, whereas areas with lower Se soil concentration have a higher distribution of granite and older metamorphic rocks (Kubota et al., 1967). Lower Se soil concentration is also due to higher acidity in the soil and areas with extremely low concentrations of soil Se are found near recent volcanic deposits or in areas with coastal deposits from a highly weathered land mass.

The concentration of Se in plants is highly dependent upon both the species of plant and the soil concentration in the area. The Plains states consistently produce crops that are adequate in their levels of Se (Kubota et al., 1967). This area of Se adequacy extends through the majority of the Plains states and west to the Rocky Mountains, and branches south-west into the Green and Colorado River basins and to the Los Angeles basin. Certain species of plants in this area are known as hyper-accumulators of Se and are responsible for toxicities experienced by grazing animals throughout the plains (Franke and Painter, 1935; Terry et al., 2000). The soil Se concentration tends to decrease with increased distance from the Plains states, with low soil concentrations in the New England and eastern Great Lakes areas and very low concentrations in the Pacific Northwest and Florida (Kubota et al., 1967). Areas of variable Se soil concentration are found adjacent to the Plains States and in the south-east.

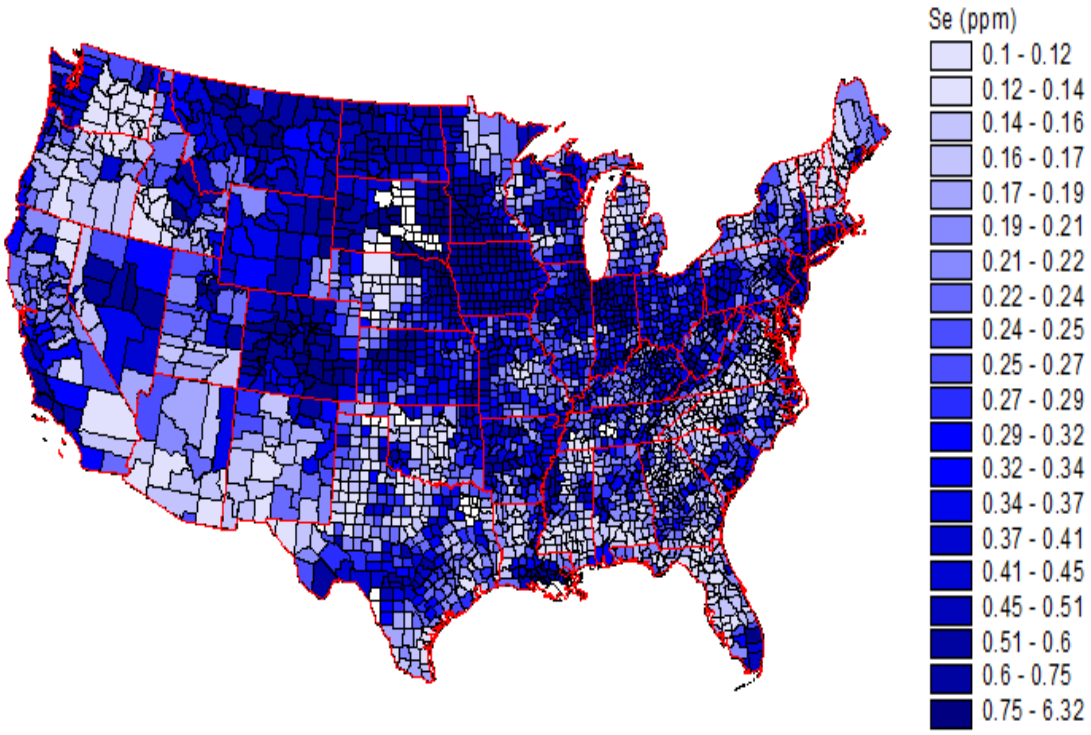


Figure 3- Selenium soil distribution in the United States (Adapted from USGS, 2008).

In the Pacific Northwest, there is still further variation in the concentration of Se in soil. The plant concentration, which is reflective of soil concentration, varies from 0 to 1.24 ppm, however two-thirds of samples from this area have plant Se concentrations below 0.1 ppm (Carter et al., 1968). The western halves of Washington and Oregon and Northern California all have the lowest concentrations, along with mountain rangelands greater than 7500 feet in northern Utah, southern Idaho and northeastern Nevada. Lower elevations surrounding these areas have adequate Se concentrations. The soil concentration increases as you move east through the states, but it is still lower than the Plains states.

Selenium Accumulation and Metabolism in Plants

As Se is essential for life in mammals, so is it essential for growth in plants. An early study in 1938 showed that plants were stimulated to grow when Se was provided in solution (Trelease and Trelease, 1938). However, the tolerance of plants for Se varies widely throughout different genera, as does Se metabolism in the plant. Selenium is taken up by plants as selenate, selenite or as organic Se, normally in the form of Sem (Virupaksha et al., 1966; Shrift and Ulrich, 1969; Sors et al., 2005). What is actually taken up by plants depends on the concentration and the chemical form of Se in the soil.

The uptake of Se by plants is both passive and active, depending upon the chemical form of the Se. Selenate and organic Se are taken up via an active process, whereas selenite is passively absorbed, as there is no evidence of mediation by a membrane transporter (Shrift and Ulrich, 1969; Terry et al., 2000; Sors et al., 2005). Selenate is moved against its electrochemical gradient, in co-transport with three hydrogen ions per ion of selenate. This uptake is in competition with sulfate and is mediated by the sulfate transporter permease (Barceloux, 1999). The salinity of sulfate in the soil inhibits selenate transport (Terry et al., 2000). However, selenate is better absorbed than either selenite or organic Se, and following uptake, it moves more easily from the roots to the shoots (Sors et al., 2005).

There are two main types of plants in terms of Se uptake, accumulators and non-accumulators. An early work in two *Astragalus* species showed that one was stimulated to grow by increasing the amount of Se provided, whereas the other was poisoned by this same treatment (Trelease and Trelease, 1939). Selenium accumulators have the ability to

accumulate extremely high levels of Se, without the threat of toxicity, whereas non-accumulators are unable to cope with high levels of Se, as seen in the early Trelease and Trelease *Astragalus* study (1939). Accumulators are found within the *Astragalus*, *Neptunia* and *Stanleya* genera, which include the legume and mustard families (Terry et al., 2000). In these plants, 10 to 15 mg Se per gram of plant dry weight can accumulate, even when grown in soils containing 2 to 10 ug Se per gram of dry weight (Virupaksha and Shrift, 1965). Comparatively, non-accumulator plants normally have no more than 25 ug Se per gram of dry weight, even in seleniferous soils (Terry et al., 2000). Selenium is hyper-accumulated in the shoots of accumulator species, as well as in the young leaves during the vegetative state of growth and the seeds in the reproductive stage. Non-accumulators have a similar content of Se in their grain and roots, with smaller amounts in the stems and leaves (Terry et al., 2000; Sors et al., 2006).

Selenium accumulator plants have a method to avoid poisoning their cells with excess Se uptake. This is done by shunting excess absorbed Se away from protein incorporation by processing it into non-protein amino acids such as Se-methylselenocysteine, γ-glutamyl-Se-methylselenocysteine and selenocystathione (Brown and Shrift, 1981; Burnell, 1981). In a comparative study between two *Astragalus* species, one an accumulator, the other a non-accumulator; different metabolic pathways of Sem were determined (Virupaksha et al., 1966). Accumulators produced Se-methyl-selenomethionine, Se-methyl-selenocysteine and selenohomocystine in the leaves of the plants, whereas non-accumulators produced only Se-methyl-selenomethionine and a selenopeptide. An earlier study had shown that Se-methyl-selenomethionine was predominant in accumulator species (Virupaksha and Shrift, 1965). Additionally, in

protein fractions extracted from accumulator plants, there was less Se than in non-accumulators, 0.46 to 0.57 ppm versus 4.17 to 5.02 ppm, indicating that in accumulator plants, the majority of Se is shifted from protein incorporation (Brown and Shrift, 1981). The negative potential of this, along with where the Se is stored in accumulator plants, leads to the potential for toxic intake levels in grazing animals in seleniferous areas.

Selenium Toxicity and Deficiency

Toxicity

Selenium toxicity, or selenosis, was first described in the 1930s. It was found that livestock ingesting grains, forages and native range plants were susceptible to Se poisoning, but signs of this poisoning sometimes did not show for months after ingestion (Beath, 1935). Once the animal started to show signs, death was likely to occur within 6 days, and the poisoning was nearly impossible to reverse. Initially, animals would go "off feed" and begin to pass blood in their urine and rapidly lose weight. In sheep fed a high Se diet, it took up to fifteen months for animals to begin showing signs of toxicity, with higher intakes manifesting sooner than lower intakes (Underwood, 1977). The signs of what was later termed selenosis were described in pigs as including loss of dermal hair and the cracking of the hooves, in a more chronic state (Miller and Schoening, 1938). In 1945, Rosenfeld and Beath examined the effect of a dietary protein on the body's elimination of Se in sheep. During early Se addition, the proportion added to the diet was proportionally eliminated, but as high intake of Se continues, elimination of Se decreases, due to damage of the kidneys and an increase of Se storage in the tissues. Within 40 days of stopping Se addition to the diet, Se excretion was equal over all of the low, medium and high Se intake treatments.

There are varying degrees of toxicity, each with their own distinct signs, also known as an 'alkali disease' (Underwood, 1977; Fan and Kizer, 1990). Chronic selenosis presents with dullness, a lack of vitality, emaciation, roughness of coat, eventually resulting in hair loss, soreness or stiffness resulting in lameness, sloughing of hooves, heart atrophy, liver cirrhosis and anemia (Underwood, 1977). Chronic selenosis occurs during steady intake of plants containing Se in concentrations of 20 to 50 ppm (Fan and Kizer, 1990). Acute selenosis, originally known by the misnomer "Blind Staggers" presents with abdominal pain, salivation, grating of teeth, paralysis, anorexia, emaciation, neurologic deterioration (blindness or ataxia) and respiratory distress (Underwood, 1977; Fan and Kizer, 1990). This form of selenosis presents after ingestion of plants containing 100 to 10,000 ppm Se. Acute exposure to Se in a laboratory setting results in respiratory distress, pulmonary edema, diarrhea, hemorrhage and liver and kidney necrosis.

The response of animals to excess Se is similar in most species of animals, but the source, whether it is organic (Sem) or inorganic (sodium selenite) and the dose will affect the response, and there are slight species variations in how they respond to source. Chicks fed a high Se diet show a reduction of feed intake, thus reducing their growth rates and egg production rates (Underwood, 1977). Breeding ring-necked pheasants show a similar response when fed a diet containing 9.3 ppm Se (Latshaw et al., 2004). Within 4 days of introducing the feed, egg production was markedly reduced and birds showed an increase in aggression. Twelve percent of the breeding hens died within one week, and following necropsy were found to have a fluid cardiomyopathy, vacuolar degeneration of hepatocytes and centrilobular hepatic necrosis. Surviving laying hens showed a 35% decrease in hatchability of eggs, even after the removal of the high Se feed

and of those eggs that did hatch, 10% had deformed beaks and abnormal eyes. A total of 50% of the eggs laid following removal of feed showed deformities of the chicks.

Most species show similar signs of deformity during embryonic development when animals are fed a high Se diet (Underwood, 1977). In a pregnancy trial, gilts were fed a series of Se supplemented diets prior to pregnancy and maintained on these diets until subsequent weaning of their offspring (Kim and Mahan, 2001b). Gilts were fed either an organic Se supplement or an inorganic Se supplement. As the concentration of Se increased, body weight gains and feed intakes decreased equally for both Se types, but that the increase in serum and liver content was greater for the organic supplement. For diets with greater than 7 ppm Se, gestation body weight and lactation feed intakes decreased. The number of live pigs born and weaned decreased and litter gains decreased, which was more marked in the organic diets. Overall, diets of greater than 7 ppm Se were toxic, and organic Se had a greater effect on reproductive performance and the inorganic Se had a greater effect on lactation. Lambs undergo depression, dyspnea and death two to three days following acute exposure to Se and showed other signs of selenosis at death (Tiwary et al., 2006). The response of the lambs was greater in those fed the organic Se supplement than the inorganic Se supplement.

In older animals, the response to diets with more than 5 ppm Se is similar. Swine fed diets containing 20 or 40 ppm Se postweaning cease feed intake within a few days and show a complete inability to coordinate their walk by 17 days (Mahan and Moxon, 1984). The pigs were able to detect the diets containing the high Se concentrations when given a choice between an unsupplemented diet and a diet supplemented with 40 ppm Se. They chose the unsupplemented diet, and even when the bins were switched, would start

feeding only from the unsupplemented bin within a few hours. All animals receiving a diet of over 5 ppm Se showed alopecia and other typical selenotic signs, although it was faster in diets containing more than 15 ppm. Steers experimentally induced to show chronic selenosis by feeding them diets containing 5, 10 and 25 ppm Se unsurprisingly showed signs of selenosis at 10 and 25 ppm (O'Toole and Raisbeck, 1995). Steers exhibited hoof lesions, hair loss, hyperplasia, acanthosis and parakeratosis, and organic supplementation exhibited its signs earlier. Pigs on a farm in Spain accidentally fed toxic levels of Se initially showed diarrhea, followed by dermatological and neurological degeneration, such as high limb paresis, cutaneous lesions and diffuse alopecia, skin necrosis, coronary band necrosis on the hooves and lesions in the cervical and lumbar intumescences of the spinal cord, along with poliomyelomalacia of the ventral horns (Casteignau et al., 2006).

Barrows fed a diet with greater than 5 ppm inorganic Se showed a more rapid decline than those fed a diet containing the same level of organic Se (Kim and Mahan, 2001a). Buffalo calves in India experienced adverse effects from Se supplemented at 2.5 ppm and showed mortality at 3.4 ppm (Deore et al., 2005). Deore et al. (2005) also noticed a correlation between whole blood Se concentration and the onset of selenosis. Additionally, animals given reduced glutathione via intravenous injection showed arrested progress of selenosis, death was prevented and whole blood Se concentration was reduced, which suggests a possible treatment option for selenotic animals.

The effects of Se toxicity are modified by the addition of various nutrients to the diet. High protein intakes appear to buffer the effect of excess Se, but the type of protein has an effect (Underwood, 1977). Additionally, the addition of various elements to the

diet can also moderate the toxicity. Arsenic, silver, mercury, copper and cadmium exert protective effects on a selenotic animal, but of course, each comes with their own set of problems in excessive or even minor doses. Arsenic has successfully been used in pigs, dogs, chickens and cattle to offset selenosis. It is thought that the addition of any of the elements causes a reaction between that element and Se in the gastrointestinal tract that forms a harmless compound.

Deficiency

Selenium deficiency presents itself in many forms and one of the more common forms of Se deficiency comes as White Muscle Disease (WMD), or more commonly termed: Nutritional Muscular Dystrophy (NMD) (Koller and Exon, 1986). Other Se related deficiencies are termed Se-associated or Se-responsive diseases. Selenium deficiency is commonly characterized by muscle weakness, especially in newborns, unthriftiness, a decrease in weight gain, diarrhea, still births, abortions and diminished fertility. Additional signs of deficiency are calf scours and pneumonia, retained placenta, nutritional myopathies, mulberry heart disease, and gastric ulcers in swine, additionally; stress may be a factor in precipitating the disease. Human Se deficiencies manifest as an increase in cancer incidence, congestive cardiomyopathies, such as Keshan-Beck disease, male subfertility, impaired thyroid hormone metabolism and progression of the Human Immunodeficiency Virus (HIV) (Chariot and Bignani, 2003). Selenium deficiencies are commonly thought to act solely upon the fetus or newborn, however the effects of Se deficiency are equally great in adults (Bates et al., 2000). In herds with marginal or deficient status, calves tend to be poorer in health and show

muscle degeneration, one of the more common symptoms of Se deficiency (Enjalbert et al., 2006).

Pathologies of Se deficiencies are not always solely due to Se, there are many disease states in which vitamin E also plays a role. Liver necrosis and exudative diathesis are prevented by additional Se and vitamin E, where the Se provides a protective effect against WMD, and vitamin E plays an indeterminate role (Muth et al., 1958). In guinea pigs, a deficiency of both vitamin E and Se caused a fatal myopathy in animals after 30 to 35 days (Hill et al., 2001). Half of the subjects had to be euthanized within this time. Animals fed the 0 Se or 0 vitamin E diets did not show the extent of the deficiency shown in animals fed the combined deficient diet. Additionally, combined vitamin E and Se supplementation decreased the duration of clinical mastitis in dairy cows by 62%, whereas Se supplementation alone decreased the duration only 46%; vitamin E supplementation alone decreased the incidence of clinical mastitis (Smith et al., 1984). The conclusion was that vitamin E deficiency increases the incidence and Se deficiency increases the duration of clinical mastitis.

The incidence of abortions is increased in animals experiencing Se deficiency. In aborted calves showing lesions of heart failure with cardiac dilation or hypertrophy and additionally a nodular liver with ascites, the liver concentration of Se is severely decreased as compared to aborted calves that do not show lesions of heart failure or from control fetuses taken from a slaughterhouse (Orr and Blakely, 1997). In aborted calves without lesions of heart failure, Se is also decreased, but not to the same extent. This leads to a conclusion that perhaps Se deficiency during pregnancy causes myocardial necrosis and heart failure, which would lead to an abortion. In Turkish women with

decreased serum Se and zinc levels and increased lead and copper levels, the incidence of neural tube defects is increased, which results in an increase in 2nd trimester induced abortions due to the neural tube defects (Cengiz et al., 2003). In women that experience recurrent spontaneous abortion (RSA), Se levels are decreased as compared to women who deliver a healthy baby at term (Koçak et al., 1999; Kumar et al., 2002). Selenium levels in women with RSA are still within a normal range, however. A correlation between Se status and pre-eclampsia in women has also been noted, and in gestating rats given a diet containing no Se, symptoms were pre-eclampsia-like, with decreased birth weights, an increase in systolic blood pressure and proteinurea levels (Vanderlelie et al., 2004). Liver and placental GPx and TrxR levels were also decreased in a dose dependent matter.

Nutritional Muscular Dystrophy is a blanket term for a myodegenerative disorder in livestock, typically caused by a nutritional deficiency. White Muscle Disease is a disease that belongs to the NMD category, and is caused by a Se deficiency (Koller and Exon, 1986). Because of the protective effects of Se and vitamin E against free radicals, deficiencies in these nutrients result in peroxidation of the lipid membranes in the cell. This eventually leads to the breakdown of the cell membrane, calcium accumulation within the cell and mitochondrial injury, which results in cell death or segmental necrosis (Thomson, 1984; Radostits et al., 1999). Clinically, animals suffering from WMD are prone to sudden death from a myocardial dystrophy, as in the aborted calves. A subacute form of the disease presents as stiffness in movement, with a weakness or trembling in the limbs, muscles are hard and swollen, and the animal often suffers from dyspnea and labored breathing if the diaphragm and intercostals are affected. Other clinical signs of

WMD/NMD present as an increase in creatine kinase and blood in the urine (Abutarbush and Radostits, 2003). In a beef cow that presented WMD at birth, administration of both Se and vitamin E alleviated the symptoms, resulting in a full recovery.

In a microarray analysis of Se responsive genes, mice were fed a Se deficient diet for several generations, and a muscle sample taken from the final treatment generation (Hooven et al., 2006). The majority of the Se responsive genes were those belonging to T-cell signal transduction, which includes transcription factors, oncogenes and various nuclear and cellular receptors. Many of the genes were related to autoimmune diseases. HIV patients often have muscular dystrophic-like symptoms, and it has been found that Se status is often impaired in these cases (Chariot et al., 1997). It is hypothesized that this Se impairment is due to a reduced intake, malabsorption or that Se is possibly being trapped by viral selenoproteins, and is no longer available for use by the patient.

Selenium and Reproduction in Swine

Many studies have been done on the effect of Se on reproductive efficiency in both the gilt/sow and the boar. For the purposes of this literature review, I will focus on the gilt and sow. With the knowledge that Se is crucial to normal health of both humans and animals comes the realization that Se is also necessary for reproductive health, but how reproduction is affected is the question. In mature sows fed a Se deficient or replete diet throughout gestation and lactation, there was no effect on reproductive performance (Wilkinson et al., 1977). The period of time with the highest Se demand was immediately following parturition, and that treatment also had an additional effect on plasma Se level at term and one month into lactation. There is also a positive dose response in serum, milk and tissue levels with inorganic Se supplementation (Mahan et

al., 1977). Pregnant sows, no matter their Se supplementation level, have lower Se levels in liver and kidney, and thus have a lower transfer to their progeny (Mahan et al., 1977; Yoon and McMillan, 2006). As supplementation level of the sow increases, tissue and serum Se concentration increases in the neonate and at weeks 2 and 4; however, at weaning, there is a decrease in serum, hepatic and kidney Se, indicating extremely short carryover of Se from the dam. Between 11 weeks of age and the age at which the animal reached 100 kg, there is an increase in Se in serum and kidney, so there is recovery of Se in the piglet as it grows. In animals maintained to be mildly Se deficient during gestation, there is a decrease in whole blood Se, liver Se and liver GPx activity, especially at days 30 and 45 for GPx activity (Hostetler and Kincaid, 2004A; Hostetler and Kincaid, 2004B). Additionally, gilts with decreased Se had increased malondialdehyde (MDA) and hydrogen peroxide concentrations in the liver. In fetuses of these gilts, liver Se decreased and MDA and hydrogen peroxide concentrations increased. Fetal liver GPx activity was not affected by maternal dietary intake but GPx decreased towards the end of gestation (Hostetler et al., 2006).

The question of Se source on reproductive performance and Se concentrations has also been asked, and there appears to be a difference between organic and inorganic Se sources. There is no direct effect upon performance in first parity gilts, but milk Se levels were increased with dietary supplementation and organic Se was highest of all (Mahan and Kim, 1996; Mahan, 2000; Kim and Mahan 2001B). Additionally, weanling piglets had higher Se concentrations with organic supplementation. There was also an effect upon colostrum, in that it increased with organic Se supplementation, but there was no effect of Se. With both sources of Se, the concentration of Se and GPx activity increased

in serum of pigs fed diets with up to 0.3 ppm during the grower and reproductive periods (Mahan and Peters, 2004). In mid to late gestation, the serum Se and GPx activity decreased and then gradually increased throughout lactation until weaning, regardless of the supplementation treatment or source (Yoon and McMillan, 2006).

In animals fed high but not toxic Se levels, there appears to be a toxic effect. In sows and piglets up to 9 weeks of age, fed between 0 and 16 ppm supplemented Se, the number of still births and surviving piglets at nine weeks was unaffected (Poulsen et al., 1989). However, in piglets fed the high Se diet body weight of the piglets was affected, with animals fed the higher intakes having lower body weights and decreased feed intake. Sows fed diets in excess of 7 ppm had lower gestation weights and feed intakes during lactation (Kim and Mahan, 2001b). An increase in organic Se supplementation, especially at high levels, decreased the number of live born and weaned compared to sows fed high inorganic diets. Piglets nursing these sows had hoof separation and alopecia, but the severity was greatest in piglets from sows fed the inorganic diets. This suggests that organic Se has greater toxicity during reproduction and that inorganic has greater toxicity during lactation.

Oxidative Stress and Reactive Species

The presence of oxygen in the atmosphere and in cellular processes is necessary to sustain life. However, the presence of oxygen also provides the possibility for serious negative consequences through the production of reactive oxygen species (ROS) and the assistance in producing reactive nitrogen species (RNS), both of which can seriously affect cellular function. This is termed oxidative stress, and it results from an increased

exposure to oxidants or from decreased protection by antioxidants, which prevent or inhibit oxidation (Benzie, 2000; Davies, 2001). At lower levels, ROS are able to mediate cell proliferation and gene expression, or when released in a controlled way, can signal apoptosis (Pierce et al., 1991; Barrett et al., 1999; Kikuta et al., 2006). Oxygen acts as the final acceptor of electrons released during oxidations in the respiratory chain in mitochondria (Bergamini et al., 2004; Genova et al., 2004). Oxygen is reduced to a dianion at complex IV at the level of the cytochrome oxidase reaction, which under normal conditions, reacts with protons to create water, in the following reaction:

$$O_2 + 4e^- \longrightarrow 2O^{2-} + 4H^+ \longrightarrow 2H_2O$$

For a schematic representation of the mitochondrial respiratory chain, please see figure 4. Of the energy produced from the reduction of this dianion, 40% goes to produce ATP. In the respiratory chain, there are potential side reactions that could result in the production of free radicals or ROS. It is theorized that the level of O_2 metabolites in the body has a role in determining the longevity of a species (Ku et al., 1993). Mitochondria are susceptible to oxidative damage from ROS actions on their lipids, proteins and DNA (Genova et al., 2004). The damage to proteins can occur in the respiratory complexes, resulting in decreased electron transfer and increased ROS production and thus a decline in energy production, leading to an aging effect. Free radicals contain unpaired electrons and function as either reducers or oxidants, and include superoxide and the peroxide anion. The presence of ROS in the mitochondria can lead to cellular damage, which, as Ku et al. (1993) showed, has an effect on aging, and also the pathogenesis of several diseases, such as atherosclerosis and the activity of the calcifying vascular cells (Mody et al., 2001). When ROS are at physiologically normal levels, they provide a role in

defending against infectious agents and in the generation of cell signals (Bergamini et al., 2004).

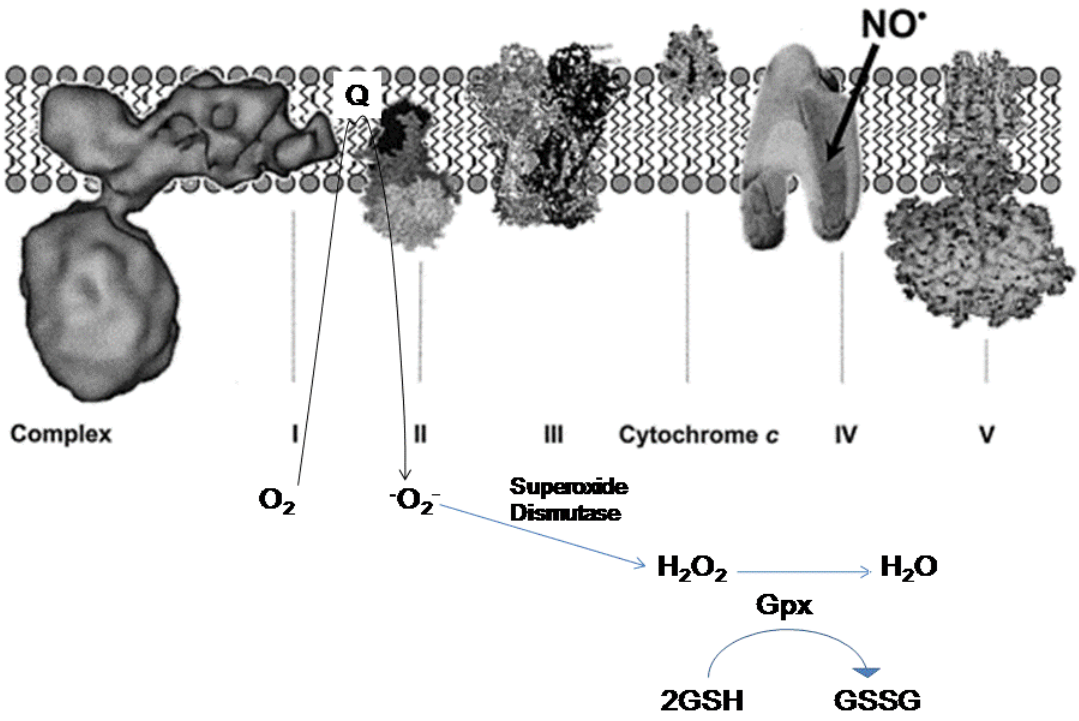


Figure 4- Electron Transport Chain of the mitochondria. All the complexes involved are listed, and sites of free radical production are included. The superoxide pathway is also included. Adapted from Brookes et al., 2002.

The production of ROS results from the reaction of oxygen with usually only one electron, and subsequently with free protons or hydroxyl radicals. Reactive oxygen species are unstable with a short lifetime in the cell and are typically unable to diffuse across cell membranes (Bergamini et al., 2004). Superoxide ($\cdot O_2^-$) is the primary ROS in the mitochondria and it is capable of both reduction and oxidation, and is able to undergo spontaneous dismutation to H_2O_2 via superoxide dismutase (SOD). Peroxide is a secondary ROS, along with hydroxyl, which is the most aggressive of the free radicals. The rate of production and decomposition of ROS is tightly controlled during steady-state

equilibrium. The superoxide anion is also capable of reacting with nitric oxide, to form peroxynitrite ($ONOO^-$), a RNS (Szabó, 2003). Peroxynitrite is a strong oxidant, and is similar in reactivity to hydroxyl, and acts upon thiols, sulfides, and transition metal complexes. The presence of peroxynitrite within the cell can inhibit the respiratory chain, Ca^{2+} pumps, and Na^+/K^+ ATP-ase activity, resulting in dysregulation of ion balance and the cell functions that are related to this balance. (Klebl et al., 1998; Muriel and Sandoval, 2000). Reactive nitrogen species are also capable of DNA cleavage and single strand breakage of the DNA (Szabó, 2003). In addition, they are also mediators of cell injury by enhancing and triggering the pro-inflammatory processes, and also mediating apoptosis.

When this system breaks down ROS production becomes detrimental to the cell's health. Production of ROS can be due to the environment, especially after exposure to ultraviolet radiation from the sun, from other chemicals, or it can be due to metabolic and enzymatic processes that occur within the cell (Bergamini et al., 2004; Svobodová et al., 2007). External stressors can also lead to the production of ROS. In mice undergoing three stress types: cold stress (CS), immobilization stress (IM) and immobilization-cold stress (ICS), there was an increase in protein oxidation, conjugated dienes and thiobarbituric acid-reactive substances, all markers of oxidative stress (Şahin and Gümüşlü, 2007). Glutathione levels decreased in liver of CS and ICS animals, while in the heart, all three stress levels decreased GSH, which leads to increased protein carbonyl levels. Additionally, there was a positive correlation between the stress marker corticosterone levels and oxidative damage markers.

Metabolic processes of ROS production can include the respiratory chain in mitochondria, degradation of purine derivatives and pathways involved in superoxide release for protection against parasitic organisms. To prevent the accumulation of ROS, mitochondria contain ROS scavengers like glutathione, which are able to detoxify the free radicals and reduce them to water through a reducing agent like GPx (Mills, 1957; Shamberger, 1983). Additionally, there are non-enzymatic compounds such as the antioxidant vitamins A, C and E that aid in breaking down the oxidative chain (Bergamini et al., 2004). Enzymatic compounds include SOD, catalase, peroxidase, GSH and transferases.

Proteins that have been inactivated by the presence of ROS or RNS can be regenerated. Thioredoxin and its reducer TrxR are capable of this, and in cultured endothelial cells treated with H_2O_2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels decreased, but the activities of Trx and TrxR were unaffected (Fernando et al., 1992). Over time, GAPDH was regenerated through the actions of Trx and TrxR. In models in which TrxR was inhibited, GAPDH levels did not recover after H_2O_2 treatment. In mice, energy restriction leads to cancer prevention through a reduction in oxidative damage to DNA (Fu et al., 1994). However, in normal weight men following a 20% energy restriction diet for 10 weeks, there was no decrease in oxidative DNA damage (Loft et al., 1995). If the metabolic rate did not decrease with weight loss, there may actually be an increase in DNA damage due to oxidants. Severely obese prepubescent children that are put on a dietary restriction weight loss program have high oxidant status, which is detrimental to their health (Mohn et al., 2005). After 6 months of dietary restriction, it has been shown that the oxidant status is normalized and that there is

also significant weight loss, resulting in healthier children. These disparate effects between prepubescent children and normal weight men suggest that oxidative stress and DNA damage are higher in obesity and that energy restriction only lowers oxidative status in obesity, and may even be detrimental when body weight is normal.

In animals with tumors and that have cancer related cachexia, the skeletal muscle actually has lower mitochondrial respiratory chain activity, an increase in protein oxidation markers and a decrease in GSH levels, due to a decrease in the redox state of the animal (Ushmorov et al., 1999; Barreiro et al., 2005). The muscles also show decreases in mass, and increased muscle wasting and muscle abnormalities due to an inefficiency of the antioxidant enzymes to neutralize ROS. The symptoms of cachexia can be reversed by the reintroduction of antioxidants or nitric oxide synthase (Buck and Chojkier, 1996). In animals that have cachexia, treatment with ornithine, a precursor to the radical scavenger spermine, causes a reversal in respiratory chain activity and thus also in the redox state (Ushmorov et al., 1999).

Selected Selenoproteins

There are at least 25 identified human selenoproteins, of which only 3 have definitive, well-defined functions in redox metabolism and thyroid hormone metabolism (Rederstorff et al., 2006). A few other selenoproteins have partially identified functions involving sperm maturation and Sec synthesis. Most of these proteins are also regulated by Se status of the body, and regulation is at the post-transcriptional site (Burk and Hill, 1993). Essential selenoproteins are probably protected during Se deficiency by the down-regulation of nonessential selenoproteins to allow for adequate translation and Sec

supply for the essential proteins. Other proteins can show maintenance of mRNA expression during deficiency, but their protein level is decreased, indicating that they may act as a supply of Se when necessary.

Glutathione Peroxidase Family

As has been previously mentioned, glutathione peroxidase-1 was the first protein to be identified as a selenoprotein in 1973 (Rotruck et al.), and since, six isoenzymes have been isolated. All of these GPx proteins use GSH to catalyze the reduction of H_2O_2 and lipid peroxides into water and other less harmful substances (Gromer et al., 2005). Each protein is localized to different areas of the body, with GPx-1 expressed throughout the body, GPx-2 mainly found in the gastrointestinal tract, GPx-3 in the kidney, and GPx-4, which is a monomer as opposed to the tetrameric form of the other GPx proteins, is mitochondrial, and is the only one to reduce phospholipid hydroperoxides (Lei et al., 2007). GPx-6 is unique as it is not a selenoprotein in rodents, lacking a Sec (Kryukov et al., 2003). The final protein, GPx-5 lacks a Sec in all orthologs.

GPx-1, or cytosolic GPx, is made up of 4 identical 22 kDa subunits, each of which contains one Sec residue and is localized mainly within the cytosol of cells in the liver and within erythrocytes, although it is expressed throughout the body tissues (Flohe et al., 1973; Cheng et al., 1997). GPx-1 functions to cope with oxidative injury and cell death mediated by both reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Lei et al., 2007). Selenium status affects both mRNA expression and activity of GPx-1, wherein a greater than 90% reduction in both is observed in the liver of the Se-deficient rat (Saedi et al., 1988). At the cellular level, a 60% reduction in mRNA

expression was observed, with a 93% reduction in activity. Upon Se replenishment, GPx-1 expression is restored, but not to maximal levels. It is theorized that GPx-1 might function as a storage unit of body Se, so that in a deficient situation, Sec can be released to supply other essential proteins, and in conditions of excess intake, GPx-1 can act as a mop to take up extra Se to prevent toxicity (Burk and Hill, 1993; Lei et al., 2007). This hypothesis has thus far been disproved since in situations in which GPx-1 is knocked-out or over-expressed; changing the expression of GPx-1 does not affect the expression of other proteins. In the knock-out model, animals became susceptible to a severe form of acute oxidative stress (Cheng et al., 1997). Conversely, in models where GPx-1 is over-expressed; there is an extra layer of protection provided towards oxidative stress (Cheng et al., 1998). The protection that GPx-1 provides appears to only be necessary in extremely severe forms of oxidative stress, since mice not expressing GPx-1, but with adequate Se levels are able to cope with moderate levels of oxidative stress (Cheng et al., 2003). Glutathione peroxidase-1 plays a role in maintaining a cell's redox status and aids in the oxidations of lipids, proteins, NADPH and NADH.

The role that GPx-1 plays with regards to RNS is slightly different from that of ROS. Nitric oxide is reactive with superoxides, which when they react together, they will form peroxynitrite (PN), a cytotoxic compound, leading to the oxidation of lipids, amino acids and DNA (Lei et al., 2007). The production of PN also leads to its reaction with lysine residues in proteins, causing protein nitration, leaving enzymes and proteins in their inactive form. GPx-1 has a promoting role in PN-induced cell death, because knock-out models show animals more resistance to PN-induced apoptosis (Jiang et al., 2000; Fu et al., 2001). In the presence of GPx-1, peroxynitrite reduction formed less

harmful nitrites, which are less harmful to cells (Sies et al., 1997). Promotion of apoptosis in oxidative stress caused by RNS contrasts with the prevention of apoptosis that occurs in ROS induced oxidative stress, where knock-out models are more sensitive to paraquat, a ROS inducing chemical (Cheng et al., 1998; de Haan et al., 1998; Cheng et al., 1999). Paraquat is lethal in a dose-dependent manner in GPx-1 knock-out mice, whereas in normal mice, paraquat transcriptionally upregulates GPx-1. A similar oxidative stress inducing drug, diquat, produces the same results as paraquat, in knock-out mice (Fu et al., 1999).

Glutathione peroxidase 2 is found mainly in the gastrointestinal tract, in the proliferative area of the intestinal tract and in the Paneth cells. It is the target of β-catenin, which functions to transfer Wnt signals for cellular proliferation and differentiation regulation, a system responsible for the regulation of cell to cell interactions (Kipp et al., 2007). Overexpression of β-catenin activates GPx-2, and the promoter region of GPx-2 for β-catenin contains five possible binding sites. In models overexpressing adenomatous polyposis coli, a genetic precursor for colon cancer, there is a decrease in basal GPx-2 promoter activity, which could have implications in the maintenance of the renewal of the intestinal epithelium (Aoki and Taketo, 2007).

A plasma GPx, GPx-3, is also found within the kidney and the thyroid, both of which have high Se content, even during a deficiency. Within the thyroid are the thyrocytes, where GPx-3 is exclusively localized, and this area has the highest selenoprotein expression of the thyroid (Schmutzler et al., 2007). GPx-3 is down-regulated in many thyroid cancer samples, and it is believed GPx-3 functions to use excess H_2O_2 and catalyze the polymerization of thyroglobulin to its storage form.

Glutathione peroxidase-4 is implicated in antioxidant protection, the formation of the mitochondrial capsule in sperm development, cerebral embryogenesis and is able to reduce lipid hydroperoxides (Sattler et al., 1994; Ursini et al., 1999; Imai and Nakagawa, 2003). Gpx-4 is a monomeric protein with no exposed loop domain, which limits the accessibility of the access site for redox regulation. Glutathione peroxidase-4 has a tendency towards polymerization, which is not the case with other glutathione peroxidase isoforms (Ursini et al., 1999). The polymerization occurs during spermatic development via the development of disulfide bonds between exposed cysteine residues that are not present in the other isoforms (Scheerer et al., 2007).

Cellular GPx activity and expression in animals are affected by Se status. In mice with increased dietary Se intake, there is increased GPx activity in the kidney, thymus and pancreas (Sun et al., 1998). Second generation mice that were maintained on a deficient diet during gestation and lactation had a 90% decrease in the original activity of GPx-1 (Sun et al., 2001). Glutathione peroxidase activity in the brain is actually increased during intake of a deficient diet, especially in the cortex, cerebellum and thalamus. Additionally, in weanling swine, at least 0.2 mg Se/kg body weight supplemented in the diet is required for maximal activity of GPx-1, GPx-3 and GPx-4 (Lei et al., 1998). In mice, dietary intake does not affect the expression of GPx in the heart, liver, lung, spleen and thyroid expression (Sun et al., 1998). In neonatal swine, GPx-1 liver expression is decreased, despite Se status of the dam (Loudenslager et al., 1986; Hostetler et al., 2006). There is a 3-fold decrease in GPx-1 expression in liver from day 45 of pregnancy to day 114, which is term.

Iodothyronine Deiodinase Family

The first recorded mention of an iodothyronine deiodinase (ID) was in 1955 in an *in vitro* system where thyroxine (T_4) was deiodinated (Sprott and MacLagen, 1955). The product of this deiodination was believed to be triiodothyronine (T_3), and that T_3 can be subsequently deiodinated. Additionally, deiodination was inhibited by antithyroxine compounds, providing further evidence that a deiodinase type of protein was acting upon thyroxine. The deiodination procedure is enzymatic, and activity is highest in liver, kidney and heart (Nakagawa and Ruegamer, 1967). The main supply of endogenous T_3 in the cortex, cerebellum and in the blood is due to this deiodination of T_4 (Kaplan and Yaskoski, 1980; Berry et al., 1991A). Kaplan and Yaskoski also believed that there was a separate ID enzyme for each of the rings in T_4, one for the phenolic and one for the tyrosyl rings.

The first evidence that ID is influenced by Se status came in the late 1980s. A 14-fold decrease in hepatic T_3 production *in vitro* was noted during Se deficiency (Beckett et al., 1987). The production of T_3 was not restored by other cofactors of T_3 production. The decrease in T_3 production was reflected in rat plasma thyroid hormone concentration, and also a subsequent T_4 increase and a T_3 decrease. Deficiency inhibited both 5- and 5'-deiodination. Prolonged deficiency in rats results in T_3 inhibition in the brain, liver and kidneys, again with an increase in plasma T_4 concentration (Beckett et al., 1989). However, the increase in T_4 does not produce the expected increased feedback inhibition on thyrotropin release from the pituitary. Following the administration of Se to the deficient animals, T_3 production returned to normal levels, and in animals administered [^{75}Se]$NaSeO_4$, a ^{75}Se containing protein fraction with ID-1 activity was detected within

four days (Arthur et al., 1990). This provided further evidence that the two described IDs were seleno-enzymes or required selenium-containing cofactors for ID activity.

In 1990, a 27.8 kDa selenoprotein was identified in the rat thyroid, liver and kidneys, which was capable of catalyzing the deiodination of T_4 to T_3 (Behne et al., 1990; Arthur et al., 1991). This protein decreased in activity in Se deficiency, similar to the response of ID, and it was found that the deiodinase subunit of the protein contained one Sec residue per molecule, which was coded by UGA in the mRNA (Berry et al., 1991A). This Sec residue was highly reactive, and necessary for 5'-deiodination (Behne et al., 1990; Berry et al., 1991B). It was unclear if both ID isoforms contained a Sec residue, but it was known that gold thioglucose was a competitive inhibitor of both ID-I and ID-II (Berry et al., 1991C). Replacing Sec with Cys decreased the sensitivity of ID-I to gold, but ID-II showed the same sensitivity without replacing the purported Sec, so it was determined that ID-II did not contain a Sec residue, but rather a Cys residue at the active site. Further evidence was provided by the fact that ID-II is not affected by Se in a concentration dependent manner and that after ^{75}Se labeling in glial cells, none of the labeled proteins corresponded with ID-II (Sofran et al., 1991). In amphibians, ID-II is homologous with other IDs, and contains an in-frame TGA (Davey et al., 1995) A response study to Sofran et al. (1991) in support of Davey et al. (1995) found an expressed sequence tag that would encode the missing SECIS element that is not present in ID-II, and that there are UGA codons in the coding sequence (Buettner et al., 1998). However, this was further countered by the bioactivation of ID-II in the brain, where ID-II was isolated (Leonard et al., 2000). The isolated protein was an essential 29 kDa subunit of ID-II, that lacked a Sec, therefore leading to the conclusion that ID-II is a non-

selenocysteine subunit of the ID family. In ID-I with a Cys replacement of Sec, enzymatic activity is maintained, but at a much lower level than in the wild type protein (Berry et al., 1992). The amino acid change also does not require the SECIS element for successful translation of the protein, and actually results in an increase in translation rate.

A third ID was described in 1994 from *Xenopus laevis* as a protein that metabolized T_3 and T_4 to inactive metabolites (St Germain et al., 1994). The protein had regions of homology with ID-I, including a UGA codon encoding Sec. In a human cDNA library, a UGA codon was pinpointed, as was a SECIS in the 3'-UTR, as it also was in the rat (Salvatore et al., 1995; Croteau et al., 1995). Iodothyronine deiodinase III is highly expressed in the placenta, to regulate circulating fetal hormones, and it is a 32 kDa protein. The serum levels of T_3 are decreased following myocardial infarction, and this alteration results from a tissue response which increases ID-III expression (Olivares et al., 2007). Myocardial infarctions induce ID-III expression within one week of the incident within the affected tissue.

Concurrent Se and iodine deficiencies have an effect on thyroid metabolism, with an increase in thyroid weight and plasma thyrotropin (Arthur et al., 1992). Measuring ID-I can be used to determine the severity of an iodine deficiency, because either a Se or an iodine deficiency will decrease ID-I expression and activity, and concurrent deficiencies affect thyrotropin and thyroid weight (Behne et al., 1992).

Thioredoxin Reductase Family

Thioredoxin reductase, like GPx and ID, was described as a protein before it was described as a selenoprotein. It was originally isolated in *Escherichia coli* and it catalyzes the reduction of the disulfide bridge in compounds such as thioredoxin, insulin

or L-cystine via reduced triphosphopyridine nucleotide or NADPH (Moore et al., 1964; Holmgren, 1977). The reaction is dependent upon thioredoxin as an intermediate electron carrier. In *Escherichia coli*, thioredoxin reductase plays a major role in metabolic oxidoreductions of protein disulfides. Thioredoxin reductase is a 66 kDa protein, which exists in a dimeric form (Thelander, 1968). The rate of reduction of small disulfides and insulin is variable, with regards to the microenvironment of disulfides (Holmgren, 1979).

Thioredoxin reductase was not described as a selenoprotein until 1996, after being isolated from a lung adenocarcinoma line (Tamura and Stadtman, 1996). This protein is a 57 kDa homodimer, which contains Se as Sec and is distinct from other described selenoproteins, such as selenoprotein P. Additionally, TrxR catalyzes a NADPH dependent reduction of insulin in the presence of Trx. This gives evidence of another Se containing protein. Around the same time, in human T-cells a homodimer of 55 kDa was isolated, with a Sec in a position corresponding with TGA (Gladyshev et al., 1996). The TGA is in a conserved C-terminal region, and the protein, TrxR, was determined to be involved in a major antioxidant enzyme system. Thioredoxin reductase from rat and bovine was sequenced and had close homology to GPx, with a conserved active site sequence between the two proteins, and a 3'-UTR that contained the SECIS element (Zhong et al., 1998; Sandalova et al., 2001). The transfer of electrons to Trx from NADPH requires little conformational change in the complex of TrxR and Trx, which makes for an easy redox reaction (Sandalova et al., 2001). Because of TrxR's similarity to GPx, it is hypothesized that TrxR evolved from GPx, and not a prokaryotic ancestor.

Thioredoxin depends on the redox status of TrxR, and is dependent on the Sec residue, which is located in the penultimate position on the C-terminus (Sun et al., 1999). Expression of TrxR-1 increases in response to ROS production by the cell because the ROS cause the oxidation of the Sec residue. This also points to a role of TrxRs in redox-regulated cell signaling, and in regulating cell growth, death and protecting against oxidative stress (Park et al., 2002). There are three described TrxRs, all of which contain a Sec residue. Thioredoxin, also responsible for cell growth, is over-expressed in some human cancers. Proper functioning of TrxR is necessary for controlled cell growth, thus making cell growth Se dependent (Sun et al., 1999; Sandalova et al., 2001). Rats fed a Se deficient diet show a decrease in TrxR activity because of decreased specific activity of the enzyme, thus altering Trx's role in cell growth (Berggren et al., 1999). The reduction in TrxR activity is to 15% of normal in rats. Additionally, providing supranutritional levels of Se increases TrxR activity through an increase in the specific activity of the enzyme. The reduction of Se intake also decreases the mRNA expression of TrxR but to a much lesser extent than the reduction in activity (Hadley and Sunde, 2001).

Thioredoxin reductase also works to protect cells from RNS, much as GPx does. Initial exposure of cells to peroxynitrite causes a decrease in TrxR activity, which is restored within 24 hours of exposure, and eventually mRNA and protein levels increase in response to RNS exposure (Park et al., 2002). TrxR also plays a role in cell death regulation by protecting cells from apoptosis. In cell lines with a Se deficient TrxR-1, cell death is rapidly induced, providing a possible link to some of the diseases observed in Se deficiencies (Anestål and Arnér, 2003). In cancers where TrxR-1 is over-expressed, cells become dependent on the Trx system and flavonoids inhibit TrxR, providing a

cancer chemoprevention (Lu et al., 2006). The inhibition of TrxR results in cell death because of the build up of free radicals in the cell. The loss or disruption of TrxR also has an effect upon the conformation of p53, a tumor suppressor protein commonly mutated in cancers. In models with altered TrxR, cells were actually more resistant to disruption of p53 conformation and therefore inducing apoptosis, providing a model as to how Se protects against cancers (Cassidy et al., 2006).

Thioredoxin reductase-2, the mitochondrial form of TrxR uses cytochrome C as a substrate. In models over-expressing TrxR2, Complex III is more resistant to impairment, because it is better able to mop-up ROS production that would inhibit Complex III (Nalvarte et al., 2004).

Selenoprotein P

The three selenoproteins already discussed have well-defined functions and modes of action. Other selenoproteins have been defined and possible functions described, but their modes of action continue to be unknown. Selenoprotein P (Sel-P) is one of these, and it was the second selenoprotein described, after GPx. Its first description was as an unknown Sec-containing rat plasma protein with a possible role in the transport of Se in the rat, and it was name Sel-P for its location in the plasma (Motsenbocker and Tappel, 1982). It was found to be a 57 kDa protein with multiple Se ions in the protein that is synthesized in the liver hepatocytes and is responsible for the transfer of Se from the liver to extrahepatic tissues, binding 60% of Se in plasma (Yang et al., 1987; Motchnik and Tappel, 1989; Hill et al., 1996). In rats it was also found that Sel-P is dependent upon Se supply for its activity and expression, but only to a certain concentration level (Yang et al., 1989; Hill et al., 1996). However, Sel-P was much less

sensitive to extremely low levels of Se intake than was GPx. The TGA codons that code for Sec were found in the open reading frame prior to the TAA termination codon, and the protein is 366 amino acids long with nine Sec in the terminal 122 amino acids (Hill et al., 1991).

Because Sel-P contains a large number of Sec, the first of any of the described selenoproteins to have more than one in a single polypeptide chain, it was postulated that Sel-P had major antioxidant properties, including the protection of cells against peroxynitrite and other RNS (Arteel et al., 1998). Selenoprotein P is capable of reducing phospholipid hydroperoxides, and so it functions as a glutathione peroxidase-like protein in the extracellular fluids (Saito et al., 1999). Cytokines have an effect on Sel-P expression in a dose dependent manner (Mostert et al., 1999). Treatment of a liver cell line with Transforming Growth Factor-β, which is responsible for many cellular functions including the induction of extracellular matrix proteins during inflammation and inhibiting lymphocyte proliferation and function, causes the induction of transcription and synthesis factors that inhibit Sel-P. This potentially affects ROS and RNS protection during inflammation, causing extra damage to already sensitive tissues.

Selenoprotein P also affects male fertility, and it is expressed in the Leydig cell fraction of the testes (Koga et al., 1998). If Leydig cells degenerate, Sel-P mRNA is no longer expressed in the testes. Selenoprotein P is induced in Leydig cells following cAMP stimulation, which could be a possible mode of protection for the testes from O_2 toxicity from an increase in testosterone production, mediated by cAMP (Nishimura et al., 2001). Male mice with a Sel-P deletion still have mature sperm, however they have severe flagellar structural defects, including a truncated mitochondrial sheath and a

hairpin bend at the midpiece (Olson et al., 2005). This Sel-P deletion has a similar phenotype as wild-type animals that are Se deficient, indicating that Sel-P is necessary for both the development of fully functioning sperm and for the delivery of Se to developing germ cells.

Patients with hypercholesteremia that are treated with LDL-apheresis show low Se concentration levels in the plasma (Persson-Moschos et al., 1995). The decrease in plasma Se results in a decrease in Sel-P level, which is due more to the treatment technique than the hypercholesteremia itself. The decrease in serum Se and in Sel-P suggests that patients treated with LDL-apheresis may need Se supplementation to prevent atherosclerosis and cardiovascular disease, for which Se deficiency is a risk factor (Salonen et al., 1982; Salonen et al., 1991). Selenoprotein P has also been implicated in prostate cancers, in which it is down-regulated in a subset of these tumors, and also in mouse tumors and prostate carcinoma cell lines (Calvo et al., 2002). The loss of Sel-P is found in both androgen-dependent and androgen-independent cell lines and results in an increase in oxidative stress.

Selenoprotein P is also necessary for motor coordination and weight maintenance. In Sel-P knockout (Sel-$P^{-/-}$) mice fed a Se deficient diet, there is a loss of motor coordination and a decrease in weight and fertility (Hill et al., 2003). In these animals, liver Se concentration was unaffected except at extremely low Se intakes, whereas in the testes, Se concentration is extremely low, independent of level of Se in the diet, indicating the Sel-P is the main provider of Se to the testes, but not to the liver. The brain also showed a decrease in Se concentrations, but did respond to higher intakes of Se. Weanling Sel-$P^{-/-}$ mice with extremely low Se intakes show spasticity and eventually

require euthanasia, unless Se intakes are increased (Hill et al., 2004). The overall dysfunction of the animal was extremely hard to reverse. Selenium concentrations were decreased in the cortex, midbrain, brainstem, cerebellum and hippocampus in Sel-$P^{-/-}$ mice and in Se deficient mice, the hippocampus was the only region of the brain that remained unaffected, indicating that Sel-P is more important in the hippocampus for maintenance of function (Nakayama et al., 2007). Selenoprotein P knockouts show disrupted special learning with an alteration in synaptic transmission, short-term plasticity and long term potentiation in the hippocampus, indicating that Sel-P is a necessary element in normal synaptic function (Peters et al., 2006). In a study inactivating liver Trsp, the Sec-tRNA gene, Sel-P is removed from the plasma, resulting in a decrease in serum and kidney Se, but no change in brain Se levels (Schweizer et al., 2005). The brain appears to be independent of plasma Se levels, so long as brain Sel-P is maintained; there are no negative neurological effects.

Selenoprotein W

Selenoprotein W (Sel-W) is a protein of unknown function that is localized mainly to muscle cell and has been implicated in white muscle disease. Selenoprotein W was first discovered following the injection of [^{75}Se] selenite into rats, where it was isolated from skeletal muscle (Vendeland et al., 1993). The protein was of low molecular weight, only 9 kDa, and there were 0.92g Se per g mol of protein, as a Sec. There had been prior evidence of a protein like Sel-W; however this was its first isolation. Its purported function is as an antioxidant in muscle cells. Within two years of its official description as Sel-W, its cDNA had been isolated and sequenced, as it was found that Sec was encoded by TGA, and there was evidence of the presence of a SECIS element in the

3'-UTR, as in other selenoproteins (Vendeland et al., 1995). It was found that dietary intake of Se affects the expression of mRNA and that the UGA codon was responsible for both Sec insertion and as a termination sequence in the mRNA, as there were two UGA codons present. Selenoprotein W is associated with glutathione, in a manner similar to GPx (Beilstein et al., 1996). The release of GSH from Sel-W occurs via reduction with dithiothreitol (Gu et al., 1999).

Selenoprotein W exists mainly within the cytosol of the muscle cells, but there is some association with the membranes (Yeh et al., 1995). The mRNA been localized to the muscle, spleen, testis and brain of various species with the highest expression in the muscle and brain (Gu et al., 2000). However, distribution is different among species, as Sel-W is expressed in the heart of sheep, but is undetectable in the heart of rats (Yeh et al., 1997A; Yeh et al., 1997B). In sheep, protein expression responds to dietary supplementation of Se, and with increased Se supplementation there is an increase in expression, whereas in low intake diets, there is a significant decline in Sel-W protein expression (Yeh et al., 1997A). Its highest expression is in the skeletal muscle and heart and its lowest is in liver. Interestingly, brain expression is unaffected by Se dietary intake in the sheep. Rat tissues respond in a similar manner with an increase in mRNA and protein in muscle with each increase in dietary Se levels (Yeh et al., 1997B). Protein expression of Sel-W is detectable in the rat heart, but only in animals that are Se sufficient. Brain Sel-W appears to be protected from deficiency in most species, especially during embryonic brain development, indicating that it may be an important antioxidant in development (Yeh et al., 1997B; Jeong et al., 2004). Selenoprotein W is expressed during early embryonic development in the brain, and in the postnatal central

nervous system, it is found in the dentate gyrus, cortex and hippocampus (Jeong et al., 2004; Loflin et al., 2006).

Selenoprotein W appears to have some antioxidant capacity. In animal models over-expressing Sel-W, there is actually an increase in H_2O_2 cytotoxicity, if only because the cells resistance to oxidative stress appears to depend upon the presence of adequate glutathione (Jeong et al., 2002; Jeong et al., 2004). In models with mutations in Sel-W, there is no resistance to H_2O_2 induced oxidative stress. Conversely, in proliferating myoblasts, there is an immediate response to oxidative stress after H_2O_2 exposure (Loflin et al., 2006). The depletion of Sel-W in the colon, which is sensitive to loss of Sel-W, causes a noticeable decrease in antioxidant protection and an increase susceptibility to the development of inflammation (Pagmantidis et al., 2005). Sel-W targets the 14-3-3 protein, and Sel-W is involved in redox regulation of the 14-3-3 family of proteins involved with peroxiredoxin (Dikiy et al., 2007). Because of the presence of Sel-W in proliferating myoblasts and the developing nervous system, it is postulated that Sel-W plays an important role in muscle and nervous system growth and differentiation.

Selenoprotein N

Selenoprotein N (Sel-N) is a much more recently described protein about which very little is known. Selenoprotein N is, however, the first selenoprotein to be implicated as a causative factor in a human genetic disorder: Rigid Spine Muscular Dystrophy (RSMD). After discovering that Sel-N is involved in several other muscular dystrophic diseases, the diseases were collectively termed SelN-1 related myopathies (Moghadaszadeh et al., 2001; Ferreiro et al., 2002; Petit et al., 2003). Sel-N was first identified after an *in silico* scan of nucleotide sequence databases in search of SECIS

related elements (Lescure et al., 1999). Following *in silico* isolation, the protein was identified *in vivo*.

The implication that Sel-N was involved in RSMD was due to evidence of linkage disequilibrium associated with Sel-N (Moghadaszadeh et al., 2001). The gene for Sel-N is located on the RSMD1 locus on human chromosome 1p, and it encodes a 70 kDa protein with one Sec residue (Petit et al., 2003). The protein is a glycoprotein that is localized within the endoplasmic reticulum, and the N-terminus contains endoplasmic reticulum-addressing and retention signals. Selenoprotein N has been detected in high levels in fetal tissue, with lower expression levels in the adult tissue, and is found in liver, brain, heart, diaphragm, skeletal muscle and stomach. In cultured myoblasts, expression is higher than in differentiating myotubes, indicating a role in early development and cell proliferation, since it is preferentially expressed in proliferating cells and growing tissues. The Sel-N mRNA levels in the adult are higher than that of the protein, but are still lower overall than in the fetus. In a zebrafish (*Danio rerio*) model of Sel-N expression, Sel-N is found to be highly expressed in the somites and notochord during early development (Deniziak et al., 2007). The inhibition of gene expression by antisense morpholinos resulted in muscle architecture disorganization and extremely reduced motility of the animal, the fate of cells was unaffected. The muscle sarcomere organization and myofiber attachment were disorganized, and the myoseptum integrity was altered. This provides further evidence that Sel-N is crucial to muscle organization and development (Deniziak et al., 2007).

Mutations involved in Sel-N related myopathies involve frameshift, nonsense and missense mutations, with the most common frameshift mutation involving the alteration

of TGA to TAA, resulting in an abbreviated mRNA sequence (Moghadaszadeh et al., 2001; Tajsharghi et al., 2005). As such, expression of Sel-N is reduced and shows irregular expression in the cytosol of patients with RSMD1 (Okamoto et al., 2006). Some Sel-N related myopathies show mutations in the SECIS region of mRNA, which decreases mRNA and protein (Allamand et al., 2005). The phenotype of this mutation is mild, but early-onset and conditions are exacerbated with pregnancy. The related symptoms of Sel-N involved myopathies are milder forms of muscular dystrophy, with no basal membrane alteration and near normal levels of serum creatine kinase, both of which can be severely altered in more severe forms of muscular dystrophy (Moghadaszadeh et al., 2001). Additionally, Sel-N myopathies show progressive nocturnal hypoventilation, which is easily treatable. In patients with desmin-related myopathies, there are intrasarcoplasmic aggregates of desmin, a subset of which includes Mallory body-like inclusions, similar to those found in liver disease (Ferreiro et al., 2004). Mallory body desmin related myopathy was recently classified as a Sel-N related myopathy, because there is a mutation in the Sel-N gene. This myopathy is characterized by neonatal hypotonia, axial and proximal muscle weakness, scoliosis, hyaline plaque and slightly elevated creatine kinase levels.

Conclusion

Since the discovery of Se in 1817, the opinion towards this element has gone through several incarnations, from a toxic element to be avoided to an absolute necessity for life. Selenium and its selenoproteins have a wide range of effects throughout the body and organ systems. Selenium's necessity in the diet, as well as its inherent toxicity, makes it an intriguing conundrum in science and livestock production. Often the

symptoms of both toxicity and deficiency are similar, making diagnosis difficult. Furthermore, the involvement of Se and its incorporation into selenoproteins exerts ever farther ranging effects. Many of these proteins are essential to life, and loss leads to embryonic lethality or gross developmental disorders. Selenoprotein N is the first selenoprotein involved in a heritable disorder, further underlining the importance of Se to life. The involvement of described selenoproteins in redox metabolism and antioxidant protection further underscores the complexity of these proteins. The study of selenium truly is a complex and fruitful field.

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CHAPTER 2

LEVELS OF mRNA FOR THREE SELENOPROTEINS IN SKELETAL MUSCLE OF FETAL AND NEWBORN PIGS

Running title: Selenoproteins in fetal pig skeletal muscle

Levels of mRNA for three selenoproteins in skeletal muscle of fetal and newborn pigs

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ABSTRACT

To determine changes in the mRNA levels of three selenoproteins during development of the fetal pig**,** prepubertal gilts were randomly assigned to either a Se-sufficient or deficient diet (n = 12 / treatment). The gilts were bred and continued to be fed their respective diets. Levels of mRNA coding for the selenoproteins glutathione peroxidase-1 (**GPx-1**), selenoprotein W (**SelW**) and selenoprotein N (**SelN**) and the concentration of Se in fetal and neonatal swine skeletal muscle (*Gluteus maximus*) were measured at time intervals throughout gestation and immediately after birth. Total RNA was isolated from skeletal muscle, and cDNA was synthesized for real-time RT-PCR analysis of the selected selenoproteins. Levels of GPx-1 mRNA in fetal skeletal muscle were unaffected by maternal Se intake nor did GPx-1 mRNA levels change during late gestation. Selenoprotein W mRNA levels increased ($P = 0.01$) in fetal skeletal muscle during late gestation but were reduced ($P = 0.05$) by low maternal intake of Se. Levels of SelN mRNA showed a gestational time by treatment interaction ($P < 0.05$). Selenium concentration in skeletal muscle of fetal and newborn pigs decreased throughout gestation ($P < 0.0001$) and was lower ($P < 0.05$) when dams were fed the low Se diet. The increased levels of SelW mRNA in skeletal muscle during late gestation, despite a decrease in Se concentration, suggest that SelW is an important antioxidant protein to the late term fetus and the newborn.

Key words: glutathione peroxidase-1, mRNA, pigs, selenoprotein N, selenoprotein W, selenium

INTRODUCTION

The selenoprotein family consists of 25 proteins, of which only 3 have well defined functions: glutathione peroxidase (**GPx**), thioredoxin reductase and iodothyronine deiodinase (Rederstorff et al., 2006). All selenoproteins contain at least 1 selenocysteine amino acid residue at the active site that provides antioxidant capability to the entire selenoprotein family (Forstrum et al., 1978; Burk, 1991).

Glutathione peroxidase-1 was the first selenoprotein to be discovered (Flohe et al., 1973; Rotruck et al., 1973). In liver, GPx-1 is sensitive to Se status with reduced activity and mRNA levels in Se deficiency (Saedi et al., 1988; Lei et al., 1998). Complete loss of GPx-1 leads to susceptibility to acute oxidative stress (Cheng et al., 1997). Selenoprotein W (**SelW**) was first isolated from skeletal muscle where it is most highly expressed, and low SelW has been implicated in white muscle disease (Vendeland et al., 1993). Expression of SelW mRNA is dependent upon nutritional Se status (Vendeland et al., 1995). The antioxidant role of SelW is supported by models with mutated SelW in which there is a loss of resistance to hydrogen peroxide (**H_2O_2**) induced oxidative stress (Jeong et al., 2002). Selenoprotein N (**SelN**) is the first selenoprotein shown to act in a human genetic disease, Rigid Spine Muscular Dystrophy (Lescure et al., 1999; Moghadaszadeh et al., 2001). SelN is more highly expressed during fetal development than in the adult (Petit et al., 2003).

Selenoproteins have important roles as biological antioxidants and reduced levels of selenoproteins affect skeletal and cardiac muscle integrity, especially in newborns. Therefore, the aim of this study was to elucidate the mRNA levels of GPx-1, SelW, and

SelN in fetal skeletal muscle and to determine if maternal Se intake affected fetal mRNA levels for these proteins.

MATERIALS AND METHODS

Diets and Animals

All animal procedures were approved by the Washington State University Institutional Animal Care and Use Committee. Diets and animal samples were prepared and collected as previously described (Table 1; Hostetler et al., 2006). In brief, diets were prepared in 900-kg batches at the Animal Feed Preparation Laboratory at Washington State University, and were composed mainly of barley and peas grown on locally Se-deficient soils. The Se adequate diet (NRC, 1998) contained 0.39 ppm. The Se-deficient diet was created by exclusion of the Se premix supplement and contained 0.05 ppm Se.

Prepubertal gilts (n = 24) were randomly assigned to 1 of the 2 treatment diets beginning 6 weeks before breeding. Animals were fed 2.25 kg of feed per head per day and water was provided ad libitum. At the onset of the second estrous, gilts were bred every 12-h until the animal was no longer detected to be in estrous. Animals were bred using freshly collected semen containing at least 4 billion sperm per insemination.

At specific periods during gestation (d 45, 70, 90), animals were humanely slaughtered via captive-bolt stunning and exsanguination ($n = 3 \cdot diet^{-1} \cdot period^{-1}$) and fetal skeletal muscle was collected (*Gluteus maximus*) and pooled from 2 fetuses per animal. The remaining 6 animals (n = 3 per treatment) completed the gestational period (d 114) and gave birth to allow for collection of neonatal tissue. Newborn piglets were killed by direct cardiac injection with an overdose of sodium pentobarbital (30 mg/kg, 4%

solution) before suckling could occur. Samples of all tissue were collected at a local abattoir and were immediately frozen in liquid nitrogen and subsequently stored at -80^0 C until analysis.

RNA Extraction

Skeletal muscle samples were taken from the *gluteus maximus* of piglets and samples were pooled by dam. Total RNA was extracted from fetal and newborn muscle with TRIzol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Approximately 0.5 g frozen muscle was crushed in liquid nitrogen with an RNase-free mortar and pestle and homogenized in 1 mL TRIzol. After room temperature incubation, 0.2 μL chloroform was added to the samples, which were shaken and incubated again at room temperature. Samples were centrifuged at 12,000 x g for 15 min at 4^0 C and the aqueous phase was transferred to fresh 1.5 mL RNase free tubes, after which 0.25 mL of RNA precipitate and 0.25 mL isopropyl alcohol were added and the solution was thoroughly mixed. Samples were centrifuged at 12,000 x g for 10 min at 4^0 C and the supernatant was removed and discarded. The remaining RNA pellet was washed twice in 75% ethanol and then a total of 1 mL ethanol was added to the pellet, vortexed and spun at 7500 x g for 5 min at 4^0 C. The supernatant was discarded and the RNA pellet was allowed to air-dry for approximately 5 min. The RNA was dissolved in RNase-free water (~20 to 100 μL) and incubated at 55^0 C. RNA concentration was quantified using a spectrophotometer measuring optical density (OD) at 260 nm and purity was estimated using OD_{260}:OD_{280} ratios. Purity was verified by 1.5% agarose, 2.2M formaldehyde gel electrophoresis using a standard procedure.

Primer Design

Primers for SelW and SelN were designed for quantitative real-time (q) PCR using Primer3 software (http://frodo.wi.mit.edu/). GPx-1 primers were previously designed by Hostetler et al. (2006) for fetal swine liver. Primers for SelW and SelN were designed based on published swine nucleotide sequences (Costa et al., 2001; GenBank Accession No. AF380118, 705 bp and Zhao et al., 2006; GenBank Accession No. EF113595, 873 bp respectively). Primers for the reference gene elongation factor-1 alpha were also designed from the published swine nucleotide sequence (Xu and Xiong, 2006; GenBank Accession No. DQ673096, 1777 bp). Specifications in Primer3 for all primers were set so that primers were approximately 20 bp in length, with melt temperatures corresponding to 58^0 to 60^0 C, resulting in amplification products approximately 100 bp in length. The resulting SelW (forward, 5'-ACTCCAAGAAGGGAGGTGAT-3'; reverse, 5'- ACATTAGCCCTGAGCCAAA-3'), SelN (forward, 5'-CATCATCCTCTCCAAAGACG-3'; reverse, 5'-CGTATAGCCACTCCATGTCC-3') and EF-1 alpha (forward, 5'-CGTGTCTGTCAAAGACGTTC-3'; reverse, 5'-CAGGATAATCACCTGAGCTGT-3') primers were synthesized at Bioneer, Inc. (Alameda, CA).

DNase Treatment and cDNA synthesis

Total RNA (2 μg) was DNase treated with DNase I (Fermentas, Glen Burie, MD) following the manufacturer's instructions. DNase treated RNA was reverse transcribed to first strand cDNA using Superscript III and oligo(dT) primers (Invitrogen). The

method followed manufacturer's protocols. In brief, 5 µL DNase treated RNA was combined with 1 µL oligo (dT) primers, 1 µL 10mM dinucleotide triphosphate (dNTP) and 3 µL diethylpyrocarbonate (DEP-C) water, for a total volume of 10 µL. Samples were incubated at 65^0 C for 5 min, and placed on ice for at least 1 min. During incubation, the synthesis mix was prepared that contained 10x RT buffer (2 µL), $MgCl_2$ (4 µL), 0.1M dithiothreitol (2 µL), 40 units RNase Out (1 µL) and 200 units Superscript III (1 uL) for a total volume of 10 µL per sample. Following the 1 min on ice, 10 µL of the synthesis mix was added to the RNA/primer mixture and the reaction was briefly centrifuged, followed by 50 min of incubation at 50^0 C. Reactions were terminated at 85^0 C for 5 min and samples were chilled on ice. Following brief centrifugation, 1 µL RNase H was added to the reaction and incubated for 20 min at 37^0 C. Resulting cDNA diluted 1:5 with DEP-C treated water. A no-RT control was also performed and diluted 1:20 with DNase treated water.

Reverse Transcription PCR

Following cDNA synthesis, RT-PCR and TBE agarose gel electrophoresis was carried out to test primers, verify fidelity of cDNA and to verify amplicon size for RT-qPCR. Amplification of cDNA was carried out using Platinum Taq (Invitrogen) to a total volume of 10 µL, containing 2 µL cDNA. RT-PCR was carried out for a total of 50 cycles with the following protocol: initial denaturation at 95^0 C for 2 min, 50 cycles of 30 s at 95^0 C, 30 s at 58^0 C and 1 min at 72^0 C, followed by a 5 min cycle of a final elongation at 72^0 C. Reactions were held at 4^0 C until removed from the thermocycler.

Following amplification, gel electrophoresis verified cDNA fidelity and initial primer functionality.

Quantification of mRNA

After completing RT-PCR, RT-qPCR was carried out to quantify mRNA levels of GPx-1, Sel-W and Sel-N in fetal and neonatal skeletal muscle. As was previously described, RNA was isolated, DNase treated and cDNA synthesized and primers designed using Primer3 software. The PCR reaction was carried out on an iCycler iQ Real Time PCR Detection System (BioRad Laboratories, Hercules, CA) and specific cDNA expression measured using iQ SYBR green Supermix fluorescence system (BioRad Laboratories). Prior to cDNA quantification, primer concentrations were optimized and primer efficiency determined using a serial dilution of a pooled cDNA standard.

Duplicate PCR reactions for each sample were run in duplicate as an additional control. Reactions were run in a final volume of 25 μL containing 12.5 μL SYBR green, 2 μL diluted cDNA and optimal primer concentrations. In order to monitor that no non-specific amplification or DNA contamination had occurred, no-template and no-reverse transcriptase reactions were included alongside reactions containing cDNA. Reactions were cycled for a total of 50 cycles using the following protocol: initial denaturation for 2:30 min at 95^0 C followed by 50 cycles of 30 s at 95^0 C, 30 s at 58^0 C and 30 s at 72^0 C. Following the completion of 50 cycles, a melt curve analysis was performed. Reactions were held at 4^0 C following completion of the entire protocol.

Muscle Selenium Analysis

To determine the effect of maternal intake of Se on the concentration of Se in fetal and newborn skeletal muscle, samples of muscle were analyzed for Se by Inductively Coupled Plasma Mass Spectrometry (Ting et al., 1989; University of Idaho Analytical Sciences Laboratory, Moscow, ID).

Statistical Analysis

Data for GPx-1, Sel-W and Sel-N were normalized to the housekeeping gene EF1A and expression quantified using the Pfaffl method (Pfaffl, 2001). The relative change in GPx-1, Sel-W and Sel-N mRNA and Se from fetal skeletal muscle was analyzed using the PROC GLM procedure of SAS (version 9.1, SAS Inst., Cary, NC). The model included Se treatment, day of gestation and the interaction of treatment and day of gestation. Pair-wise comparisons of the least squares means were carried out using the *pdiff* command in SAS, which is a protected t-test.

RESULTS AND DISCUSSION

The marginal Se deficiency that was induced by feeding a low-Se diet 42 d prior to breeding and throughout gestation provided an opportunity to examine the effects of maternal Se intake and gestational age upon mRNA levels for several selenoproteins in fetal skeletal muscle. Previous studies have shown the effect of maternal intake upon selenoprotein expression in fetal and maternal liver, as well as Se in whole blood and upon lipid peroxides and H_2O_2 (Hostetler and Kincaid, 2004a,b; Hostetler et al., 2006). The Se concentration in maternal whole blood was decreased by the reduced intake of Se,

which also lowered concentrations of Se in maternal and fetal liver (Hostetler and Kincaid, 2004a). The decreased Se in maternal whole blood was enough to classify the gilts as Se deficient, but as only marginally Se deficient based upon concentration of Se in maternal liver. The marginal Se deficiency reduced liver GPx-1 activity without affecting levels of GPx-1 mRNA (Hostetler and Kincaid, 2004b; Hostetler et al., 2006). Similarly, in the current study GPx-1 mRNA levels in fetal skeletal muscle was not affected by maternal dietary Se (Figure 1). In fetal liver there was a 3-fold decrease in GPx-1 mRNA levels between d 45 and 114 of gestation (Hostetler et al., 2006). In the current study, there was no change in GPx-1 mRNA levels in fetal skeletal muscle during late gestation. Thus GPx-1 mRNA levels in fetal skeletal muscle were less responsive to gestational age than was GPx-1 mRNA levels in fetal liver. This maintenance of GPx-1 mRNA levels in skeletal muscle may help modulate oxidative stress, by maintaining the redox capability of the cell.

Selenoprotein W mRNA levels and protein expression are generally sensitive to Se status (Yeh et al., 1997; Gu et al., 2002; Pagmantidis et al., 2005). In the current study, the level of Sel-W mRNA was decreased ($p < 0.05$) in fetal skeletal muscle by the marginal Se deficiency in the dams (Figure 2). Of particular interest is that Sel-W mRNA increased 58% between gestational d 90 and 114 ($p = 0.01$; Figure 3). Thus, increased Sel-W mRNA levels during late fetal development, accompanied by maintained mRNA levels of GPx-1 may provide skeletal muscle with antioxidant protection against lipid peroxidation, muscle myopathies and calcification (Bostedt and Schramel, 1990).

Selenoprotein N is only recently recognized as a member of the selenoprotein family, and as such, its function is poorly understood. Sel-N may play a significant role in preventing some muscular dystrophies, a group of diseases referred to as SepN1 related myopathies, where SepN1 refers to the gene name of the Sel-N protein (Moghadaszadeh et al., 2001; Petit et al., 2003). Various genetic point and frameshift mutations of SepN have been implicated in all of the SepN related myopathies. Sel-N is integral in the development of muscular architecture, and in zebrafish where Sel-N has been severely reduced, muscular sarcomere organization, myofiber attachment and myoseptum integrity are disrupted (Deniziak et al., 2007). In the current study, maternal Se intake had no effect upon the fetal muscle Sel-N mRNA level, nor did gestational age affect Sel-N mRNA (Figures 4, 5). However, there was an interaction of maternal Se intake and gestational age d 90 of gestation (Figure 6). At d 90, the low Se group tended to express Sel-N mRNA at a higher relative level than the adequate Se group ($p=0.07$). For all of the SepN related myopathies the onset of disease occurs soon after birth. Sel-N may protect against muscle disorganization, because muscle maturation is still occurring at this point, in the number of myofibers developing, in terminal development of the myofibers and in size, as well as in expression of myosin isoforms (Swatland, 1973; Lefaucheur et al., 1995).

The concentration of Se in fetal skeletal muscle was reduced with gestational age and further reduced by low maternal intake of Se (Figures 7, 8). Similarly, Se concentrations in fetal liver decreased from early gestation to late gestation, and were reduced by low maternal Se intake (Hostetler and Kincaid, 2004a). In the current study, the concentration of Se in fetal skeletal muscle significantly dropped between d 45 and

70, and between d 90 and term. It is not surprising that the concentration of Se in fetal skeletal muscle decreased in a manner similar to fetal liver. The liver is the main storage site for Se in swine, and behind the kidney has the highest concentration of Se (Groce et al., 1973; Mahan and Moxon, 1978). The liver is also a main site for Se transfer to the body tissues during deficiency, as it has the largest reservoir of stored Se. As Se stores are decreased in the liver, the supply of Se to the muscle would be decreased.

IMPLICATIONS

Despite the decrease in Se concentration in the fetal skeletal muscle at the end of gestation, mRNA levels for Sel-W increased, further supporting the role of Sel-W as an important antioxidant in the early life of the piglet by protecting against muscular myopathies. Maternal Se intake did not affect fetal GPx-1 mRNA levels, which were maintained in skeletal muscle at a constant level throughout pregnancy. The maintenance of fetal GPx-1 mRNA levels during induced maternal Se deficiency suggests GPx-1 is important to muscle redox metabolism. Levels of mRNA for Sel-N were unaffected by both maternal Se intake and gestational age. Thus Sel-W and GPx-1 appear to be important in fetal and newborn skeletal muscle to protect against lipid peroxides, and thus protect against muscular myopathies.

Table 1. Composition of maternal gestation diet[a], as-fed basis

Ingredient, %	Low Se	Adequate Se
Barley	81.95	81.8
Cull peas[b]	12.0	12.0
Animal fat	2.0	2.0
Dicalcium phosphate	1.85	1.85
Limestone	0.9	0.9
Salt	0.5	0.5
Trace mineral[c]	0.1	0.1
Vitamin premix[d]	0.5	0.5
Mg sulfate	0.2	0.2
Se premix[e]	-	0.15

[a]The diet contained 13% CP, 0.55% lysine, 0.79% Ca, and 0.67% P, as fed (calculated values). The Se concentrations (by analysis) were 0.05 and 0.39 ppm on a DM basis.

[b]Cull peas contained 0.03 ppm Se by analysis, DM basis.

[c]Provided the following per kilogram of diet: 100 mg Fe as $FeS0_4$; 100 mg Mn as MnO; 100 mg Zn as ZnO; 10 mg Cu as $CuSO_4$; and 0.3 mg I as ethylenediamine dihydroiodide.

[d]Provided the following per kilogram of diet: 4400 IU vitamin A, 330 IU vitamin D_3, 22 IU vitamin E, 4 mg vitamin B_2, 13.2 mg niacin, 13.2 mg d-pantothenate, 495 mg choline, 0.02 mg vitamin B_{12}, 1.0 mg menadione sodium bisulfite, 1.1 mg folic acid, 0.1 mg d-biotin and 50 mg ethoxyquin.

[e]Provided 0.3 mg Se as sodium selenite per kg of diet.

Figure Legends

Figure 1- Effect of dietary Se on GPx-1 mRNA expression in fetal and neonatal skeletal muscle (n = 3 sows/diet per period). The housekeeping gene elongation factor-1 alpha was used to normalize the relative quantities of GPx-1 mRNA.

Figure 2- Effect of dietary Se upon total SelW mRNA expression in fetal and neonatal skeletal muscle (n = 12 sows per treatment). The housekeeping gene elongation factor-1 alpha was used to normalize the relative quantities of SelW mRNA. There was a significant effect of treatment on SelW mRNA level ($P < 0.05$).

Figure 3- Effect of maternal Se intake and gestational age on SelW mRNA expression in fetal and neonatal skeletal muscle (n = 3 sows/treatment per period). The housekeeping gene elongation factor-1 alpha was used to normalize the relative quantities of SelW mRNA. There was a significant effect of gestational age on SelW mRNA level ($P < 0.01$). ^{ab}Different superscripts differ $P = 0.01$.

Figure 4- Effect of dietary Se upon total SelN mRNA expression in fetal and neonatal skeletal muscle (n = 12 sows per treatment). The housekeeping gene elongation factor-1 alpha was used to normalize the relative quantities of SelN mRNA.

Figure 5- Effect of gestational age upon total SelN mRNA expression in fetal and neonatal skeletal muscle (n = 3 sows per period). The housekeeping gene elongation factor-1 alpha was used to normalize the relative quantities of SelN mRNA.

Figure 6- The interaction of gestational age and treatment upon SelN mRNA level (n = 3 sows/treatment per period). The housekeeping gene elongation factor-1 alpha was used to normalize the relative quantities of SelN mRNA. There was a significant effect of the interaction between gestational age and treatment. * Indicates $P < 0.05$ between treatments. † Indicates $P < 0.07$ between treatments, and indicates a trend.

Figure 7- Effect of gestational age on skeletal muscle Se concentration in fetal and neonatal skeletal muscle (n = 3 sows/treatment per period). There was a significant effect of gestational age on skeletal muscle Se concentration ($P < 0.0001$). Values are expressed on a wet weight basis. ^{abc}Different superscripts differ $P < 0.007$.

Figure 8- Effect of maternal Se intake on skeletal muscle Se concentration in fetal and neonatal skeletal muscle (n = 3 sows/treatment per period). There was a significant effect of maternal Se intake on skeletal muscle Se concentration ($P = 0.0164$). Values are expressed on a wet weight basis.

Figure 1

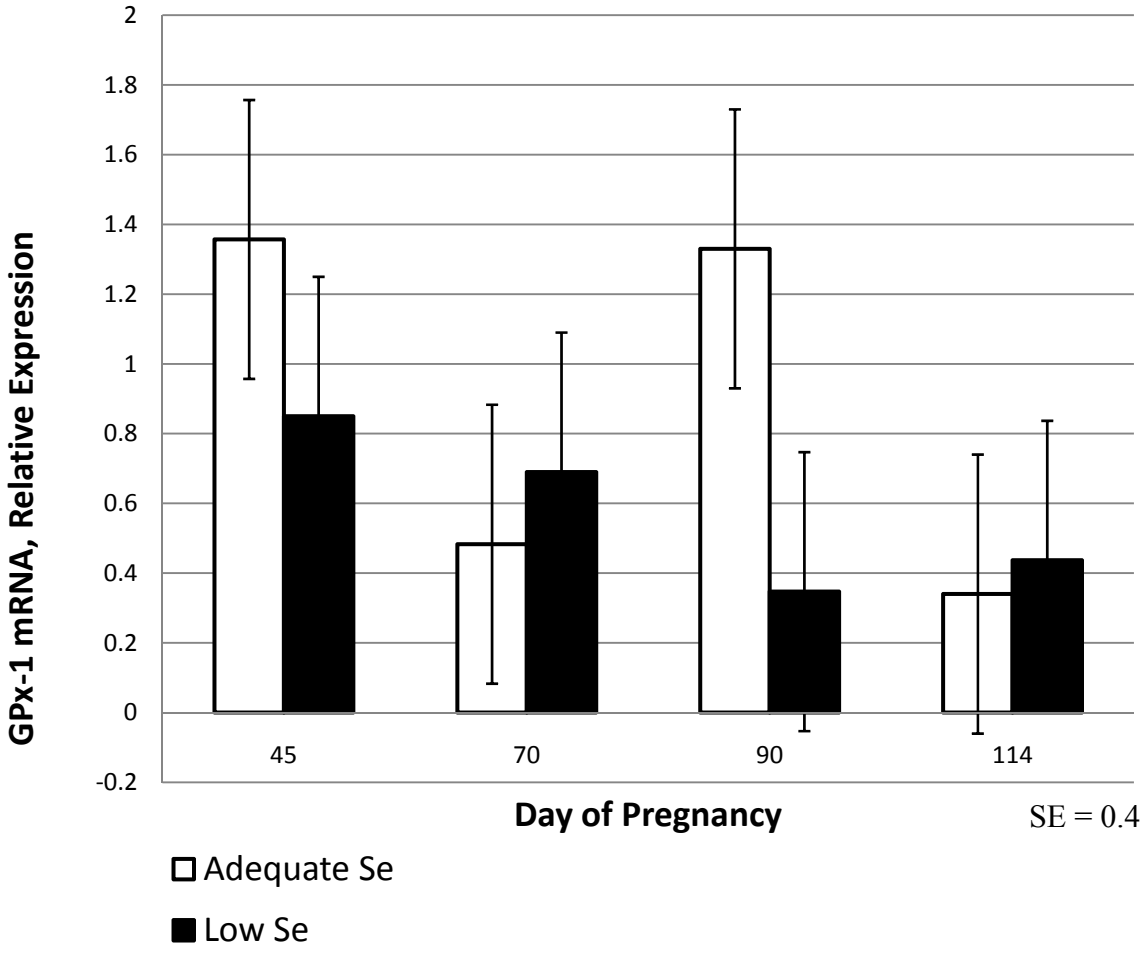
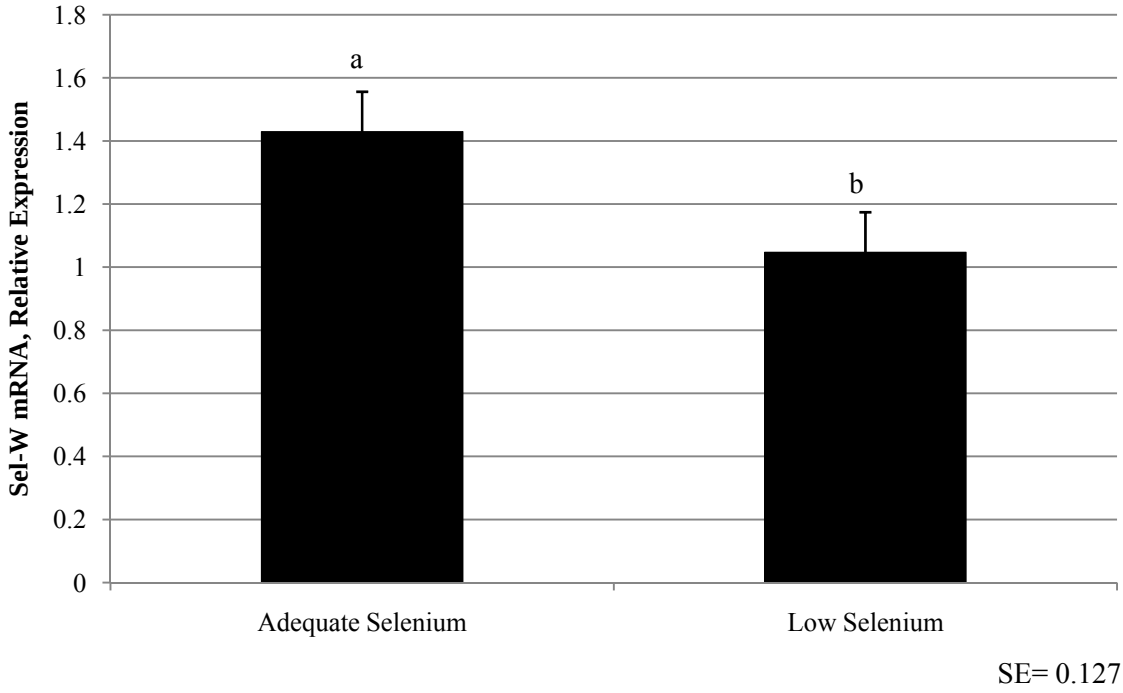

GPx-1 mRNA, Relative Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
45
70
90
114
Day of Pregnancy
SE = 0.4
Adequate Se
Low Se

Figure 2



SE= 0.127

Figure 3

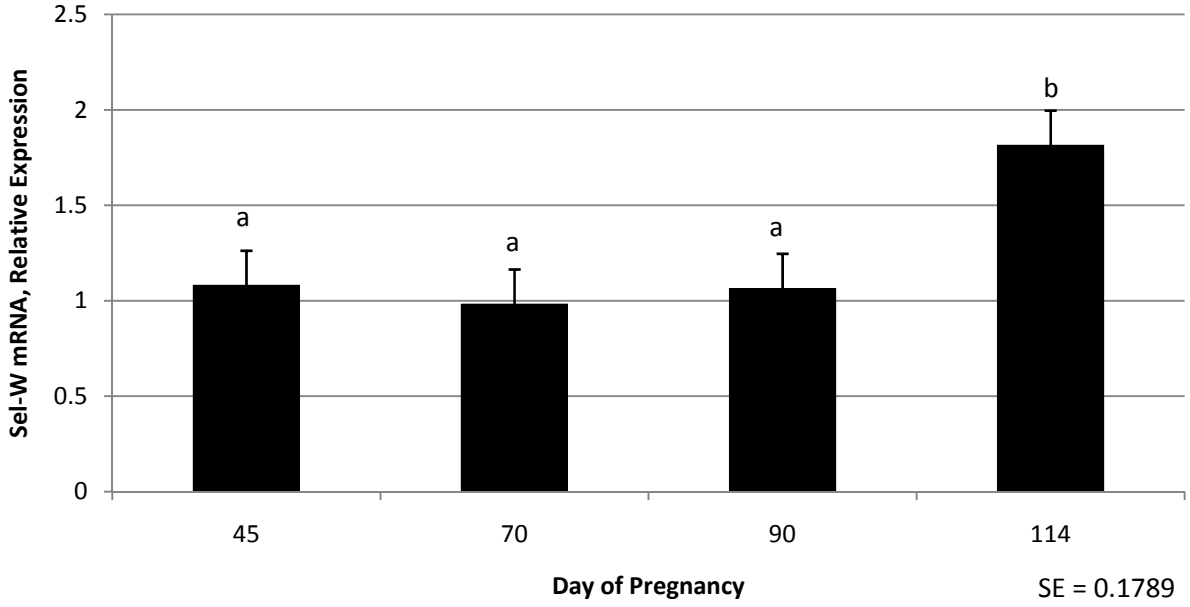
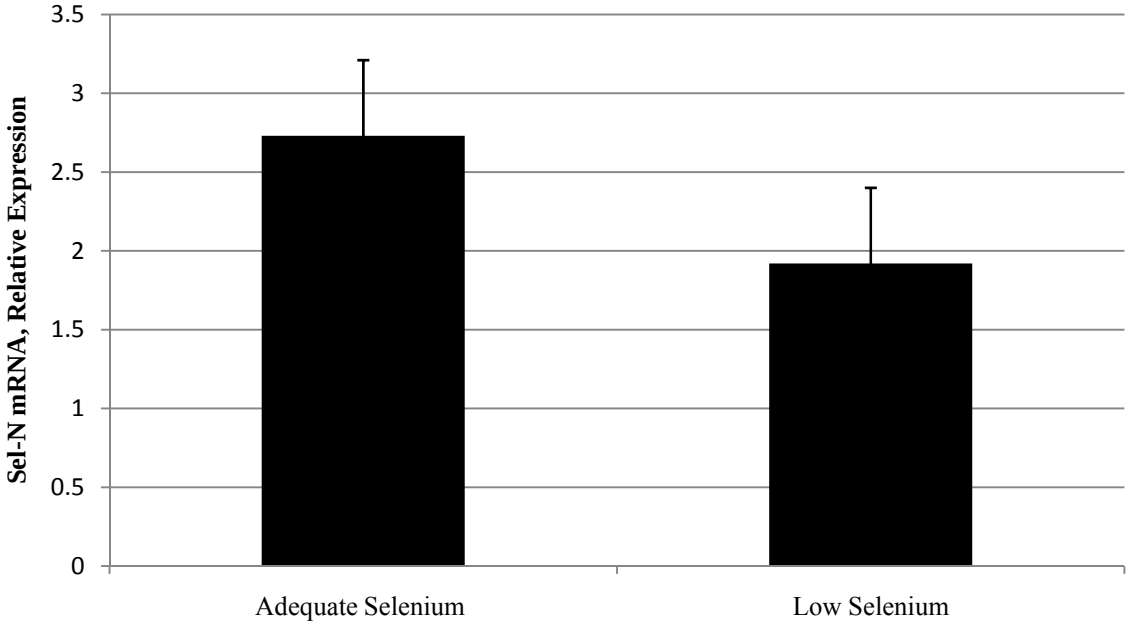

2.5
2
1.5
1
0.5
0
Sel-W mRNA, Relative Expression
a
a
a
b
45
70
90
114
Day of Pregnancy
SE = 0.1789

Figure 4



SE = 0.48

Figure 5

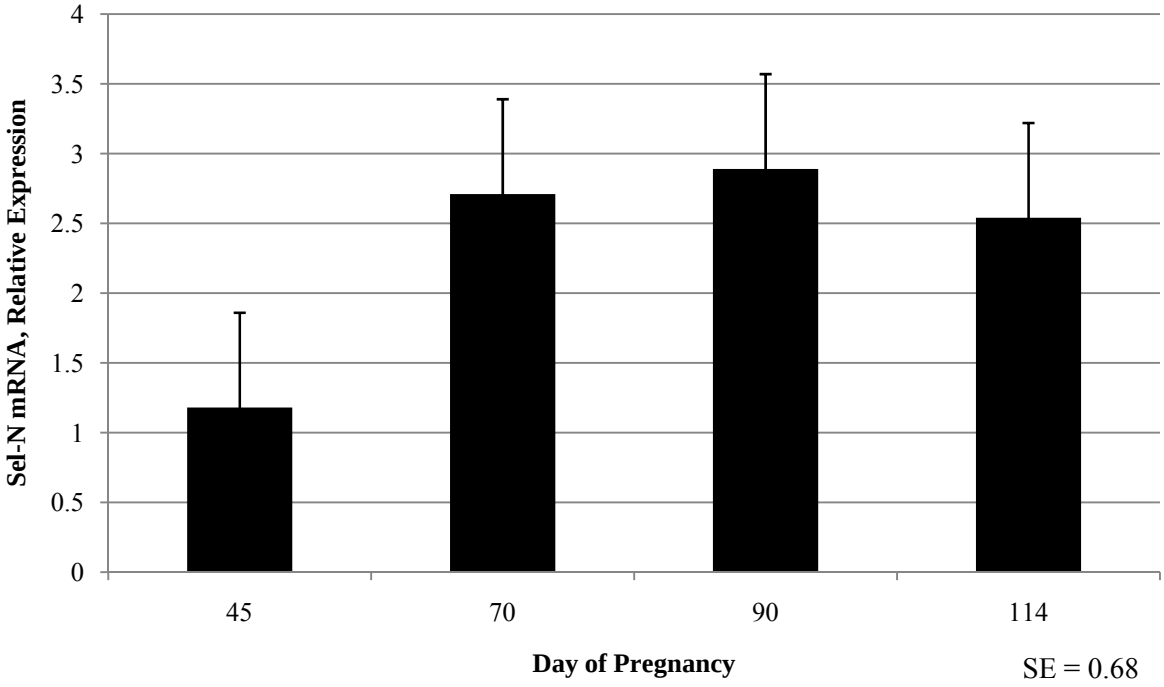

4
3.5
3
2.5
2
1.5
1
0.5
0
Sel-N mRNA, Relative Expression
45
70
90
114
Day of Pregnancy
SE = 0.68

Figure 6

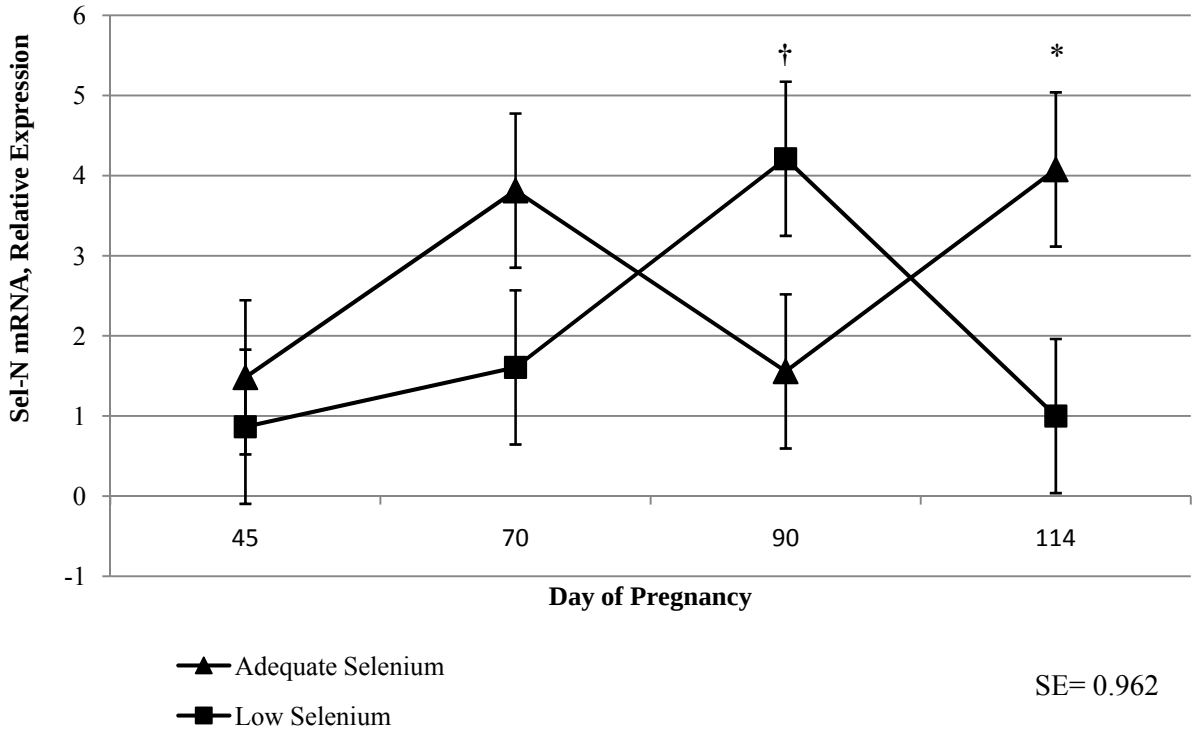

6
5
4
3
2
1
0
-1
Sel-N mRNA, Relative Expression
†
*
45
70
90
114
Day of Pregnancy
Adequate Selenium
Low Selenium
SE= 0.962

Figure 7

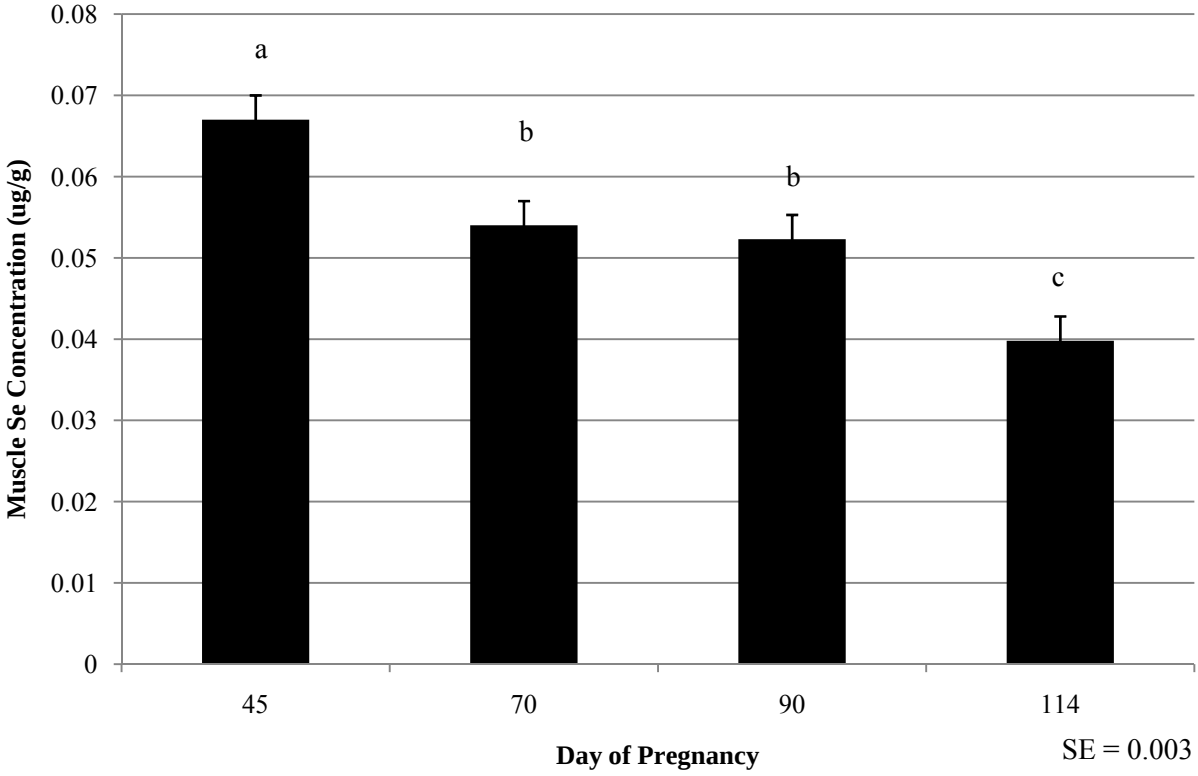
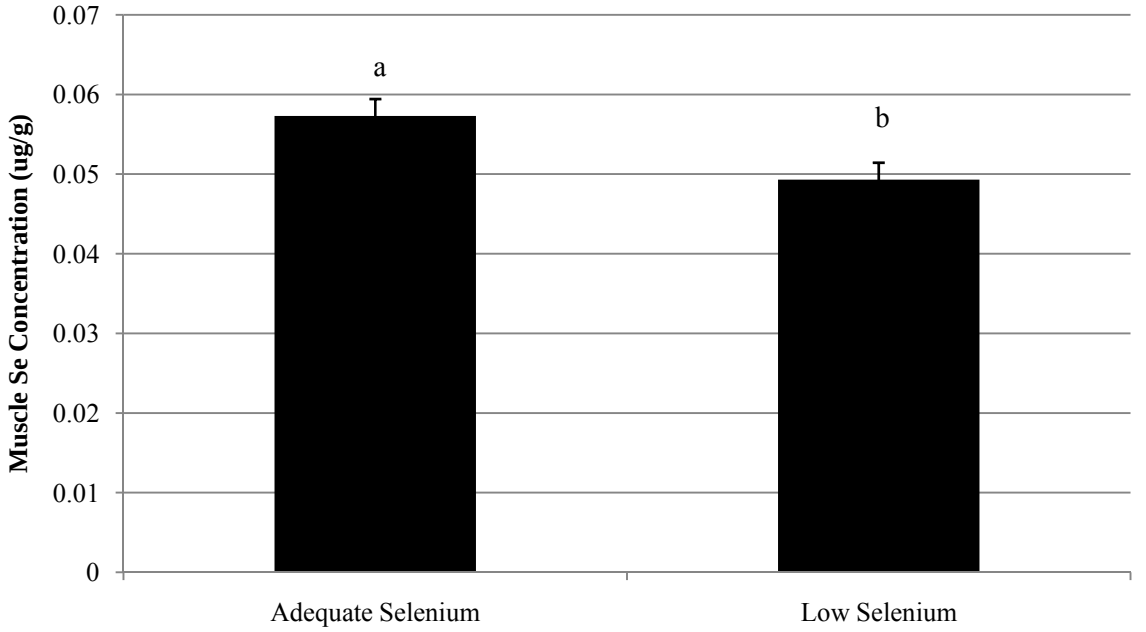

0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Muscle Se Concentration (ug/g)
a
b
b
c
45
70
90
114
Day of Pregnancy
SE = 0.003

Figure 8



SE = 0.0021

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CHAPTER 3

LEVELS OF mRNA FOR SELECTED SELENOPROTEINS IN BEEF COWS DURING WEIGHT LOSS

ABSTRACT

Oxidative stress is a metabolic state that can lead to metabolic disorders if uncompensated for by the body. Parturition and lactation induce oxidative stress by mobilizing body fat for milk synthesis. The expressional response of the selenoprotein antioxidants glutathione peroxidase-1 (GPx-1), thioredoxin reductase-1 (TrxR-1) and selenoprotein W (Sel-W) were measured in skeletal muscle (*Biceps femoris*) of 19 Angus or Angus-cross cows during weight loss and maintenance. RNA was extracted from muscle samples and cDNA synthesized for real-time quantitative PCR analysis. Expression of GPx-1 mRNA was not increased as a result of oxidative stress, however, expression of mRNA for TrxR-1 and Sel-W were significantly increased ($p < 0.05$). The increased levels of mRNA for of TrxR-1 and Sel-W suggest that these two antioxidant selenoproteins are up-regulated to help protect skeletal muscle against oxidative stress and muscle degeneration during weight loss.

INTRODUCTION

The selenoprotein family of proteins has a wide range of functions, including redox metabolism and maintenance of cellular antioxidant status. Only a few selenoproteins have well defined cellular functions and methods of action, but from these, it can be theorized how the other undefined selenoproteins function (Gromer et al., 2005). The antioxidant functionality of the selenoproteins is provided by selenocysteine (Sec) in the active site, where all selenoproteins contain at least one Sec (Burk, 1991). The well-defined selenoproteins include the glutathione peroxidase (GPx), thioredoxin reductase (TrxR), and iodothyronine deiodinase (ID) families. Of these, GPx and TrxR function mainly as antioxidants, whereas the ID family functions first as a means to reduce thyroxine to triiodothyronine and thus affect thyroid hormone activity (Sprott and MacLagan, 1955; Gromer et al. 2005). Selenoprotein W (Sel-W) appears important in preventing White Muscle Disease and cellular management of oxidative stress in early postnatal swine (Terry et al., in preparation).

Thioredoxin reductase-1 is responsible for the reduction of the disulfide bridge in thioredoxin, via nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) (Moore et al., 1964; Holmgren, 1977). As in all selenoproteins, the active site for TrxR-1 is a Sec, encoded by TGA, and inserted with the aid of the selenocysteine insertion sequence (SECIS), conserved across all selenoproteins (Gladyshev et al., 1996; Zhong et al., 1998; Sandalova, 2001). Thioredoxin reductase-1 expression increases in response to the increased presence of reactive oxygen species, which contain an unpaired electron and are unable to diffuse across cell membranes (Sun et al., 1999; Bergamini et al., 2004). In addition to responding to changes in reactive oxygen species (ROS) production, TrxR-1

is also responsible for cell signaling, cell death and is required for cell growth (Park et al., 2002).

As with TrxR-1, GPx-1 also responds to changes in redox status in the cell and is mainly responsible for the reduction of lipid peroxides, some proteins and NADPH via the use of glutathione (GSH) (Gromer et al., 2005; Cheng et al., 2003). In animal models containing a GPx-1 knockout, the animal becomes susceptible to acute oxidative stress, which could be mediated by sufficient provision of Se in the diet (Cheng et al., 1997). Conversely, in animals that overexpress GPx-1, there is an additional layer of protection against oxidative stress (Cheng et al., 1998). Selenoprotein W, which also associates with GSH, does not confer extra protection in animal models overexpressing Sel-W, most likely due to a limited amount of GSH present in the cell (Jeong et al., 2002; Jeong et al., 2004). In animal models in which Sel-W is mutated, there is no resistance to H_2O_2 induced stress (Loflin et al., 2006).

Selenoproteins protect against oxidative stress in the cell, which can be brought about by weight loss due to increased oxygen requirements to support increased metabolic levels (Keaney et al., 2003). In dairy cows, there is an increase in oxidative markers in late gestation and early lactation, corresponding to high milk production and fat mobilization (Grummer, 1993). Additionally, cows with a higher body condition score prior to calving have a greater loss of body condition score post-calving, causing higher levels of reactive oxygen species (ROS) in blood (Bernabucci et al., 2005). The activities of GPx increase and TrxR-1 decrease in the first 30 days in milk, indicating that GPx provides a more robust antioxidant response against increased ROS levels (Bernabucci et al., 2005; Sordillo et al., 2007).

Oxidative stress has negative effects on cell survival and metabolic integrity. The effect of periparturient weight loss and subsequent oxidative stress in beef cattle has received little attention. Thus, the aim of this study to determine the expression changes of three selenoproteins, GPx-1, TrxR-1 and Sel-W, in skeletal muscle of cows during weight maintenance and weight loss.

MATERIALS AND METHODS

Animal Treatments and Sample Collection

All animal procedures were approved by the Washington State University Institutional Animal Care and Use Committee. Animal treatments, sample collection and RNA isolation were prepared and collected as previously described in Brennan et al. (in process). In brief, to understand the effect of weight loss on antioxidant status, 26 Angus or Angus-cross cattle underwent two 60 day periods of observation: weight maintenance or minor weight gain immediately following weaning and weight loss throughout lactation. Animals were fed a maintenance diet of bluegrass straw and alfalfa, approximately 2% body weight per day (NRC, 2000). Tissue samples from the *Biceps femoris* muscle were aseptically collected at the end of each 60 d period and immediately frozen in liquid nitrogen.

Total RNA was extracted from approximately 0.3 g of frozen muscle tissue ground in liquid nitrogen using the TRIzol method (Invitrogen, Carlsbad, CA) as described in the manufacturer's instructions. Crushed tissue as homogenized in 1 ml TRIzol, allowed to incubate at room temperature and 0.2 μL chloroform was added to the samples. Following the addition of chloroform, samples were shaken and again allowed

to incubate at room temperature, after which they were centrifuged at 12,000 x g for 15 min at 4^0 C. The aqueous phase was transferred to fresh 1.5 mL RNase free tubes and 0.25 mL RNA precipitate and isopropyl alcohol were added and the total solution was thoroughly mixed. Following mixing, samples were centrifuged at 12,000 x g for 10 min at 4^0 C and the resulting supernatant was discarded. The collected RNA pellet was washed twice with 75% ethanol and a total of 1 mL 75% ethanol was added to the pellet and vortexed. Samples were stored overnight at -20^0 C. Following overnight incubation, samples were spun at 7500 x g for 5 min at 4^0 C and the supernatant discarded. The remaining RNA pellet was allowed to air dry for approximately 5 min and then dissolved in RNase-free water and incubated at 55^0 C. Following dissolution, RNA concentration was measured by optical density (OD) at 260 nm and the purity of RNA was estimated using OD_{260}:OD_{280} ratios. To verify the purity of the RNA, 1.5% agarose, 2.2M formaldehyde gel electrophoresis was performed using a standard procedure. A subsample of 2 μg total RNA (n=18 animals / time period) was used.

DNase treatment and synthesis of cDNA

The subsample of total RNA (2 μg) was DNase treated with Turbo DNase (Ambion, Inc, Austin, TX). In brief, RNA was mixed on ice with 1 μl each of Turbo DNase and a 10x Turbo DNase buffer provided, and nuclease free water was added to make up the solution to 10 μl. RNA mixtures were incubated 30 min at 37^0 C. Following incubation, 2 μl DNase inactivation reagent was added and the samples were incubated at room temperature for 2 min, then vortexed two to three times during incubation, after which, samples were centrifuged for 1.5 min at 10,000 x g. Resulting supernatant was

transferred to a new nuclease free tube. Following DNase treatment, first strand cDNA was synthesized using Superscript III and oligo(dT) primers (Invitrogen). DNase treated RNA (5 μL) was combined with 1 μL each of oligo(dT) primers, 10 mM dinucleotide triphoasphate (dNTP) and 3 μL diethylpyrocarbonate (DEP-C) water, to a total volume of 10 μL. Newly mixed samples were incubated at 65^0 C for 5 min, and placed on ice for a minimum of 1 min. A synthesis mix was prepared, containing 10x RT buffer (2 μL), $MgCl_2$ (4 μL), 0.1M dithiothreitol (2 μL), 40 units RNase Out (1 μL) and 200 units Superscipt III (1 μL) for a total volume of 10 μL per sample. After the ice bath incubation was completed, 10 μL of the synthesis mix was added to the RNA/primer mixture and briefly centrifuged. Samples were then placed in a 50^0 C water bath for 50 min, after which the reaction was terminated at 85^0 C for 5 min. Samples were subsequently chilled on ice and briefly centrifuged. Following centrifugation, 1 μl RNase H was added to the samples and incubated at 37^0 C for 20 minutes. After incubation was complete, the cDNA was diluted 1:5 in DEP-C treated water. A no-RT control was also performed and diluted 1:20 in DEP-C treated water.

Primer Design

Primers for Sel-W were previously designed for swine tissue and primers for GPx-1 were previously designed using published bovine sequences (Hostetler et al., 2006; Terry et al., in preparation). Swine Sel-W has 89% homogeneity with bovine Sel-W (swine GenBank Accession No. AF380118, 705 bp; bovine GenBank Accession No. BC103044, 750 bp). Primers for the reference gene β-actin were also previously designed (Brennan et al., in process). Primers for TrxR-1 were designed for quantitative

real-time (Q) PCR using Primer 3 software (http://frodo.wu.mit.edu) based on published bovine nucleotide sequences (Sakurai et al., 2004; GenBank Accession No. NM174625, 3535 bp). Primer3 specifications were set such that primers were each approximately 20 bp in length and had melt temperatures between 58^0 C and 60^0 C, with resulting amplification products 100 bp in length. TrxR-1 primers (forward, 5'-AGGAGAAAGCTGTGGAGAAA-3'; reverse, 5'- TTATCCCTTGATGGAATCGT-3') were synthesized at Bioneer, Inc. (Alameda, CA).

Reverse Transcription PCR

Following the synthesis of cDNA, the initial effectiveness of primers on cDNA was performed using RT-PCR and TBE agarose gel electrophoresis. In addition to testing the efficacy of primers, RT-PCR verified the fidelity of cDNA and amplicon size for eventual RT-qPCR. PCR amplification of cDNA was carried out using the Platinum Taq system (Invitrogen) in a total volume of 10 µL, containing a total of 2 µL cDNA. RT-PCR was carried out over 50 cycles, with the following protocol: initial denaturation at 95^0 C for 2 min., 50 cycles of 30 s at 95^0 C, 30s at 58^0 C an 1 min at 72^0 C. Following the 50 cycles, samples underwent 5 min at 72^0 C for final elongation. After completion of RT-PCR, reactions were held at 4^0 C until TBE agarose gel electrophoresis could be carried out.

Real-time RT-PCR

To quantify mRNA levels of Sel-W, GPx-1 and TrxR-1 from tissue samples, RT-qPCR was used. RNA had previously been isolated, DNase treated and from this cDNA

was synthesized. Primers for RT-qPCR were designed using Primer3 software. Specific cDNA expression was measured using the iQ SYBR Green Supermix fluorescence system (BioRad Laboratories, Hercules, CA) and the iCycler iQ Real Time PCR Detection System (BioRad Laboratories). Prior to quantification of cDNA samples, primers were optimized and their relative efficiency was determined using a 1:5 dilution of pooled cDNA samples from maintenance subjects.

Each PCR reaction was run in duplicate and each reaction was run in a total volume of 25 μL, of which 12.5 μL was SYBR Green, 2 μL was cDNA and optimal primer concentrations (200 nM for GPx-1, β-actin and Trx-R1; 300 nM for Sel-W). To control for non-specific amplification and DNA contamination, no-template and no-reverse transcriptase reactions were included for each primer or sample respectively. RT-qPCR reactions were cycled using the following cycling protocol: initial denaturation for 2:30 min at 95^0 C, 50 cycles of 30s at 95^0 C, 30s at 58^0 C and 30s at 72^0 C. After 50 amplification cycles have been completed, a melt curve analysis was performed and samples were held at 4^0 C.

Statistical Analysis

Quantification data for GPx-1, TrxR-1 and Sel-W were normalized to the housekeeping gene β-actin, and maintenance samples were used as a standard control, where each animal was its own block, using the Pfaffl method (Pfaffl, 2001). Data were analyzed using the PROC GLM procedure of SAS (version 9.1, SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Weight loss in beef cows results in increases in oxidative stress, determined using nonesterified fatty acid (NEFA) concentrations in red blood cells (Brennan et al., in progress). As a result of increases in ROS, antioxidant levels increase to a certain extent in order to maintain antioxidant protection against cellular degeneration and death (Frei et al., 1988; Trevisan et al., 2001; Castillo et al., 2004; Bernabucci et al., 2005).

In beef cows undergoing weight loss, TrxR-1 and Sel-W mRNA levels significantly increased in skeletal muscle (Figures 1 and 2) relative to a period of weight maintenance when animals are not undergoing oxidative stress. The period of weight maintenance occurred after weaning. The increase in expression of TrxR-1 mRNA in skeletal muscle beef cows differed from the response in peripheral blood mononuclear cells in dairy cows during a similar period in their lifecycle (Sordillo et al., 2007). In the peripartum in dairy cows studied by Sordillo et al. (2007), TrxR activity decreased, and although the mRNA response was not measured, it is unlikely that mRNA levels of TrxR increased as enzyme activity of TrxR decreased. Unfortunately, TrxR-1 expression and activity in skeletal muscle have not been measured in peripartum dairy cows. In lactating rats, TrxR activity is increased in the liver and kidneys as compared to pregnant and virgin rats, indicating that lactation affects TrxR's antioxidant status (Taylor et al., 2005).

During oxidative stress, the TrxR-1 system is necessary for activation of other non-Se dependent antioxidants such as heme oxygenase-1 in aortic endothelial cells (Trigona et al., 2006). The dependence of some antioxidants upon TrxR-1 activity supports the importance of TrxR-1 for maintenance of redox metabolism throughout the entirety of oxidative stress. In instances of Se deficiency, mRNA expression of TrxR-1

and overall activity of TrxR-1 are reduced (Berggren et al., 1999; Hadley and Sunde, 2001). Presumably, in cases of Se deficiency during pregnancy, not only would the health of the fetus be affected, but the maternal ability to cope with the onset of oxidative stress with lactation would also be compromised.

The significant increase in Sel-W suggests that this particular selenoprotein antioxidant is also important in the management of oxidative stress in skeletal muscle of cattle during weight loss. In proliferating myoblasts *in vitro*, Sel-W responds to induced oxidative stress, and is also highly expressed during development even under non-stress conditions (Loflin et al., 2006). Selenoprotein W appears to be important in both development and maintaining redox status throughout life. In ruminants where Sel-W has been compromised, the animal is susceptible to white muscle disease, a degenerative muscle disease resulting in muscle calcification (Vendeland et al., 1993). Increases in Sel-W mRNA expression during weight loss would help maintain antioxidant status while providing extra protection against muscle degeneration.

Unlike Sel-W and TrxR-1, GPx-1 expression is not significantly affected by changes in oxidant status during weight loss (Figure 3). Glutathione peroxidase-1 activity in early lactation in dairy cows is increased in erythrocytes (Bernabucci et al., 2002; Bernabucci et al., 2005; Sordillo et al., 2007). This could potentially be the case in beef cows, although GPx-1 activity was not measured in the current study. When GPx-1 mRNA expression has been measured in leukocytes in dairy cows, the expression was unchanged from prepartum through 22 days in milk (Colitti and Stefanon, 2006). This further supports that even though expression is not affected by oxidative changes, activity might still be increased. In mice, overexpression of GPx-1 results in insulin resistance

and weight gain, so in oxidative stress, only minor increases of GPx-1 would protect against the onset of these problems (McClung et al., 2004). Glutathione peroxidase-1 not only functions as an antioxidant but also possibly acts as an additional site for Se storage for times of Se deficiency, or as a way to mop up extra Se (Burk and Hill, 1993; Lei et al., 2007). Maintaining expression levels of GPx-1 while altering activity levels could be the method of action in order to help maintain Se storage capability but also allows for compensation against ROS.

IMPLICATIONS

Oxidative stress during weight loss can lead to metabolic malfunction and disease, further affecting long term health. In humans, there is a connection between obesity and subsequent oxidative stress and atherosclerosis (Morrow, 2003). The antioxidant system has evolved to cope with the production of ROS. Thioredoxin reductase-1 and Sel-W both increase expression in response to increased oxidative stress, demonstrating that TrxR-1 and Sel-W are vital to healthy cellular function. Selenoprotein W increases at the end of gestation in fetal skeletal muscle of swine, showing that it is an important antioxidant throughout life (Terry et al., 2008). Thioredoxin reductase-1 appears to be important in protecting skeletal muscle against oxidative stress. The lack of change in GPx-1 mRNA expression during weight loss suggests that this protein is not up-regulated in skeletal muscle in response to oxidative changes. Thus, the up-regulation of the antioxidants TrxR-1 and Sel-W aid in protecting muscle cells from oxidative damage during lactation in beef cattle.

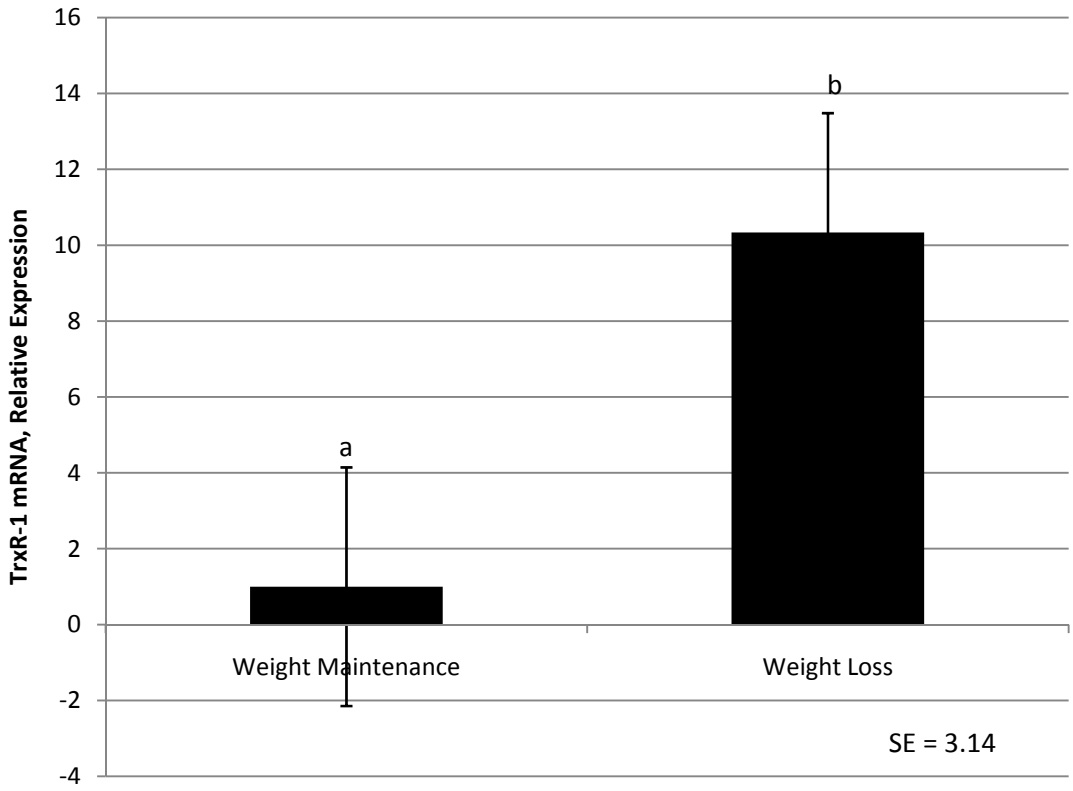

Figure 1- Effect of body condition on total expression of TrxR-1 mRNA in bovine skeletal muscle (n=18 cows/period). The housekeeping gene β-actin was used to normalize the relative quantities of TrxR-1 mRNA. There was a significant effect of body condition period on TrxR-1 expression (p= 0.043). [ab]Different superscripts differ p=0.043.

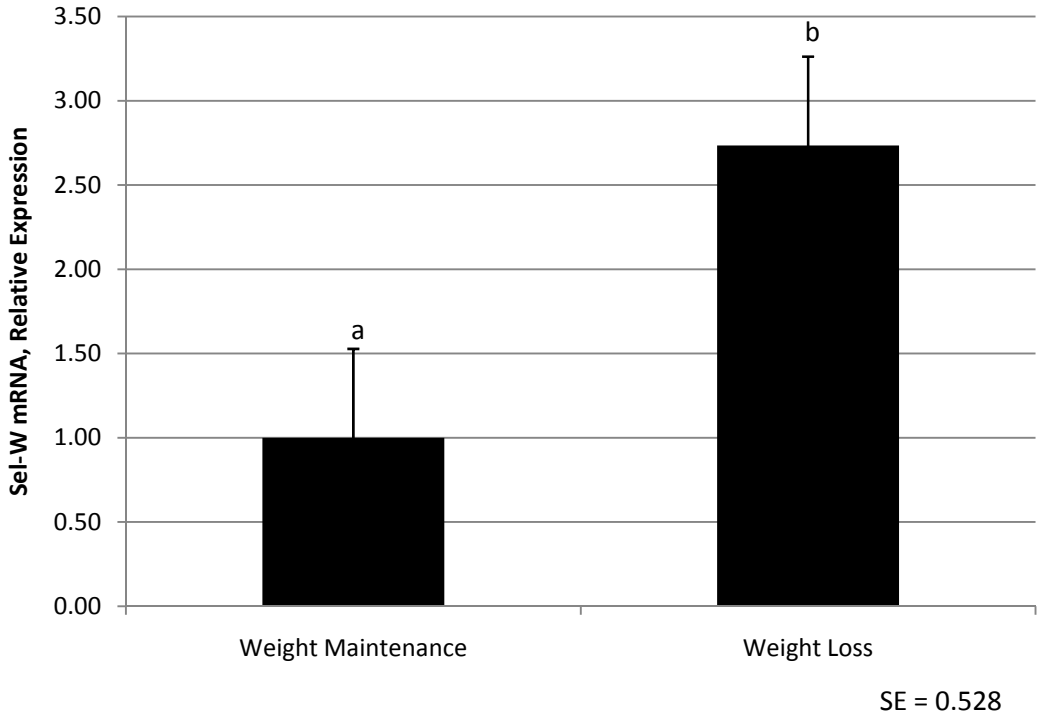

Figure 2- Effect of body condition on total expression of Sel-W mRNA in bovine skeletal muscle (n=18 cows/period). The housekeeping gene β-actin was used to normalize the relative quantities of Sel-W mRNA. There was a significant effect of body condition period on Sel-W expression (p= 0.026). [ab]Different superscripts differ p=0.026.

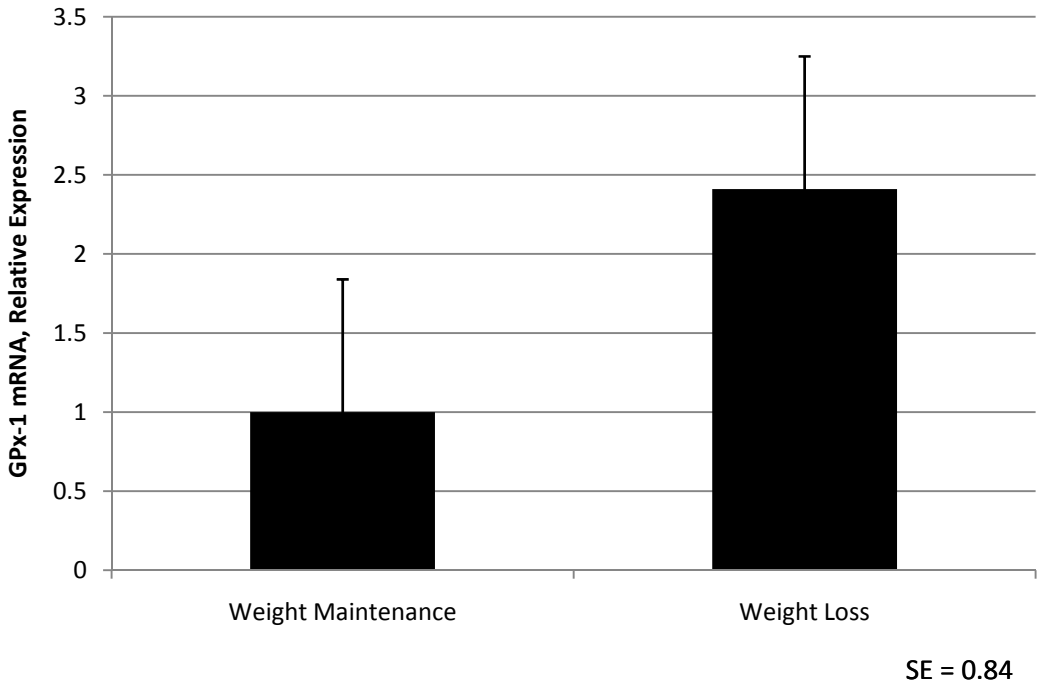

Figure 3- Effect of body condition on total expression of GPx-1 mRNA in bovine skeletal muscle (n=18 cows/period). The housekeeping gene β-actin was used to normalize the relative quantities of GPx-1 mRNA.

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CHAPTER 4

THESIS CONCLUSION

THESIS CONCLUSION

Selenoproteins, synthesized via the cotranslational incorporation of selenocysteine (Sec), play important roles from prenatal development and throughout life. In fetal pigs, oxidative stress is induced by a marginal maternal Se deficiency, which results in varied responses of select selenoproteins in fetal liver and skeletal muscle (Hostetler and Kincaid, 2004a,b; Hostetler et al., 2006; Terry et al., 2008). Similar varied responses to oxidative stress by selenoproteins also occur in adult cow skeletal muscle.

In fetal pigs, gluathione peroxidase-1 (GPx-1) mRNA levels declined in late gestation in liver but not in skeletal muscle. Similarly, activity of GPx-1 was decreased in pig fetal liver in late gestation (Hostetler et al., 2006). In adult cow skeletal muscle, the onset of oxidative stress from weight loss did not significantly affect GPx-1 expression. Collectively, these studies show GPx-1 expression in fetal pigs and adult cattle is not influenced by increased reactive oxygen species (ROS) production (Bernabucci et al., 2002; Hostetler and Kincaid, 2004b; Castillo et al., 2005; Sordillo et al., 2007). Maintenance of GPx-1 expression is needed to prevent cell damage due to increased ROS production. Glutathione peroxidase-1 is a member of the selenoprotein family, with four Sec residues, as GPx-1 is a tetramer (Flohe et al., 1973; Cheng et al., 1997). The presence of 4 Sec residues puts forth the as yet unproven theory that GPx-1 may also function as a storage site for Se, for use in both a deficiency and in times of excessive intake (Burk and Hill, 1993; Lei et al., 2007). Maintenance of GPx-1 mRNA levels in muscle during a marginal Se deficiency and in oxidative stress, would maintain selenoprotein antioxidant activity and thus prevent any potential muscle degeneration (Koller and Exon, 1986).

Further protection to skeletal muscle in the fetal pig and adult cow is provided by selenoprotein W (Sel-W). Expression of Sel-W in fetal tissue was decreased by maternal Se deficiency, but regardless of treatment, expression levels increased in late gestation, indicating the Sel-W has an important role in protecting against oxidative stress in the neonate, a time when animals are particularly susceptible to nutritional muscular dystrophies (Bates et al., 2000). However, Sel-W expression was also upregulated during oxidative stress in bovine skeletal muscle during weight loss, indicating that Sel-W plays an important role in antioxidant protection during adulthood as well. The pattern of Sel-W expression in both fetal and adult tissue provides evidence that this protein is important throughout life, first to protect against early onset of nutritional muscular dystrophies, and second to protect against oxidative stress in the adult.

In the bovine tissue, upregulation of Sel-W was paired with increased thioredoxin reductase-1 (TrxR-1) expression. Activity of TrxR-1 is reported to decrease during lactational onset of oxidative stress as a result of weight loss in dairy cows (Sordillo et al., 2007). Activity of TrxR-1 was not measured in the current bovine study, but because of the increased expression levels, it is probable that activity was either maintained or increased to compensate for the change in oxidant status. Conversely, in swine fetal liver, TrxR-1 expression was affected by maternal intake of Se but unaffected by gestational age (Hostetler et al., 2006). Likewise, TrxR-1 expression in fetal pig liver was not altered to compensate for a change in oxidant status, as was observed in the bovine muscle tissue. This suggests that the antioxidant role of TrxR-1 is not as uniform as Sel-W or GPx-1, but that TrxR-1 has a defined role in protecting beef cows from oxidative stress as a result of lactational weight loss.

The expression of selenoprotein N (Sel-N), a recently described selenoprotein, was measured in fetal swine tissue. Selenoprotein N has a distinctive expression pattern whereby it is most highly expressed in fetal and neonatal tissue (Petit et al., 2003). Although expressed in adult tissue, Sel-N expression is at a much lower level than in fetal tissue. The implication is that Sel-N is more important during development and muscle maturation than it is in the maintenance of muscle integrity during adulthood. As such, Sel-N was not examined in the adult bovine tissue. However, in the fetal swine tissue, Sel-N showed an interesting pattern of expression, wherein it was more highly expressed in tissue from Se deficient dams at 90 days of pregnancy than it was in dams with sufficient Se intake. For the rest of gestation, both before and after day 90, this is not the case; the expression of Sel-N was higher in fetal muscle from Se sufficient dams than it is in fetal muscle from Se deficient dams. It is unclear as to why this expression difference at d 90 of gestation occurred, a possible explanation is that because muscle maturation occurs during this period, an increase in Sel-N prevents damage to muscle cells (Swatland, 1973; Lefaucheur et al., 1995). Damage to maturing muscle cells could be a method of action for the onset of SepN1 related myopathies in the neonate in which Sel-N is mutated (Moghadaszadeh et al., 2001; Petit et al., 2003). By increasing expression of Sel-N in already susceptible cells, there is further protection against damage that may occur as a result of insufficient maternal Se intake.

The Se status of the animal affects responses in selenoprotein expression due to oxidative stress. In the current study, Se status of the beef cows was adequate. Activity of neither GPx activity nor superoxide dismutase was affected by the oxidative stress. In the swine trial, gilts were fed a Se deficient diet for 6 weeks prior to breeding and

throughout gestation. The concentration of Se in fetal pig tissue was decreased as a result of low maternal intake of Se. Additionally, Se concentration in fetal pig tissues decreased throughout gestation, regardless of maternal Se intake. The reduction of Se in fetal skeletal muscle reduced expression of Sel-W mRNA, however, mRNA expression of GPx-1 and Sel-N were unaffected. Thus, Sel-W mRNA expression was more sensitive than GPx-1 and Sel-N mRNA to the lower Se in muscle. However, fetal expression of Sel-W increased during late gestation, most likely as additional protection was needed against neonatal oxidative stress. These varied reactions of selenoproteins to changes in Se status suggest that these selenoproteins act independently to fulfill their role within the cell to protect against oxidative stress and potential cellular damage.

The role of Se in health and oxidative stress is a fruitful field in which much research still needs to be done. There are many selenoproteins that are defined but their specific cellular roles are largely unknown. Included in this list are Sel-W and Sel-N, both of which have been implicated in serious muscular dystrophic like diseases (Bostedt and Schramel, 1990; Moghadaszadeh et al., 2001; Petit et al., 2003). Once the mechanisms of action of these proteins are understood, better methods of prevention and treatment may be developed for these diseases. Additionally, for the proteins that have been well defined, such as GPx-1 and TrxR-1, there is not a definitive consistency among animal species, and even sub-species, in how these proteins respond to Se deficiencies and oxidative stress. Age and physiological state also appear to affect expression of these selenoprotein. Thus, there is still much to be understood about the role of selenoproteins in nutrition and health.

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