

The Effect of α -Aminoadipate δ -Semialdehyde Synthase Knockdown on the Lysine Requirement and Urate Oxidase Knockdown on Oxidative Stress in a Murine Hepatic Cell Line

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ABSTRACT

The Effect of α -Aminoadipate δ -Semialdehyde Synthase Knockdown on the Lysine Requirement and Urate Oxidase Knockdown on Oxidative Stress in a Murine Hepatic Cell Line

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The technique of RNA interference was applied in two different systems. In system I, RNA interference was used to knockdown expression of α -aminoadipate δ -semialdehyde synthase (AASS). AASS is the bifunctional enzyme containing the lysine α -ketoglutarate reductase (LKR) and saccharopine dehydrogenase activities responsible for the first two steps in the irreversible catabolism of lysine. A rare disease in humans, familial hyperlysinemia, can be caused by very low LKR activity and, as expected, reduces the lysine “requirement” of the individual. This concept was recreated in a murine hepatic cell line (ATCC, FL83B) utilizing RNA interference to achieve AASS mRNA knockdown. Cells were antibiotic selected for stable transfection of two plasmids that express different short hairpin RNA sequences for AASS knockdown. Compared to the wild type cell line, AASS mRNA abundance was reduced $79.0 \pm 6.4\%$ ($p < 0.05$), resulting in a $29.8 \pm 5.2\%$ ($p < 0.05$) reduction in AASS protein abundance, $46.6 \pm 7.7\%$ ($p < 0.05$) less LKR activity, and a reduction in lysine oxidation by $51.7 \pm 11.8\%$. To determine the effect of AASS knockdown on lysine requirement, cells were grown in media containing 12.5, 25.0, 50.0, 100, or 200 μM lysine. Using a segmented model approach for growth rate analysis, the lysine requirement of the cell line with AASS silencing was $43.4 \pm 1.7 \mu\text{M}$, approximately 26% lower ($p < 0.05$) than the lysine requirement of the wild type cell line. These results indicate AASS knockdown decreases the lysine requirement of the cell via a reduction of lysine catabolism through the saccharopine pathway, providing the initial proof in principle that RNA interference can be used to reduce the nutrient requirement of a system. In system II, RNA interference was used to knockdown urate oxidase (UOX) expression. Humans, birds, and higher primates do not express the uric acid degrading enzyme UOX and, as a result, have plasma uric acid concentrations higher than UOX expressing animals. Although high uric acid concentrations are suggested to increase the antioxidant defense system and provide a health advantage to animals without UOX, knockout mice lacking UOX develop pathological complications including gout and kidney failure. As an alternative to the knockout model, RNA interference was used to decrease UOX expression using stable transfection in a mouse hepatic cell line (ATCC, FL83B). Urate oxidase mRNA was reduced 66% compared to wild type, as measured by real time RT-PCR. To determine if UOX knockdown resulted in enhanced protection against oxidative stress, cells were challenged with hexavalent chromium (Cr(VI)) or 3-morpholinosydnonimine hydrochloride (SIN-1). Compared to wild type, cells with UOX knockdown exhibited a $37.2 \pm 3.5\%$ reduction ($p < 0.05$) in the electron spin resonance signal after being exposed to Cr(VI) and displayed less DNA fragmentation ($P < 0.05$) following SIN-1 treatment. Cell viability decreased in wild type cells ($P < 0.05$), but not cells with UOX knockdown, after treatment with SIN-1. These results are consistent with an increased intracellular uric acid concentration and an increased defense against oxidative stress.

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LITERATURE REVIEW

Prologue

The following literature review is divided into three sections. Part I introduces a laboratory technique that I have applied to two very different physiological systems which will be discussed in part II and part III of the literature review. The two systems represent two of my primary interests; amino acid nutrition, specifically lysine metabolism, and oxidative stress, and although the status of one can impact the other in a biological system, here they are discussed separately.

PART I

RNA Interference

An approach traditionally used to study protein function has been the loss-of-function model in which the protein is “knocked out” of the genome and the resulting phenotype is observed. The disadvantages of this model are its high cost of production, limitation of species in which it can be easily applied, and possibility for lethality during early stages of life. A potentially preferable technique that can replace knock-out models is termed RNA interference (**RNAi**), in which the expression of a protein of choice is “knocked down” to, potentially, 5% of wild type expression. The advantages of this method over the knock-out method are its relatively low cost, success in nearly all species tested, versatility in inducing various levels of knockdown, and ability to cause either transient or stable protein knock-down.

RNA interference was initially recognized in *Caenorhabditic elegans* as a response to exogenous dsRNA that resulted in sequence-specific silencing (Fire et al, 1998). Since this initial discovery, RNAi has been successfully applied in insects (Pal-Bahdra et al, 2002), plants (Chuang and Meyerowitz, 2000), chickens (Sato et al., 2004), pigs (Zhang et al., 2005), and human cell culture (Gondi et al., 2006), and has even been achieved in transgenic mice and rats (Hasuwa et al, 2002). The role of RNAi in a biological system is to protect against transposon or viral infection (reviewed in Hannon, 2002) or, in the case of microRNAs, regulation of gene expression (reviewed in Shivdasani, 2007). Therefore RNAi could be a treatment option for virally induced diseases in humans. This type of treatment is still in its experimental stages but has already met with some success (Coburn and Cullen, 2002 and Jiang and Milner, 2002).

The biochemistry of the RNA interference reaction mechanism is still being investigated, however, the basic scheme is recognized. In the cytoplasm, long dsRNA molecules are processed into 21 to 25-nucleotide short interfering RNAs (**siRNA**). The processing occurs by an RNaseIII-like enzyme called Dicer that is highly conserved across many species (Susi et al, 2004). *In vitro*, these fragments are injected into the cytoplasm or processed into siRNA from a short hairpin RNA (**shRNA**) in mammalian cells to avoid an interferon response from the cell. The siRNA is incorporated into a RNA interfering silencing complex (**RISC**) with several proteins that target the mRNA complementary to the siRNA sequence. The RNase activity of the RISC targets the mRNA for degradation, preventing both message translation (reviewed in Wilson and Richardson, 2003) and message inclusion into processing bodies (P-bodies) (reviewed in Anderson, 2005).

RNA interference has been tested as a treatment means for metabolic diseases, including diabetes, obesity, and high cholesterol (reviewed in Rondinone, 2006). RNAi may also be used for treatment of diseases in lysine metabolism, to better understand the regulation of lysine oxidation, or to improve the efficiency of lysine use for protein synthesis. Utilizing RNAi for these applications would provide a greater understanding of lysine nutriture and possibly provide a means for treatment of diseases of lysine metabolism.

One example where utilizing the knock-out approach would not be practical is for research with urate oxidase. Knocking out urate oxidase in mice is fatal, causing death within weeks of birth from negative effects of hyperuricemia (Wu et al., 1994). As a replacement for the knock-out model, RNAi may be utilized to avoid the extreme hyperuricemia caused by eliminating urate oxidase expression, thus providing insight into the advantages afforded by marginally higher concentrations of uric acid during “basal” conditions or conditions associated with increased oxidative stress. It may also offer information regarding the relationship between hyperuricemia and hypertension or cardiovascular disease.

PART II

History of Protein Nutrition

Research in the requirement of food consumption and health was arguably first undertaken in the 1700's by William Stark whose purpose was to use himself as a test subject to distinguish between foods that were “hurtful” and those that were “innocent and pleasant”. The first nutritional experiments using animals is credited to Francois

Magendie in the early 1800's who fed dogs, rabbits, and guinea pigs various combinations of foods to emphasize the value of nitrogenous foods. This was the first of numerous experiments during that century that established the critical role of protein in the diet and that the value of the protein changed with variations in amino acid content and the physical state of the protein (reviewed in Himwich, 1951) .

In 1911, the initial body of work investigating the capability of various amino acids and proteins to support growth and maintenance was published (Osborne and Mendel). The first evidence that lysine was an essential amino acid for growth came in 1914 (Osborne and Mendel) when rats fed a low-lysine diet failed to thrive until their diet was supplemented with lysine. It was not apparent until 1945 that lysine was an essential amino acid for maintenance (Neuberger and Webster) when rats fed a lysine-devoid diet continually lost weight while rats fed the identical diet with added lysine maintained their weight. Further exploration established the distinction between essential and nonessential amino acids, and determining the physiological and biochemical functions of amino acids became, and still remains, an area of active research.

Lysine Metabolism

In animals, lysine is an essential amino acid. Lysine is found primarily as a component of body protein, and is approximately 6% of total body protein by weight. It acts both as a structural and a functional component of protein. One functional role of protein-bound lysine is its capacity to undergo acetylation in histones (Megee et al., 1990), which relaxes the DNA-histone interaction, allowing for DNA replication, gene expression, and nuclear division. An additional example of the functional role of lysine

residues is their ability to be cross-linked in structural proteins like collagen (Kahn et al., 1994), contributing to the distinct physical properties of this protein.

Intracellular and circulating free lysine also play functional roles in whole-body homeostasis. Lysine is strictly ketogenic since it is converted to acetyl-CoA, which is subsequently oxidized in the TCA cycle (Noda and Ichihara, 1976). An additional role of free lysine is to serve as a precursor for functional metabolites generated from pathways of lysine oxidation. Glutamate (Papes et al., 2001) and carnitine (Hoppel et al., 1980) are produced in such pathways and play crucial roles in central nervous system function and lipid metabolism, respectively.

Grossly speaking, research examining role of lysine as a functional nutrient could be grouped into one of three categories: 1) the effects of dietary lysine on growth and metabolism, 2) the pathways of lysine degradation and their regulation, and 3) the effects of inborn errors of lysine catabolism. A discussion of each of these major categories follows.

Effects of Dietary Lysine on Growth and Metabolism

The limiting amino acid is defined as the essential amino acid in shortest dietary supply relative to its requirement. Lysine is frequently the first limiting amino acid for growth in human (Young and Pellett, 1990) and swine (Mavromichalis et al., 1998) cereal grain based diets. Lysine is the second limiting amino acid in such diets in poultry (Fernandez et al. 1994) and fish (Cheng et al., 2003). Therefore, protein deficiencies that result in these cereal grain-consuming populations are often the result of an insufficient intake of dietary lysine, rather than a shortage of amino acids as a whole. When a dietary

lysine deficiency occurs growth, feed efficiency and the overall health of an animal are compromised (Smiriga et al., 2004, Smiriga et al., 2002, Fernandez et al., 1994, Si et al., 2001). Therefore, meeting the lysine requirement is imperative in a livestock production system to avoid the monetary losses associated with suboptimal animal performance and the cost of feeding amino acids as an energy source.

The lysine (and protein) requirement of an individual is inversely proportional to age and directly proportional to growth rate. For example, the lysine requirement of young pigs that weigh between 3-5 kg is 1.5%, a value that decreases with age and weight to a requirement of 0.6% dietary lysine for finishing pigs between 80-120 kg (NRC, 1998). However, environmental factors, health status, gender, level of production, and genotype can all affect the nutritional status and change the lysine requirement of an animal.

Different methods have been used to determine the lysine requirement of an animal. Traditional methods involve feeding groups of animals diets that vary only in lysine content and measure growth rate (Robbins et al., 1979), nitrogen balance (Dourmad and Etienne, 2002), plasma urea (Coma et al., 1995), and plasma amino acid concentrations (Stockland et al., 1970) as indices of nutritional status. Determining the dietary lysine concentration at which the growth rate and nitrogen retention are maximal or the plasma urea and lysine concentrations, which are suggestive of amino acid oxidation, are minimal is indicative of the lysine requirement.

A newer method used to determine amino acid requirements that is less labor intensive but more costly than conventional methods is the indicator amino acid oxidation technique (Bertolo et al., 2005). This technique utilizes the concept that essential amino

acids being consumed in excess of the requirement are oxidized when the diet is deficient in an essential amino acid. Oxidation of the excess amino acids decreases and plateaus as the dietary inclusion of the essential amino acid of interest approaches the requirement. Oxidation is measured using either a stable or radioactive indispensable amino acid tracer, usually phenylalanine. Lysine requirements determined using the indicator amino acid oxidation technique are comparable to those determined by conventional methods (reviewed in Pencharz and Ball, 2003).

Accurately determining the amino acid requirement of an animal is crucial for optimal growth and performance. This is particularly a concern in livestock operations where achieving the most efficient growth is vital for financial success. If a lysine deficiency occurs, negative impacts on health transpire and economic losses occur.

Pigs that consume a lysine deficient diet gain less weight than pigs consuming a lysine-adequate diet. Pigs consuming lysine-deficient diets also have lower fractional and absolute protein synthesis rates in muscle, a lower capacity for protein synthesis, and synthesize less protein per gram of RNA than do pigs consuming lysine adequate diets (Rivera-Ferre et al., 2005). Additional studies have examined the effects of dietary lysine deficiencies on growth-related hormones, which may modulate the observed effects of reduced growth and lower rates of protein synthesis. Lactating sows (Mejia-Guadarrama et al., 2002) and growing pigs (Katsumata et al., 2002) consuming lysine-deficient diets exhibited lower levels of plasma insulin-like growth factor-1 (**IGF-1**) and IGF-binding protein-3 but not growth hormone, indicating that the effects of a dietary lysine deficiency on metabolism may be mediated by changes in the somatotropic axis.

As in swine, the effects of a lysine deficiency in chickens are a reduction in weight gain and reduced tissue weights (Tesseraud et al., 1996). However, the observed effects on protein turnover observed in pigs contradict those seen in chickens, indicating that responses to dietary amino acid deficiencies may be species-specific. Chickens consuming an inadequate amount of lysine have an increase in muscle turnover evidenced by increases in both fractional rates of protein synthesis and breakdown and an increased capacity for protein synthesis (Tesseraud et al., 1996 and Tesseraud et al., 2001). However, due to differences in tissue size, absolute protein synthesis rates remained lower in muscles of lysine deficient birds. The somatotrophic axis was not as responsive in chickens as in swine, as IGF-I decreased during severe, but not moderate, lysine deficiencies while IGF-II did not change with any level of dietary lysine (Carew et al., 2005).

Increasing the dietary lysine concentration beyond the requirement does not positively impact growth in chickens (Sklan and Noy, 2004) or swine (Salter et al., 1990). Nitrogen and amino acid retention increases until dietary lysine reaches adequate levels, at which retention values plateau. In pigs, whole-body protein synthesis increased to a greater extent than did protein degradation with adequate and excess levels of dietary lysine, leading to an increase in protein accretion rate. There is typically a metabolic cost associated with catabolism of excess amino acids, which may negatively impact feed efficiency and growth if dietary amino acids are in severe excess. A severe excess of dietary lysine has the ability to inhibit growth partially via antagonism, a type of competitive inhibition, in dogs (Czarnecki et al., 1985) and chickens (Austic and Scott, 1975) but has not been observed in swine (Edmonds and Baker, 1987). To understand

the metabolic consequence of alterations in dietary lysine, an appreciation of the pathways of lysine catabolism and their regulation is required.

Pathways of Lysine Degradation and Their Regulation

α -Aminoadipate δ -Semialdehyde Synthase

In 1965, it was determined that in rat liver mitochondria, lysine is degraded to saccharopine in the presence of α -ketoglutarate by the enzyme lysine α -ketoglutarate reductase (**LKR**, E.C. 1.5.1.8.; figure 1) (Higashino et al., 1965). Also, it was hypothesized that α -aminoadipic δ -semialdehyde is the product of saccharopine degradation in the same metabolic pathway (Higashino et al., 1965). Lysine α -ketoglutarate reductase was purified in 1968 (Hutzler and Dancis, 1968) and saccharopine dehydrogenase (**SDH**, E.C. 1.5.1.9; figure 1), the enzyme that degrades saccharopine, was purified in 1970 (Hutzler and Dancis, 1970), both from human liver preparations. Conflicting reports indicated that LKR and SDH may or may not exist as one bifunctional protein (Fjellstedt et al., 1975 and Dancis et al., 1976) until 1984 when LKR and SDH activities were purified to apparent homogeneity from bovine liver. Results from this experiment indicated that both enzyme activities exist on a single, bifunctional protein which was named aminoadipic semialdehyde synthase (**AASS**, figure 1; Markovitz et al., 1984).

Aminoadipic semialdehyde synthase is expressed in numerous tissues in chickens (Manangi et al., 2005), humans (Sacksteder et al., 2000), mice (Papes et al., 1999), and pigs (Pink et al., 2004). In mammals and birds, the liver has the highest level of LKR activity on a per gram basis (Miller et al., 1962, Hutzler and Dancis, 1968, Manangi et

al., 2005). However, during late embryonic development in the rat, the activity of LKR is higher in brain than liver, and this relationship slowly reverses itself through at least 30 days of age (Rao et al., 1992). Since glutamate is a product of lysine degradation via AASS, the requirement for glutamate in the developing brain may be the cause for upregulation of LKR at this site during embryonic development.

In addition to being under developmental control, LKR activity is under dietary and hormonal regulation at both a transcriptional and post-translational level. Glucagon is known to up-regulate LKR and SDH activity (Rao et al., 1992, Scislowski et al., 1994, Shinno et al., 1980), while insulin down-regulates LKR and SDH activity (Shinno et al., 1980). In rodents, high dietary protein will increase LKR activity and lysine oxidation (Chu and Hegsted, 1976, Foster et al., 1993, Blemings et al., 1998, Kiess et al., 2005), as will lysine injections (Papes et al., 1999). Mice fed a high protein diet had an increase in AASS mRNA and LKR activity, however, AASS protein abundance remain unchanged (Kiess et al., 2005), indicating that post-translational modifications to the AASS protein may be responsible for regulating its activity. Similar evidence for post-translational modification to AASS was observed in chickens (Kiess et al., 2006) and fish (Higgins et al., 2006). Findings in plants are consistent with the apparent post-translational regulation of AASS activity in animals. In soybean (Miron et al., 1997) and tobacco seeds (Karchi et al., 1995), post-translational modifications to AASS can occur as phosphorylations, which affect enzyme activity.

Sequence analysis of the AASS protein indicates that the first 30 amino acids code for a mitochondrial localization signal that targets the translated protein to mitochondria. In fact, in rat liver, AASS is found only in the mitochondrial matrix,

(Blemings et al., 1994) implying that lysine must be transported into mitochondria for degradation via AASS. Authors have suggested that the rate of mitochondrial lysine uptake is a limiting step for lysine oxidation (Blemings et al., 1998), therefore the lysine transporter(s) may be a potential target for regulation of lysine catabolism. Additional investigation into the regulation of mitochondrial lysine uptake may provide further insight into the unique aspects of lysine metabolism, namely the capacity of animals to “conserve” lysine and the ability of lysine to be fed “out-of-phase” (reviewed in Benevenga and Blemings, 2007).

Lysyl Oxidase

The metabolite generated by AASS, amino adipic semialdehyde, is also generated by another enzyme, lysyl oxidase (E.C. 1.4.3.13; Pinnell and Martin, 1968). However, unlike the intramitochondrial AASS, lysyl oxidase is found in the extracellular matrix fibers in numerous mammalian (Gacheru et al., 1990 and Maki and Kivirikko, 2001) and avian (Marayanan et al., 1974 and Opsahl et al., 1982) tissues. In these tissues, lysyl oxidase is responsible for oxidizing lysine residues in elastin and collagen, generating peptidyl α -amino adipic- δ -semialdehyde. This residue can then spontaneously condense with adjacent aldehydes or amino groups to form inter- and intra-chain covalent Schiff-base crosslinks. Increasing the number of crosslinks in elastin and collagen will convert the protein into insoluble connective tissue (reviewed in Kagan and Trackman, 1991). Although the enzyme acts primarily on protein-bound lysine, evidence indicates that it is able to oxidize free lysine as well (Trackman and Kagan, 1979).

Lysyl oxidase activity is under dietary and hormonal regulation. Lysyl oxidase is a metalloenzyme requiring a single copper atom per monomer that is essential for its catalytic activity and possibly its structural integrity (Gacheru et al., 1990). Increases in dietary copper from 0.3 ppm to 16 ppm linearly increased lysyl oxidase activity in chick tendon (Opsahl et al., 1982), indicating that copper concentrations may be limiting for enzyme activation. Follicle stimulating hormone and androgens were found to decrease both lysyl oxidase mRNA and enzymatic activity in a rat granulosa cell line (Harlow et al., 2003). Transforming growth factor- β superfamily members (Harlow et al., 2003 and Gacheru et al., 1997), interleukin-1 β (Roy et al., 1996), and platelet derived growth factor (Green et al., 1995) induce lysyl oxidase activity, likely via cAMP signalling (Ravid et al., 1999).

L-Amino Acid Oxidase

L-Amino acid oxidase (**AAOX**, E.C. 1.4.3.2) is an enzyme capable of oxidizing lysine (Scannone et al., 1964), producing the intermediate pipecolic acid (Grove et al., 1968) which is converted to the metabolite common to other pathways of lysine oxidation, amino adipic semialdehyde (Struck and Sizer, 1960). The enzyme is found in microsomes in the brain and liver of various mammals (Nakano et al., 1968, Murthy and Janardanasarma, 1999) and birds (Boulander et al., 1957, Struck and Sizer, 1960) and in snake venom (Wellner and Hayes, 1968). Amino acid oxidase is capable of oxidizing numerous amino acids and, not surprisingly, the affinity of AAOX for different amino acids varies. In chicken liver, AAOX had the highest affinity for L-leucine, followed by L-lysine, with almost no affinity for D-leucine (Struck and Sizer, 1960). L-leucine was

also the amino acid with the highest affinity for the enzyme in rat tissue (Blanchard et al., 1944).

There is very little data regarding the regulation of AAOX activity. Chickens fed a lysine deficient diet show a trend for lower AAOX activity and mRNA levels compared to chickens fed a lysine adequate diet (Kiess et al., 2006), suggesting that the enzyme may be affected by dietary changes.

Ornithine Transcarbamoylase

The enzyme ornithine transcarbamoylase (**OTC**, EC 2.1.3.3), most often associated with its role in the formation of citrulline in the urea cycle, can also catalyze the formation of homocitrulline from free lysine (Jasiorowska and Kleczlowski, 1970). Lysine transcarbamoylase activity has been localized outside the inner mitochondrial membrane in rat liver (Carter et al., 1984). Ornithine transcarbamoylase activity was not affected by increasing levels of dietary lysine in pigs (Edmonds and Baker, 1987), so regulation of the enzyme appears to be independent of lysine availability.

Effects of Inborn Errors of Lysine Metabolism

Any protein or enzyme involved in lysine metabolism, since it is the product of a DNA template, is subject to mutations. Mutations that cause changes in protein activity may affect lysine metabolism and can cause serious consequences in an individual. A number of diseases have been identified in humans that occur as a result of mutations in proteins involved in lysine metabolism. The phenotype of such mutations is either

hyperlysinemia or hypolylinemia, and in either case, the health of the individual may be compromised.

Hyperlysinemia

Type I familial hyperlysinemia is characterized by a defective AASS enzyme, in which both LKR and SDH enzyme activities are reduced to below 10% of normal levels (Dancis et al., 1979). Type II familial hyperlysinemia is distinguished by a low LKR activity (30-40% of controls), while SDH activity is undetectable (Fellows and Carson, 1974). In both diseases, lysine levels in the blood are high, having been detected between 685 μM and 1500 μM with normal levels ranging from 71 μM to 150 μM (Woody et al., 1966 and Fellows and Carson, 1974). However, in type II familial hyperlysinemia, patients also demonstrate hypersaccharopinuria, since the LKR enzyme is slightly more active in type II patients as compared to type I (Fellows and Carson, 1974). Patients with hyperlysinemia typically have high plasma pipercolic acid (Dancis and Hutzler, 1986), which likely result from an increased flux of lysine through the amino acid oxidase pathway. This alternate pathway of lysine degradation, in addition to the lysyl oxidase pathway, are unable to degrade lysine at a rate capable of preventing hyperlysinemia from occurring, indicating that the AASS is likely responsible for the large majority of whole-body lysine degradation.

Other symptoms of familial hyperlysinemia can be moderate to severe mental and motor retardation (Woody et al., 1966). Patients can also develop normally and even exhibit above average intelligence (Dancis et al., 1983). It is unknown exactly why hyperlysinemia can cause developmental problems but several hypotheses have been

suggested. Hyperlysinemia can be accompanied by hyperammonemia, which can have a negative impact on central nervous system function. The production of glutamate via lysine degradation in the brain during embryonic development is likely to be significantly reduced in individuals suffering from familial hyperlysinemia, which may impair central nervous system development (Papes et al., 2001).

A mutation in AASS has been identified in a single patient with type I familial hyperlysinemia that is responsible for the reduction of AASS activity. The patient was homozygous for an out-of-frame 9-bp deletion in exon 15, which results in a premature stop codon at position 534 in the region of the SDH portion of the protein (Sacksteder et al., 2000). It has not been determined whether the same or a similar mutation exists in the other documented cases of familial hyperlysinemia.

Treatment of patients with familial hyperlysinemia has been consumption of a low-lysine diet (Gregory et al., 1989). This treatment is successful at reducing the plasma lysine levels to near-normal levels but unsuccessful at reducing ammonia concentrations. Therefore, if hyperammonemia is the cause of the developmental problems that accompany familial hyperlysinemia then reducing the lysine intake will do little to improve health. However, since some, although unpredictable, success was observed in patient health, restricting lysine intake remains the primary treatment option for patients with familial hyperlysinemia.

Hyperlysinemia can occur in individuals with normal levels of LKR and SDH activities. Two documented cases of hyperlysinemia report the cause of the disease as a possible defect in lysine transport into mitochondria, which would prevent lysine access to AASS (Oyanagi et al., 1986). Other enzyme deficiencies that result in hyperlysinemia

via limiting α -ketoglutarate availability are a deficiency in pyruvate carboxylase (Wong et al., 1986), which causes low levels of plasma α -ketoglutarate, a requirement for AASS activity, or deficiencies in urea cycle enzymes, which causes the majority of α -ketoglutarate to be sequestered in glutamine during hyperammonemia (Wong et al., 1986).

Hypolysinemia

Multiple protein defects have been identified as causes of hypolysinemia. Hypolysinemia has similar effects on health as consumption of a lysine deficient diet. Growth is limited primarily because protein accretion does not occur at a normal rate since lysine is limiting protein synthesis. However, this effect is secondary to other symptoms associated with hypolysinemia, such as hyperammonemia, that are of a much greater concern.

Lysinuric protein intolerance is a disease caused by a defective dibasic amino acid transporter in epithelial and hepatic cells in which transport of dibasic amino acids, including lysine, is severely reduced (reviewed in Palacin et al., 2004). Absorption of lysine in the intestine is poor (Rajantie et al., 1980) and reabsorption in the renal proximal tubule is very low (Kato et al., 1982), leading to a massive urinary excretion of lysine, resulting in a low plasma concentration. The transport of arginine and ornithine, two additional dibasic amino acids, is also reduced (Rajantie et al., 1980 and Kato et al., 1982), which leads to urea cycle malfunction and subsequent hyperammonemia (Kato et al., 1984). Symptoms of lysinuric protein intolerance include aversion to dietary protein, muscle weakness, osteoporosis, and moderate mental retardation (reviewed in Palacin et

al., 2004). The disease can be treated by oral supplementation with lysine (Lukkarinen et al., 2003) and citrulline (Rajantie et al., 1980), which improves protein tolerance.

Hypolysinemia has been identified in a patient with an α -ketoglutarate dehydrogenase deficiency, which results in high plasma concentrations of α -ketoglutarate (Kamoun et al., 2002) and presumably a greater flux of lysine through the saccharopine pathway. Interestingly, this indicates that the concentration of α -ketoglutarate in mitochondria is, in part, limiting the rate of LKR activity and lysine degradation. Most research has focused on the regulation of lysine degradation induced by changes in dietary protein or lysine concentrations. However, it seems that the availability of α -ketoglutarate for LKR also has the potential to regulate lysine degradation.

In chapter 2 of this dissertation I utilized the RNAi technique to provide insight into the nutrient-gene regulation that occurs in an animal. This insight could lead to more efficient feeding of livestock through better utilization of available lysine also will have relevance to human health. Human health will benefit as better treatment of diseases of lysine metabolism may be possible and lysine nutriture of human populations could be improved.

PART III

Theories of Aging

Aging is defined as the gradual alterations in structure and function that occur over time, eventually leading to an increased probability of death not associated with disease or trauma. What causes these “gradual alterations” and how to reduce their rate

of accumulation to, in effect, slow the rate of aging has been a topic of controversy and research for many decades.

One of the first theories of aging set forth by Harman (1956) attributes aging to damage inflicted on macromolecules caused by free radicals. This theory is evidenced by a significant inverse correlation between metabolic rate, or rate of oxygen utilization, and life span (Cutler, 1991). As the metabolic rate increases in mammals so does the rate of oxygen consumption and oxygen radical production (Ku et al., 1993), indicating the potential role that free radicals have on the rate of aging. As scientific knowledge rapidly progressed, several other theories were promulgated that include the glycation hypothesis (Cerami, 1985) and the Maillard theory of aging (Monnier, 1990), which suggest that tissue and collagen cross-linking impair protein structure and function, which defines the aging process. Another theory that combines the previous three hypotheses is one that suggests a synergistic effect is present between the components of the systems. The synergistic theory of aging (Kristal and Yu, 1992) hypothesizes that age-related deterioration results from damage triggered by interacting free radical producing systems, glycation, and Maillard products.

Substantial evidence indicates that oxidative stress is a significant contributor to the aging process. Oxidative stress refers to the “situation of a serious imbalance between production of reactive oxygen species and reactive nitrogen species and antioxidant defense” (Halliwell and Gutteridge, 1999) that favors the former. Damage induced by such stress is often referred to as oxidative damage and can be identified as an age-dependent accumulation of lipid peroxidation, protein nitrosylation, pentosidine, and modified guanine residues in DNA and mtDNA in nearly every tissue of the body.

Harman's mitochondrial theory of aging (Harman, 1985) states that accumulating oxidative damage in mitochondria, especially mtDNA mutations, is the promoter of aging in humans.

Oxidative Stress and Disease

A localized increase in the production of free radicals can occur in certain diseases and the resultant elevation in oxidative stress can exacerbate the disease condition. Neurodegenerative diseases, like Alzheimer's, Parkinson's, and Huntington's disease, are all characterized by a gradual reduction of mitochondrial integrity, which compromises energy transduction and stimulates reactive oxygen species release. The subsequent increase in oxidative stress advances oxidative damage to the brain, further promoting pathogenesis (reviewed in Mancuso et al., 2007). In vascular diseases, like atherosclerosis and hypertension, there is a marked increase in production of reactive species and inflammation (Kotur-Stevuljevic et al., 2006), which contributes to vascular damage.

Just as a condition can be worsened by an increase in oxidative stress, disease can result from high levels of oxidant production caused by persistent exposure to reactive species. Various environmental factors or habits, like tobacco smoke (Howard et al., 1998), asbestos (Panduri et al., 2004), and ozone (Wang et al., 2006) exposure in lung tissue or exposure to high doses of UV light in melanocytes (Marrot et al., 2005) can induce oxidative stress and increase the rate of accumulation of oxidative damage, which may accelerate the onset of disease. Patients with chronic obstructive pulmonary disease (**COPD**) show evidence of increased oxidative stress, indicating that causes of oxidative

stress, like smoking or asbestos exposure, are risk factors for COPD development (Bowler et al., 2004). Cancers and mitochondrial dysfunction may also be caused by oxidative modifications to DNA and mtDNA, which is accelerated by exposure to inducers of oxidative stress (Howard et al., 1998).

Involvement of Antioxidants in Aging

Much research has been geared toward retarding the aging process by slowing the accumulation of oxidative damage. A positive correlation between mammalian life span and cellular resistance to stress (Kapahi et al., 1999) indicates the potential role that antioxidants have in defending the cell from oxidative damage, thereby extending life span. Antioxidants and antioxidant enzymes are the body's natural defense against reactive species-mediated tissue injury. Therefore, much effort has been placed on learning what role antioxidants and antioxidant enzymes play in preventing oxidative damage as well as their subsequent contribution to longevity.

It is generally expected that longer lived species will have higher antioxidant concentrations and higher antioxidant enzyme activities to combat oxidative stress, thereby reducing oxidative damage and retarding the aging process. However, research has shown that most antioxidant concentrations and enzyme activities are negatively correlated with life span. Although this is not always true, since these relationships can change with tissue type and the variables applied. Lung superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activities, measured per mg of lung protein, and glutathione and ascorbic acid, on a per gram of tissue basis, negatively correlate with maximum life span in various vertebrate species (Perez-Campo et al.,

1994). In liver this same relationship was detected for catalase, glutathione peroxidase, and glutathione (Lopez-Torres et al., 1993). The correlation of liver superoxide dismutase activity and maximum life span potential is controversial since studies, depending on the sample size and units applied, have observed positive, negative, or no correlation (Lopez-Torres et al., 1993 and Cutler, 1985) between the two variables. The plasma concentration of α -tocopherol per specific metabolic rate (cal/g/day) (Cutler, 1991) and serum carotenoid concentrations correlate positively with life span in mammalian species (Cutler, 1984), which lends support to Harman's free radical theory of aging.

Role of Uric Acid in Aging

There exists a positive correlation between plasma urate per specific metabolic rate and life span among both primate and non-primate mammalian species (Cutler, 1984). However, examining this relationship in primates and non-primates independently reveals contrasting observations. The role of uric acid as an antioxidant and its involvement in longevity is strongly supported in Hominoidea species. In this group, the value of plasma urate concentration per specific metabolic rate changes largely due to differences in the numerator since metabolic rate stays relatively consistent between primates. In fact, in primates there is a positive correlation between plasma urate concentration and life span even without scaling the urate concentration to specific metabolic rate. This correlation does not exist in non-primates. Plasma urate concentrations are very similar in non-primates, therefore, the value of plasma urate concentration per specific metabolic rate changes largely due to differences in the

denominator. These expected differences in specific metabolic rate in non-primates generally occur from vast size dissimilarities (consider a mouse versus a cow), which result in a positive correlation between plasma urate concentration per specific metabolic rate and life span in non-primates. For that reason, the positive correlation between plasma urate concentration and life span in non-primates may simply be a function of metabolic body size.

The average serum uric acid concentration of primates is higher than non-primate mammalian species (Cutler, 1984), presumably due to the loss of uricolytic activity or complete silencing of the urate oxidase gene during primate evolution (Friedman et al., 1985). The inactivation of the hominoid *Uox* gene for urate oxidase appears to have been a two-step process; first, in the promoter region and second, in the coding region, both existing as independent nonsense or frameshift mutations (Oda et al., 2002). Without this enzyme, humans and primates have achieved a state of hyperuricemia that almost reaches the saturation point, as uric acid is the end product of purine degradation (Christen et al., 1970; Friedman et al., 1985). Humans also express a urate transporter in kidney tissue, which contributes to the elevation of plasma uric acid by reabsorbing uric acid from the tubule (Enomoto et al., 2002). As a result, some humans are predisposed to gouty arthritis and renal stones, as well as other metabolic diseases associated with increased uric acid concentrations (Gutman, 1965; Smyth, 1975).

Because there are clear disadvantages of hyperuricemia, the functional advantage of the loss of urate oxidase remains unclear. One suggested advantage that increased plasma uric acid may provide primates is an increased antioxidant capacity for free radical scavenging, which may reduce oxidative damage and extend life span (Ames et

al., 1981). Another possible advantage of uric acid accumulation is the prevention of peroxynitrite-induced tissue damage caused by an inflammatory response and oxidative damage in the brain (Scott and Hooper, 2001). An additional, largely unsupported, theory that addresses the possible role that uric acid may have played in stimulating intellectual capacity (Orowan, 1955) was formulated primarily from the close structural similarity that uric acid has to the cerebral stimulants caffeine and theobromine.

Ames's theory regarding the advantage of higher serum uric acid concentrations (Ames et al., 1981) is supported by many studies that demonstrate the potent antioxidant capacity of uric acid. Uric acid can protect erythrocyte membranes and free fatty acids from lipid peroxidation caused by various free radical production systems (Matsushita et al., 1963, Kellogg and Fridovich, 1977, and Smith and Lawing, 1983). Uric acid inhibits DNA strand cleavage induced by free radicals (Cohen et al., 1984, Miura et al., 1993, and Singh et al., 1998) and may also repair oxidatively damaged DNA bases (Simic and Jovanovic, 1989). Administration of uric acid to rats prior to cerebral artery occlusion or reperfusion resulted in a reduction of ischemic damage to the brain and improved behavioral outcome (Yu et al., 1998). Supplementing poultry diets with inosine, a precursor of uric acid reduced tissue injury caused by free radical damage and enhanced growth performance (Simoyi et al., 2002). Uric acid also modulates superoxide dismutase activity *in vivo*, indicating it may contribute to reducing oxidative stress both by scavenging reactive species and enhancing protective effects of other antioxidants (Hink et al., 2002). *In vitro* and *in vivo* evidence lends support to Ames's theory that uric acid provides an antioxidant defense to reduce oxidative damage caused by several reactive species.

To generate a model of primate hyperuricemia and to determine whether urate oxidase is an essential enzyme in lower mammals, a knockout mouse for urate oxidase was developed that resulted in a 10-fold increase in serum uric acid (Wu et al., 1994), a concentration about twice the value found in normal humans (Mikkelsen et al., 1965). As a result, uric acid crystals developed and caused obstructive nephropathy, leading to early death in 65% of the mutant mice. These mice are reported to have a defect in the ability to concentrate urine, which leads to nephrogenic diabetes insipidus, a condition prevented by PEG-uricase treatment (Kelly et al., 2001). It was not determined if the mice had a greater capacity to scavenge free radicals and prevent oxidative damage since the negative effects of hyperuricemia overshadow any positive implication it may have on reducing oxidative stress. Therefore the exact role that uric acid plays in promoting health and life span extension is unclear. Due to severe complications of the high uric acid levels, the applicability of knock-out mice as models for urate oxidase-lacking humans is questionable.

Role of Uric Acid in Disease

Despite numerous reports that high levels of uric acid are beneficial for reduction of oxidative stress and promotion of health and longevity, a body of evidence demonstrates a positive correlation between serum uric acid and coronary artery disease, cardiovascular disease, and hypertension (reviewed in Wannamethee, 2005 and Schachter, 2005). Much debate questions whether elevated uric acid is a risk factor for development of disease or if hyperuricemia is only associated with the etiological factors for disease development. Numerous studies have found that, especially in women, an

elevated level of serum uric acid is independently associated with cardiovascular disease (Fang and Alderman, 2000 and Levine et al., 1989). However, risk factors for coronary artery disease and cardiovascular disease include hypertension, dyslipidemia, insulin resistance, and obesity, all of which can cause hyperuricemia either by increasing uric acid synthesis or enhancing its reabsorption from the kidney. Potentially harmful effects of uric acid that may exacerbate the onset of cardiovascular disease include the reduction of endothelial function via reduction of nitric oxide availability (Waring et al., 2000), induction of proglomerular vascular disease (Mazzali et al., 2002), and an increase in the inflammatory response by activating transcription factor NF- κ B (Kanellis et al., 2003).

If hyperuricemia causes pathological changes that contribute to disease development, then do methods of uric acid reduction have a potential for therapeutic implications? There is contradicting evidence regarding whether reducing uric acid improves hypertension in rat models (Minami et al., 2005 and Kanellis et al., 2003). A single study in adolescent humans, despite a very small sample size (n=5), found that reducing serum uric acid via allopurinol treatment ameliorated the onset of hypertension (Feig et al., 2004), significantly reducing systolic blood pressure from 140 mm Hg to 131 mm Hg, suggesting that hyperuricemia plays a key role in development of hypertension. Since animal studies have contradicting results and given there is no documentation yet involving manipulation of uric acid in an adult population, the exact role that hyperuricemia plays in pathogenesis of hypertension or cardiovascular disease remains unidentified.

The unknowns surrounding the involvement of uric acid as an antioxidant and its contribution to longevity as well as the potential causal relationship between

hyperuricemia and disease development indicate the need for further research in these areas. Additionally, there is potential for the use of uric acid as a therapeutic agent to treat diseases characterized by excessive oxidative stress. To further investigate these topics it would be very helpful to create an animal model that exhibits uric acid concentrations similar to that of humans. Since knocking out UOX in mice is fatal, RNA interference may provide a useful alternative for reducing UOX expression, allowing for moderately increased uric acid concentrations and further investigation into the effects of uric acid elevation on human health.

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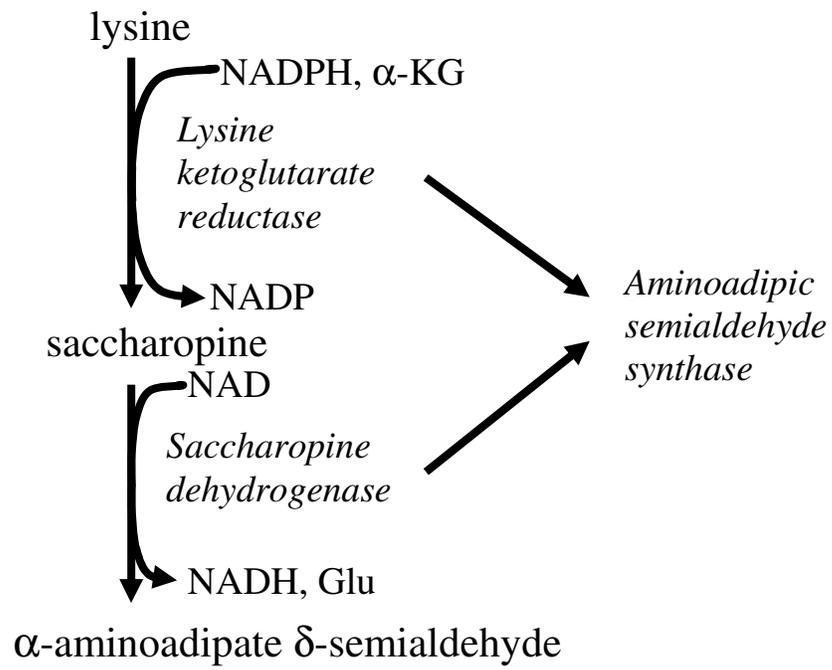


Figure 1. The enzymes, substrates, and cofactors of the major pathway of lysine oxidation.

CHAPTER ONE

α -Aminoadipate δ -Semiaidehyde Synthase mRNA Knockdown Reduces the Lysine Requirement of a Murine Hepatic Cell Line

ABSTRACT

α -Amino adipate δ -semialdehyde synthase (AASS) is the bifunctional enzyme containing the lysine α -ketoglutarate reductase (LKR) and saccharopine dehydrogenase activities responsible for the first two steps in the irreversible catabolism of lysine. A rare disease in humans, familial hyperlysinemia, can be caused by very low LKR activity and, as expected, reduces the lysine “requirement” of the individual. This concept was recreated in a murine hepatic cell line (ATCC, FL83B) utilizing RNA interference to achieve AASS mRNA knockdown. Cells were antibiotic selected for stable transfection of two plasmids that express different short hairpin RNA sequences for AASS knockdown. Compared to wild type cells, AASS mRNA abundance was reduced $79.0 \pm 6.4\%$ ($p < 0.05$), AASS protein abundance was reduced $29.8 \pm 5.2\%$ ($p < 0.05$), and LKR activity decreased $46.6 \pm 7.7\%$ ($p < 0.05$). To determine the effect of AASS knockdown on lysine requirement, cells were grown in media containing 12.5, 25.0, 50.0, 100, or 200 μM lysine. Using a segmented model approach for growth rate analysis, the lysine requirement of the cell line with AASS silencing was $43.4 \pm 1.7 \mu\text{M}$, approximately 26% lower ($p < 0.05$) than the lysine requirement of the wild type cell line. These results indicate AASS knockdown decreases the lysine requirement of the cell via a reduction of lysine catabolism through the saccharopine pathway, providing the initial proof in principle that RNA interference can be used to reduce the nutrient requirement of a system.

Keywords: α -Amino adipate δ -Semialdehyde Synthase, Lysine α -Ketoglutarate Reductase, RNA interference, lysine, familial hyperlysinemia

INTRODUCTION

Lysine is an essential amino acid in animals that is frequently the first or second limiting amino acid for growth in human (Young and Pellett, 1990), swine (Mavromichalis et al., 1998), poultry (Fernandez et al. 1994), and fish (Cheng et al., 2003) cereal grain-based diets. Therefore, protein deficiencies that occur in these cereal grain-consuming populations are often the result of an insufficient amount of dietary lysine, rather than a shortage of amino acids as a whole. When dietary lysine does not meet the requirement the well-being of the organism is compromised (Smirga et al., 2004, Smirga et al., 2002, Fernandez et al., 1994, Si et al., 2001). Therefore, meeting the lysine requirement is imperative in humans for optimal health and in livestock production to avoid the monetary losses associated with suboptimal animal performance. Additionally, improving lysine nutriture would improve health in humans consuming a lysine limiting diet and decrease the feed costs in animal production systems, which generally account for between fifty and seventy-five percent of production costs.

To improve lysine nutriture, a clear understanding of lysine catabolism and its regulation would be helpful. The main pathway for the irreversible degradation of lysine is catalyzed by the enzymes lysine ketoglutarate reductase (LKR, E.C. 1.5.1.8.) and saccharopine dehydrogenase (SDH, E.C. 1.5.1.9), which are located on the bifunctional protein, α -aminoadipate δ -semialdehyde synthase (AASS) (Markovitz et al., 1984). Hyperlysinemia, a rare genetic disease found in humans, can be caused by a defective AASS enzyme and is characterized by a significant reduction in lysine degradation through the AASS pathway (Dancis et al., 1969). As a result of a lower oxidative flux of

lysine through the saccharopine pathway, lysine accumulates in the blood to a concentration 10 times higher than normal (Fellows and Carson, 1974). Individuals with familial hyperlysinemia often have motor and mental handicaps that tend to advance with age (Dancis et al., 1983). Hyperlysinemic children who consume a diet that would ordinarily be deficient in lysine exhibit a drop in plasma lysine concentrations to near-normal levels and growth and development improve (Gregory et al., 1989). This indicates that individuals with familial hyperlysinemia have a lower lysine requirement, presumably from a reduction in lysine oxidation through the AASS pathway.

Despite the symptoms, familial hyperlysinemia represents a condition in which the body uses lysine more efficiently by significantly reducing lysine degradation. Application of this condition to production animals would provide for considerable economic savings as feed can be reformulated to contain less lysine to meet a lower lysine requirement. Reducing dietary protein also means feeding less excess amino acids, subsequently reducing the nutrient content of animal manure and attenuating the environmental impact of farming. In 1995, the cost of adding synthetic lysine to swine diets accounted for about 1.5% of total feed costs (Taylor, 2001), which translates to approximately one hundred million dollars per year that swine producers spend on feed-grade lysine (data collected from USDA). Therefore, the swine industry is particularly poised to benefit considerably from a reduction in the animal's lysine requirement. Likewise, the costs of production of other species can be decreased. There is also potential for improving health of human populations consuming lysine-deficient diets, since the nutritional and immunological status of cereal-protein consuming populations

improves with consumption of lysine-fortified wheat flour (Hussain et al., 2004, Zhao et al., 2004).

Our objective was to reduce the lysine requirement of a murine hepatic cell line by utilizing RNA interference (RNAi) to decrease AASS mRNA. Here we report that murine hepatic cells with reduced AASS expression and slower rates of LKR activity and LOX have a lower lysine requirement than wild type cells. Therefore, we have provided the initial proof in principle that RNAi can be used to decrease nutrient requirements. Extrapolation of these results to livestock production indicates the incredible potential for improved economic gain and reduced environmental impact.

METHODS AND MATERIALS

Plasmid Construction

Two plasmids, p*Silencer*4.1-CMVneo and p*Silencer*4.1-CMVpuro (Ambion, Austin, TX), were selected for stable expression of short hairpin RNA sequences in mammalian cells while conferring resistance to different antibiotics. Sequences used for RNA interference were generated against mouse AASS using the siRNA Selection Server (Yuan et al., 2004). The following four sense sequences were chosen 1) 5'-AUGUCCUGAAUUACCACAC-3', 2) 5'-AUCUUGUGAUCAGCUUGUU-3', 3) 5'-UCACUGCAAGCTACAUUAC-3', and 4) 5'-ACUCAUCAACAGAGAAGCA-3'. The loop sequence, CUUGCUC, joined the sense and antisense sequences in the hairpin structure. Oligonucleotides coding for the shRNA sense and antisense strands were generated with BamHI and HindIII restriction sites flanking the shRNA to facilitate ligation into the p*Silencer*4.1-CMVneo and p*Silencer*4.1-CMVpuro plasmids. Plasmid sequences were verified by direct sequencing. An additional "scrambled" shRNA sequence that was designed by Ambion to have limited similarity to the mouse genome database served as a negative control and was ligated into p*Silencer*4.1-CMVneo and p*Silencer*4.1-CMVpuro vectors.

Cell Culture and Transfection Conditions

A mouse hepatic cell line was purchased from ATCC (FL38B: ATCC, CRL-2390, Manassas, VA) and grown in F-12K media (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum, 500 I.U. penicillin/mL, and 500 ug streptomycin/mL.

Initial Transfection. On day one, 24×10^3 cells were plated in a well of a 24-well plate. On day two, immediately before transfection, growth media was replaced with antibiotic-free growth media, 0.4 μg p*Silencer*4.1-CMVneo, and 1 μL Lipofectamine2000 (Invitrogen, Carlsbad, CA) per transfection reagent protocol. The transfection was completed for each of the four shRNA sequences for AASS knockdown and the nonsense shRNA sequence. Six hours post-transfection the media was replaced with antibiotic containing growth media. Twenty-four hours post-transfection, cells were expanded to a 60-mm cell culture plate with growth media supplemented with 900 μg G418/mL media. Surviving colonies were selected and expanded. Cells that express shRNA sequences for AASS are named for the shRNA sequence they express and are indicated as shRNA1, shRNA2, shRNA3, or shRNA4. Cells that express the nonsense shRNA sequence are indicated as shRNAN.

Double Transfection. The nonsense sequence and the shRNA sequence that yielded the second highest AASS mRNA knockdown (5'-AUGUCCUGAAUUACCACAC-3') were ligated into p*Silencer*4.1-CMVpuro vectors. The p*Silencer*4.1-CMVpuro vector with the nonsense shRNA sequence was transfected into cells from the initial transfection that express the nonsense shRNA from the p*Silencer*4.1-CMVneo plasmid. The p*Silencer*4.1-CMVpuro with the AASS shRNA sequence was transfected into the cell line with the highest amount of AASS knockdown from the initial transfection. The conditions for the second transfection were as follows: 60×10^3 cells per well were plated with antibiotic-free growth media on a 24-well plate with 48.5 μL Opti-MEM media (Invitrogen), 1.5 μL XP-sport (Ambion) and 0.5 μg p*Silencer*4.1-CMVpuro with either the shRNA to AASS or the nonsense sequence per

transfection reagent protocol. Antibiotic selection and colony expansion proceeded as above with 450 ng puromycin per mL media. Three cell lines that were used in subsequent experiments were the wild type cell line, the double-nonsense transfected cell line (shRNANN), and the cell line that exhibited the greatest AASS knockdown after the second transfection (shRNA335).

Real Time RT-PCR

Real time RT-PCR was used to analyze mRNA knockdown in cell lines using acidic ribosomal protein (ARP) as a reference gene. A T-75 flask of cells was washed with 5 mL of cold Hanks balanced salt solution and layered with 3 mL Trizol LS (Invitrogen). After 5 minutes the Trizol was collected and 600 μ L chloroform was added. Samples were vortexed, incubated for 5 minutes, then centrifuged at 4°C and 15,000 x g for 15 minutes. The supernatant was collected and RNA was precipitated with isopropanol and pelleted by spinning at 15,000 x g for 15 minutes at 4°C. RNA was washed twice with 70% ethanol and resuspended in nuclease free water. RNA was quantified and the quality was determined using the $A_{260}:A_{280}$ ratio. Two μ g of RNA was DNase (Promega, Madison, WI) treated and reverse transcribed using random primers (Invitrogen) and MMLV (Promega) per manufacturer's protocol. Complementary DNA was diluted 1:4 with nuclease free water and 2 μ L was used in a 20 μ L PCR reaction with 10 μ L 2X SYBR Green Supermix (BioRad, Hercules, CA), 1.25 μ M forward ARP primer (5'-CAACCCAGCTCTGGAGAAAC-3') and 1.25 μ M reverse ARP primer (5'-GTGAGGTCCTCCTTGGTGAA-3') or 0.625 μ M forward AASS primer (5'-TGGAGACTTCAACGGCTTCT-3') and 0.625 μ M reverse AASS primer (5'-

TGGCCCATAGATCTCCTTTG-3'). The real time PCR was performed on a BioRad iCycler IQ Detection System using a "3-step step-down" procedure. The procedure began with a "hot-start" at 95°C for 5 minutes, followed by a cycle of 95°C denaturing for 15 seconds, 70°C annealing for 30 seconds, and 72°C extension for 30 seconds. The next four PCR cycles followed the same temperature sequence and time lengths except the annealing temperature was reduced by 1°C with each subsequent cycle. The final thirty-five PCR cycles proceeded at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Melt curve analysis confirmed a single PCR product in each reaction. The products were sequenced and confirmed as AASS or ARP. Real-time PCR data was analyzed using the efficiency corrected relative expression method (Pfaffl, 2001).

Western Blotting

Cells were harvested from flasks using trypsin and 3×10^6 cells were pelleted by centrifugation at 300 x g for 10 minutes at 4°C. The cell pellet was resuspended in 100 uL lysis buffer (20 mM HEPES, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 50 mM β -glycerophosphate, 5 mM EDTA, 1 mM sodium orthovanadate, 2 mM benzamidine, 100 μ M leupeptin, 100 μ M pepstatin, 250 μ M soybean trypsin inhibitor, 200 μ M phenylmethylsulfonyl fluoride, 0.50% Triton-X100, pH 7.4) and incubated for 1 hour at 4°C on a Nutator. The lysed cells were centrifuged at 300 x g and the cell lysate was removed for western blotting. Analysis of AASS abundance was done on an equal number of cell basis and not per μ g protein. Therefore, only the protein concentration of the wild type lysate was determined using Pierce Coomassie Plus Protein Assay Reagent with BSA as a standard. SDS-PAGE and western blotting were performed using a

published protocol (Switzer and Garrity, 1999) with 15 µg wild type cell lysate protein and an equal volume of shRNANN and shRNA335 cell lysate (i.e. an equal number of cells). Primary antibodies were generated in rabbits (Invitrogen; Kiess et al., 2005) to a 15-amino acid residue of mouse AASS. The secondary antibody was goat anti-rabbit conjugated with horseradish peroxidase and detected with Pierce SuperSignal West Pico Chemiluminescent substrate (Pierce Biotechnology Inc, Rockford, IL). The band intensity was quantified using densitometry (Flouorochem 8000, Alpha Innotek Corporation, San Leandro, CA).

Lysine α -Ketoglutarate Reductase Assay

Lysine α -ketoglutarate reductase activity was determined in mitochondria isolated from individual cell lines using a modification of a procedure performed by Blemings et al. (1994). To isolate mitochondria, 40×10^6 cells were harvested from flasks with trypsin and collected at $500 \times g$ for 5 minutes at 4 °C. Cells were resuspended with ice cold homogenization buffer (200 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA, 0.05% BSA, 5 mM 2-mercaptoethanol, pH 7.4) and homogenized with 70 strokes using a small clearance pestle. The homogenate was centrifuged at $3,000 \times g$ for 5 minutes. The supernant was collected, spun at $10,000 \times g$ for 5 minutes, and the pellet containing mitochondria was resuspended in 60 µL homogenization buffer.

Lysine α -ketoglutarate reductase activity was measured by determining the rate of disappearance of NADPH (absorbance at 340nm) in a sample of 100 µL buffer (150 mM HEPES, 135 mM mannitol, 45 mM sucrose, 5 mM 2-mercaptoethanol, 0.05% bovine serum albumin, 0.25 mM NADPH, 15 mM α -ketoglutarate, 0.05% Triton-X100, 50 mM

L-lysine-HCl, pH 7.8) and 10 μ L of the mitochondrial suspension using a SpectraMax Plus³⁸⁴ microplate reader (Molecular Devices). A blank sample was included that contained all assay components except L-lysine-HCl to subtract for background NADPH disappearance.

Lysine Oxidation Assay

Lysine oxidation was measured by adapting a procedure previously described (Blemings et al., 1994). Radiolabeled lysine ¹⁴C-labeled only at the 1 carbon (American Radiolabeled Chemicals, L-[1-¹⁴C]), a generous gift from Dr. Norlin Benevenga (University of Wisconsin, Madison), was purified using a cation exchange Dowex-50 column. Before correcting for the percent column recovery, which was typically 85-95%, the specific activity of the lysine was 2368 Bq/nmole. One mL of lysine oxidation buffer (0.1 mM L-lysine, 314 mM mannitol, 78 mM sucrose, 20 mM HEPES, 6 mM Mg₂Cl, 0.4 mM EDTA, 30 mM α -ketoglutarate, and 1 mM NADPH, pH 7.0) was combined with 3.5×10^6 cells resuspended in 1 mL homogenization buffer in a 25 mL Erlenmeyer flask. Incubations lasted 30 minutes in a 37 °C shaking water bath set to 60 oscillations per minute, after which samples were acid killed with by injection with 0.5 mL 35% perchloric acid. The incubation following the acid kill continued for at least 180 minutes during which carbon dioxide was collected in a hanging well (0.6 mL eppendorf tube) containing methylcellosolve and ethanolamine (2:1 ratio) as a “base trap” that was suspended with a piano wire from a rubber stopper that the sealed the mouth of the flask. After this incubation the eppendorf tube was carefully removed and counted for

radioactivity. The rate of lysine oxidation was determined in duplicate less an acid killed control that was injected with perchloric acid prior to the initial 30 minute incubation.

Determination of the Lysine Requirement

Media containing 12.5, 25.0, 50.0, 100 or 200 μM lysine supplemented with 10% dialyzed fetal bovine serum and antibiotics was created by mixing F-12K media lacking lysine (SAFC Biosciences, Lenexa, KA) with unmodified F-12K media (ATCC). On day zero, 60,000 cells were plated with treatment media in each of 4 wells on a 12-well plate with one plate dedicated to each cell line by treatment combination. Cells in one well from each plate were counted using a hemacytometer every 12 hours after the initial seeding for 48 hours. Growth rates were determined by plotting the hours after the initial seed versus the log (cell count) and generating a line of best fit. The slope of the line represented the growth rate of the cells for each cell line by treatment combination. The maximal growth rate was determined by averaging the growth rate of the cells treated with media containing lysine concentrations higher than the requirement of the cell. The experiment was performed five times.

Statistics

Data were analyzed by analysis of variance (ANOVA) with PC-SAS general linear models procedure for significant differences between treatment means. In the event of a significant F-value, the least-significant-difference (LSD) procedure was used for means comparisons. To determine the lysine requirement of cells, cell growth rates were fitted to an NLIN SAS segmented model curve. The intersection of the linear

segments estimates the lysine requirement of the cell line, as indicated in figure 3. A bootstrap analysis of replicates and subsequent ANOVA and LSD procedure was completed to determine differences in the lysine requirement and growth rates between cell lines.

RESULTS

The relative abundance of the AASS mRNA and protein of expanded cell colonies that resulted in the greatest AASS mRNA reduction for each shRNA sequence (shRNA1-4), the negative control nonsense shRNA sequence (shRNAN), and the wild type cells are shown in figure 1. There was no difference in mRNA abundance between the wild type cell line, the shRNAN cells, or the shRNA4 cells ($P>0.05$). Three of the four shRNA sequences targeting AASS silenced AASS mRNA ($P<0.05$), with cells expressing sequence 3 (shRNA3) exhibiting numerically the greatest knockdown to $32.5 \pm 7.0\%$ of wild type AASS mRNA levels. Cells expressing sequence 1 (shRNA1) exhibited knockdown to $47.3 \pm 7.0\%$ of wild type AASS mRNA abundance. Although there was a significant reduction in AASS mRNA levels across the different cell lines, the AASS protein abundance was not different between cell lines (figure 1, $P>0.05$).

Amino adipate semialdehyde synthase mRNA and protein abundance and LKR activity in wild type, shRNANN, and RNA335 cells is shown in figure 2. There was no difference ($P>0.05$) in AASS mRNA abundance, protein abundance, LKR activity or LOX between wild type and shRNANN cells. The shRNA335 cell line that expresses both shRNA sequences 1 and 3 exhibited a reduction in AASS mRNA abundance to $21.0 \pm 6.4\%$ ($P<0.05$), a reduction in AASS protein abundance to $71.2 \pm 5.2\%$ ($P<0.05$), a reduction in LKR activity to $54.4 \pm 7.7\%$ ($P<0.05$), and a rate of lysine oxidation that was $48.3 \pm 11.8\%$ slower ($p<0.05$) compared to wild type levels.

The lysine requirement and growth rate of the wild type cell line, shRNANN and shRNA335 are shown in table 1. The shRNA335 cell line had a lysine requirement

25.6% lower than the wild type cell line ($P < 0.05$) while cell growth rates at lysine concentrations above the requirement were not different across the three cell lines.

Figure 3 shows the SAS graph that generates lines to fit the growth rates from the wild type cells and the corresponding estimation of the lysine requirement.

DISCUSSION

RNA interference was used to reduce expression of the AASS gene at the mRNA, protein, and activity levels. Amino adipate semialdehyde synthase mRNA abundance was silenced to 21% of wild type levels in the shRNA335 cell line. Amino adipate semialdehyde synthase protein abundance decreased approximately 30% while LKR activity and LOX were reduced to approximately 50% of wild type levels. As a result of less lysine oxidation and not a slower growth rate, the lysine requirement of the cells exhibiting AASS silencing was reduced almost 26% compared to wild type cells. This study demonstrates that RNA interference can be used to modify the nutrient requirements of a system. More specifically, these data indicate the potential for AASS silencing as a mechanism for improving the efficiency of lysine utilization for protein synthesis in livestock, especially swine and poultry, in which lysine is a first and second limiting amino acid, respectively.

Despite the marked reduction in AASS mRNA, AASS protein abundance and LKR activity and LOX did not decrease to numerically comparable levels in shRNA335 cells. While this is surprising, previous research in plants indicates that post-translational modifications to the AASS protein are primarily responsible for changes in its activity. In tobacco seeds, regulation of LKR activity operates through protein phosphorylation and intracellular signaling cascades with calcium as a second messenger (Karchi et al., 1995). Research in soybeans indicates that LKR activity decreases with dephosphorylation of the AASS enzyme (Miton et al., 1997), lending further support for the proposed role of post-translational modifications as a regulator of AASS activity. In

animals it is unknown if AASS phosphorylation occurs, but in fish (Higgins et al., 2006) diet mediated changes in AASS mRNA and LKR activity suggest post-translational modifications as a mechanism for regulation of AASS activity. Therefore, in the present experiment the reduction in AASS mRNA unaccompanied by comparable changes in AASS protein abundance and LKR activity supports the model that, in animals, post-translational modifications to AASS are dominant regulators of AASS activity.

The lysine requirement of the cell lines was determined using SAS NLIN to fit growth rates to a two straight-line, one-breakpoint model in which the lysine concentration where the breakpoint occurred estimates the lysine requirement. An additional model that was considered for use was the quadratic broken-line model that fits data below the requirement to a quadratic curve, rather than a straight line, which tends to yield a higher requirement than the straight-line one-breakpoint model (Robbins et al., 2006). Data were fit to both models and while the quadratic broken-line model gave lysine requirements numerically higher (data not shown) than the two straight-line, one-breakpoint model, both models determined that the shRNA335 cell line had a significantly lower lysine requirement.

Phenotypes of familial hyperlysinemia in humans may be small stature (Dancis et al., 1983) and physical and mental retardation but normal development is also reported. Treatment for familial hyperlysinemia is the consumption of a low-protein or synthetic diet that limits lysine intake (Gregory et al., 1989), resulting in reduction of the plasma lysine concentration with unpredictable improvements in development. Why hyperlysinemia causes the described developmental phenotypes or why the phenotypic severity is sometimes reduced with a reduced consumption of lysine is unknown.

Existing theories suggest that the absence of a metabolite of lysine degradation or hyperammonaemia as possible mechanisms that cause the developmental phenotype of familial hyperlysinemia. The former theory is supported by the proposed role that AASS has in central nervous system development since LKR activity is higher in brain than in liver during late embryonic development in rats (Rao et al., 1992). Glutamate is a product of the SDH reaction and the requirement for glutamate in neurogenesis (reviewed in Schlett, 2006) may be the cause for upregulation of AASS during late embryonic development. Therefore, the reduction of glutamate production in the brain of familial hyperlysinemics may impair central nervous system development and contribute to the onset of the hyperlysinemic phenotype. However, this does not explain why reducing plasma lysine concentration during childhood via dietary protein or lysine restriction can improve health and development. What may partially account for developmental improvement is the reduction of plasma ammonia levels that occur from a low lysine intake (Gregory et al., 1989). High circulating and intracellular concentrations of lysine may inhibit urea cycle function through arginine antagonism (Czarnecki et al., 1984) and inhibition of arginase (Ameen and Palmer, 1987).

Since reducing the AASS mRNA abundance decreased the lysine requirement in cell culture, the economic potential for the incorporation of AASS knockdown into livestock production becomes conceptually feasible. In the present experiment, AASS knockdown to 20% of wild type mRNA levels resulted in only a 50% reduction in LKR activity and LOX, compared to 90% or greater reduction in LKR activity seen in humans with familial hyperlysinemia. Short interfering RNA sequences silence genes with varying efficacy, which provides an element of control over the extent to which AASS

mRNA or activity is reduced. Therefore, in livestock, the increase in the plasma lysine concentration would likely be less severe with AASS mRNA knockdown and the developmental phenotypes observed in humans with familial hyperlysinemia would likely be eliminated. Additionally, the objective behind AASS knockdown in livestock is to reduce the lysine requirement, with the rationale that reducing the lysine requirement permits the consumption of a cheaper low-lysine diet, which would prevent the onset of hyperlysinemia.

Reducing the lysine requirement in poultry and swine by utilizing AASS RNA interference seems plausible. RNA interference has been successful in avian embryos and tissues (Sato et al., 2004, Harpavat and Cepko, 2006) and porcine cell culture (Zhang et al., 2005, Hirano et al., 2004). However, there have been no reports of the generation of a transgenic livestock animal that exhibits RNA interference. Literature reporting development of transgenic mice displaying stable germ-line transmittance of “genes” for gene-specific RNA interference has been available for several years (Tiscornia et al., 2003, Rubinson et al., 2003). The lentiviral-based system used to generate these transgenic gene-silencing mice has been successfully employed for generation of transgenic livestock (Chapman et al., 2005, Hofmann et al., 2003) so the methodologies required for creating livestock transgenic for gene silencing are available. Recently, genetically modified pigs have been produced with a nonviral vector (Manzini et al., 2006), which may curb concerns from the public regarding the safety of the consumption of products that come from genetically modified animals created using lentiviral methods.

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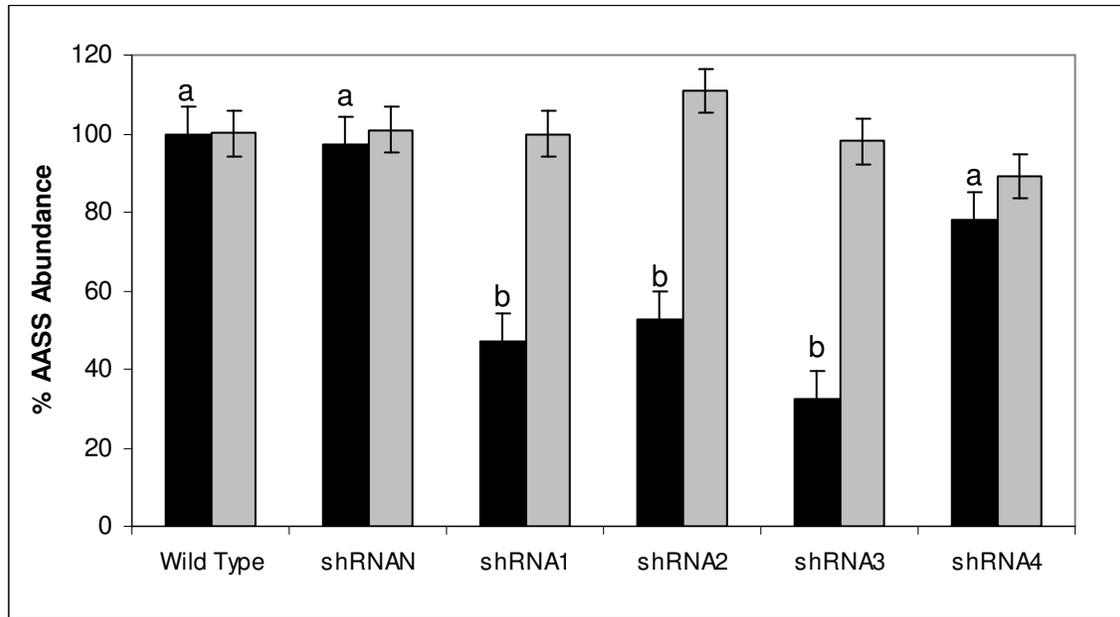


Figure 1. Aminoadipate semialdehyde synthase mRNA (black bars) or protein (gray bars) abundance in wild type cells or cells transfected with a plasmid expressing a nonsense shRNA (RNAN) or a shRNA for AASS knockdown (shRNA1, shRNA2, shRNA3, shRNA4). Aminoadipate semaldehyde synthase mRNA and protein abundance is scaled to wild type levels, which is set at 100%. Bars represent means \pm SEM (n=2). Different letters represent significant differences ($P < 0.05$) among cell lines.

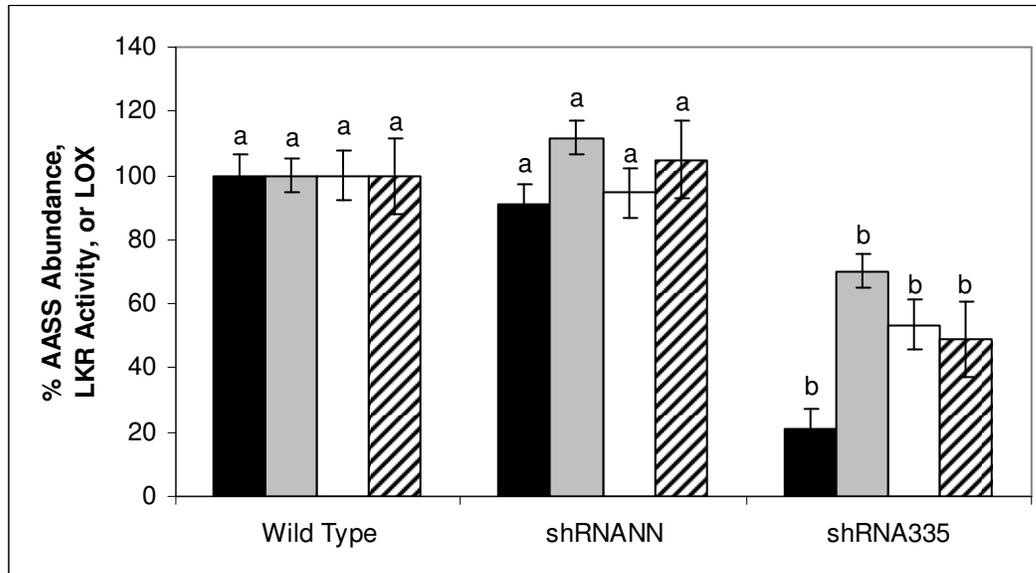


Figure 2. Aminoadipate semialdehyde synthase mRNA (black bars; n=2) or protein (gray bars; n=2) abundance, LKR activity (white bars; n=3), or LOX (hatched bars; n=3) in wild type cells or cells transfected with two plasmids expressing either nonsense shRNAs (shRNANN) or shRNA sequences 1 and 3 for AASS knockdown (shRNA335). Bars represent means \pm SEM. Different letters represent differences between cell lines ($P < 0.05$). Data from shRNANN and shRNA335 cell lines are scaled to wild type values, which were set at 100%.

Cell Line	Lysine Requirement in Media (μM)	Growth Rate At or Above Requirement (Log (cell count/hour)*1000)
Wild Type	58.4 \pm 1.7 ^a	13.5 \pm 0.7
shRNANN	61.9 \pm 1.7 ^a	14.3 \pm 0.7
shRNA335	43.4 \pm 1.7 ^b	12.7 \pm 0.5

Table 1. Numbers are means \pm SEM. Numbers with superscripts of different letters are different ($P < 0.05$).

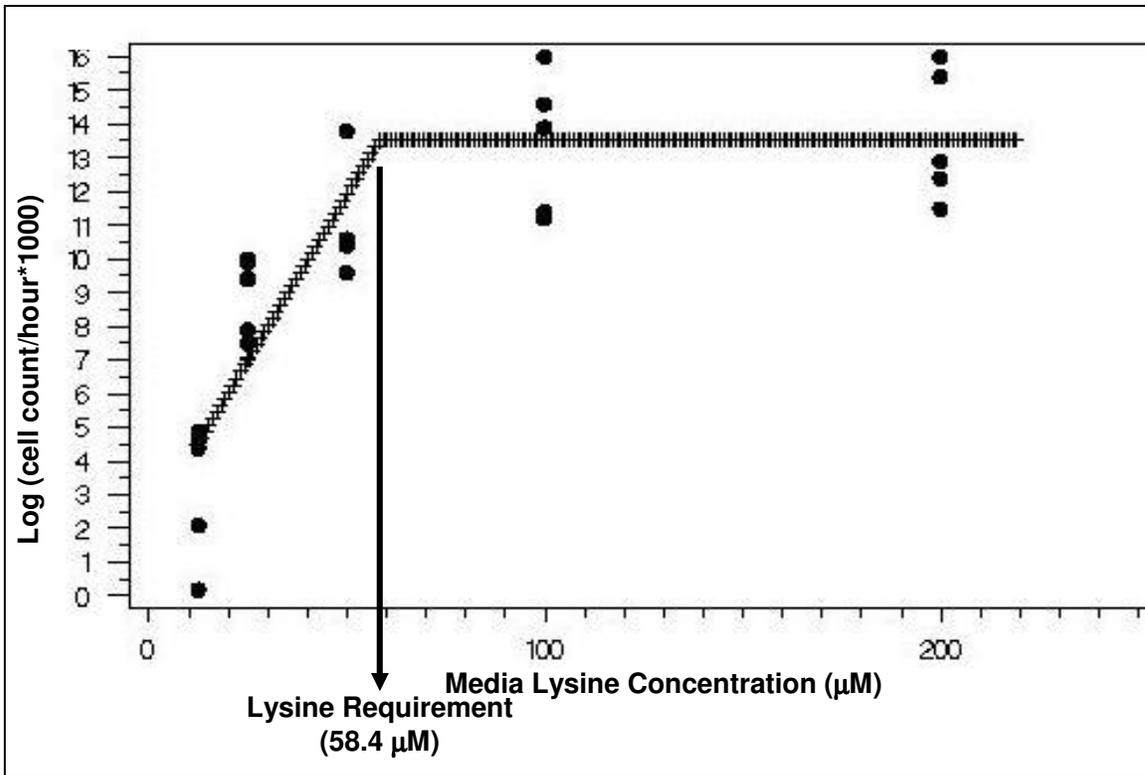


Figure 3. SAS output graph for data from wild type cells growing in media with varying concentrations of lysine. The arrow indicates the x-value at which the intersection of the two lines occurs, estimating the lysine requirement of the cells.

CHAPTER TWO

Using RNA Interference to Reduce Urate Oxidase Expression Decreases Oxidative Stress in a Murine Hepatic Cell Line

ABSTRACT

Humans, birds, and higher primates do not express the uric acid degrading enzyme urate oxidase (UOX) and, as a result, have plasma uric acid concentrations higher than UOX expressing animals. Although elevated uric acid concentrations are suggested to increase the antioxidant defense system and provide a health advantage to animals without UOX, knockout mice lacking UOX develop pathological complications including gout and kidney failure. As an alternative to the knockout model, RNA interference was used to decrease UOX expression using stable transfection in a mouse hepatic cell line (ATCC, FL83B). Urate oxidase mRNA was reduced 66% compared to wild type, as measured by real time RT-PCR. To determine if UOX knockdown resulted in enhanced protection against oxidative stress, cells were challenged with hexavalent chromium (Cr(VI)) or 3-morpholinopyridone hydrochloride (SIN-1). Compared to wild type, cells with UOX knockdown exhibited a $37.2 \pm 3.5\%$ reduction ($p < 0.05$) in the electron spin resonance signal after being exposed to Cr(VI) and displayed less DNA fragmentation ($P < 0.05$) following SIN-1 treatment. Cell viability decreased in wild type cells ($P < 0.05$), but not cells with UOX knockdown, after treatment with SIN-1. These results are consistent with an increased intracellular uric acid concentration and providing an increased defense against oxidative stress.

Keywords: Urate Oxidase, oxidative stress, uric acid, RNA interference

INTRODUCTION

In the genomes of both prokaryotes and eukaryotes lies the gene for the uric acid degrading enzyme, urate oxidase (**UOX**, EC 1.7.3.3.), however, not all organisms express this gene. Among those organisms in which its expression is silenced are humans, birds, some primates, terrestrial reptiles, and most insects. In these organisms, uric acid is excreted as the terminal product of purine degradation. Urate oxidase further degrades uric acid to allantoin, the terminal product of purine degradation in UOX expressing mammals. When UOX is expressed, the plasma concentration of uric acid is much lower than in those animals that do not express the enzyme. For example, the normal range of uric acid in the blood of humans is 200 to 400 μM , which is similar to that found in birds (Tsahar et al, 2006) and 10 to 20 times that found in UOX expressing animals. Without this enzyme, humans and primates have achieved a state of hyperuricemia that approaches the saturation point (Christen et al, 1970; Friedman et al, 1985) and, as a result, gout is a prevalent disease in humans.

The controversial association of hyperuricemia with hypertension (reviewed in Schachter, 2005) and cardiovascular disease (reviewed in Wannamethee, 2005) in humans, as well as the pathological complications resulting from uric acid crystallization in the kidneys and joints makes the functional advantage of a lack of UOX expression unclear. One widely accepted theory is that increased plasma uric acid may provide primates with an increased antioxidant capacity for free radical scavenging, which may reduce the accumulation of oxidative damage and extend life span (Ames et al, 1981). Supporting this theory is the positive correlation between plasma urate and life span

among the primate species (Cutler, 1984). Also, numerous reports cite the ability of uric acid to protect cellular components from reactive oxygen and nitrogen species (reviewed in Becker, 1993 and Glantzounis et al., 2005), which may contribute to animal longevity.

To further understand the potential role that uric acid has in longevity, disease development, and disease treatment, a mouse model for hyperuricemia was created that lacks urate oxidase expression (Wu et al., 1994 and Kelly et al., 2001). These mice were severely hyperuricemic as they exhibited serum uric acid concentrations ten times higher than that found in wild type mice and twice that found in humans. Uric acid crystals developed in the kidneys and led to urate nephropathy and nephrogenic diabetes insipidus, resulting in dehydration and death of most mice within several weeks of birth. The extremely high uric acid concentrations and resultant pathological complications obviate the use of these mice, therefore a more appropriate animal model of human hyperuricemia should be established.

The objective of this experiment was to utilize RNA interference to “knockdown” urate oxidase expression in a mouse hepatic cell line and determine the effect on the oxidative stress response. This will provide a direct link between reduced UOX expression and the resultant increased protection this affords from oxidative damage by reactive species. The advantage of using small interfering RNAs is that this approach affords an element of control over gene expression and the degree to which serum uric acid concentrations are elevated.

METHODS AND MATERIALS

Plasmid Construction

Plasmid p*Silencer*4.1-CMVneo (Ambion, Austin, TX), was selected for stable expression of short hairpin RNA sequences in mammalian cells while conferring resistance to antibiotics. Sequences used for RNA interference were generated against mouse UOX and the following four sense sequences were chosen 1) 5'-AGCCUUCCGAACAUCACU-3', 2) 5'-ACCUCAAGGUCUUGAAAAC-3', 3) 5'-GGACUGAUCAACAAGGAAG-3', and 4) 5'-ACCUACACGGUGAUAAUUC-3'. The loop sequence, TTCAAGAGA, joined the sense and antisense sequences in the hairpin structure. Oligonucleotides coding for the shRNA sense and antisense strands were flanked with BamHI and HindIII restriction sites to facilitate ligation into the p*Silencer*4.1-CMVneo plasmid. Construct sequences were verified by direct sequencing. An additional "scrambled" shRNA sequence that was designed by Ambion to have limited similarity to the mouse genome database served as a negative control and was ligated into p*Silencer*4.1-CMVneo

Cell Culture and Transfection Conditions

A mouse hepatic cell line was purchased from ATCC (designation: FL83B, Manassas, VA) and grown per supplier's instructions in F-12K media (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum and 500 I.U. penicillin/mL, and 500 ug streptomycin/mL. On the day of transfection, 6×10^4 cells were plated per well with antibiotic-free growth media in a 24-well plate with 48.5 μ L Opti-MEM media

(Invitrogen), 1.5 μ L XP-sport (Ambion) and 0.5 μ g p*Silencer*4.1-CMVneo with the shRNA to either UOX or the nonsense sequence per transfection reagent protocol. The transfection was completed for each of the four shRNA sequences for UOX knockdown and the nonsense shRNA sequence. Six hours post-transfection, the media was replaced with antibiotic containing growth media. Twenty-four hours post-transfection, cells were expanded to a 60-mm cell culture dish with growth media supplemented with 900 μ g G418/mL media. Cells were exposed to G418 and surviving colonies were selected and expanded.

Real Time RT-PCR

Real time RT-PCR was used to analyze mRNA knockdown in cell lines using acidic ribosomal protein (ARP) as a reference gene. A T-75 flask of cells was washed with 5 mL of cold Hanks balanced salt solution and layered with 3 mL Trizol LS (Invitrogen). After 5 minutes the Trizol was collected and 600 μ L chloroform was added. Samples were vortexed, incubated for 5 minutes, then centrifuged at 15,000 x g for 15 minutes (4°C). The supernant was collected and combined with an equal volume of acid phenol:chloroform (Ambion, Austin, TX). After centrifugation at 15,000 x g for 15 minutes (4°C), the RNA in the aqueous layer was precipitated with isopropanol and pelleted at 15,000 x g for 15 minutes (4°C). RNA was washed twice with 70% ethanol and resuspended with nuclease free water. RNA was quantified and the quality was determined using $A_{260}:A_{280}$. Two μ g of RNA was reverse transcribed using oligo-dT primers (Promega) and MMLV (Promega) per manufacturer's protocol. Complementary DNA was diluted 1:4 with nuclease free water and 10 μ L (for UOX PCR) or 5 μ L (for

ARP PCR) was used in a 50 μ L PCR reaction with 25 μ L 2X SYBR Green Supermix (BioRad, Hercules, CA), 1.25 μ M forward ARP primer (5'-CAACCCAGCTCTGGAGAAAC-3') and 1.25 μ M reverse ARP primer (5'-GTGAGGTCCTCCTTGGTGAA-3') or 1.25 μ M forward UOX primer (5'-TGGAGACTTCAACGGCTTCT-3') and 1.25 μ M reverse UOX primer (5'-TGGCCCATAGATCTCCTTTG-3'). The real time RT-PCR protocol was performed on a BioRad iCycler IQ Detection System. The protocol began with a "hot-start" at 95°C for 5 minutes, followed by a cycle of 95°C denaturing for 15 seconds, 60°C annealing for 30 seconds, and 72°C extension for 30 seconds. The denaturing-annealing-extension cycle was completed forty times, followed by melt curve analysis to confirm the existence of a single product. The PCR products were sequenced and confirmed as UOX or ARP. Real-time RT-PCR data was analyzed using the efficiency corrected relative expression method (Pfaffl, 2001). The cell line demonstrating the greatest UOX knockdown, the negative control cell line, and the wild type cell line were characterized in subsequent experiments.

Electron Spin Resonance

Electron spin resonance (**ESR**) spin trapping was used to detect short-lived reactive oxygen intermediates using a technique modified from Leonard et al. (2000). The intensity of the signal is used to quantify the amount of free radicals produced in the reaction and hyperfine splitting of the spin-adduct is used to identify the trapped radicals. All ESR measurements were conducted using a Bruker EMX spectrometer (Bruker Instruments Inc., Billerica, MA) with a flat cell assembly and an Acquisit program was

used for data acquisition and analysis. The ESR spectrometer settings were: receiver gain, 5.02×10^4 ; time constant, 40.96 ms; modulation amplitude, 0.50 G; scan time, 41.94 s; magnetic field 3480 ± 100 G. Experiments were performed at room temperature, under ambient air. Signal intensity was quantified by measuring average distance (mm) using the peak to peak method. Each sample (500 μ L) contained 5×10^5 cells in phosphate buffered saline, 200 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and 0.0 or 200 μ M hexavalent chromium (Cr(VI)) to induce radical formation. Samples (n=5) were incubated for 37 °C for 5 minutes before loading into the flat cell assembly for free radical detection.

Cell Viability

Cell viability was used to determine the ability of the cells to survive a reactive nitrogen species challenge. On day one, 1×10^4 cells were plated in a well of a 96-well plate and grown overnight. On day two, the media was replaced with 100 μ L media containing 0, 100, 200, 300, 400, 500, or 1000 μ M 3-morpholinopyridone hydrochloride (SIN-1). After 24 hours of SIN-1 exposure the cell viability was determined using the MTT assay (Cayman Chemical, Ann Arbor, MI) per manufacturer's protocol.

DNA Fragmentation

Cells were seeded, 1.5×10^5 per well, in a 12-well plate and allowed to grow overnight. On day 2, media was replaced with media that contained 0, 250, 500, or 1000 μ M SIN-1. After 3 hours of SIN-1 exposure, cells were harvested and resuspended in

500 μ L of ice-cold PBS. Cell treatment, harvest, and the Comet Assay were completed under minimal lighting to avoid additional DNA denaturation. Fragmented DNA was detected using the Comet Assay per manufacturer's protocol (Trevigen, Gaithersburg, MD). Briefly, cells were suspended in agarose and plated on a microscope slide, lysed, and subjected to electrophoresis. DNA was stained using SYBR Green and visualized using an Olympus AX70 Photomicroscope (Olympus America, Center Valley, PA). The image was captured with a Retiga 2000R camera (QImaging, Burnaby, BC) and SimplePCI software (Compix Inc., Imaging Systems, Sewickley, PA). The extent of DNA fragmentation was determined by measuring tail length beginning at center of the cells to the left rim of SYBR green fluorescence using Optimas 6.5 Image analysis software (Media Cybernetics, Silver Spring, MD)

Lipid Peroxidation

Lipid peroxidation was measured by quantifying the cellular malondialdehyde (MDA) concentration using a "thiobarbituric acid reactive substances" colorimetric assay kit (Cayman Chemical) per manufacturer's protocol. Cells were seeded in a 25 cm² flask and grown to approximately 100% confluency. Media was removed and replaced with media containing 0, 250, or 500 μ M SIN-1. After 3 hours of SIN-1 exposure, cells were harvested and 2×10^6 cells were lysed by four freeze-thaw cycles and the entire cell lysate was analyzed for MDA content. Standards and reagent blanks were used to generate a standard curve.

Statistics

Data were analyzed by regression analysis, with SIN-1 concentration as the independent variable and percent cell viability as the dependent variable, or analysis of variance with PC-SAS general linear models procedure for significant differences among treatment means. In the event of a significant *F* value, the LSD procedure was used for means comparisons. Differences were considered significant at $P < 0.05$.

RESULTS

Real time RT-PCR analysis indicated that in the cell line expressing shRNA sequence 1 (5'-AGCCUCCGAACAUCACU-3') there was a 66% reduction in UOX mRNA for UOX silencing (Fig 1) ($P < 0.05$). No difference in UOX mRNA abundance between the wild type cell line and the cell line serving as the negative control was detected.

Representative ESR spectra are shown in figure 2. The intensity of the signal is used to quantify the amount of free radicals produced in the reaction and hyperfine splittings of the spin-adduct are used to identify the trapped radicals. This spectrum consists of a 1:2:2:1 quartet with hydrogen and nitrogen splitting constants of $a_H = a_N = 14.9$ G. Based on these splitting constants the quartet was assigned to a DMPO/OH adduct, which can also be generated by decomposed superoxide free radicals. There was no measurable signal in cells not exposed to Cr(VI). When exposed to Cr(VI), cells with UOX knockdown exhibit a 37% smaller ($P < 0.05$) free radical signal than wild type cells.

Data presented in figure 3 was tested for fit to a regression line to determine if SIN-1 exposure had an effect on cell viability. In both the wild type and negative control cell lines, cell viability decreased with increasing concentrations of SIN-1 ($P < 0.01$). However, no decrease in cell viability occurred in the cell line with UOX knockdown ($P > 0.05$). Across all cells lines, after 24 hours of exposure to 1000 μ M SIN-1, there was no cell survival (data not shown).

Tail length, which is measured in arbitrary units and indicative of the extent of DNA fragmentation, from all cell line by treatment combinations was quantified and is

presented in figure 4. Analysis of variance indicated a main effect of cell line and SIN-1 concentration, as well as a cell line by SIN-1 concentration interaction, on tail length ($P < 0.05$). Pooled across all cell lines, comet tail length increased with an increase in SIN-1 concentration ($P < 0.05$). Across SIN-1 concentrations, the overall tail length of wild type cells and the negative control cell line was 27.7 ± 0.7 and 26.7 ± 0.9 , respectively, which were longer ($P < 0.05$) than the 22.1 ± 0.8 overall tail length in the cells with UOX silencing. There was no difference in tail length between cells not exposed to SIN-1. The only concentration of SIN-1 at which the UOX knockdown cells had a significantly shorter tail length ($P < 0.05$) than both wild type and negative control cells was $1000 \mu\text{M}$. When exposed to $500 \mu\text{M}$ SIN-1, cells with UOX knockdown had shorter tails ($P < 0.05$) than only the negative control cell line. At the $250 \mu\text{M}$ SIN-1 concentration, cells with UOX silencing had shorter tails ($P < 0.05$) than wild type cells but only exhibited a trend ($P = 0.08$) towards shorter tail length relative to negative control cells.

Lipid peroxidation that occurred at various levels of SIN-1 exposure is indicated in figure 5. No effect of SIN-1 concentration or cell type on lipid peroxidation was detected ($P > 0.05$).

DISCUSSION

In the present experiment, short interfering RNAs were used to silence UOX mRNA approximately 66% compared to wild type. To determine if UOX silencing had any effect on the cells oxidative stress response, cells were exposed to reactive species and indices of oxidative stress and oxidative damage were measured. Exposure to Cr(VI) resulted in a less intense ESR signal in cells with UOX silencing, indicative of lower hydroxyl and superoxide radical concentrations. In wild type and negative control cells, cell viability decreased with increasing concentrations of the peroxynitrite producing chemical, SIN-1, up to the 500 μ M level. This relationship was not observed in cells with urate oxidase silencing. The comet assay, used to detect DNA fragmentation induced by SIN-1, indicated that cells with UOX silencing had less DNA denaturation when exposed to SIN-1. These aforementioned findings are consistent with increased protection from oxidative damage in UOX silenced cells.

The effect of reducing UOX mRNA on intracellular uric acid concentrations is unknown. Attempts were made to assess concentration differences but technical difficulties prevented accurate quantification. However, because of existing differences in the physiological responses to oxidative stimuli between the cell lines, it is likely that an increase in the intracellular concentration of uric acid occurred with UOX mRNA silencing. Additionally, neither the transfection conditions nor the expression of the shRNA affected the handling of oxidative stress, since the effects of oxidative stress in the negative control cell line remain similar to wild type cells. The only difference between the negative control cell line and the cell line exhibiting UOX knockdown is the

sequence of the shRNA expressed by the plasmid. Additionally, reducing urate oxidase activity levels with oxonic acid, a competitive inhibitor of the enzyme, is effective in increasing circulating uric acid concentrations (Roncal et al., 2007). Therefore, assuming the intracellular concentration of uric acid is increased in cells with UOX silencing, these results support the role of uric acid as an antioxidant.

Hexavalent chromium is a carcinogen in both human and animal models (reviewed in Costa and Klein, 2006). The most documented evidence of Cr(VI) toxicity is from inhalation of the molecule, which is most apparent in the increased rate of lung (Gibb et al., 2000), kidney, prostate, and bladder cancers (Cohen et al., 1993) and mental illness (Gibb et al., 2000) in those exposed in the workplace. Once in the cell, Cr(VI) is reduced by intracellular antioxidants like glutathione, ascorbic acid, and cysteine, or through the intermediates Cr(V) and Cr(IV) to trivalent chromium (Cr(III)) (reviewed in Zhitkovich, 2005). Trivalent chromium can interact with DNA to form Cr-DNA adducts (Zhitkovich et al., 1995), which can induce apoptosis or mutagenesis. Interaction of the hexavalent chromium with cellular components can induce production of hydroxyl and superoxide free radicals detected by ESR, inducing oxidative stress and causing oxidative damage (Leonard et al., 2002, Ding and Shi, 2002). Previous studies have used ESR detection to demonstrate the ability of uric acid to scavenge superoxide generated by xanthine oxidase (Stinefelt et al., 2005), hydroxyl radicals generated by Fenton chemistry (Stinefelt et al., 2005), and peroxy radicals (Muraoka and Miura, 2003). The present study indicates that cells with UOX silencing are exposed to less reactive oxygen species than wild type cells when treated with Cr(VI). This supports an existing that, *in vitro*, uric acid was effective at reducing DNA damaged induced by Cr(III) exposure

(Burkhardt et al., 2001). The probable increase in intracellular concentration of uric acid either contributes to greater free radical scavenging or reduces the rate of free radical production, or a combination of both, thus protecting cellular components from Cr(VI)-induced reactive oxygen species damage. This raises the possibility that uric acid may be a therapeutic agent for Cr(VI) exposure, a concept that warrants further investigation.

In addition to Cr(VI), SIN-1 was used in the present experiment to induce oxidative stress. Decomposition of SIN-1 in solution at pH > 5 causes production of superoxide and nitric oxide. Together, these chemicals spontaneously form peroxynitrite, which is detected both in the media (Doulias et al., 2001) and inside cells (Saito et al., 2006). It has been well established in numerous cell types that treating cells with SIN-1 causes intracellular oxidative stress and accumulation of intracellular markers of oxidative damage in a dose-dependent manner, resulting in a reduction in cell viability.

Exposure of human lymphoid blastoma cells to SIN-1 concentrations between 1 mM and 5 mM caused a dose-dependent increase in cell death (Li et al., 2002). This is supported by data in human premonocytic cells where a reduction in viability was found after treatment of cells with 1 mM and 2 mM SIN-1 (Yang and Park, 2007). Interestingly, rodent cell culture exhibits a lower tolerance to SIN-1-induced reduction in viability. In the present experiment, wild type cell viability began to decrease when the SIN-1 concentration in the media was 100 μ M and decreased to approximately 50% viability when the concentration reached 500 μ M, while a 1 mM concentration resulted in no viable cells after 24 hours. The 50% reduction in cell viability at 500 μ M was also observed in rat adrenal gland cell culture (Choi et al., 2004). It is unknown why rodent cells are more sensitive to SIN-1 compared to human cell lines. The presence of higher

concentrations of endogenous antioxidants, like uric acid, or antioxidant enzymes in human cells may be responsible for quenching some reactive species that are formed, enabling a higher SIN-1 tolerance. Biological fluids also have a quenching effect on reactive species produced by SIN-1 (Pascual and Reinhart, 1999) so the origin of animal sera used to supplement growth media likely influences SIN-1-induced peroxynitrite exposure.

Incubating cells with antioxidants or antioxidant enzymes reduces the toxicity of SIN-1 in cell culture. Exposure to 1 mM or 5 mM uric acid (Choi et al., 2004, and Li et al., 2004) or a combination of catalase and superoxide dismutase (Li et al., 2004) limited the reduction in cell viability or cell death caused by SIN-1. In the present experiment, the maintenance of cell viability between 100 μ M and 500 μ M SIN-1 that occurred with UOX silencing supports the concept that intracellular uric acid is increased in these cells, protecting the cellular components from oxidative damage that leads to cell death.

One of the specific sites of SIN-1 induced oxidative damage is denaturation of genomic DNA. The data presented here shows that there was an increase in DNA denaturation that was dose-dependent on SIN-1 concentration. This agrees with numerous reports utilizing the comet assay as an indicator of DNA damage induced by SIN-1 in human lymph cells (Doulias et al., 2001, Li et al., 2004) and Chinese hamster fibroblasts (Muzandu et al., 2006) that was preventable by inclusion of catalase (Doulias et al., 2001), lycopene, or β -carotene (Muzandu et al., 2006) in the cell culture media. The ability of antioxidants or antioxidant enzymes to limit the strand breaks implies that an increased antioxidant capacity is present in cells with UOX silencing and presumably this is an increased uric acid concentration.

Exposure of cells to supraphysiological concentrations of uric acid will attenuate the protective effects that uric acid otherwise exerts on free radical-induced lipid peroxidation (Stinefelt et al., 2005). It is likely that a free radical intermediate formed during the reaction of high concentrations of uric acid with a reactive species may further propagate this oxidative damage (Santos et al., 1999). Since uric acid can have either an antioxidant or a prooxidant effect on lipid peroxidation, it was a concern that the effect of UOX silencing on intracellular uric acid concentrations would lead to increased basal levels of lipid peroxidation. However, the results of the present experiment indicate that the uric acid concentration in cells with UOX knockdown did not reach levels that were prooxidative.

Previous research demonstrated that SIN-1 induced lipid peroxidation is decreased by antioxidant exposure (Stevens et al., 2003, Choi et al., 2004). In the current study, up to 500 μ M SIN-1 exposure did not increase lipid peroxidation. Without evidence of SIN-1 mediated lipid peroxidation, it was not possible to determine if UOX silencing was able to reduce lipid peroxidation as a marker of oxidative stress.

As demonstrated in this study, RNA interference can be used in a mouse-derived cell culture system to reduce UOX expression, which resulted in increased protection against oxidative stress. The next logical experiment would be to apply this concept in a living mouse, which is feasible since the technology is available. Literature reporting development of transgenic mice displaying stable germ-line transmittance of “genes” for gene-specific RNA interference has existed for several years (Tiscornia et al., 2003, Rubinson et al., 2003). The advantage of retaining a certain level of UOX expression, as opposed to eliminating it completely as in the knockout models, is that uric acid

degradation is not completely abolished. This would likely result in moderate increases in circulating uric acid concentrations proportional to UOX silencing, a variable controlled by the experimenter.

Urate oxidase silenced mice would allow for further investigation into the role that uric acid may play in disease treatment and prevention. Such mice would also help clarify the relationship between uric acid levels and risk factors for various diseases. Common methods currently employed to increase circulating uric acid in animals include treatment with uric acid precursors inosine or hypoxanthine (Simoyi et al., 2002 and Scott et al., 2002) or the urate oxidase inhibitor oxonic acid (Roncal et al., 2007). Therefore, reducing UOX activity either through the use of an inhibitor or through RNA interference is an additional technique to manipulate uric acid levels and alter oxidative stress in rodents, permitting their use as models of disease.

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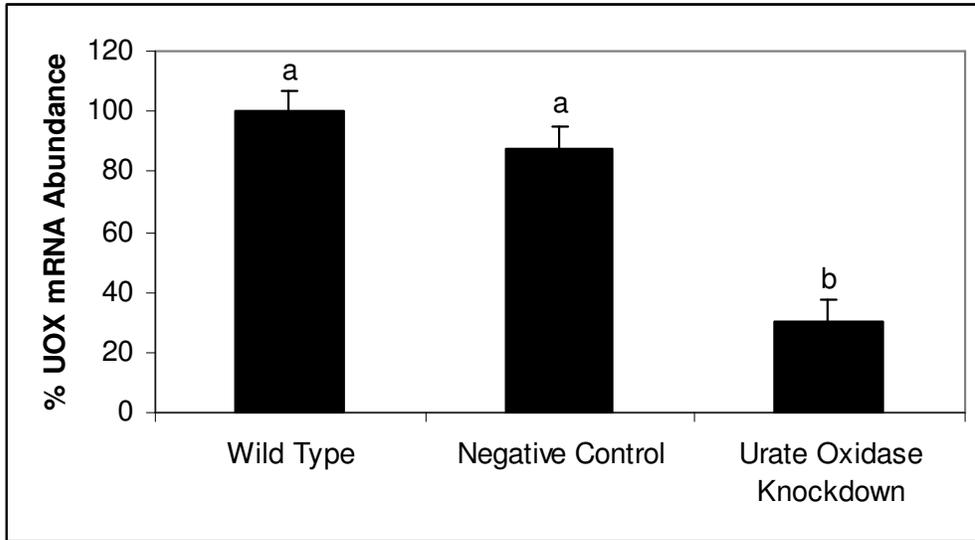


Figure 1. Urate oxidase mRNA abundance in wild type cells, negative control cells, or cells with urate oxidase knockdown. Urate oxidase mRNA abundance is scaled to wild type levels, which is set at 100%. Error bars represent means \pm SEM, n=2. Different letters indicate differences ($P < 0.05$) between cell lines.

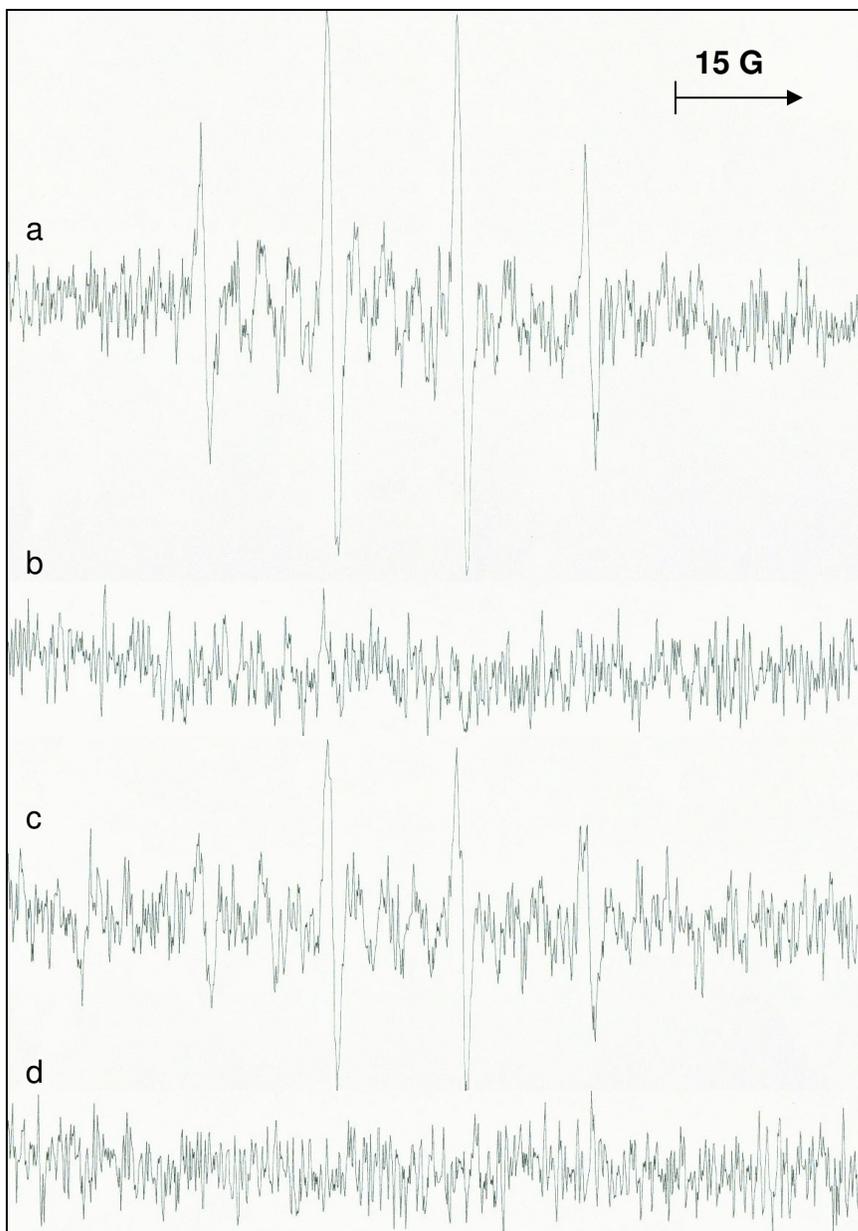


Figure 2. Electron spin resonance in wild type and cells with urate oxidase silencing. Spectra (a) and (b) were generated in the presence of wild type cells and spectra (c) and (d) were generated in the presence of cells with urate oxidase knockdown. Only cells in spectra (a) and (c) were exposed to Cr(VI) at 200 μ M.

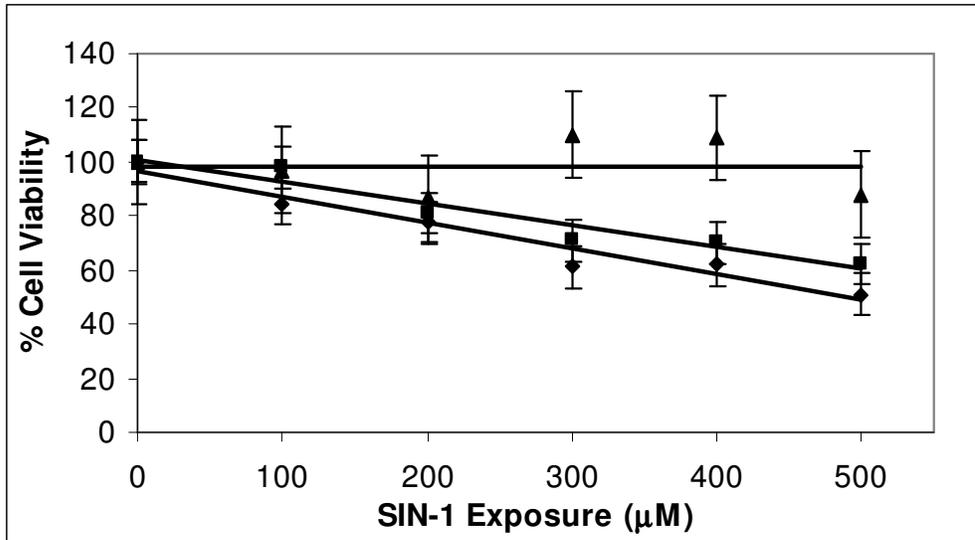


Figure 3. Cell viability of wild type (diamonds), negative control (squares) and urate oxidase knockdown (triangles) cell lines exposed to various concentrations of SIN-1 for 24 hours. Data points represent means \pm SEM, n=2. Data within each cell line were scaled to the viability of cells exposed to 0 μ M SIN-1, which was set at 100% viability. The regression line for each cell type is indicated. Wild type: $P < 0.0001$, $R^2 = 0.78$; Negative Control: $P < 0.0001$, $R^2 = 0.51$; UOX Knockdown: $P > 0.9$, $R^2 < 0.1$.

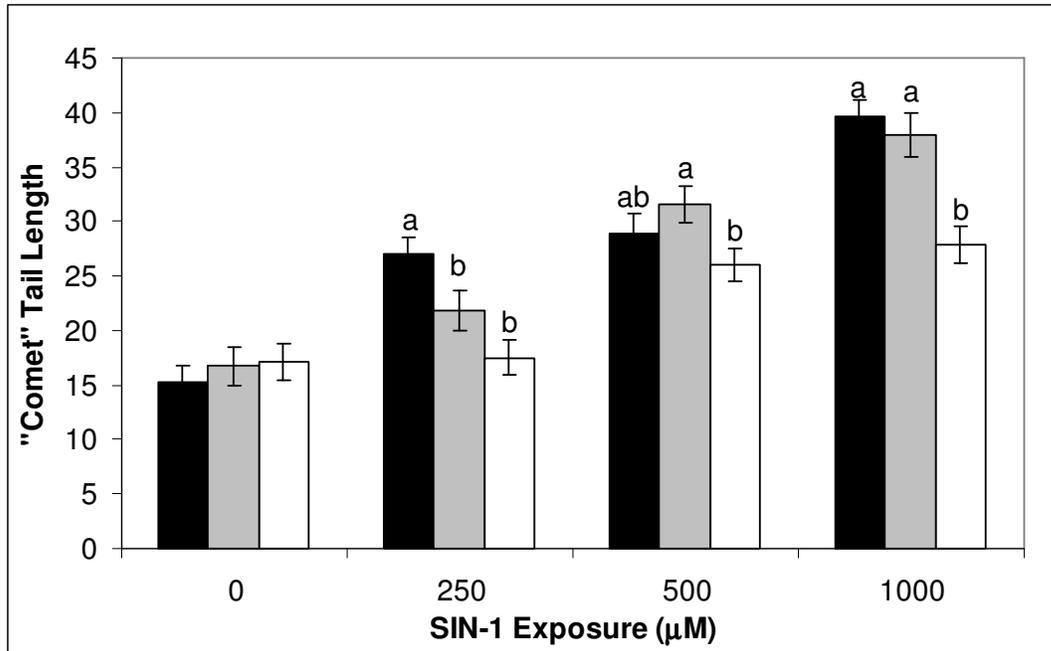


Figure 4. Comet tail length (arbitrary units) of the wild type (black bars), negative control (gray bars) and urate oxidase knockdown (white bars) cell lines exposed to various concentrations of SIN-1. Bars represent means \pm SEM. Letters represent significant differences between cell lines within the same concentration of SIN-1.

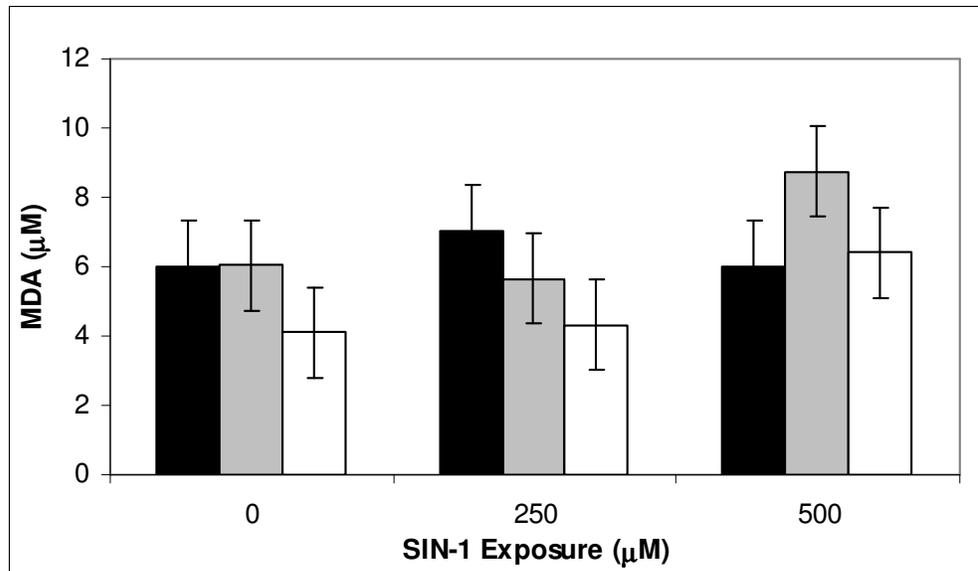


Figure 5. Lipid peroxidation of the wild type (black bars), negative control (gray bars) and urate oxidase knockdown (white bars) cell lines exposed to various concentrations of SIN-1. The concentration of the lipid peroxide, malondialdehyde (MDA, μM), was used as an indicator of lipid peroxidation. Bars represent means \pm SEM, $n=2$.

APPENDIX

Section 1. AASS mRNA Abundance in Isolated Colonies After Initial Transfection

Numerous cell colonies were selected from each transfection since AASS knockdown was expected to be quite variable. Variability in knockdown likely results from promoter interactions with the host genome since the integration into the DNA occurs randomly. The greatest knockdown from each shRNA sequence was reported in the body of the dissertation. Bars in figure 1 through figure 4 represent expanded cell colonies selected for resistance to G418 after transfection with a plasmid for shRNA sequence 1 (figure 1), sequence 2 (figure 2), sequence 3 (figure 3), or sequence 4 (figure 4) expression, except where indicated as wild type. The AASS mRNA abundance in isolated colonies was scaled to wild type levels, which was set at 100%.

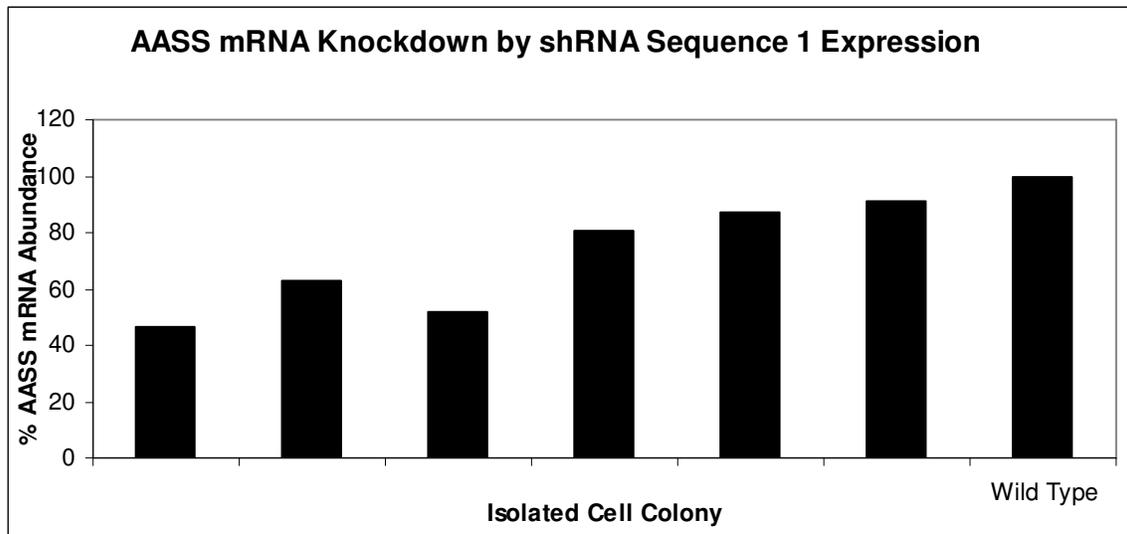


Figure 1.

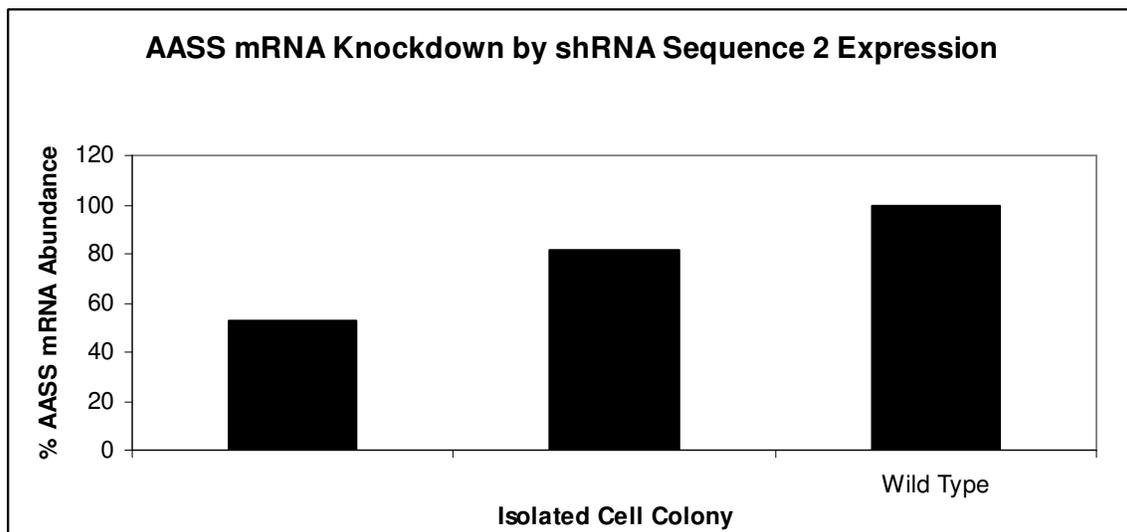


Figure 2.

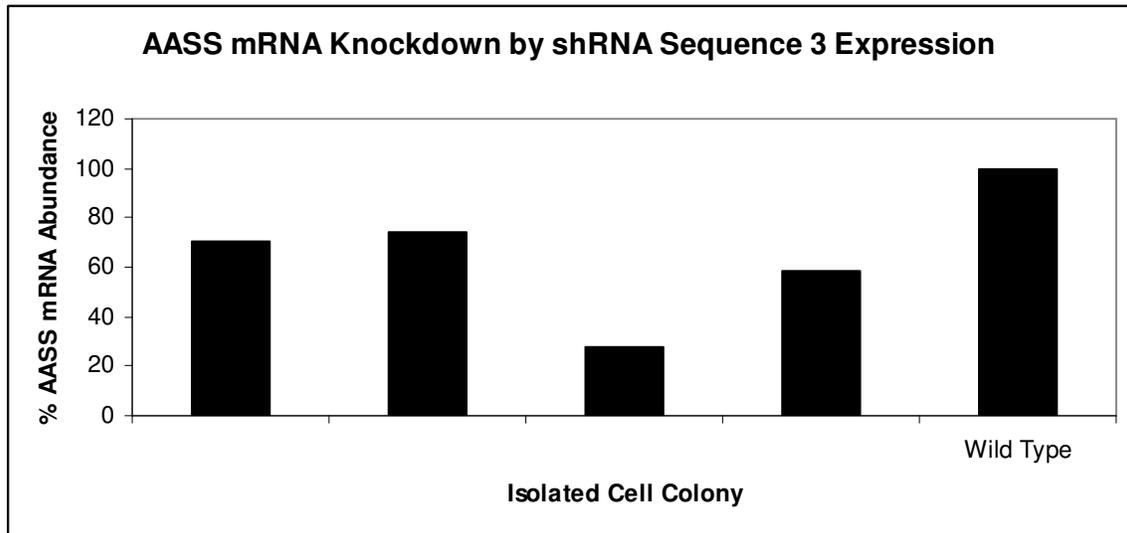


Figure 3.

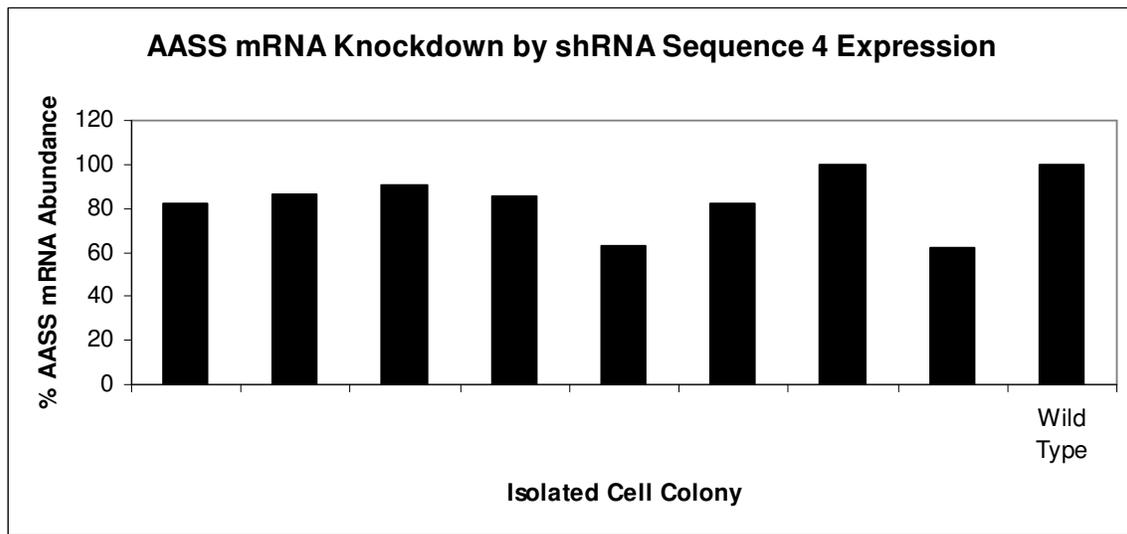


Figure 4.

Section 2. AASS mRNA Abundance in Isolated Colonies After Transfection with Purchased Sequences for AASS Knockdown

New shRNA sequences were purchased (Ambion, Austin, TX) that would, theoretically, knockdown down AASS mRNA 75% or more in at least one of the three sequences. The sequences are as follows:

Sequence 5: 5' - CGCAUUCAAUCCUUUCAAUU - 3'

Sequence 6: 5' - GGAAGUCUUUAAUGAGCUAAU - 3'

Sequence 7: 5' - GGAAUCCGGCUCAUUGAUUU - 3'

Sequences were incorporated into p*Silencer*4.1-CMVneo plasmids and the plasmids were transfected into cells as in the previous experiment. An additional transfection was done that transfected a mix of plasmids that express all three sequences. The bars in the following figures, except when indicated as wild type, represent AASS mRNA abundance in colonies selected for resistance to G418 resulting from transfection of plasmids expressing sequence 5 (figure 5), sequence 6 (figure 6), sequence 7 (figure 7), and sequences 5, 6, and 7 (figure 8). None of the sequences resulted in the expected 75% or greater knockdown in AASS mRNA.

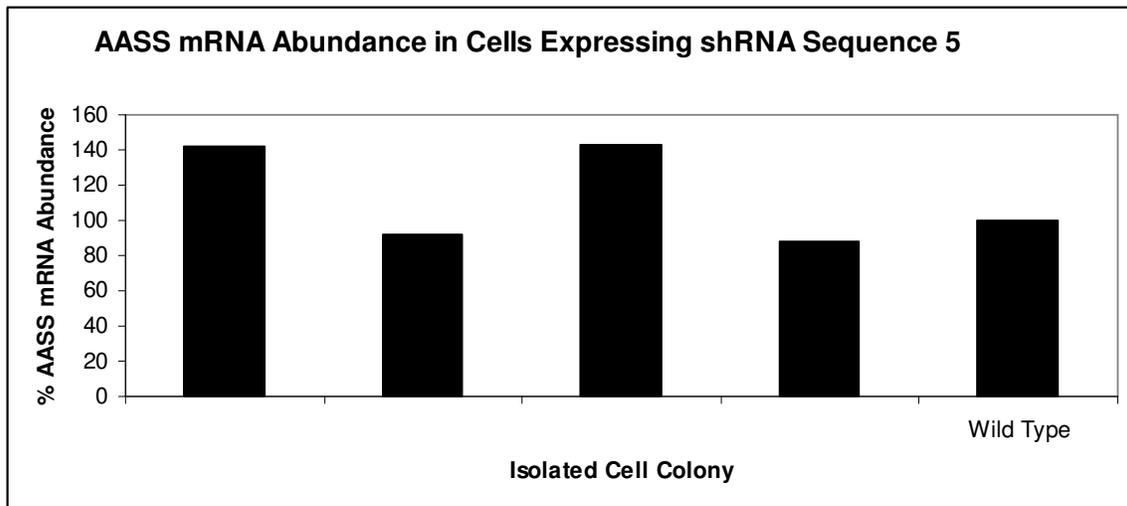


Figure 5.

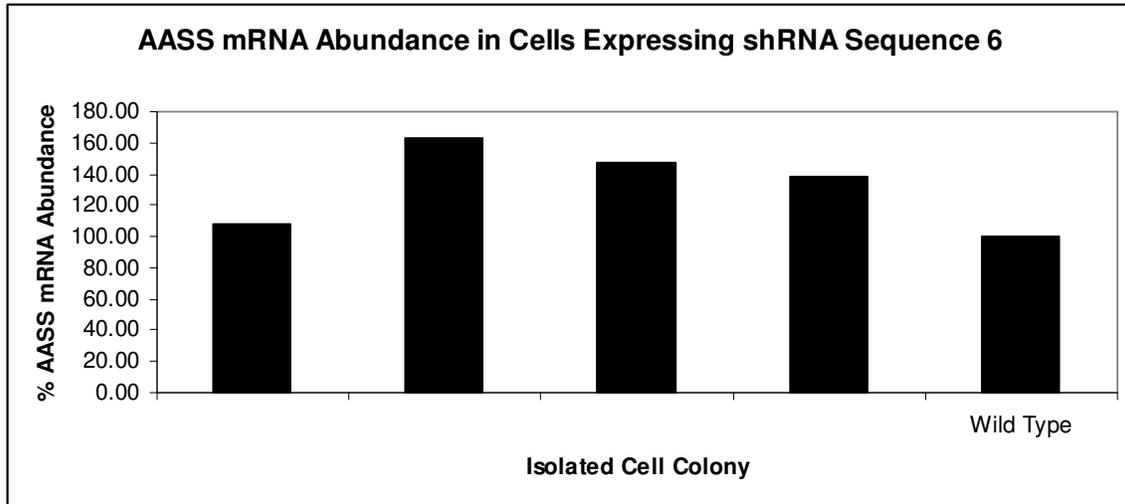


Figure 6.

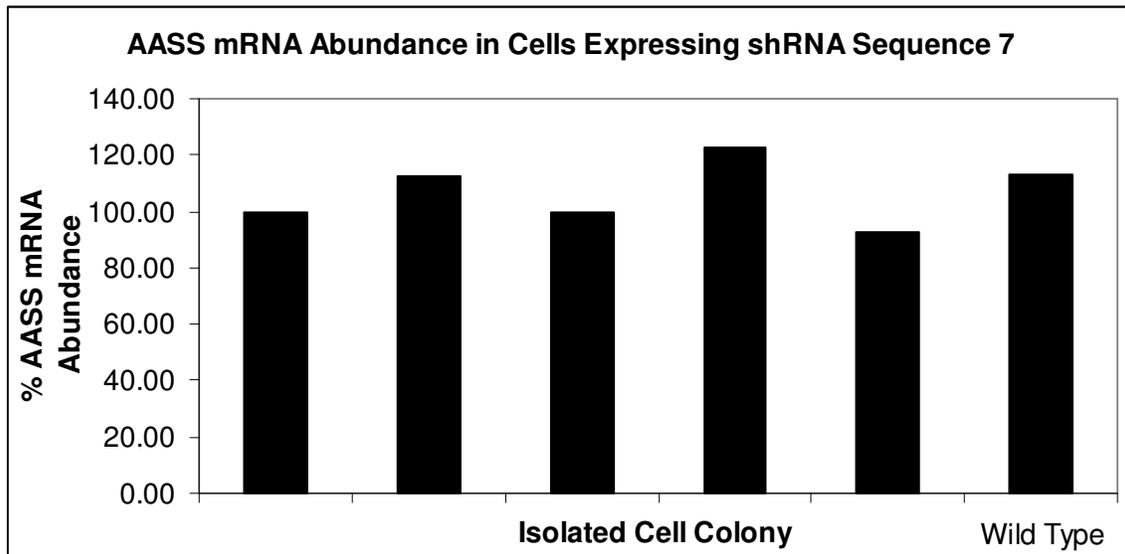


Figure 7.

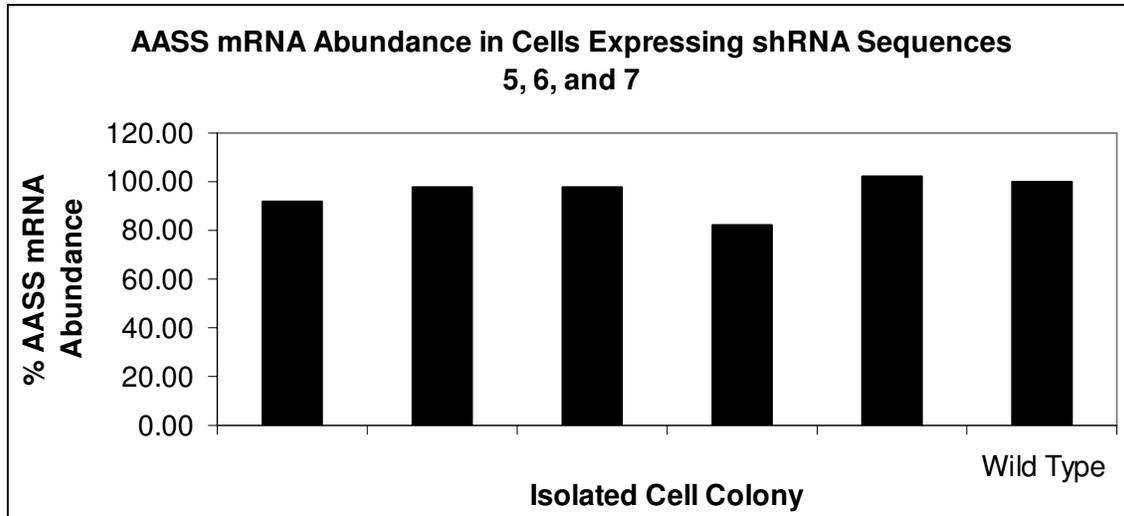


Figure 8.

Section 3. AASS mRNA Abundance After Double Transfection

Since the purchased shRNA sequences (5, 6, and 7) did not achieve levels of AASS mRNA knockdown higher than those attained after the initial transfection, a double transfection approach was utilized. Sequences 1 and 3, which singly resulted in the greatest levels of knockdown, were incorporated into p*Silencer*4.1-CMVpuro plasmids. The p*Silencer*4.1-CMVpuro plasmid expressing shRNA sequence 1 was transfected into the cell colony already expressing sequence 3 from the p*Silencer*4.1-CMVneo plasmid and the p*Silencer*4.1-CMVpuro plasmid expressing shRNA sequence 3 was transfected into the cell colony already expressing sequence 1 from the p*Silencer*4.1-CMVneo plasmid. The bars in figures 9 and 10 represent AASS mRNA levels of colonies selected for puromycin resistance, except where indicated, and analyzed for AASS mRNA knockdown.

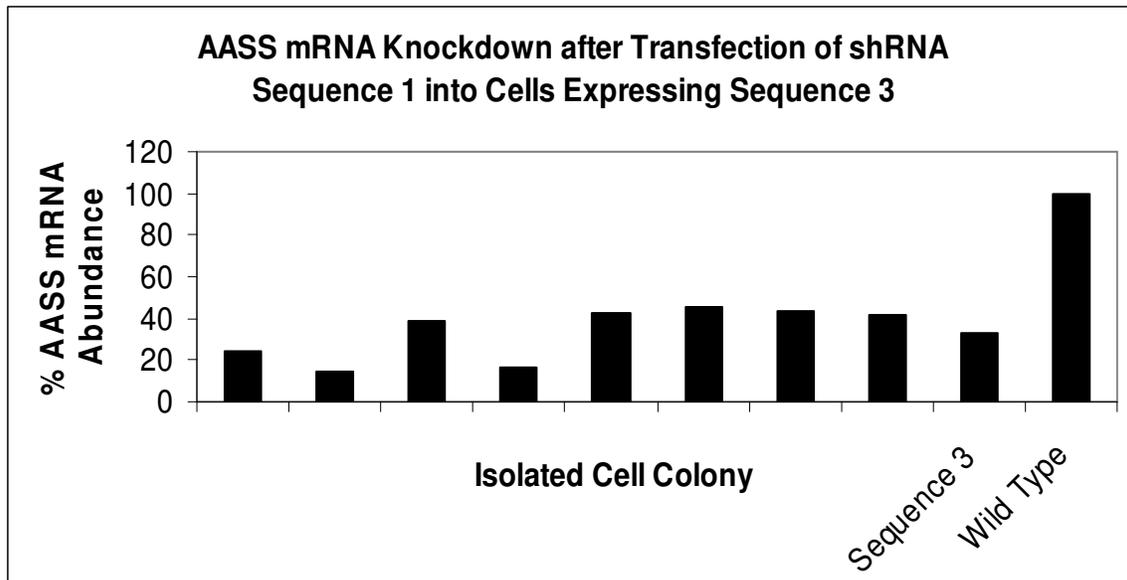


Figure 9.

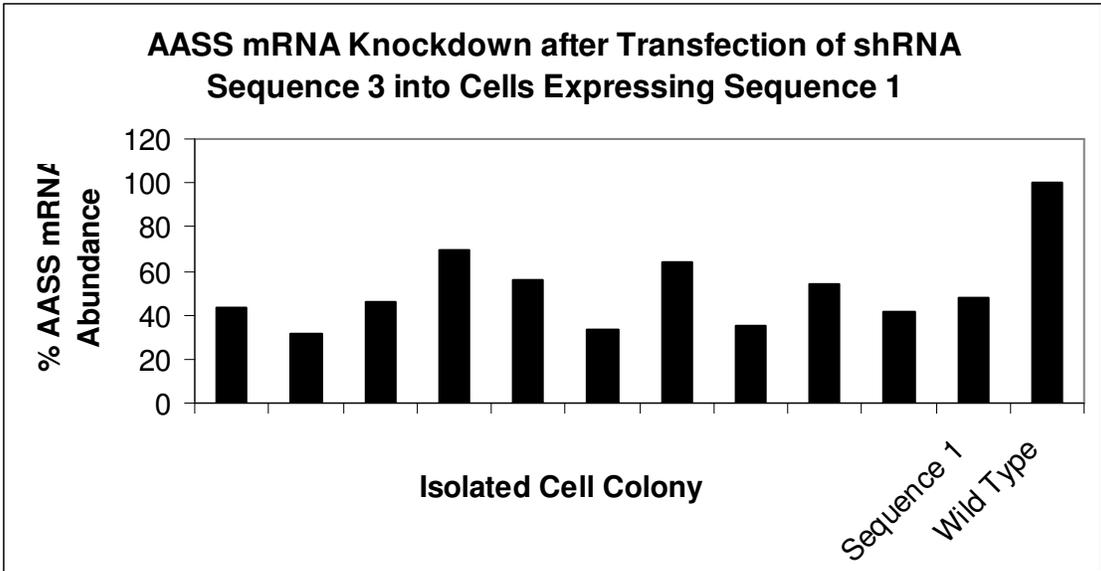


Figure 10.

Section 4. SAS Programs for Data Analysis

Sample SAS Program used to determine treatment effects

```
options pageno=1 pagesize=60;
filename data dde 'excel|sas|r3c8:r36c10';
title 'Cell Growth assay';
data cellgrowth;infile data; input cell conc growth;
proc print;

proc glm data = cellgrowth;
classes cell;
model growth conc= cell;
lsmeans cell/ pdiff stderr;
means cell /lsd lines;
run;
```

Sample SAS Program used for broken-line analysis of lysine requirement

```
Data one; *iso req study;
input x y;
datalines;

;

data fill; *generates;
do x=.0122 to .42 by .01; y=.; output; end;
run;
data one; set one fill; run;
proc sort data=one; by x;
proc nlin data=one; *straight broken-line;
parameters L=.0153 U=-2853 R=.085;
z1=(x<R)*(R-x);
model y = L + U*(z1);
output out=ppp p=predy;
run;

proc gplot;
title2 '2 linear broken lines';
goptions hpos=35 vpos=35 ftext=swiss;
symbol1 v=dot c=black;
plot y*x predy*x/overlay;
run;
```

Sample SAS Program for quadratic-with-plateau analysis of lysine requirement

```
title 'Quadratic model with plateau';
data a;
input x y;
datalines;

;

proc print data=a;

proc nlin;
parms a=.45 b=.05 c=-.0025;

x0=-.5*b / c;
if x<x0 then
model y=a+b*x+c*x*x;
else
model y=a+b*x0+c*x0*x0;

if _obs_=1 and _iter_=. then do;
plateau=a+b*x0+c*x0*x0;
put / x0= plateau= ;
end;
output out=b predicted=yp;
run;

legend1 frame cframe=ligr label=none cborder=black
position=center value=(justify=center);
axis1 label=(angle=90 rotate=0) minor=none;
axis2 minor=none;

proc gplot;
plot y*x yp*x/frame cframe=ligr legend=legend1
vaxis=axis1 haxis=axis2 overlay;
run;
```

Section 5. List of Optimized Real Time RT-PCR Conditions

Optimized Real-Time PCR Programs

- All PCR products confirmed as expected products via sequencing
- All PCR reactions were optimized using BioRad SuperMix Sybr Green
- Except where noted, DNase treat 2 μ g RNA (per Promega protocol), Reverse Transcribe with random primers (per Promega protocol), and dilute cDNA at least 1:4 with nuclease-free water before using 2 μ L cDNA per 20 μ L PCR reaction

Mouse Liver

Acidic Ribosomal Protein

Forward Primer (ARPF2): TGCCCACTCCATCATCAAT

Reverse Primer (ARPR2): CTTTTCAGCAAGTGGGAAGG

Product Length: 83 bases

Efficiency: 1.86

Final Primer Concentration in PCR Reaction: 2.5 μ M

Amino adipate Semialdehyde Synthase

Forward Primer (MLKRF3): TGGAGACTTCAACGGCTTCT

Reverse Primer (MLKRR3): TGGCCCATAGATCTCCTTTG

Product Length: 135 bases

Efficiency: 2.2

Final Primer Concentration in PCR Reaction: 1.25 μ M

Program Protocol:

Step 1: 95 °C for 5 min

Step 2: 95 °C for 15 sec (denaturing)

70 °C for 30 sec (annealing)

72 °C for 30 sec (extension)

Step 3: Repeat step 2 ten times, decreasing the annealing temperature by 1 °C each time.

Step 4: 95 °C for 15 sec

60 °C for 30 sec

72 °C for 30 sec

Step 5: Repeat step 4 thirty times

Pig Liver

Acidic Ribosomal Protein

Forward Primer (PigARP40S2): GCTAAGGTGCTCGGTTCTTC

Reverse Primer (PigARP151A2): GTGCGGACCAATGCTAGG

Product Length: 93 bases

Efficiency: 2.12

Final Primer Concentration in PCR Reaction: 1.25 μ M

Aminoadipate Semialdehyde Synthase

Forward Primer (PLKRF2): ACAGGAACAGCAGTCAGGCT

Reverse Primer (PLKRR2): GGGTCCTATTGACTTTGGCA

Product Length: 86 bases

Efficiency: 2.13

Final Primer Concentration in PCR Reaction: 1.25 μ M

Program Protocol

Step 1: 95 °C for 5 min

Step 2: 95 °C for 15 sec (denaturing)

60 °C for 45 sec (annealing and extension)

Step 3: Repeat step 2 thirty-nine times

Mouse Hepatocytes (ATCC, designation FL38B, catalog #CRL-2390)

Acidic Ribosomal Protein

Forward Primer (ARPF3): CAACCCAGCTCTGGAGAAAC

Reverse Primer (ARPR3): GTGAGGTCCTCCTTGGTGAA

Product Length: 75 bases

Efficiency: 1.99

Final Primer Concentration in PCR Reaction: 1.25 μ M

Amino adipate Semialdehyde Synthase (primers amplify in SDH region)

Forward Primer (MLKRF3): TGGAGACTTCAACGGCTTCT

Reverse Primer (MLKRR3): TGGCCCATAGATCTCCTTTG

Product Length: 135 bases

Efficiency: 1.86

Final Primer Concentration in PCR Reaction: 0.625 μ M

Amino adipate Semialdehyde Synthase (primers amplify in LKR region)

Forward Primer (MLKRF7): AGGGTCTCGGATAGTGGCTT

Reverse Primer (MLKRR7): AGGCGTATGATGTCCCAAAG

Product Length: 106 bases

Efficiency: 1.91

Final Primer Concentration in PCR Reaction: 0.625 μ M

Glyceraldehyde Phosphate Dehydrogenase

Forward Primer (MGAPDHF2): AACTTTGGCATTGTGGAAGG

Reverse Primer (MGAPDHR2): GGATGCAGGGATGATGTTCT

Product Length: 132 bases

Efficiency: 2.05

Final Primer Concentration in PCR Reaction: 1.25 μ M

Mitochondrial Cationic Amino Acid Transporter - 2

Forward Primer (MCATF2): AGGTACCAACCTGGCTTGTG

Reverse Primer (MCATR2): TGGGACTCGCTCTTCAAAGT

Product Length: 98 bases

Efficiency:

Final Primer Concentration in PCR Reaction: 1.25 μ M

Program Protocol:

Step 1: 95 °C for 5 min

Step 2: 95 °C for 15 sec (denaturing)

70 °C for 30 sec (annealing)

72 °C for 30 sec (extension)

Step 3: Repeat step 2 five times, decreasing the annealing temperature by 1 °C each time.

Step 4: 95 °C for 15 sec

60 °C for 30 sec

72 °C for 30 sec

Step 5: Repeat step 4 thirty-five times

Urate Oxidase

Forward Primer (UOXF6): AACAGGGACAGTCAAGAGGAAG

Reverse Primer (UOXR6): GCAGGCAAGCATAACAGTATTCC

Product Length:

Efficiency: 2.08

Final Primer Concentration in PCR Reaction: 1.25 μ M

Urate Oxidase (primers span an intron, used to detect gDNA contamination since samples are not DNase treated)

Forward Primer (UOXF2): TGTGAGGTGGAGCAGATGAG

Reverse Primer (UOXR2): CACCTCAGGGAGGGTAGTGA

Product Length: 132 bases

Efficiency: 2.21

Final Primer Concentration in PCR Reaction: 1.25 μ M

Urate Oxidase (and ARP) Sample Preparation: (described more thoroughly in body of dissertation)

- To isolate RNA: use phenol:chlorophorm solutions to reduce gDNA contamination
- Do not DNase treat RNA
- Use oligo-dT primers for reverse transcription
- Dilute cDNA 1:3 for PCR reaction
- Reaction volume is 50 μ L
- Use 10 μ L cDNA for UOX reaction
- Use 5 μ L cDNA for ARP3 reaction (scale up master mix described above)
- Use PCR protocol listed above

Chicken Liver

Acidic Ribosomal Protein

Forward Primer (CARPF3): TGTGCAGCTGATTAAGACCG

Reverse Primer (CARPR3): GATGTCCAGCACTTCAGGGT

Product Length: 148 bases

Efficiency: 1.99

Final Primer Concentration in PCR Reaction: 2.5 μ M

Lysyl Oxidase

Forward Primer (CLOXF3): CTTATCGGGCGGATGTTAGA

Reverse Primer (CLOXR3): AGGCCTGCTTGGTAGGAAAT

Product Length: 101 bases

Efficiency: 1.95

Final Primer Concentration in PCR Reaction: 1.25 μ M

L-Amino Acid Oxidase

Forward Primer (CAAOF2): TCATCTTGGCCTCCTACACC

Reverse Primer (CAAOR2): GGTGGATGTCCGACAAGTCT

Product Length: 105 bases

Efficiency: 1.94

Final Primer Concentration in PCR Reaction: 1.25 μ M

Amino adipate Semialdehyde Synthase

Forward Primer (CLKRF3): CTTATCGGGCGGATGTTAGA

Reverse Primer (CLKRR3): AGGCCTGCTGGTAGGAAAT

Product Length: 147

Efficiency: 1.91

Final Primer Concentration in PCR Reaction: 1.25 μ M

Program Protocol

Step 1: 95 °C for 5 min

Step 2: 95 °C for 15 sec (denaturing)

61 °C for 30 sec (annealing)

72 °C for 30 sec (extension)

Step 3: Repeat step 2 thirty-nine times

Section 6. Lysine Ketoglutarate Reductase Activity Assay Using Supramax Plus³⁸⁴ Plate Reader

The LKR assay was optimized and demonstrated to be linear within the range of mitochondria indicated in figure 15. Mitochondria from 40×10^6 cells were isolated as described above and resuspended in 60 μL buffer.

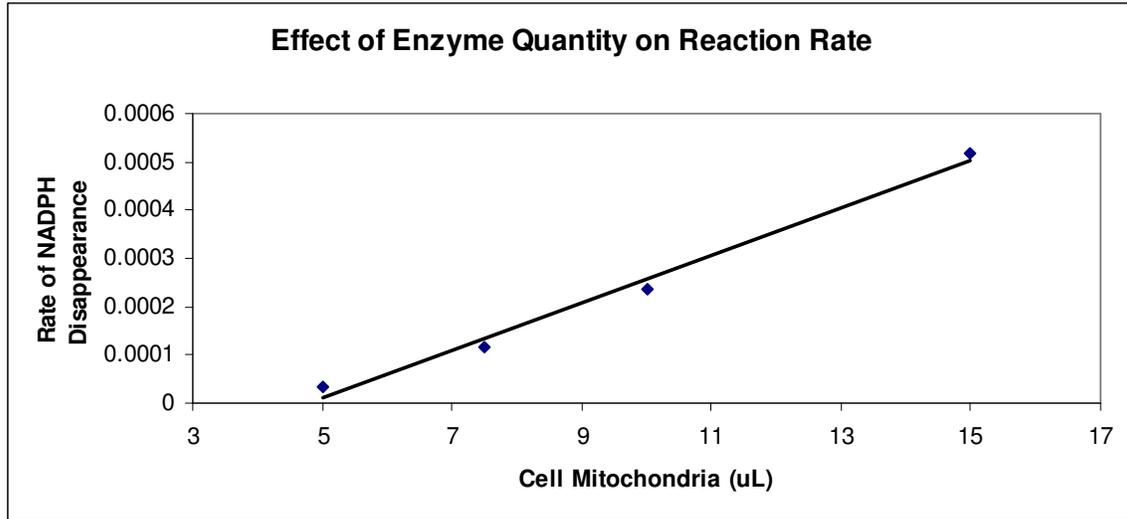


Figure 11.
 $y = 5\text{E-}05x - 0.0002$
 $R^2 = 0.9906$

Section 7. Brightfield Images of Cell Morphology

Brightfield images were acquired using a Nikon TE2000-S inverted microscope with a 20x Plan Fluor objective, a Photometrics CoolSnap HQ CCD camera and the MetaMorph imaging software package. Panels (a) and (d) are shRNA335 cells, panels (b) and (e) are wild type cells, and panels (c) and (f) are shRNANN cells.

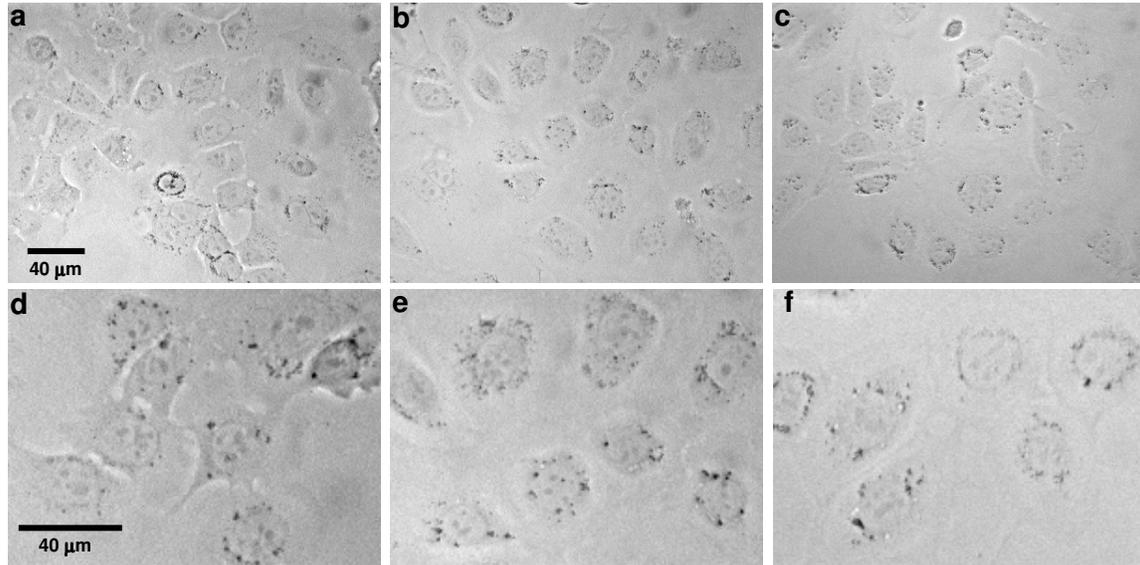


Figure 12.