UNDERSTANDING THE GENETIC REGULATION AND FUNCTION OF SELENOPROTEIN P

by

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ABSTRACT

Selenium is an essential trace element that has been linked to beneficial health effects in multiple disease states. These effects have been attributed to antioxidant activity of selenoproteins; proteins containing selenium incorporated as the amino acid selenocysteine during translation of the protein.

Selenoprotein P is an extracellular glycoprotein containing multiple selenocysteine residues. It is the primary selenium distribution protein of the body as well as the major selenium containing protein in serum. An antioxidant function has been observed for this protein. The experiments presented in this dissertation were designed to further characterize the mechanisms of selenoprotein P regulation and function and test the hypothesis that mechanisms regulating the expression of selenoprotein P provide for modulation of this protein so it may function to provide antioxidant protection in extrahepatic tissues.

When stimulated with ecdysone analogs, selenoprotein P expression was increased with the use of a fusion transcription factor that contains the glucocorticoid receptor DNA binding domain, an ecdysone ligand-binding domain, and a strong transactivation domain as well as the retinoid X receptor. *In silico* analysis of the selenoprotein P promoter identified putative glucocorticoid and retinoid responsive binding sites. Luciferase reporter assays

and quantitative PCR were used to measure selenoprotein P transcription in engineered HEK-293 cells. The native glucocorticoid receptor inhibited selenoprotein P transactivation, and selenoprotein P was further attenuated in the presence of dexamethasone.

These studies also aimed to determine if selenoprotein P possessed hydroperoxidase activity against lipid hydroperoxides generated from the metabolism of arachidonic acid by 15-lipoxygenase-1. Enzymatic reduction of 15-hydroperoxyeicosatetraenoic acid (15-HpETE) by selenoprotein P was observed in a NADPH-coupled biochemical assay. Diphenylpyrenylphosphin was used to measure lipid hydroperoxides in human embryonic kidney cells treated with selenoprotein P following exposure to 15-HpETE. Cellular oxidation increased with 15-HpETE treatment and selenoprotein P reduced this effect. These results suggest that selenoprotein P can function as an antioxidant enzyme during inflammation.

An increased understanding of the mechanisms regulating selenoprotein P expression and activity could provide insight into the way in which selenium exerts its physiological effects.

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AA	arachidonic acid
ApoER2	apolipoprotein E receptor-2
COX	cyclooxygenase
DBD	DNA binding domain
Dex	dexamethasone
DMSO	dimethylsulfoxide
DPPP	diphenylpyrenylphosphin
EcR	*Drosophila* ecdysone receptor
eEFsec	seleneocysteine elongation factor
EtOH	ethanol
EMSA	electrophorectic mobility shift assay
FOXO1a	forkhead box O1a
GATHER	Gene Annotation Tool to Help Explain Relationships
GPx	glutathione peroxidase
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HEK-293	human embryonic kidney cells
HETE	hydroxy-eicosatetraenoic acids
HNF-4α	hepatic nuclear factor 4α

HpETE	hydroperoxy-eicosatetraenoic acids
ICP	inductively coupled plasma spectrometry
LacZ	β-galactosidase
LBD	ligand binding domain
LOX	lipoxygenase
MEME	Multiple EM for Motif Elicitation
$NaSeO_3$	sodium selenite
PCR	polymerase chain reaction
PCG-1α	peroxisomal proliferator activated receptor-γ coactivator 1α
PLPC-OOH	phospholipid hydroperoxide
PonA	ponasterone A
ppm	parts per million
PTM	Pavlidis Template Matching
RARγ	retinoic acid receptor gamma
RRE	retinoid responsive element
ROS	reactive oxygen species
RXR	retinoid X receptor
SBP2	selenocysteine insertion sequence (SECIS) binding protein 2
Sec	selenocysteine
SECIS	selenocystine insertion sequence
SelP	selenoprotein P protein product
SEPP1	selenoprotein P gene

SLA	soluble liver antigen
t-BHP	*tert*-butyl hydroperoxide
TESS	Transcriptional Element Search Software
TFBS	transcription factor binding site
TRED	Transcriptional Regulatory Element Database
$tRNA^{[Ser]Sec}$	selenocysteine specific transfer RNA
VgEcR	synthetic transcription factor based on the *Drosophila melanogaster* ecdysone receptor (EcR)

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CHAPTER 1

RATIONALE FOR EVALUATING REGULATORY AND FUNCTIONAL MECHANISMS OF SELENOPROTEIN P

Introduction

The effects of supplemental selenium intake have been evaluated in multiple chronic and acute diseases, including cancer, cardiovascular disease, and inflammatory conditions such as sepsis, trauma, and burns. The mechanism by which selenium exerts its effects during disease conditions is not completely understood; however, it has been hypothesized to be due to the antioxidant activity of selenoproteins. These proteins contain selenium incorporated as the amino acid selenocysteine (Sec) during translation of the protein. Selenoprotein P (SelP) is an extracellular selenoprotein containing multiple Sec residues. It is the major source of plasma selenium and a majority of the protein is synthesized in the liver for the purpose of selenium distribution. However, the mRNA is detected in almost all tissues, leading to a proposed antioxidant function for the protein.

Evidence exists for changes in plasma selenium levels following glucocorticoid administration, with both increases and decreases observed under

different conditions, and it is believed these changes result from redistribution of selenium between tissue and plasma. Additionally, SelP is decreased in the plasma of critically ill patients, a population that tends to have increased levels of free plasma cortisol. This led to the question of whether there might be a role for the glucocorticoid receptor (GR), a nuclear hormone receptor, in regulating SelP expression.

Data suggests that the antioxidant activity of SelP may be specific for lipid-derived substrates, as opposed to other sources of reactive oxygen stress. Inflammation results in the production of numerous reactive lipid intermediates as a result of arachidonic acid (AA) metabolism by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 pathways. These reactive metabolites can damage cellular macromolecules and contribute to the pathogenesis of multiple disease states, including cancer. The ability of SelP to exert enzymatic activity against these metabolites is unknown; therefore, experiments were designed to determine if SelP displayed lipid hydroperoxidase activity directed at 15-LOX-1-generated metabolites.

The experiments presented in this dissertation were designed to further characterize the mechanisms of SelP regulation and function. An increased understanding of SelP may provide evidence of the mechanisms by which selenium exerts its physiological effects. This knowledge can then be considered and applied in the design and execution of *in vitro,* animal, and clinical studies aimed at examining the beneficial health effects of selenium.

Selenium and Selenoproteins

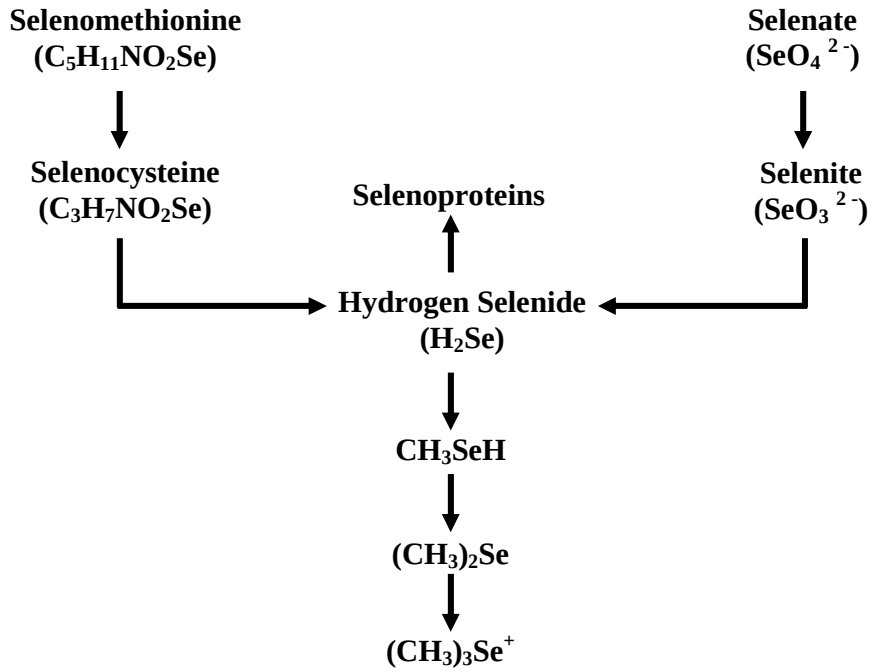
Selenium was first discovered by the Swedish chemist JJ. Berzelius in 1817 (Alissa et al., 2003) and was demonstrated as an essential trace element in 1957 (Schwarz and Flotz, 1957). In 1973, selenium was found to be part of the active site of the enzyme glutathione peroxidase (Rotruck et al., 1973) and the vital role of the element in humans was first documented in a case-study where supplementation was used to successfully treat a muscular dystrophy patient receiving long-term parenteral nutrition (Van at al., 1979).

Selenium is distributed throughout the earth's crust in rocks, minerals, fossil fuels and volcanic material (Lockitch, 1989; Alissa et al., 2003). Foods supply the majority of natural selenium to humans, with selenomethionine as the major dietary form of the element (Tinggi, 2008). Dietary sources of selenium include Brazil nuts, kidneys, meats and fish, and breads and cereals (Alissa et al., 2003; Rayman, 2000). Selenium concentration in food is dependent on the soil content in which plants are grown or animals are raised; therefore, serum selenium levels within human populations vary geographically (Ge and Yang, 1993). Low levels exist in Finland, New Zealand, and regions of China (Salonen et al., 1982; Thomson and Robinson, 1980; Ge and Yang, 1993), while toxic soil levels have been noted in the Enshi County of China (Yang et al., 1989). Estimated selenium intake in the United States ranges from 60-220 μg/day, higher than the daily recommended value of 55μg (Combs, 2001; Bleys et al., 2008; Food and Nutrition Board, Institute of Medicine 2000).

A narrow therapeutic index exists between the essential nutrient effects of selenium and the toxicity of this trace element (Daniels, 1996). Deficiency has been attributed to the development of Keshan disease, a potentially fatal form of cardiomyopathy that was first documented in a selenium-deficient region of northeast China (Ge and Yang, 1993). Alternatively, acute selenium intoxication results in conditions such as hypotension, tachycardia, abdominal pain, pulmonary edema, coma and death, while chronic selenium poisoning presents as alopecia and nail changes (Nuttall, 2006).

Both inorganic and organic selenium are utilized as nutrients in mammals (Alissa et al., 2003) and are readily metabolized to various forms of selenium metabolites (Brenneisen et al., 2005, Ip, 1998) (Figure 1.1). Hydrogen selenide plays a central role in this metabolism (Brenneisen et al., 2005), as inorganic selenate and selenite are reduced to hydrogen selenide by selenoglutathione and glutathione selenopersulfide and the organic compounds selenomethionine and Sec are metabolized to hydrogen selenide by beta-lyase (Ip, 1998). Further metabolism of hydrogen selenide leads to methylation products that are excreted in the breath or urine. Alternatively, hydrogen selenide can serve as a selenium precursor in selenoprotein synthesis (Brenneisen et al., 2005, Ip, 1998).

Selenoproteins are selenium containing proteins in which selenium atoms are incorporated as the amino acid Sec. Sec differs from cysteine in containing a selenium, rather than sulfur, atom. While these two amino acids share many chemical properties, the lower pK_a of Sec makes it much more reactive than cysteine (Tinggi, 2008). Sec residues are encoded by a UGA codon (Berry et al.,



Adapted from Ip 1998

Figure 1.1 Selenium metabolism. Hydrogen selenide (H_2Se) is formed either by reduction of the inorganic compounds selenate and selenite or by metabolism of the organic compounds selenomethionine and selenocysteine. H_2Se serves as a selenium precursor for selenoprotein synthesis or can be methylated to excretion products.

1991). As this codon is typically translated as a termination codon, several factors are required to ensure specific incorporation of Sec. Key players in this process include the Sec specific transfer RNA ($tRNA^{[Ser]Sec}$) (Lee et al., 1989), the Sec insertion sequence (SECIS) found in the 3' untranslated region of selenoprotein mRNAs (Berry et al., 1991), the Sec specific elongation factor (eEFsec) (Fagegaltier et al., 2000), and the SECIS binding protein 2 (SBP2) (Copeland et al., 2000). Additional factors shown to be involved include the ribosomal protein L30 (Chavette et al., 2005), soluble liver antigen (SLA) (Gelpi et al., 1992), and SECp43 (Ding and Grabowski, 1999). These factors work together to form protein complexes that recruit $tRNA^{[Ser]Sec}$ to the mRNA and present the tRNA to the ribosome, allowing for Sec translation rather than termination at the UGA codon (Small-Howard et al., 2006) (Figure 1.2).

Twenty-five selenoproteins have been identified in the human genome thus far; however, the functions of many of these are still unknown (Lu and Holgren, 2009). Functions that have been identified suggest selenoproteins act in a variety of biological processes. The deiodinases are selenoproteins that function in production and activation of thyroid hormones (Kuiper et al., 2005; Bianco and Larsen, 2005). Several selenoproteins are also involved in providing cellular antioxidant defense (Steinbrenner and Sies, 2009). The glutathione peroxidases (GPx) are some of the best characterized antioxidant selenoproteins, protecting cells from reactive oxygen and nitrogen species including hydrogen peroxide, nitric oxide, peroxynitrite, free fatty acid hydroperoxides, and phospholipid hydroperoxides (Klotz et al, 2003; Valko et al., 2006; Brigelius-Flohé and Flohé, 2003). The thioredoxin reductases

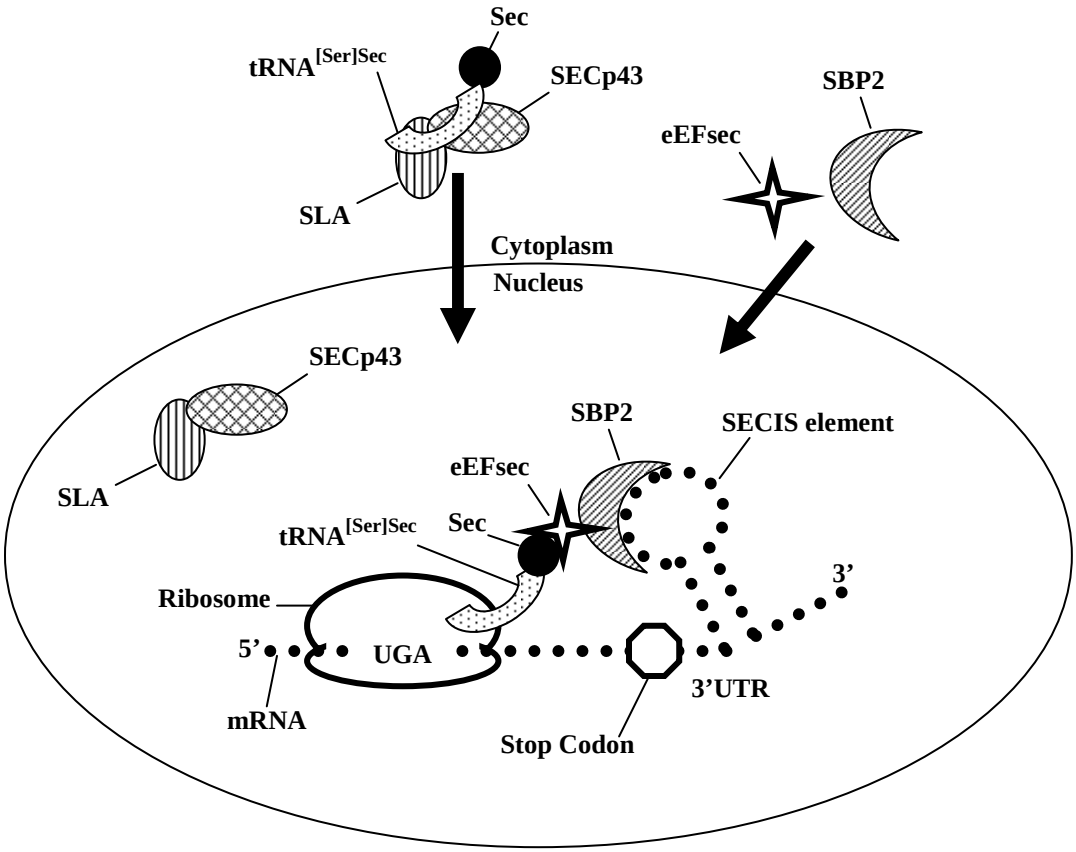


Figure 1.2 Selenocysteine (Sec) translation. Soluble liver antigen (SLA) and SECp43 associate with the Sec transfer RNA ($tRNA^{[Ser]Sec}$) and the complex undergoes nuclear transport, as does the Sec specific elongation factor (eEFsec) and the SECIS binding protein 2 (SBP2). The stem loop structure making the Sec insertion sequence (SECIS) element is found downstream of the stop codon. The SECIS binds SBP2, which subsequently binds eEF_{Sec}. SLA and SECp43 dissociate from $tRNA^{[Ser]Sec}$ and eEF_{Sec} recruits the $tRNA^{[Ser]Sec}$ carrying selenocysteine. The transfer RNA is presented to the ribosome as it translates the UGA codon, allowing for Sec translation rather than termination.

also function in cellular redox homeostasis by reducing thioredoxin and other substrates (Tamura and Stadtman, 1996). Evidence has also suggested an antioxidant function for SelP (Burk et al., 1995; Arteel et al., 1998; Saito et al., 1999; Traulsen et al., 2004). Other selenoproteins that have been less extensively characterized include selenoprotein Sep15, selenoprotein R, and selenoprotein W, which play roles in glycoprotein folding, reduction of methionine sulfoxides, and muscle function, respectively (Lu and Holmgren, 2009; Allan et al., 1999).

A hierarchy exists among selenoproteins for utilization of available selenium (Brigelius-Flohé, 1999). This hierarchy is twofold during selenium-deficiency in that 1) there is preferential tissue expression and activity of selenoproteins and 2) specific selenoproteins are preferentially synthesized within a particular tissue (Gross et al., 1995; Lu and Holmgren, 2009). Preference for selenium retention or accumulation is observed in tissues such as the brain, reproductive organs, and endocrine glands (Burk et al., 1972; Behne et al., 1988), while the activities of most selenoproteins are decreased in the liver, kidney, and lung under selenium deficient conditions (Lu and Holmgren, 2009). Cytosolic GPx mRNA and protein levels decrease rapidly in the liver when selenium levels are low; however, phospholipid hydroperoxide GPx and thioredoxin reductase are maintained at higher levels (Brigelius-Flohé, 1999). In porcine epithelial kidney cells, preference for 5'-deiodinase expression over cytosolic GPx was observed when selenium supply was low (Gross et al., 1995). In the liver of rats, SelP and 5'-deiodinase mRNA levels remain higher than cytosolic GPx when animals were fed a selenium-deficient diet (Hill et al., 1992). Additionally, the level of selenium required to increase plasma

SelP concentration in rats is lower than the level required to restore cytsolic GPx activity following selenium-deficiency (Yang et al., 1989). This hierarchal expression of selenoproteins may be based on biological significance of the protein, with changes in activity likely being the result of a decrease in selenium incorporation into the protein (Gross et al., 1995). Adequate selenium intake is therefore important in maintaining proper translation and function of the selenoproteins (Wingler et al., 1999; Bermano et al., 1995).

Beneficial Health Effects of Selenium

Supplemental selenium intake has been evaluated in multiple chronic and acute diseases (Clark et al., 1996; Mark et al., 2000; Nomura et al., 2000; Brown & Arthur, 2001; Angstwurm & Gaertner, 2006; Angstwurm et al., 2007) and one mechanism by which selenium is hypothesized to exert its beneficial health effects is through the enzymatic activity of selenoproteins. (Diwadkar-Navsariwala & Diamond, 2004; Irons et al., 2006, Diwadkar-Navsariwala, 2006). Meta-analysis has shown reduced all-cause mortality when supplements containing selenium were used (Bjelakovic et al., 2007); however, it appears this effect may be specific to individuals with low plasma selenium levels (Bleys et al., 2008).

Serum selenium levels have been shown to be inversely correlated to the incidence of certain cancers (Clark et al., 1996; Clark et al., 1993; Mark et al., 2000). Dietary supplementation with 200μg/day selenium in the form of enriched yeast has led to decreased cancer mortality and a lower incidence of various types of secondary cancers (Clark et al., 1996). These results led to the Selenium and

Vitamin E Cancer Prevention Trial (SELECT), a double-blind, randomized, placebo-controlled clinical trial designed to determine whether selenium, vitamin E, or the combination could prevent prostate cancer (Lippman et al., 2009). The trial was terminated early when it was found that there was no difference in the rates of prostate cancer between the treatment groups and a nonsignificant increase in diabetes mellitus was observed in the selenium group.

Despite the increased incidence of type 2 diabetes following long-term supplementation with selenium (Stranges et al., 2007; Lippman et al., 2009), there is evidence suggesting potential beneficial effects of selenium in diabetes. Relative selenium deficiency was previously suggested to be associated with diabetes (Rajpathak et al., 2005). Additionally, animal studies have shown that supplementation with low doses of selenium may delay complications of diabetes through an improvement in glucose metabolism (Stapleton, 2000; Sheng et al., 2004; Mueller and Pallauf, 2006). The incidence of vascular complications of diabetes was also shown to be decreased with selenium supplementation (Faure et al., 2004).

Low serum selenium and decreased SelP concentrations have been associated with an increased risk of cerebrovascular events (Koyama et al., 2009). While meta-analysis shows an inverse correlation between selenium concentration and coronary heart disease (Flores-Mateo et al., 2006), no association has been found between selenium levels and cardiovascular mortality (Bleys et al., 2008).

Adequate selenium availability also appears to be important in proper immune function, with supplementation enhancing proliferation and activity of immune cells

(Rayman, 2000). Specifically, plasma selenium levels are significantly decreased in critically ill patients and this correlates with severity of disease (Forceville et al., 1998). A meta-analysis evaluating the effect of antioxidants in critically ill patients revealed a trend toward reduced mortality with selenium supplementation (Heyland et al., 2005), and this was confirmed in the Selenium in Intensive Care trial, which showed a 10.3% reduction in 28-day mortality rate in patients receiving 1000μg sodium selenite per day (Angstwurm et al., 2007).

While selenium is acknowledged as an essential nutrient for humans, the exact role of this element in various disease states is not yet fully understood. Despite the beneficial effects that have been noted under numerous disease conditions, it appears that much of the data are conflicting and inconclusive. Therefore, further studies are required in order to fully elucidate the mechanism and extent of selenium's health effects.

Selenoprotein P

SelP was the second animal selenoprotein to be identified, following cytosolic GPx (Burk and Hill, 2005). It was first discovered in rat plasma in 1977 (Herrman, 1977) and found to contain selenium in the form of Sec in 1982 (Motsenbocker and Tappel, 1982). Purification of the protein was finally achieved in 1987 using immobilized monoclonal antibodies (Yang et al., 1987). Because no function could be attributed to the protein at the time of its discovery, the letter P was used to signify its localization to the plasma (Burk and Hill, 2005).

Genomic sequences have been reported for the both the murine and human SelP gene (SEPP1) (Steinert et al., 1998; Yasui et al., 1996), and cDNA encoding analogs in additional species have been sequenced (Hill et al., 1991; Saijoh et al., 1995; Kryukov and Gladyshev, 2000). SEPP1 appears to be expressed only in vertebrates, as the gene was not found in *Caenorhabditis elegans* or *Drosophila melanogaster* (Kryukov et al., 2003). The human gene is 12kb and contains five exons, with the translational start site in the second exon (Yasui et al., 1996). The gene has ten UGA codons coding for Sec, with one found in the second exon and the remaining nine found in the fifth exon. Two functionally distinct SECIS elements also exist in the fifth exon (Berry et al., 1993). These features are unique to SEPP1, as other selenoproteins contain just one Sec residue and one SECIS element. A complex translational process involving an inefficient decoding step at the N-terminal UGA by the 3'-proximal SECIS is required to ensure the incorporation of multiple Sec residues into SelP (Stoytcheva et al., 2006). This inefficiency at the first UGA seems to serve as a checkpoint at which the presence of components required for Sec incorporation can be verified prior to translation of the remaining nine Sec residues by the additional SECIS element. If conditions are not favorable for Sec translation, such as might occur during selenium deficiency, then the mRNA will undergo nonsense-mediated decay.

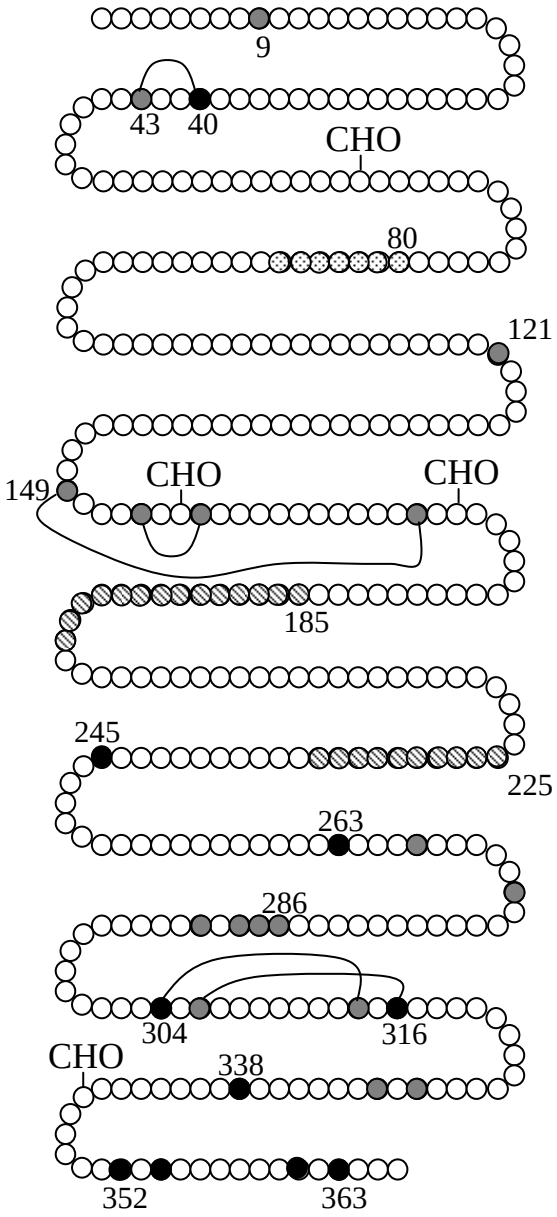
The amino acid sequence of SelP deduced from rat liver cDNA contains 366 residues with a predicted peptide weight of 41,052 Da (Hill et al., 1991). There are 10 Sec, 17 cysteine, and 28 histidine residues within the polypeptide (Read et al., 1990; Hill et al., 1991). Two domains exist with regard to selenium content. The

N-terminal 244 residues include one Sec while the 122 amino acid C-terminal domain contains nine (Saito et al., 2004). Two histidine-rich regions exist at residues 185-198 and residues 225-234, and multiple disulfide and selenenylsulfide linkages are found throughout the protein (Burk and Hill, 2005; Ma et al., 2003, Ma et al., 2005). SelP binds heparin at pH 7.0 and becomes unbound as pH is raised toward 8.5 (Chittum et al., 1996). A motif located at residues 80-95, a region that includes three lysine and three histidine residues, is responsible for the majority of heparin binding (Hondal et al., 2001). Three *N*-glycosylation sites and one *O*-glycosylation site have been identified (Ma et al., 2003). These carbohydrate additions to the protein account for discrepancies in molecular mass measurements between the predicted weight and weight observed by mass spectrometry or SDS-PAGE, which show the native protein at approximately 57,000 Da (Read et al., 1990; Ma et al., 2003) (Figure 1.3).

Protein purified from rat serum showed an average of 7.5 $\pm$ 1 atom of selenium per molecule SelP (Read et al., 1990). Inconsistency between the number of predicted Sec residues and the number of selenium atoms measured in purified protein has been attributed to the existence of multiple SelP isoforms (Himeno et al., 1996; Ma et al., 2002). In addition to the full length protein with ten Sec incorporated, three shorter isoforms have been observed that all share the N-terminus sequence of the full length protein, but terminate at the second, third, or seventh UGA (Ma et al., 2002). Because these isoforms share an N-terminal sequence and only one SEPP1 mRNA is known to exist in mammals, it has been postulated that these three shorter

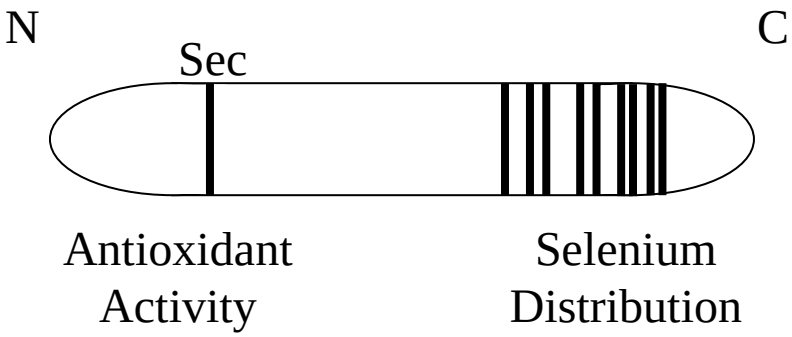
Figure 1.3 Selenoprotein P protein features. (A) Representation of the amino acid sequence of rat selenoprotein P. Selenocysteine residues are shown in black and cysteine residues are shown in gray. The heparin-binding site is indicated by spotted circles and histidine rich regions are shown with hashed circles. CHO indicates *N*- and *O*- glycosylation sites. Selenenylsulfide and disulfide bonds are represented by lines connecting the respective amino acid residues. (B) Two domains exist in regards to the selenium content of selenoprotein P. One Sec residue is found in the N-terminal domain that is believed to be responsible for the antioxidant activity of the protein, while the remaining nine Sec residues are clustered in the C-terminal domain believed to function in selenium distribution.

A



Adapted from Burk & Hill, 2005

B



Adapted from Saito et al., 2004

isoforms result from alternative translation of the UGA codons as Sec or stop codons (Burk and Hill, 2005).

The regulation of SEPP1 expression is an active area of investigation with changes noted under a broad spectrum of biological processes. Promoter activity has been shown to be inhibited by cytokines including interleukin 1β, tumor necrosis factor α, interferon γ, and transforming growth factor β_1 (Dreher et al., 1997; Mostert et al., 2001). Decreased SEPP1 expression has been observed with neoplastic progression from normal tissue to carcinoma to metastatic disease in cells of prostate origin (Dhanasekaran et al., 2001). Evaluation of SEPP1 expression in the Oncomine database (Rhodes et al., 2004) also identifies decreased SEPP1 expression in melanoma, lung, and colon cancer compared to normal tissue suggesting that decreased SEPP1 expression may be a common feature of malignancies. Increased expression has been observed in differentiating myeloid, pulmonary, and Sertoli cells (Tabuchi et al., 2005; Ghassabeh et al., 2006; Wade et al., 2006). Alternatively, promoter activity is stimulated in hepatic cells through a mechanism involving the collaboration of the coactivator peroxisomal proliferator activated receptor-γ coactivator 1α (PCG-1α), the forkhead box transcription factor FOXO1a, and the hepatic nuclear factor 4α (HNF-4α) transcription factor (Speckmann et al., 2008; Walter et al., 2008).

The majority of SelP is derived from hepatic sources; however, the mRNA can be detected in almost all tissues, with appreciable concentrations observed in the kidney, heart, lung, brain, skeletal muscle, and testis (Burk and Hill, 2005). SelP is, for the most part, an extracellular protein; however, intracellular

localization within human astrocytes (Steinbrenner et al., 2006 a) and Purkinje cells (Schweizer et al., 2004) has been reported. The plasma concentration of SelP is approximately 5-6 mg/L in humans (Burk and Hill, 2005) and the protein accounts for more than 50% of the selenium content of human plasma (Saito and Takahashi, 2002). A plasma half-life of 4 hours is consistent with a high turnover rate for this protein (Burk et al., 1991).

SelP is strongly associated with endothelial cells in the liver, kidney, and brain (Burk et al., 1997) and saturable binding to membranes has been observed in various organs (Wilson et al., 1993). Receptor-mediated uptake of SelP has been confirmed in the testis and kidney, where apolipoprotein E receptor-2 (ApoER2) and megalin, are responsible for protein uptake, respectively (Olson et al., 2007; Olson et al., 2008). Additionally, ApoER2 has been suggested to interact with SelP in the brain (Burk et al., 2007). Heparin binding by SelP is also thought to provide a mechanism for localizing or binding SelP to specific structures for the purpose of functioning in distinct biological processes. Specifically, as a result of the pH dependence of heparin binding, SelP may localize under acidic conditions, such as sites of inflammation (Hondal et al., 2001)

SelP appears to be a bifunctional protein with two functionally distinct domains (Saito et al., 2004; Burk and Hill, 2009). The N-terminal domain possesses the first Sec residue and is thought to be responsible for antioxidant activity. The remaining nine Sec are found in the C-terminal domain that is thought to function in selenium distribution (Saito et al., 2004) (Figure 1.3).

SelP functions as a selenium supplier and has been shown to be more effective in supplying selenium to cells than plasma GPx, selenocystine, sodium selenite, or selenomethionine (Saito and Takahashi, 2002). SelP knockout mice display altered selenium distribution, particularly to the testes and brain (Hill et al., 2003; Burk et al., 2006). The knockout phenotype also consists of significant neurological dysfunction and male infertility when the animals are fed a selenium-deficient diet (Hill et al., 2003). Dietary supplementation prevents neurological impairment (Hill et al., 2004); however, male infertility, resulting from structural defects during spermiogenesis, persists regardless of selenium status (Olsen et al., 2005). This suggests that the brain has an alternative mechanism beyond SelP for acquiring selenium, but that the testes do not. The C-terminal region of SelP was shown to be critical in the delivery of selenium to the brain and testes (Hill et al., 2007), lending further support to the selenium distribution function of this domain. Additionally, transgene expression of hepatic SEPP1 in knockout mice restores selenium transport to these two tissues and prevents neurological disturbances and male infertility (Renko et al., 2008).

Biochemical data has supported a role for SelP as a phospholipid hydroperoxidase (Saito et al., 1999; Takebe et al., 2002), with the N-terminal domain implicated in this antioxidant activity (Saito et al., 2004). Further evidence of the antioxidant activity of SelP includes protection against diquat-induced oxidative liver damage in rats (Burk et al., 1995) and inhibition of low-density lipoprotein oxidation (Traulsen et al., 2004). Lipid hydroperoxides have been shown to increase in myofibroblasts when SelP expression is knocked down

(Kabuyama et al., 2007). Additionally, SelP protected against *tert*-butyl hydroperoxide (*t*-BHP)-induced cytotoxicity in endothelial cells and astrocytes when the cells were maintained in selenium deficient medium (Steinbrenner et al., 2006 a; Steinbrenner et al., 2006 b).

Lastly, SelP is also proposed to function as an acute phase protein due to its negative regulation by cytokines (Dreher et al., 1997; Mostert et al., 2001). This hypothesis is supported by the observation that plasma SelP concentration is reduced in septic patients (Hollenbach et al., 2008; Forceville et al., 2009). These patients tend to have increased levels of free plasma cortisol (Hamrahian et al., 2004), introducing the possibility that glucocorticoids could potentially play a role in regulating SelP during the acute phase of inflammation.

Glucocorticoids and Nuclear Receptors

The glucocorticoid receptor is a member of the nuclear receptor superfamily, a family of which there are more than 150 different members ranging across various evolutionary species (Mangelsdorf et al., 1995). Additional receptors of this superfamily include mineralocorticoid, estrogen, progesterone, and androgen steroid receptors, the retinoic acid receptor (Giguére et al., 1987; Petkovich et al., 1987), the retinoid X receptor (RXR) (Mangelsdorf et al., 1990), a receptor for thyroid hormones (Sap et al., 1986; Weinberger et al., 1986), and the *Drosophila* ecdysone receptor (EcR) (Koelle et al., 1991).

These receptors contain three functional domains: a variable N-terminal domain, a DNA-binding domain (DBD), and a C-terminal ligand binding domain

(LBD) (Mangelsdorf et al., 1995). The N-terminal domain contains a strong transactivation domain thought to be involved in gene regulation; the DBD specifies receptor binding to target DNA sequences through two highly conserved zinc fingers; and the LBD ensures specific and selective physiological consequences of receptor activation by mediating ligand recognition (Mangelsdorf et al., 1995; Lu et al., 2006; Kumar and Thompson,1998).

A key characteristic of these receptors is their ability to function as ligand-inducible transcription factors (Germain et al., 2006; Teboul et al., 2008). In the absence of ligand, the GR remains localized to the cytoplasm, where is bound by various chaperone proteins. Ligand binding results in conformational changes that release chaperone proteins and induce translocation of the receptor to the nucleus (Schimmer and Parker, 2009). The receptor then dimerize as homo- or hetero-dimers, recruit transcriptional coactivator proteins, and bind DNA response elements composed of two core hexameric motifs (Schimmer and Parker, 2009; Aranda and Pascual, 2001). This mechanism allows for modulation of gene expression, with target genes dictated by cell and tissue specific conditions (Lu et al., 2006) (Figure 1.4).

The GR, as well as other steroid receptors, bind to palindromes of the consensus sequence AGAACA. The consensus sequence for non-steroid nuclear receptors is AG(G/T)TCA (Aranda and Pascual, 2001). Multiple nuclear receptor types can bind these sequences and mediate transcriptional activity, allowing for differential control of overlapping gene networks (Bedo et al., 1989; Umesono et al., 1991). Additionally, a single GR acting through a half-site of the typical

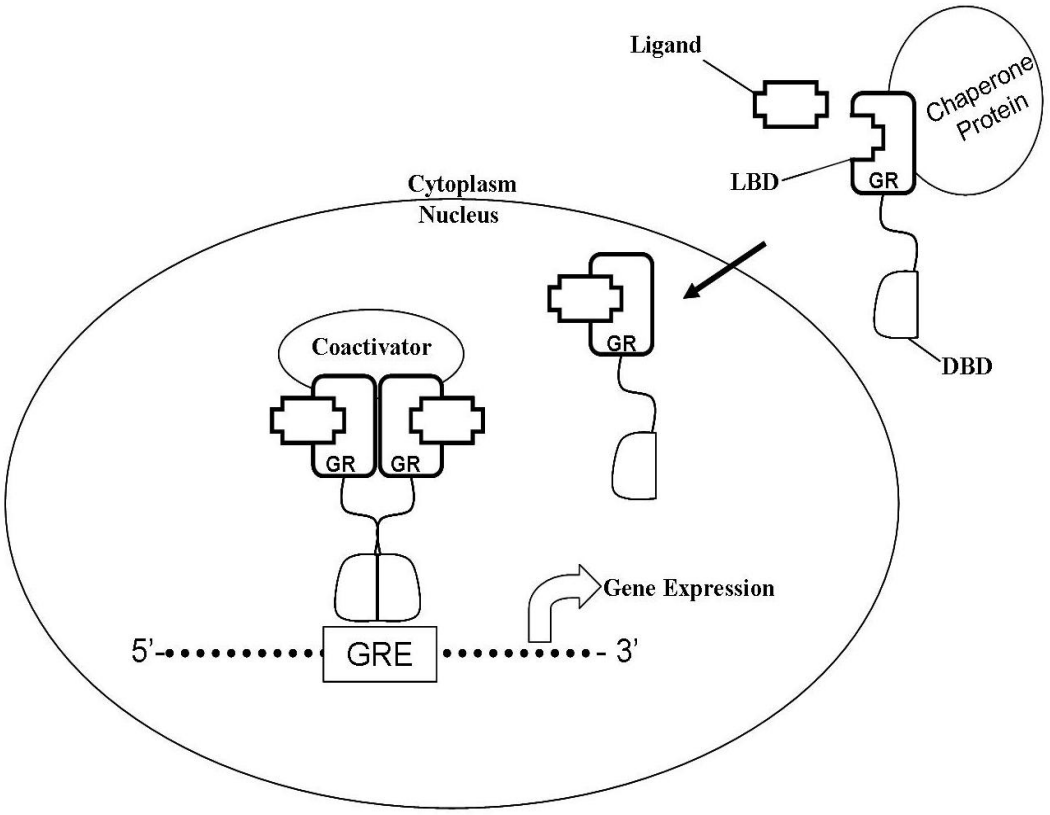


Figure 1.4 Mechanism of glucocorticoid receptor signaling. The GR consists of a ligand binding domain (LBD) and DNA binding domain (DBD). Under basal conditions, the receptor is maintained in the cytoplasm through interactions with chaperone proteins. Ligand binding results in loss of chaperone protein interactions and translocation of the receptor into the nucleus. Once in the nucleus, the receptors dimerize, recruit coactivator proteins, and bind to glucocorticoid response elements (GRE) in the promoter region of target genes. Resulting modulation of gene expression produces the varying physiological and therapeutic effects of glucocorticoids.

glucocorticoid response element (GRE) has been shown to be sufficient for activity when acting synergistically with other transcription factors or when the response element is located in close proximity to the TATA box (Strähle et al., 1988).

Alternative splicing leads to multiple isoforms of the GR, with GRα and GRβ among the best characterized (Lu and Cidlowski, 2005). The GRα isoform appears to be responsible for inducing transcription of target genes through its ability to bind both ligands and GREs. Alternatively, the GRβ isoform does not bind DNA despite being capable of dimerization. Heterodimerization of GRβ with GRα interferes with the function of the α isoform (Lu and Cidlowski, 2004). The GR is expressed in almost all tissues; however, tissue-specific expression of the isoforms has been observed (Pujols et al., 2001; Oakley et al., 1996; Lu and Cidlowski, 2005).

Glucocorticoid synthesis and secretion occurs in the adrenal cortex through a process regulated by negative feedback on the hypothalamic-pituitary-adrenal axis. Cortisol is the major endogenous glucocorticoid in humans and a total of approximately 10 mg is secreted daily. Secretion occurs in a diurnal manner with total serum concentration ranging from 16 μg/dL in the morning to 4 μg/dL in the evening (Schimmer and Parker, 2009). Cortisol is highly protein bound in the plasma and only free circulating cortisol is considered to be biologically active (Mueller and Potter, 1981; Rhen and Cidlowski, 2005). Consequentially, alterations in serum protein levels can increase the availability of free cortisol capable of exerting activity on target cells (Hamrahian et al., 2004).

Through their ability to modulate gene expression, nuclear receptors play a pivotal role in some of the most fundamental aspects of physiology (Lu et al., 2006). Specifically, glucocorticoids work in regulation of carbohydrate, protein, and fat metabolism; preservation of normal function of the cardiovascular system, the immune system, the kidney, skeletal muscle, the endocrine system, and the nervous system; and protection against stressful stimuli such as injury, hemorrhage, severe infection, major surgery, hypoglycemia, cold, pain, and fear. Glucocorticoids also play a vital role in growth and development (Schimmer and Parker, 2009).

As a result of their diverse biological activity, glucocorticoids are used therapeutically in several disease states. Chemical modification of the cortisol molecule has led to the development of synthetic glucocorticoids, such as prednisone, methylprednisolone, and dexamethasone (Dex), which have characteristics favorable for therapeutic use, including increased specificity, potency, and duration of action. With the exception of treatment for adrenal insufficiency, the therapeutic use of glucocorticoids is considered empirical (Schimmer and Parker, 2009). Immunomodulatory activity of glucocorticoids lends to their use in infections, allergies, pulmonary disease, and inflammatory conditions (Lu et al., 2006; Schimmer and Parker, 2009). They are also used in the treatment of certain leukemias and added to chemotherapeutic regimens for their antiemetic, antiedema, and palliative properties (Schimmer and Parker, 2009). Prolonged therapy can cause serious side effects, including immunosuppression, osteoporosis, glaucoma, metabolic syndrome, impaired development, and

psychological disturbances (Rhen and Cidlowski, 2005; Schimmer and Parker, 2009).

Evidence exists for changes in plasma selenium levels following glucocorticoid administration, with both increases and decreases noted under different sets of conditions (Peretz et al., 1987; Marano et al., 1990; Watanabe et al., 1997). Although the effect of glucocorticoids on selenium levels has not been fully characterized, it is believed that these changes result from redistribution of selenium between tissue and plasma. Tissue-specific modifications in selenium concentration have been observed in mice treated with Dex, with increases seen in the plasma and cerebrum, decreases observed in the liver, and no effect observed in the kidney, muscle, heart, cerebellum, or brain stem (Watanabe et al., 1997). Selenium redistribution to high priority organs has also been proposed as a mechanism for changes in plasma selenium observed in critically ill patients (Forceville et al., 1998) and these patients tend to have increased levels of free plasma cortisol (Hamrahian et al., 2004). It is unknown what role SelP may play in glucocorticoid-induced selenium redistribution; however, a reduction in plasma SelP concentration is observed in septic patients (Hollenbach et al., 2008; Forceville et al., 2009).

Inflammation and Oxidative Stress

Inflammation occurs in response to tissue injury resulting from insults such as infection or mechanical injury (Burke et al., 2009). It is a localized response aimed at destroying, diluting, or walling-off the site of injury (Gallin and Snyderman,

1999). Activation and migration of leukocytes to the site of damage is a hallmark feature of inflammation (Coussens and Werb, 2002; Gallin and Snyderman, 1999). Cytokines including tumor necrosis factor α and transforming growth factor β work to orchestrate the inflammatory response through chemoattraction of specific leukocyte populations and phagocytic cells (Burke et al., 2009; Coussens and Werb, 2002).

During this process, cytokines, as well as other inflammatory stimuli, also initiate the release of polyunsaturated fatty acids, such as arachidonic acid (AA), from membrane phospholipids (Fitzpatrick and Soberman, 2001). Once released from the cell membrane by phospholipase A_2 enzymes, these lipids are metabolized to bioactive eicosanoids through one of four separate pathways (Smyth and FitzGerald, 2009) (Figure 1.5). Prostaglandin synthesis occurs through metabolism by the cyclooxygenase (COX) pathway (Parente and Perretti, 2003). The 5-, 12-, and 15-lipoxygenase (LOX) enzymes metabolize AA to hydroperoxy-eicosatetraenoic acids (HpETE), which are rapidly converted to hydroxy derivatives (HETEs) and leukotrienes (Natarajan and Nadler, 2004; Sordillo et al., 2008). AA is converted to hydroxy- and epoxy-eicosatrienoic acids by specific cytochrome P450 isozymes and the isoeicosanoids are formed by nonenzymatic peroxidation of AA (Smyth and FitzGerald, 2009).

The oxidative metabolites produced by these pathways are known as reactive oxygen species (ROS). In addition to the lipid radicals formed from AA, alternative ROS include the superoxide anion, hydroxyl radicals, hydrogen peroxide, peroxynitrite, and singlet oxygen (Steinbrenner and Sies, 2009). At low

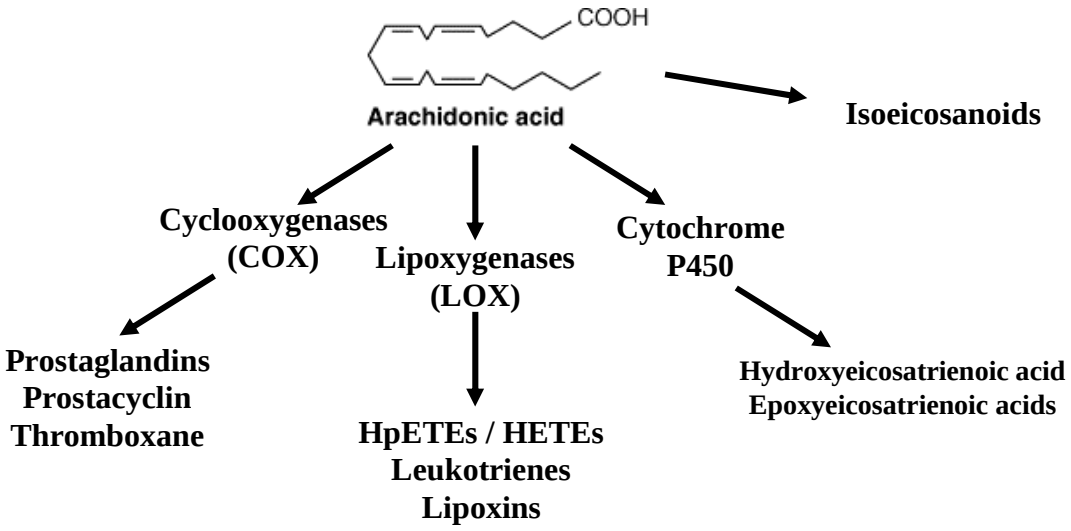


Figure 1.5 Arachidonic acid metabolism. Nonenzymatic peroxidation of AA produces isoeicosanoids. Oxidation by COX, LOX, or cytochrome P450 enzymes results in the generation of various bioactive eicosanoids.

levels, ROS can modulate signal transduction pathways; however, an overabundance of ROS can lead to oxidative stress that damages cellular macromolecules including DNA, protein, and lipids (Brenneisen et al., 2005). For this reason, ROS have been linked to the pathogenesis of multiple diseases, including cardiovascular disease, neurodegenerative diseases, and cancer (Steinbrenner and Sies, 2009).

Excessive inflammation resulting from a prolonged inflammatory reaction or abnormal recognition of an injury is therefore considered a risk factor for disease development as a result of ROS formation (Coussens and Werb, 2002; Gallin and Snyderman, 1999). Specifically, a relationship between chronic inflammation and carcinogenesis has been noted in numerous malignancies, including colon cancer and hepatocellular carcinoma (Itzkowitz et al., 2004; Macarthur et al., 2004). It is believed that leukocytes and other phagocytic cells involved in the inflammatory process may lead to an induction of DNA damage in proliferating cells through the production of reactive oxygen and nitrogen species (Maeda and Akaike, 1998; Coussens and Werb, 2002). Additionally, enzymes expressed during the inflammatory process have been shown to be upregulated in certain cancers (Gupta et al., 2001; Kelavkar et al., 2000) and membrane lipids released and metabolized during inflammation have been linked to various malignancies, including prostate cancer (Hursting et al., 1990). In addition, end products of lipid peroxidation have been implicated as being mutagenic (Ray et al., 2002), further contributing to evidence that inflammation may result in carcinogenesis through its ability to increase the oxidative tone of the cellular environment.

Cells possess a series of enzymatic and non-enzymatic antioxidant systems for detoxifying ROS and repairing the oxidative damage they cause. In addition to superoxide dismutases and catalase, the selenoproteins, and specifically the glutathione peroxidases, are among the most important intracellular antioxidant enzymes (Steinbrenner and Sies, 2009). An antioxidant function has been observed for SelP (Burk et al., 1995; Arteel et al., 1998; Saito et al., 1999; Traulsen et al., 2004), and while this activity seems to be specific for phospholipid hydroperoxides versus other forms of oxidative stress, it is unknown whether SelP exerts this effect against AA metabolites formed during inflammation.

Research Objectives

Selenoprotein P is an extracellular glycoprotein that functions both in selenium distribution and has antioxidant activity. The following studies were designed to further characterize this protein, both in regards to mechanisms regulating expression and antioxidant function.

Chapter 2 describes the *in silico* evaluation of putative transcription factor binding sites within the selenoprotein P promoter. The results of this evaluation were confirmed *in vitro* with the use of luciferase reporter assays, quantitative polymerase chain reaction, and electrophoretic mobility shift assay. These experimental approaches aimed to characterize the regulation of selenoprotein P through glucocorticoid response elements and the results are described in Chapter 3 of this dissertation.

Chapter 4 outlines work aimed at determining the role of selenoprotein P in regulating the cellular oxidative stress induced by reactive hydroperoxylipid intermediates. Biochemical reduction of 15-HpETE by SelP, as well reduction of lipid hydroperoxides in cells exposed to 15-HpETE were evaluated.

Collectively, the studies presented were aimed at testing the hypothesis that mechanisms regulating the expression of selenoprotein P provide for modulation of this protein so it may function to provide antioxidant protection in extrahepatic tissues.

Major Research Findings

Chapter 2

Electronic database analyses were able to identify multiple putative transcription factor binding sites in the selenoprotein P promoter. Specifically, glucocorticoid and retinoid responsive elements that could be involved in gene induction by the fusion transcription factor VgEcR were identified.

Chapter 3

When stimulated with ecdysone analogs, selenoprotein P expression was increased with the use of a fusion transcription factor that contains the glucocorticoid receptor DNA binding domain, an ecdysone ligand-binding domain, and a strong transactivation domain as well as the retinoid X receptor. The native glucocorticoid receptor inhibited selenoprotein P transactivation, and selenoprotein P was further attenuated in the presence of dexamethasone. Putative glucocorticoid

and retinoid responsive elements in the selenoprotein P promoter were responsible for the observed transactivation.

Chapter 4

Enzymatic reduction of 15-HpETE by selenoprotein P was observed in a NADPH-coupled biochemical assay. Lipid hydroperoxides increased with 15-HpETE treatment of cells, and SelP reduced this affect both when the protein and metabolite were added simultaneously, and in a transcellular assay when 15-LOX-1 is metabolically active.

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CHAPTER 2

DISCOVERY OF PUTATIVE TRANSCRIPTION FACTOR BINDING SITES IN SELENOPROTEIN P USING ELECTRONIC DATABASE ANALYSIS

Introduction

Gene regulation at the transcriptional level is activated or repressed by binding of transcription factors to short DNA sequences known as transcription factor binding sites (TFBS) (Novina and Roy, 1996; Sandelin and Wasserman, 2004). These sites typically range in size from ~5-12 base pairs and can show significant variability in sequence, while still remaining a functional binding site for transcription factors (Sandelin et al., 2004). Difficulty arises when attempting to discover these regulatory regions within the promoter region of a gene of interest, as binding sites tend to be short and degenerate, while being widely distributed over several thousand base pairs. Additionally, promoter regions in general can often be difficult to precisely identify (Sandelin et al., 2004; Bailey et al., 2006). Therefore, multiple computational systems have been developed to aid in identifying putative TFBS within a given DNA sequence (Elnitski et al., 2006).

When used in concert, these systems can work synergistically to provide multiple lines of evidence regarding the regulatory mechanisms controlling expression of a gene or a series of genes. TRED (Transcriptional Regulatory Element Database) is a database of both *cis*- and *trans*- regulatory elements that serves as a resource for studying gene regulation and function. TRED cross-references with other databases, including PubMed, GenBank, GeneCards, and TRANSFAC, so as to provide users with more complete information regarding genes of interest (Jiang et al., 2007). TRANSFAC is a complementary database to TRED, providing factor-site interaction data for multiple species (Matys et al., 2003). TESS (Transcriptional Element Search Software) is a web-based software tool that uses TRANSFAC as a source of raw data about transcription factors and preprocesses the TRANSFAC files to create indexed tables that are easier to access and analyze. TESS works to align model binding sites with a user-defined DNA sequence, allowing for identification of possible TFBS in DNA sequences (Schug and Overton, 1997).

GATHER (Gene Annotation Tool to Help Explain Relationships) integrates information from multiple data sources so as to elucidate a biological context for molecular signatures produced from high-throughput assays, such as microarrays (Chang and Nevins, 2006). This creates annotations that identify potential shared regulatory mechanisms and functions among the genes of a particular molecular signature. The inclusion of a Bayesian statistical model provides a novel analytical method that increases the accuracy of annotations defined by GATHER. A positive Bayes factor indicates that evidence supports the association between the annotation

and the signature, and the magnitude corresponds to the strength of the evidence for the association, where higher values are stronger. A Bayes factor of at least 6 has been recommended to represent a significant annotation (Chang and Nevins, 2006).

MEME (Multiple EM for Motif Elicitation) allows for the discovery of signals, or "motifs", within DNA sequences of interest (Bailey and Elkan, 1994). Users may evaluate a set of sequences for shared sequence signals, with these signals potentially indicating TFBS shared among coexpressed genes. Identification of these motifs is accomplished by searching for repeated, ungapped sequence patterns occurring within the user-defined sequences (Bailey et al., 2006). Results are presented as block diagrams, which show the relative positions of the motifs in each of the input sequences, as well as which positions in the motif, displayed as columns, are most highly conserved. Columns are colored according to the majority category of the letters occurring in that column of the alignment and a multilevel consensus sequence is created based on these probabilities (Bailey, 2002). The amount of information contained in each position of the motif is measured in bits, with highly conserved positions having high information and positions where all bases are equally likely having low information. A sum of the information content for each position of the motif provides the total information content of the motif, which serves as a measure of the usefulness of the motif in database searches such as TESS. (Bailey and Elkan, 1994; Bailey, 2002).

Use of these *in silico* methods can aid in the identification of potential binding sites within the promoter region of a gene, but the discovery of such sites does not necessarily mean that the site will prove to be a functional regulatory element either

in vitro or *in vivo*. Phylogenetic footprinting is a technique that can be used to improve the detection of functional elements in DNA sequences (Sandelin et al., 2004; Zhang and Gerstein, 2003). This technique is based on the concept that the selective pressure of evolution will produce a preferential conservation of functional regulatory regions in noncoding gene sequences than in regions that have no sequence-specific function. Therefore, if a sequence has remained highly conserved during evolution, it is likely that this sequence is functional (Duret and Buchert, 1997). ConSite is a web-based tool that uses this type of comparative sequence analysis in the identification of regulatory regions (Lenhard et al., 2003; Sandelin et al., 2004). The major components of the ConSite analysis include aligning the orthologous input sequences, calculating the degree of conservation in the alignment, scanning the sequences for transcription factor binding profile models, filtering the initial sets of sites using phylogenetic footprinting, and presenting the results in user-selected output formats (Sandelin et al., 2004).

When used together, each of the databases described above can serve overlapping and complementary functions that provide powerful evidence of mechanisms regulating expression of a gene of interest. This chapter describes the use of these computational tools for the identification of potential regulatory regions in the selenoprotein P (SEPP1) promoter. Coexpressed genes were identified through microarray analysis and the promoter sequences of these genes were entered into the databases in order to identify homologus motifs, determine potential TFBS, and search the potential binding sites for evolutionary

conservation. When taken together, this evidence provides specific gene regions that can be used in further experimental analysis.

Methods

Materials

The human embryonic kidney (HEK-293) cell line was purchased from American Type Culture Collection (Manassas, VA). Advanced DMEM, CD-293, pVgEcR, zeocin and geneticin were purchased from Invitrogen (Carlsbad, CA). Ovalbumin was purchased from Fisher Scientific (Pittsburgh, PA). Ponasterone A was purchased from A.G. Scientific (San Diego, CA). Arachidonic acid was purchased from Nu-Check Prep (Elysian, MN). RNeasy Mini Kit was obtained from Qiagen (Valencia, CA).

Cell Culture

pVgEcR encodes the fusion transcription factor used to generate ecdysone-inducible cells. HEK-293 cells were transfected with pVgEcR and selected for zeocin resistance to generate stable expression of the VgEcR gene product. Ecdysone-inducible cells that conditionally express 15-LOX-1, ΔIle^{662} 15-LOX-1, or 12-LOX have been previously described (Yu et al., 2004; Cordray et al., 2007). Conditional expression of β-galactosidase (LacZ) in HEK-293 cells was achieved using similar methods. These engineered cells were maintained at 37°C in a humidified incubator with 5% CO_2 in Advanced DMEM supplemented with 2% fetal bovine serum and 2mM L-glutamine.

cDNA Microarray

Microarray experiments were performed using Agilent 44K (human whole genome) oligonucleotide microarrays (Agilent, Santa Clara, CA) and processed on site in the Microarray Resource located within the Huntsman Cancer Institute. Engineered HEK-293 cells were plated on a 6-well plate at a concentration of $5x10^5$ cells/well in CD-293 medium containing 2 mM L-glutamine and 0.1% ovalbumin. After 24 hours cells were treated with 10 μM ponasterone A (PonA) and incubated for 24 hours at 37^0C to induce expression of 15-LOX-1, ΔIle^{662} 15-LOX-1, 12-LOX, or LacZ. Cells were then treated with 20μM arachidonic acid (AA) for 4 hours prior to RNA collection. Sufficient total RNA was recovered using the Qiagen RNeasy minikit protocol and RNA concentration was determined with a Nanodrop spectrophotometer for the gene expression analysis. The quality of the RNA was monitored using an Experion automated electrophoresis station (BioRad, Hercules, CA) with standard sensitivity RNA chips. Agilent labeling kits were utilized to amplify and generate Cy-dye labeled cRNA for hybridization to Agilent oligonucleotide arrays. The samples from ponasterone A, arachidonic acid, and ponasterone A and arachidonic acid combination treated cells were all labeled with Cy-5 and compared against the Cy-3 labeled EtOH control.

Transcript levels were assessed on each channel and quantified by Agilent Feature Extraction software. This software preprocessed the data as follows: local background was subtracted, irregular spots were flagged, global linear regression (lowess) normalization was performed, and this ratio was log transformed. Data was imported into TIGR MEV 3.1 software for further analysis. A supervised

strategy was used to identify the genes with expression profiles that were similar or reciprocal to SEPP1 gene expression using Pavlidis Template Matching (PTM) (Pavlidis and Noble, 2001). The six genes matched most strongly with SEPP1 were identified and the three best annotated of these genes were used in further analyses.

Electronic Database Analyses

Genes matched with SEPP1 expression, as measured by PTM analysis of the microarray data, were analyzed in GATHER. The TRANSFAC component of GATHER worked to detect the presence of shared potential TFBS within the promoters of the genes. Significance was measured with a Bayes factor and a factor greater than 6 was considered statistically significant.

Among the six genes found to match SEPP1 expression most strongly by PTM analysis, the three best annotated of these were identified. These gene names, as well as SEPP1, were entered into TRED. Two kilobase sequences surrounding the transcriptional start sites (1700 bp upstream, 330 bp downstream) were retrieved in FASTA format. These promoter sequences were entered into the MEME database and analyzed for homologous motifs. Identified motifs that contained regions of the SEPP1 promoter and had information content greater than 20 bits were chosen for further analysis. The sequences of all sites represented in each of these motifs were entered into TESS. Results were examined in tabular format to view details regarding the putative binding sites that were identified, including the start position, sense, similarity scores, sequence, factor name, and accession numbers. TESS results were manually sorted in order to identify putative

binding sites found within all genes represented within a particular motif. Evaluation of evolutionary conservation among these sites was accomplished using ConSite. The genomic sequences of human and murine SEPP1 were entered into the ConSite database in FASTA format and these orthologous sequences were analyzed for sequence homology.

Results

A custom spotted cDNA microarray was utilized to identify gene expression changes following induced expression of 15-LOX-1 and arachidonic acid treatment. Only a small number of genes were found to have changes in expression under these conditions, with an expression analysis showing SEPP1 to be the most upregulated gene on the array (Figure 2.1). Importantly, results were only compared with the vehicle treated control and not compared to controls in which either enzyme induction or arachidonic acid treatment were controlled for individually.

A commercial human whole genome microarray was run with these control conditions included. Additionally, a 12-LOX expressing cell line was included for comparison and a 15-LOX mutant construct (15-LOX-ΔI) and the LacZ gene were used as control cell lines. The 15-LOX mutant lacks the C-terminal isoleucine responsible for coordinating the nonheme iron that functions in the enzymatic activity of all LOX enzymes (Chen et al., 1995). Results suggested that activation of the VgEcR gene expression system by ponasterone A was sufficient to induce

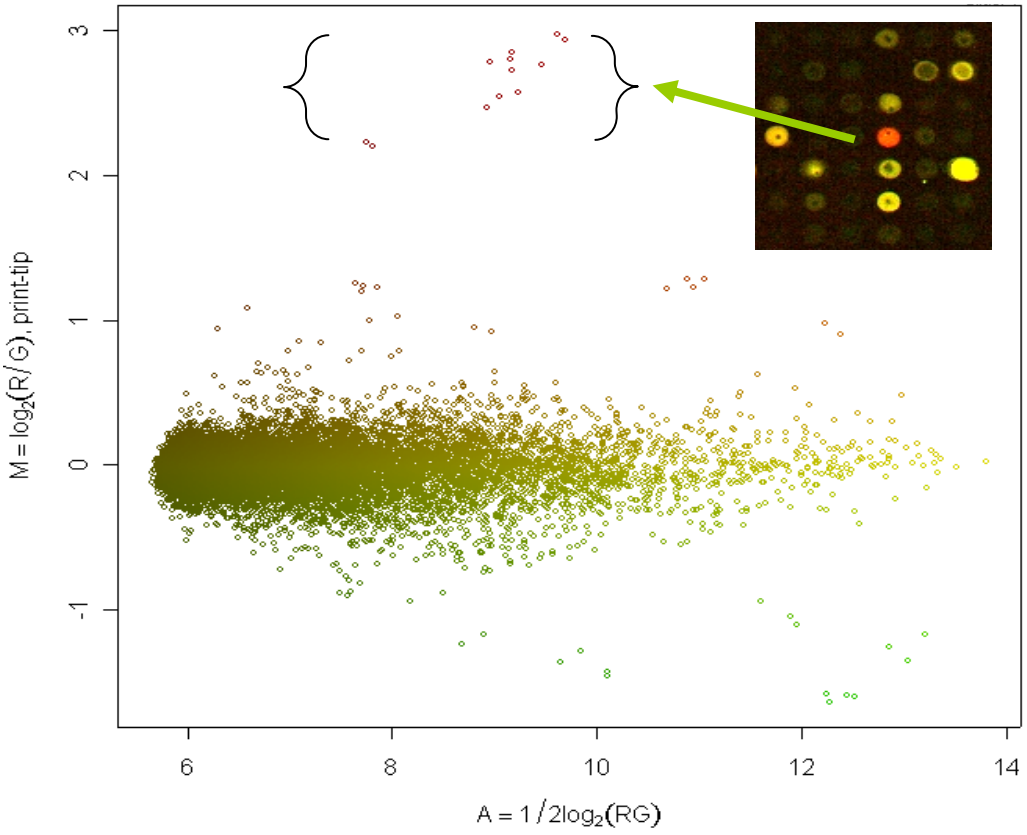


Figure 2.1 15-LOX metabolism of arachidonic acid induces SEPP1. M versus A plot of microarray data comparing induced 15-LOX cells in the presence of arachidonate for 4 hours to uninduced cells. Induced genes (in brackets) were found to be consistent with SEPP1 upon sequencing. Inset shows raw data for one spot.

expression of SEPP1, regardless of the overexpressed gene or whether the lipid substrate arachidonic acid had been added (Figure 2.2).

In order to gain information regarding the regulatory mechanisms involved in SEPP1 expression, multiple electronic database tools were used to search for shared regulatory regions between SEPP1 and co-expressed genes identified in the microarray results (Figure 2.3). PTM analysis of microarray data revealed 149 matched and 41526 unmatched genes in relation to SEPP1 expression following treatment of ecdysone-inducible HEK-293 cells. This molecular signature was analyzed using GATHER in an attempt to identify shared TFBS annotated by TRANSFAC. Results of this analysis did not yield Bayes factors greater than 6; therefore, annotations were not considered significant and TFBS shared among the genes were not identified by this method.

This led to a search for unidentified sequence patterns within the promoters of the co-expressed genes. For simplicity of analysis, the number of genes evaluated was reduced by identifying the six genes matched most strongly with SEPP1 through PTM analysis and choosing the three best annotated of these for further analysis. These three genes included Tissue factor pathway inhibitor 2 (TFPI2), Semenogelin (SEMG1), and Megalencephalic leukoencephalopathy with subcortical cysts 1 (MLC1). TFPI2 is proteinase inhibitor that acts against a wide range of serine proteases (Chand et al., 2004). SEMG1 is the predominate protein component of human semen (Lilja et al., 1989). The exact function of MLC1 is unknown; however, mutations in the gene have been associated with the neurological condition megalencephalic leukoencephalopathy with subcortical cysts

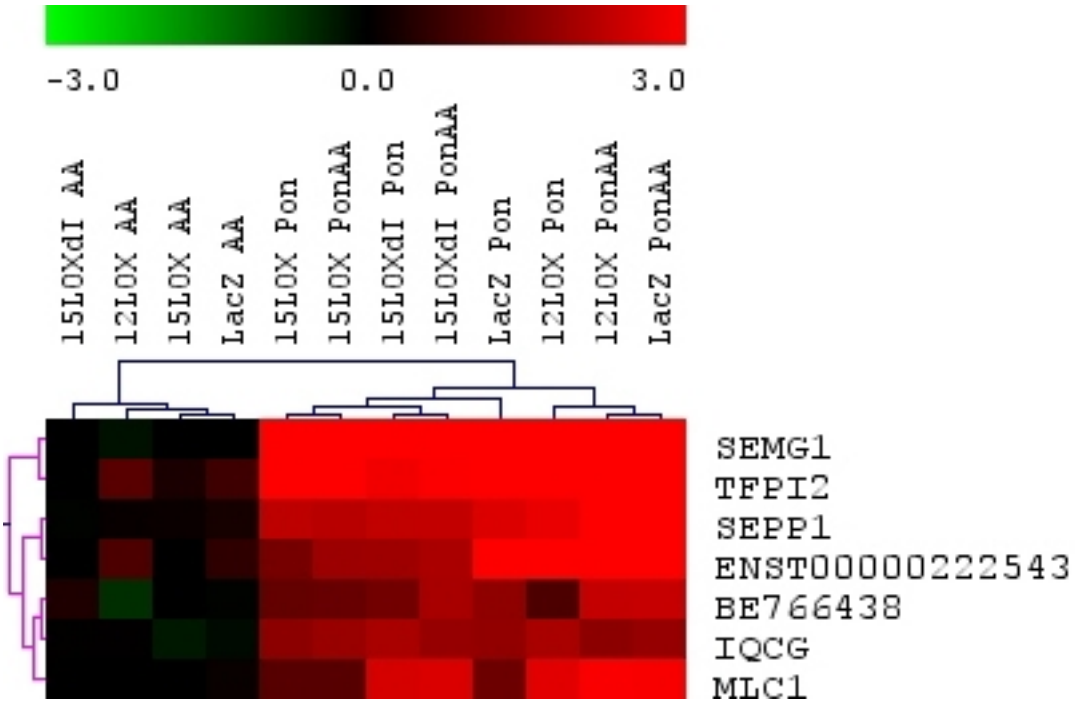


Figure 2.2 Activation of VgEcR induces host gene expression. Pavlidis Template Matching of microarray data compared induced 15-LOX, 12-LOX, 15-LOX-ΔI, or LacZ cells to uninduced cells in the presence or absence of arachidonate for four hours. The heat map displayed is a representation of the six genes that best matched the SEPP1 expression profile. Activation of VgEcR by PonA was sufficient for gene expression changes regardless of the overexpressed gene or arachidonate treatment status.

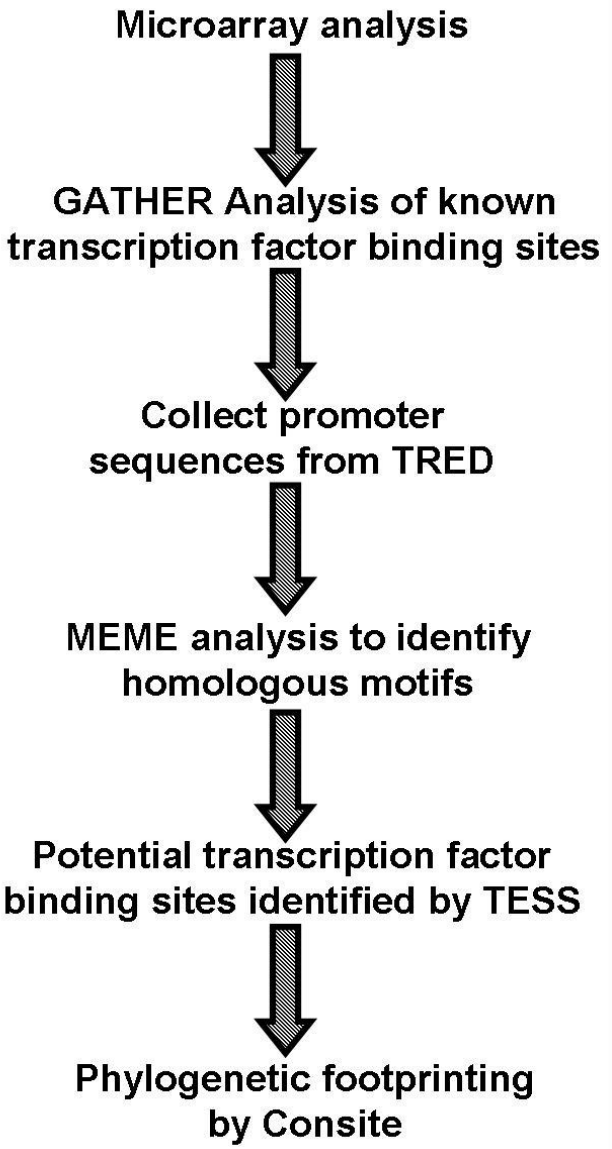


Figure 2.3 Flow diagram of electronic database analyses of putative TFBS. Coexpressed genes were identified by microarray analysis following treatment of ecdyone-inducible cells with PonA. The GATHER database worked to identify regulatory relationships between the coexpressed genes, but failed to find shared TFBS in the gene sequences. Promoter sequences of coexpressed genes were collected from TRED and entered into the MEME database to identify homologous sequence motifs. These homologous sequences were entered into TESS to identify putative TFBS. Evolutionary conservation of motifs identified in the SEPP1 promoter was evaluated by ConSite.

(Leegwater et al., 2001). These genes seem functionally unrelated to SEPP1, which works in selenium distribution to extrahepatic tissues (Hill et al., 2003; Renko et al., 2008). Gene expression changes observed in the microarray results for these four genes are outlined in Table 2.1.

In order to identify regions of sequence similarity among these four co-expressed genes, promoter regions (2 kilobase sequences surrounding the transcriptional start site) of the genes were retrieved from TRED (ID #34663 for SEPP1, ID #39081 for TFPI2, ID #26315 for SEMG1, and ID #28529 for MLC-1) and searched using MEME. Of the 10 motifs presented in the MEME results, eight included sequence signals from SEPP1 and three included signals from all four of the genes analyzed. Each motif displayed varying degrees of sequence conservation with total information content ranging from 14.2 to 39.9 bits. A representative block diagram is shown for a motif that included signals from all analyzed genes (Figure 2.4).

For each MEME motif that included signals from SEPP1 and had information content greater than 20 bits, the sequences of all sites represented in the motif was entered into TESS. Table 2.2 lists putative binding sites identified within each of the analyzed MEME motifs when these sites were found to exist in all genes represented within that particular motif. Because these sites were represented across the coexpressed genes, they were thought to be the most likely candidates for functional binding sites. Of particular interest were the putative glucocorticoid receptor (GR) and retinoic acid receptor gamma (RARγ) sites identified in Motif 4

Table 2.1

Fold Changes in Gene Expression of Coexpressed Genes
Following Treatment of Ecdysone-Inducible
HEK-293 Cells with Ponasterone A

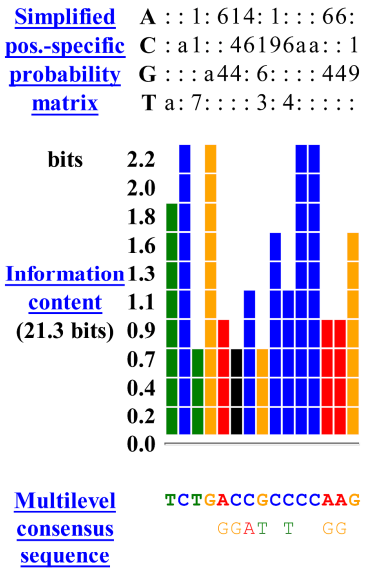
	TFPI2[a]	SEMG1[b]	SEPP1[c]	MLC1[d]
15-LOX PonA	3.27	3.13	2.24	1.03
15-LOX PonA+AA	3.53	3.17	2.18	1.04
15-LOX-ΔI PonA	2.86	4.03	2.29	2.52
15-LOX-ΔI PonA+AA	2.98	3.13	2.37	2.58
12-LOX PonA	3.92	3.58	2.78	2.69
12-LOX PonA+AA	4.24	3.68	3.27	2.96
LacZ PonA	3.79	3.32	2.61	1.29
LacZ PonA+AA	4.41	3.41	3.66	2.95

[a] Tissue factor pathway inhibitor 2

[b] Semenogelin

[c] Selenoprotein P

[d] Megalencephalic leukoencephalopathy with subcortical cysts 1



NAME	STRAND	START	P-VALUE	SITES
TFPI2:chr7:93118291	+	1866	7.61e-09	CCAACAGGTA **TCTGGCCGCTCCAGG** AGCTTCTCTC
MLC1:chr22:48705809	+	1489	2.47e-08	CTAGTTGGGT **TCTGACAGCTCCGAG** GCAGTGGTTT
MLC1:chr22:48705809	-	1845	6.64e-08	CATTGATCCA **TCTGGGCCCTCCAGG** TGCAACACCT
SEPP1:chr5:42812134	+	41	1.13e-07	TGCCTTAAAA **TCCGAGCTCCCCGAG** TGCACAATTT
SEMG1:chr20:44474104	-	1502	1.13e-07	TTTTCTTTCT **TCTGGCAGCCCCAGC** AAATTTTTAT
MLC1:chr22:48705809	+	1426	1.64e-07	GCTTCTTTCT **TCTGAGAGACCCGAG** CTCTGTGGCG
TFPI2:chr7:93118291	+	191	4.85e-07	GGTCAAATCT **TCAGAACTCCCCAAG** GCTATCTCTC

Figure 2.4 Sample MEME output. Representative motif in which sequence from all four input genes are included. The sites identified as belonging to the motif are indicated, with the consensus sequence shown above them. The color-coded bar graph shows conservation at each position in the motif. The level of conservation is measured in bits, and a sum of the bits across the motif provides information content for the entire motif.

Table 2.2

Representative Putative Transcription Factor Binding Sites Identified in the SEPP1 Promoter by TESS

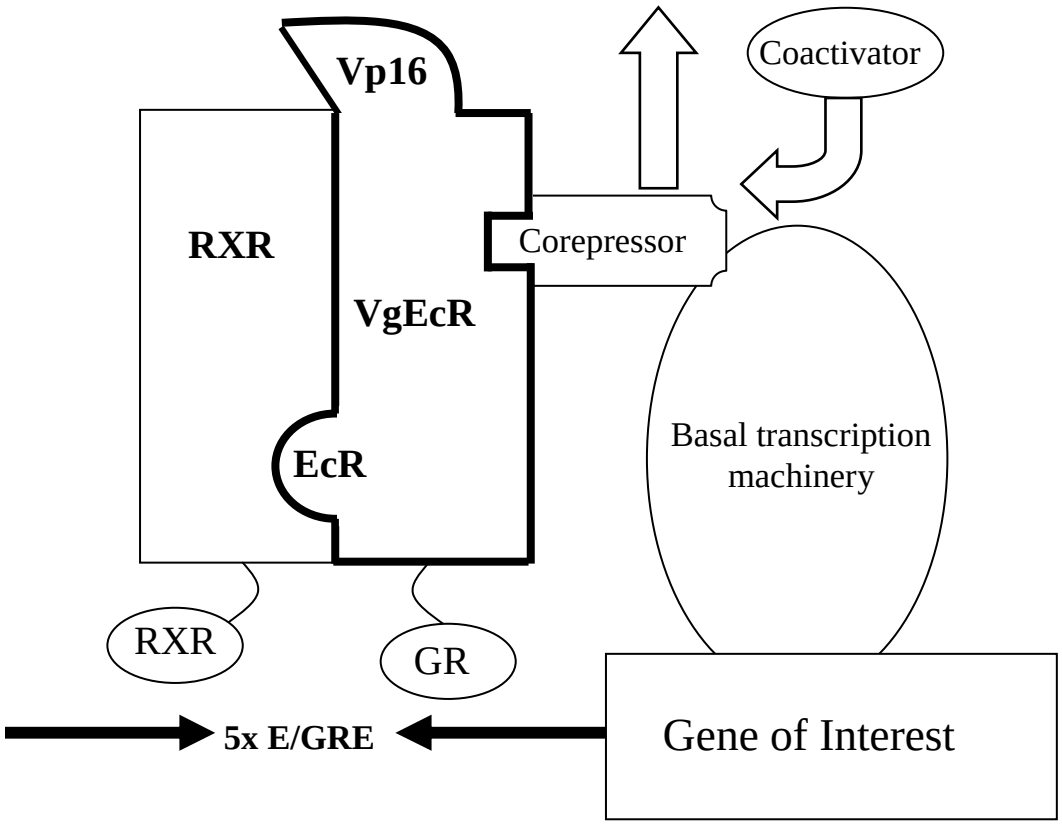
Motif 1	**Motif 6**
CAC-binding protein	CAC-binding protein
NF1	C/EBPalpha
LBP-1	RAR-gamma
CP2	GR
LVc	MZF-1
NF-S	TEF2
	SRY
Motif 3	R2
LBP-1	V$CAP_01
IL-6.RE-BP	CF1
CP2	c-Myb
NF1	Elk-1
GAGA	Ttk
AP-4	SP1
erg	TFII-I
	Erg
Motif 4	T-Ag
GATA-1	NF-IL6
HSTF	PEB1
NF1	Zmhoxla
Eve	
Dof3	**Motif 8**
TCF-1	Ttk
OBF	ADR1
C/EBPalpha	Sp1
GR	TCF-1
TFII-I	
PEB1	
Elk-1	

and 6 on the SEPP1 promoter, as transcriptional activation by VgEcR involves binding of a heterodimer formed between GR and RXR (Saez et al., 2000). VgEcR is a synthetic receptor produced by the fusion of the ligand-binding and dimerization domain of the *Drosophila* ecdysone receptor (EcR), the DNA-binding domain of the GR, and the transcriptional activation domain of herpes simplex virus VP16. Upon exposure to ponasterone A, VgEcR dimerizes with the RXR, corepressors are released, coactivators are recruited, and the complex becomes transcriptionally active (Figure 2.5) While the VgEcR system is not expected to transactivate host genes by itself, such as was observed here, changes in endogenous gene levels have been previously observed in mammalian cells treated with EcR ligands (Oehme et al., 2006; Panguluri et al., 2007).

Phyologenetic footprinting analysis by ConSite revealed that the GR and RARγ sites of Motif 6 were located in a region of the SEPP1 that shares approximately 80% sequence homology with the murine gene (Figure 2.6). ConSite also identified a concentration of potential TFBS within this region of the gene.

Discussion

Understanding the mechanisms responsible for regulating gene activity is a primary goal of the post-genomic era of biology (Sandelin et al., 2004). The function of regulatory elements is mediated by DNA-protein interactions; therefore, focus has centered on the identification of protein binding sites, and in particular TFBS, within the human genome (Elntiski et al., 2006). While experimental



Adapted from http://www.stratagene.com/manuals/217468.pdf

Figure 2.5 Regulation of transcription by the VgEcR system. The synthetic VgEcR transcription factor is a fusion of the ligand-binding domain of the *Drosophila* EcR, the DNA-binding domain of the GR, and the transcriptional activation domain of herpes simplex virus Vp16. The EcR dimerizes with RXR and binds to multiple copies of a synthetic ecdysone-responsive element (E/GRE). Ligand binding of the EcR leads to the release of corepressors and recruitment of coactivators, allowing the system to become transcriptionally active.

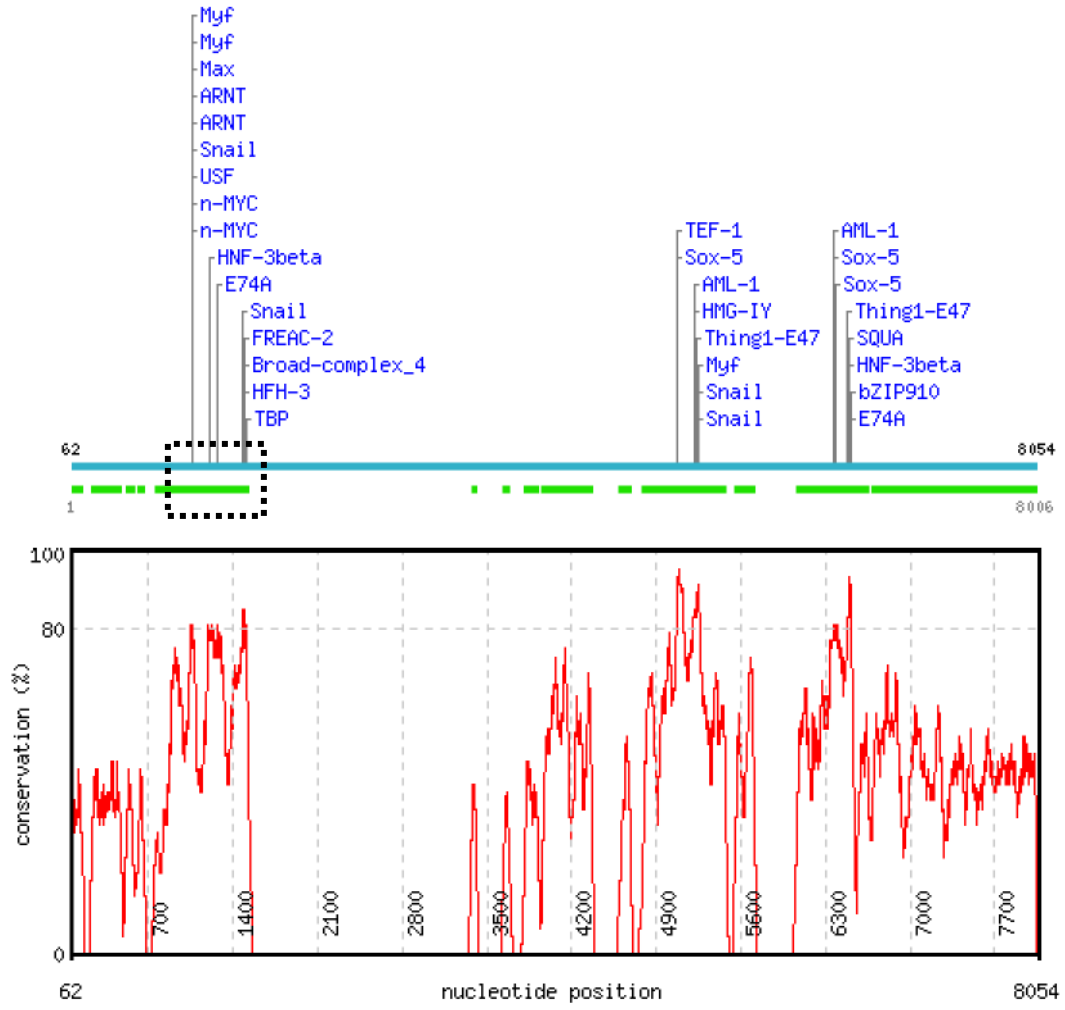


Figure 2.6 Phylogenetic footprinting of SEPP1 genomic sequence. Evolutionary conservation of the SEPP1 gene was evaluated using ConSite. The murine sequence, indicated in green, was aligned with the human sequence, indicated in blue. Level of conservation was measured at each nucleotide position and reported as a percentage of sequence homology. Conserved putative binding sites were also reported by ConSite. The dashed boxed indicates the position of the putative GR and RARγ binding sites identified by TESS.

approaches such as DNase footprinting, gel shift assays, and microarrays can be used to identify molecules working cooperatively to affect a biological process (Galas and Schmitz, 1978; Garner and Revzin, 1981; Schena et al., 1995), they do not provide direct evidence of the promoter regions controlling changes in gene expression.

To aid in the discovery of TFBS, multiple computational tools for modeling and predicting gene regulatory elements have been developed (Elnitski et al., 2006). While each of these tools seem to offer their own niche capabilities, overlapping and complementary functions allow the various databases to be used together in a way that strengthens evidence identifying a binding site model within the promoter region of a gene or group of genes of interest. The collaborative use of these databases also reduces the rate of false-positives that occurs when a single tool is used as the sole means for modeling and predicting binding sites (Jolly et al., 2005; Tompa et al., 2005).

Despite accelerating the discovery of transcriptional regulatory mechanisms, the usefulness of these tools is limited by their inability to factor in the contribution of biological function, such as tissue-specific effects, on gene expression (Elnitski et al., 2006). The cellular environment ultimately dictates which events occur during transcription. For this reason, experimental confirmation of computational predictions is considered prudent and remains the best form of validation of *in silco* data (Elnitski et al., 2006).

In recognizing the unique capabilities and limitations of experimental and computational techniques, the synergism of these two approaches becomes

apparent. In results presented here, experimental data from microarray analysis provided information regarding genes that were coexpressed in ponasterone A-induced HEK-293 cells. Database analysis then identified sequence motifs shared between the promoter regions of these co-expressed genes and predicted TFBS within the sequences. The identification of these putative sites has guided further analysis of the mechanisms regulating expression of SEPP1 on a transcriptional level. Experimental techniques, such as promoter analyses and electrophoretic mobility shift assays (EMSA) were used to confirm the TFBS predictions reported here and the findings of these experiments are described in Chapter 3 of this dissertation.

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CHAPTER 3

SELENOPROTEIN P REGULATION BY THE GLUCOCORTICOID RECEPTOR

Introduction

Selenoprotein P (SelP) is an extracellular glycoprotein that carries approximately 40% of plasma selenium (Akesson et al., 1994). SelP is unique among the selenoproteins in that it can possess up to 10 selenocysteine residues in mammals (Burk & Hill, 2005). SelP primarily functions in selenium distribution (Hill et al., 2003; Renko et al., 2008), with knockout mice displaying altered selenium distribution, particularly to the testes and brain (Hill et al., 2003; Burk et al., 2006). The majority of SelP is derived from hepatic sources, however; the mRNA can be detected in almost all tissues, with appreciable concentrations observed in the kidney, heart, lung, brain, skeletal muscle, and testis (Burk & Hill, 2005)

The regulation of selenoprotein P gene (SEPP1) expression is an active area of investigation with changes in SEPP1 noted under a broad spectrum of biological processes. In HepG2 cells and primary rat hepatocytes, promoter activity has been shown to be inhibited by cytokines including interleukin 1β, tumor necrosis factor

α, interferon γ, and transforming growth factor β_1 (Dreher et al., 1997; Mostert et al., 2001). This inhibition suggests that the SEPP1 gene product may function as a negative acute-phase protein in response to inflammation. Alternatively, promoter activity is stimulated in hepatic cells through the FOXO1a and HNF-4α transcription factors (Speckmann et al., 2008; Walter et al., 2008).

In addition to inflammation, microarray analyses have revealed changes in SEPP1 expression during development and following alterations in the differentiation state of extrahepatic cells. Elegant developmental studies have demonstrated SEPP1 ortholog spatiotemporal expression in both zebrafish (Thisse et al., 2003) and murine model systems (Lee et al., 2008). Increased expression has been observed in differentiating myeloid, pulmonary, and Sertoli cells (Tabuchi et al., 2005; Ghassabeh et al., 2006; Wade et al., 2006). Conversely, SEPP1 expression is decreased with neoplastic progression from normal tissue, to carcinoma, to metastatic disease in cells of prostate origin (Dhanasekaran et al., 2001). Evaluation of SEPP1 expression in the Oncomine database (Rhodes et al., 2004) also identifies decreased SEPP1 expression in melanoma, lung, and colon cancer compared to normal tissue suggesting that decreased SEPP1 expression may be a common feature of malignancies. Indeed, work in colorectal cancer suggests that specific selenoenzymes are reduced, indicating that changes in SEPP1 is not a general alteration in nutrition or decreased selenium (Al-Taie et al., 2004).

In this chapter, induction of SEPP1 in human cells stably transfected with the ecdysone inducible system (VgEcR-RXR) is reported. Due to VgEcR's glucocorticoid receptor DNA binding domain, as well as evidence of SEPP1

modulation during development and inflammation, regulation of SEPP1 by the glucocorticoid receptor or the retinoid X receptor was evaluated. In addition, evidence exists for changes in plasma selenium levels following glucocorticoid administration, with both increases and decreases noted under different sets of conditions (Peretz et al., 1987; Marano et al., 1990; Watanabe et al., 1997). Although the effect of glucocorticoids on selenium levels has not been fully characterized, it is believed that these changes result from redistribution of selenium between tissue and plasma. It is unknown what role SelP may play in this glucocorticoid-induced selenium redistribution. Therefore, the glucocorticoid responsiveness of the SEPP1 promoter was examined in this chapter, and it was found that the glucocorticoid receptor (GR) inhibits the expression of SEPP1.

Methods

Materials

The HEK-293 cell line was purchased from American Type Culture Collection (Manassas, VA). Advanced DMEM, T4 DNA ligase, *HindIII*, *XhoI*, and *SstI*, Accuprime Pfx DNA polymerase, SuperScript III reverse transcriptase reagents, OneShot Top 10 chemically competent cells, zeocin, geneticin, Lipofectamine 2000, and Ni-NTA agarose were purchased from Invitrogen (Carlsbad, CA). Ponasterone A (PonA) was purchased from A.G. Scientific (San Diego, CA). Dexamethasone (Dex) was purchased from EMD Chemicals (Gibbstown, NJ). RNeasy Mini Kit and EndoFree Maxi-and Mini-prep Kits were obtained from Qiagen (Valencia, CA). Lightcycler 480 SYBR Green I master mix

was purchased from Roche Diagnostics (Indianapolis, IN). Biolase DNA polymerase, dNTPs, magnesium chloride, and NH_4 reaction buffer were purchased from Bioline (Taunton, MA). SYBR Green I was purchased from Cambrex (East Rutherford, NJ). Human genomic DNA and the Dual Luciferase Reporter Assay System was purchased from Promega (Madison, WI). NE-PER nuclear extraction reagents, Biotin 3' end DNA labeling kit, and Lightshift chemiluminescent EMSA Kit were purchased from Pierce (Rockford, IL).

Plasmids

pVgEcR (Invitrogen) encodes the fusion transcription factor used to generate ecdysone-inducible cells. pRL-RSV and pGL4.21 (Promega) were used in the luciferase reporter assays; pRL-RSV constitutively expresses *Renilla reniformis* luciferase, and SEPP1 promoter fragments were cloned into the pGL4.2.1 plasmid that contains firefly luciferase. The pLTRluc glucocorticoid reporter plasmid and pDsRed-hGR glucocorticoid receptor expression plasmid were gifts from Dr. Carol Lim (University of Utah).

Cell Culture

Human embryonic kidney line HEK-293, as well as all subsequently engineered cells, were cultured in Advanced DMEM medium containing 2% fetal bovine serum and 2mM L-glutamine. Cells were maintained at 37°C in a humidified incubator with 5% CO_2.

HEK-293 were transfected with pVgEcR and selected for zeocin resistance to generate stable expression of the VgEcR gene product and are referred to as 293-EcR. 293-EcR cells that conditionally express 15-LOX-1 and ΔIle^{662} 15-LOX-1 were previously described (Yu et al., 2004; Cordray et al., 2007). Conditional expression of LacZ in the 293-EcR was achieved using similar methods.

An expression vector, pDsRed-hGR, that constitutively expresses a DsRed2-labeled, functional human GR was generously provided by Dr. Carol Lim, University of Utah. The 293-EcR cells were stably transfected with this expression vector and selected for neomycin resistance in order to study the effects of GR signaling in HEK-293 cells. These cells are referred to as EcR-GR.

Polymerase Chain Reaction (PCR)

The Transcription Regulatory Element Database (Jiang et al., 2007) was used to identify the ~2 Kb sequence surrounding the transcriptional start site of SEPP1, promoter ID #34663 (1770 bp upstream of start site, 300 bp downstream) and used as an electronic template to generate promoter constructs. A 1.9 Kb sequence was amplified from human genomic DNA by PCR using the primer pair 5'-TAGGTACCCCAGTTCTTTCCGGTGTTCA-3' and 5'-TACTCGAGCGCA-CTGGGAACTTCACCTA-3'. The PCR product was digested with *XhoI* and *SstI* and cloned into the pGL4.21 luciferase reporter vector. This construct is referred to as -1652 to +247 and was utilized as template DNA in subsequent PCR reactions used to synthesize smaller fragments of the SEPP1 promoter region of interest. A *HindIII* digestion of the -1652 to +247 construct generated -1652 to -385 and -391

to +247 promoter fragments. The fragments were cloned into the pGL4.21 vector following *HindIII* digestion. Due to the use of the *HindIII* site in the pGL4.21 vector, the -391 to +247 fragment was only subcloned in the reverse orientation, and despite several attempts, no colonies were obtained with this fragment in the forward orientation. The -109 to +247 and the -53 to +247 fragment were generated using PCR and cloned into the pGL4.21 vector using *XhoI* and *SstI* digestions.

Quantitative PCR was used to assess SelP mRNA expression. 293-EcR and EcR-GR cells were treated with 10 μM ponasterone A 24 hours prior to mRNA collection, and 10 nM dexamethasone was then added at 8 or 16 hours prior to mRNA purification. Vehicle treatments with ethanol (EtOH) or dimethyl sulfoxide (DMSO) were used as controls. The Qiagen RNeasy Mini Kit was used to collect and purify mRNA from cells. First strand cDNA was synthesized using Superscript III reverse transcriptase and these cDNA samples were run in triplicate as 1:5 dilutions. Standards were run in duplicate at concentrations between 10^3 to 10^8 copies/μl and β_2 microglobulin was run as a reference gene. The SEPP1 amplicon consisted of the 100 bp spanning the final intron of the genomic sequence. The primer pair 5'-TTCGGGCAGAGGAGAACA-3' and 5'-CTGGCACTGGCT-TCTGTG-3' were used to amplify this region. Average threshold copy number was used to calculate changes in expression level as compared to vehicle treated controls.

Site-directed Mutagenesis

Putative response elements of interest were mutated using a PCR-based strategy. The putative GRE sequence CAAGAATGA**AC**ATTGAACT at position -87 of the SEPP1 promoter (GRE #1) was mutated to the sequence CAAGAATGA**CT**ATTGAACT using the primer 5'-GGTCACTGCAAGAA-TGA**CT**ATTGAACTTTGGACTATAC-3' and its complementary sequence (exchanged nucleotides are bold and underlined). The putative GRE sequence TCAGAG**TG**TGCT at position -24 of the SEPP1 promoter (GRE #2) was mutated to the sequence TCAGAG**GA**TGCT using the primer 5'-GGACTATAA-ATATCAGAG**GA**TGCTGCTGTGGCTTTGTG-3' and its complementary sequence. These mutations should eliminate activity of potential GRE half sites (Nordeen et al. 1990). The putative retinoid responsive element sequence ACAT**TGA**ACTTTGG at position -73 of the SEPP1 promoter (RRE) was mutated to the sequence ACAT**CTT**ACTTTGG using the primer 5'-CTGCAAG-AATGAACAT**CTT**ACTTTGGACTATACCTGAGG-3' and its complementary sequence. The FOXO1a binding sequence GTAAA**CA**A at position -46 of the SEPP1 promoter was mutated to the sequence GTAAA**TC**A using the primer 5'-CCTGAGGGGTGAGGTAAA**TC**ACAGGACTATAAATATCAGAG-3' and its complementary sequence.

Luciferase Reporter Assay

Reporter assays were quantified using a Dual Luciferase reporter assay. SEPP1 promoter constructs cloned into pGL4.21 or a mouse mammary tumor virus

promoter reporter construct (pLTRluc) were co-transfected with the pRL-RSV plasmid that serves as an internal control for transfection efficiency. Cells were seeded into 6-well plates at a concentration of 5×10^5 cells/well. Each well was cotransfected with approximately 1 μg of firefly reporter plasmid along with 50 ng of the pRL-RSV vector. Twenty-four hours after transfection, medium was replaced. Cells transfected with SEPP1 promoter constructs were treated with either 10 μM of the ecdysone analong ponasterone A, 10 nM dexamethasone, or a combination of both for an additional 24 hours. Vehicle treatment with EtOH and/or DMSO served as negative controls. Cells transfected with pLTRluc were treated with either DMSO or 10 nM dexamethasone for 24 hours. Following treatments, cells were collected in 200 μl of Passive Lysis Buffer and stored at -80°C at least overnight to allow for cell membrane disruption. Cell lysates were diluted in Passive Lysis Buffer and each sample was quantified in triplicate on Perkin-Elmer Victor^3 V plate reader. The sequential addition of Luciferase Assay Reagent II and Stop & Glo reagent allowed for the measurement of firefly and *Renilla* luciferase activity, respectively.

Immunoblotting

EcR-GR cells were supplemented with 1 μM sodium selenite and treated with EtOH as a vehicle control, 10 μM ponasterone A, 10 nM dexamethasone, or a combination of both for 24 hours. SelP was partially purified from the culture medium of these cells using Ni-NTA agarose. Culture medium was mixed with the Ni-NTA agarose and the mixture was incubated on a nutating mixer at 4°C

overnight. The Ni-NTA beads, along with any bound proteins, were collected by centrifugation, washed twice with 500 µl cold PBS, and then mixed with loading buffer and separated by NuPAGE 4-12% Bis-Tris gels. Proteins were transferred to a polyvinyl difluoride membrane. Membranes were blocked with 5% nonfat dry milk in TBS-T and then probed for SelP (antibody specific for SelP was a gift from Drs. Kris Hill & Raymond Burk, Vanderbilt University). A peroxidase conjugated secondary antibody was used to detect chemiluminescence indicative of protein expression.

Electrophoretic Mobility Shift Assay

Nuclear fractions were collected from 293-EcR and EcR-GR using NE-PER nuclear extraction reagents. Gel shift assays were run using the Lightshift chemiluminescent electrophoretic mobility shift assay kit. Double-stranded 5'-biotinylated oligonucleotides (5'-GGTCACTGCAAGAATGAACATTGAACTT-TGGACTATAC-3') corresponding to the wild-type sequence of GRE #1 was used as a probe. Following end-labeling with biotinylated UTP, complementary oligonucleotides in equimolar amounts were heated to 95°C for 1 minute, cooled to 65°C, and then stored at -20°C. Binding reactions were performed in a 20 µl volume containing 20 fmol labeled probe, 5 µg nuclear proteins, 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 2.5% glycerol, 0.05% NP-40, 1 µg herring sperm DNA, and 1 µg bovine serum albumin. Where indicated, 4 pmol of unlabeled competitor probe was added to reactions. For supershift experiments, 1 µg anti-glucocorticoid receptor antibody (BuGR2; Calbiochem,

Gibbstown, NJ) was added 10 minutes after addition of biotinylated probe and nuclear extract and incubated for an additional 20 minutes at room temperature. Reactions were then loaded onto an 8% TBE gel in 22.25% Tris, pH 8.4, 22.25% boric acid, 0.5 mM EDTA and electrophoresed at 22°C. DNA was transferred to a positively charged nylon membrane, UV cross-linked, probed using Lightshift chemiluminescent EMSA reagents, and detected on a Kodak Image Station 440CF.

Statistical Analysis

GraphPad Instat, version 3.06, was used to evaluate the statistical significance of the results. Two-tailed student's t-tests were used to determine statistical significance when comparing two data sets. In cases where multiple data sets were compared, statistical significance was determined by one-way ANOVA with Tukey or Tukey-Kramer multiple comparison post hoc tests, and differences were considered significant for $p<0.05$.

Results

Previous results using 293-EcR cells with ecdysone-inducible 15-LOX-1, when supplemented with an appropriate substrate, like arachidonate, show an inhibition of the selenoprotein thioredoxin reductase activity by ~50% (Yu et al., 2004). This raised the question of whether other selenoenzymes might demonstrate altered expression under similar conditions. Quantitative PCR experiments performed using 293-EcR cells with stable, ecdysone-inducible 15-LOX-1, as well as the control cell lines with inducible ΔIle^{662} 15-LOX-1, and LacZ, demonstrated

enhanced expression of SEPP1 following ponasterone A treatment (Figure 3.1). Since SEPP1 demonstrated increased expression in all these cell lines, even without substrate for the 15-LOX-1, it is likely that the changes in SEPP1 expression resulted from components of the ecdysone-inducible system rather than a response to 15-LOX-1 catalysis.

The SEPP1 promoter was examined, from -1652 to +247, based on promoter ID #34663 in the Transcriptional Regulatory Element Database, to determine the region of the promoter responsible for this ecdysone-inducible transcription. Fragments of the promoter were tested using the luciferase reporter assay in the 293-EcR cells. Fragments included -1652 to +247, -1652 to -385, -391 to +247, -109 to +247, and -53 to +247 (Figure 3.2A). The greatest level of transcriptional activation following treatment with ponasterone A was observed on the -109 to +247 fragment, suggesting that a site within this region of the promoter may bind a component of the ecdysone-inducible system and induce transcription of SEPP1 (Figure 3.2B).

VgEcR is a synthetic transcription factor that is a fusion of the ligand-binding and dimerization domain of the *Drosophila* ecdysone receptor, the DNA-binding domain of the GR, and the transcriptional activation domain of herpes simplex virus VP16. This gene expression system is designed to activate transcription upon dimerization of VgEcR with RXR, and binding of the heterodimer transactivates a synthetic ecdysone-responsive element (Saez et al., 2000) (Figure 2.5).

Many of the nuclear hormone receptors have similar DNA binding sites. The VgEcR-RXR binds the sequence AGTGCATTGTTCTC in the synthetic response

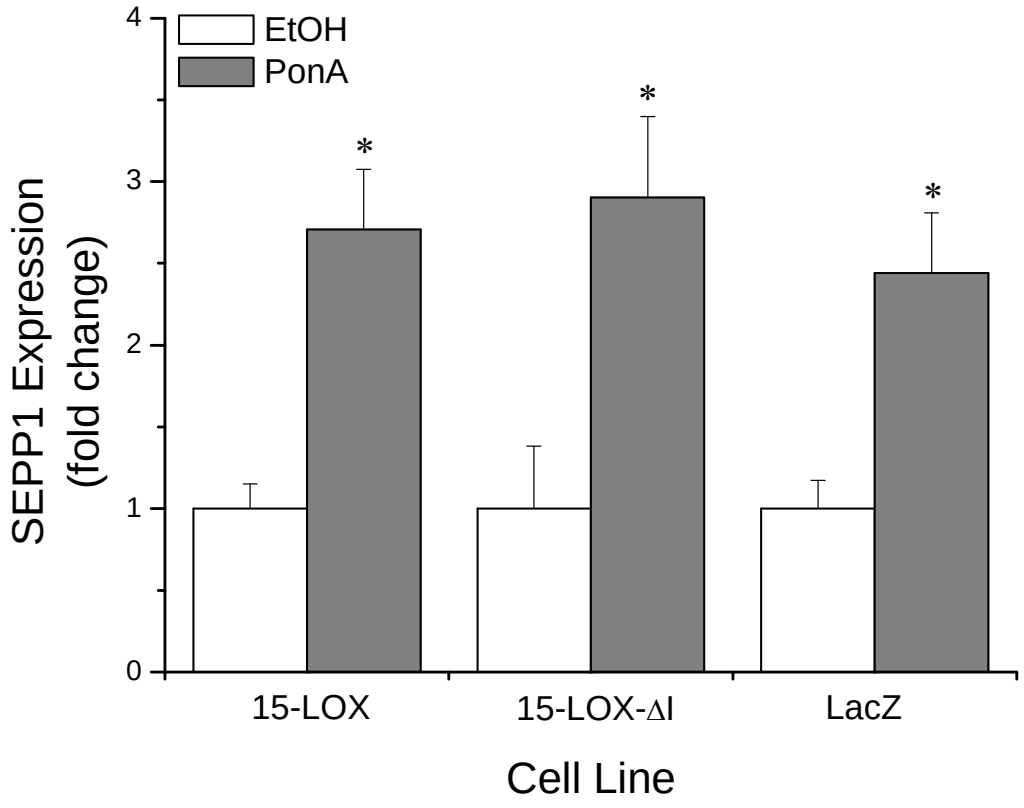
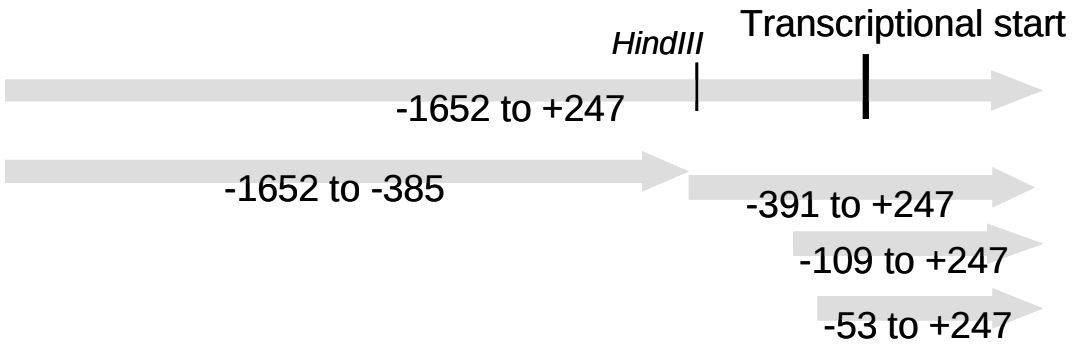


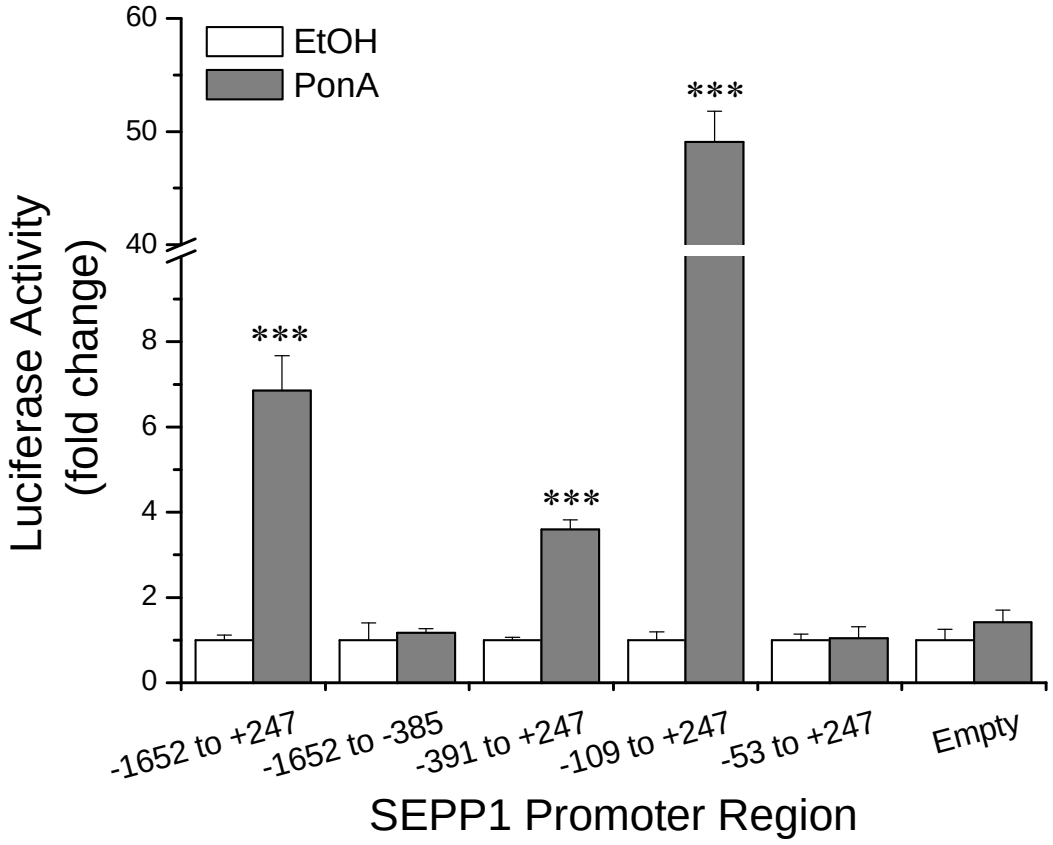
Figure 3.1 Quantitative PCR analysis of SEPP1 expression in HEK-293 EcR-15-LOX and control cell lines. Ecdysone inducible expression of 15-LOX, 15-LOX-ΔI (ΔIle^{662} 15-LOX-1), or LacZ was achieved through a stable co-transfection of pVgEcR into HEK-293 cells. Cells were treated with EtOH (white) or 10 μM PonA (grey) for 24 hours prior to mRNA purification. SEPP1 expression was measured by quantitative PCR. The data are presented as the mean ± standard error of relative gene expression changes observed over a minimum of three experiments and demonstrate differential expression as assessed by a two-tailed t-test (*, $p<0.05$).

Figure 3.2 PonA induction of SEPP1 luciferase reporter constructs. (A) Schematic of SEPP1 promoter fragments that were synthesized by PCR and cloned into the pGL4.21 vector. **(B)** 293-EcR were engineered through a stable transfection of pVgEcR into HEK-293 cells. 293-EcR cells were transfected with SEPP1 reporter constructs. Twenty-four hours after transfection, medium was replaced and cells were treated with EtOH (white) or 10 μM PonA (grey) for an additional 24 hours. Cells were lysed and relative firefly luciferase activity was measured using a Dual Luciferase reporter assay. Technical replicates were run in each experiment, and data are presented as in Figure 3.1 but representing the relative activity changes observed over a minimum of three distinct biological experiments and demonstrate differential luciferase activity as assessed by a two-tailed t-test (***, $p<0.001$).

A


HindIII
Transcriptional start
-1652 to +247
-1652 to -385
-391 to +247
-109 to +247
-53 to +247

B


EtOH
PonA
Luciferase Activity
(fold change)
60
50
40
8
6
4
2
0
***
***
***
-1652 to +247
-1652 to -385
-391 to +247
-109 to +247
-53 to +247
Empty
SEPP1 Promoter Region

element (the binding sites for RXR and the GR DNA binding domains are underlined), the GR binding sequence is TGT(T/C)CT(G/T/C) (Beato et al., 1989; Nordeen et al., 1990), and, for comparison, the endogenous ecdysone receptor binds the sequence (A/G)G(G/T)T(C/T)A (Vogtli et al., 1998; Panguluri et al., 2007). It is also worth noting that RXR and HNF-4α can bind with similar affinity to direct repeats of (A/G)G(G/T)TCA with one base spacing (Nakshatri and Chambon, 1994; Nakshatri & Bhat-Nakshatri, 1998). Due to the similarities in the response elements, it seemed prudent to evaluate cellular responses to both VgEcR and GR as well as to evaluate RXR DNA-binding sequences.

In order to evaluate the interplay between the VgEcR-RXR system and the GR on the SEPP1 promoter, the 293-EcR cell line was engineered to express a DsRed2-labeled, functional human GR. The pLTRluc reporter assay confirmed that the GR is activated by dexamethasone in these EcR-GR cells, with minimal activity in HEK-293 or 293-EcR cells (Figure 3.3). To evaluate possible cross-talk between VgEcR-RXR and GR, ponasterone A was used to treat 293-EcR or EcR-GR cells, transiently transfected with pLTRluc, and only background reporter activity was seen (data not shown).

When the luciferase reporter assay was run in the EcR-GR cells to test activation of the SEPP1 promoter constructs, the GR exerted a repressive effect on this promoter (Figure 3.4). Even in the absence of dexamethasone activation, ponasterone A-induced activity was attenuated in the EcR-GR cells, as compared to 293-EcR cells with no active GR. In order to verify that these results were not due to translocation of the GR to the nucleus in the absence of ligand, fluorescent

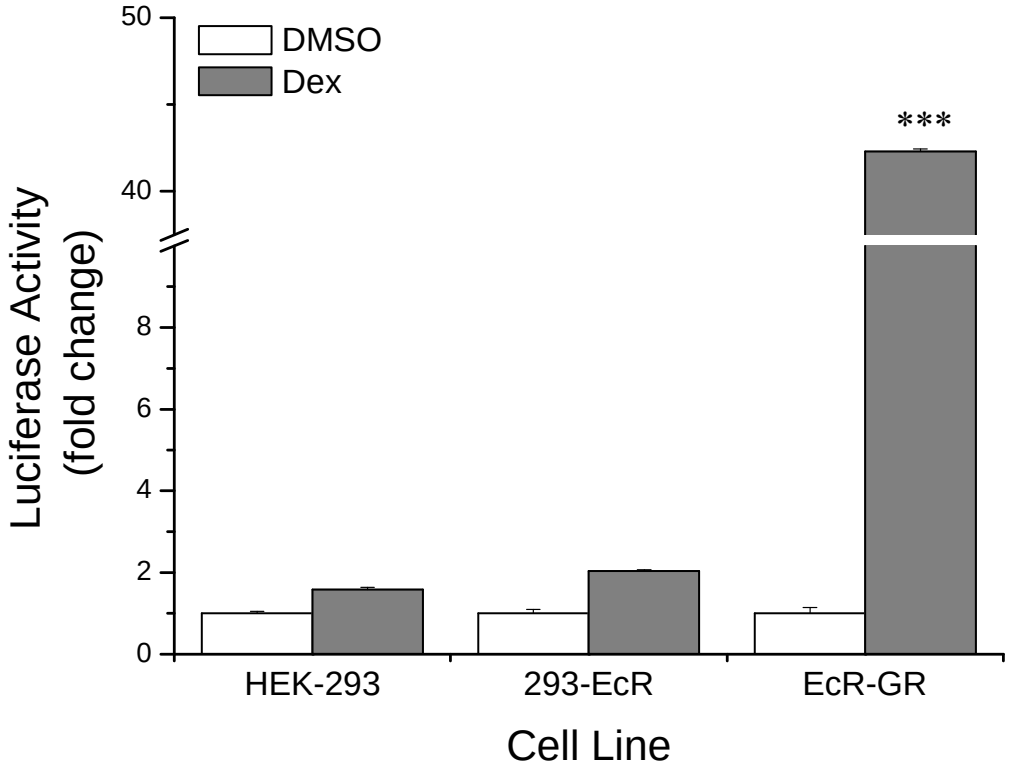
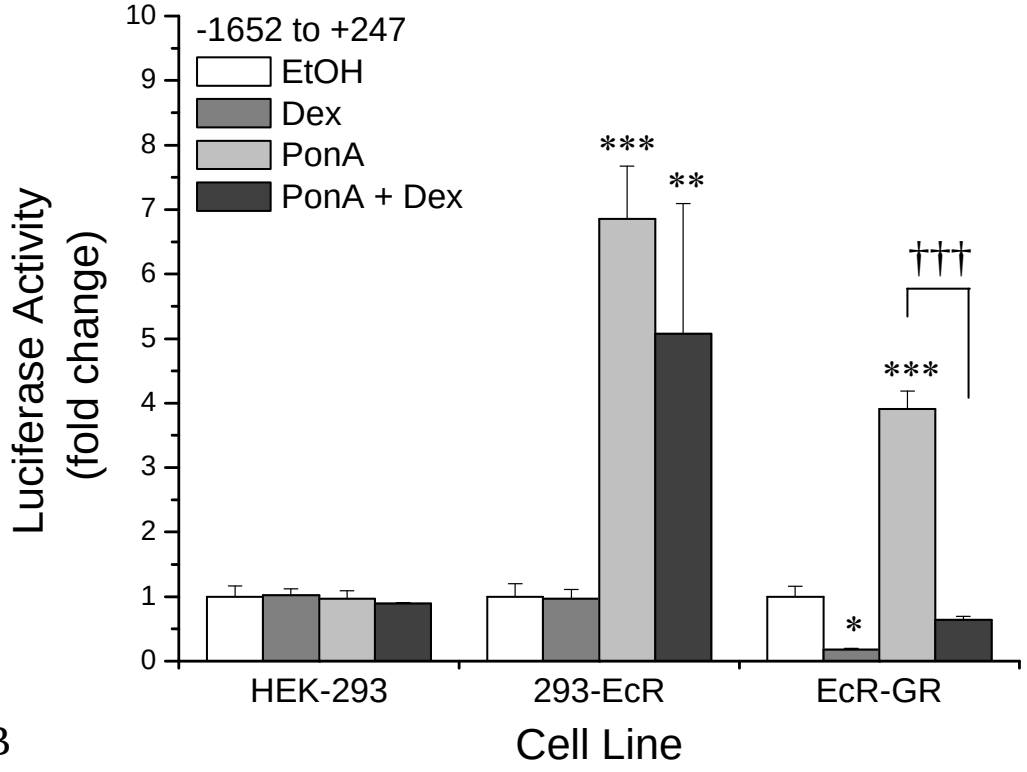


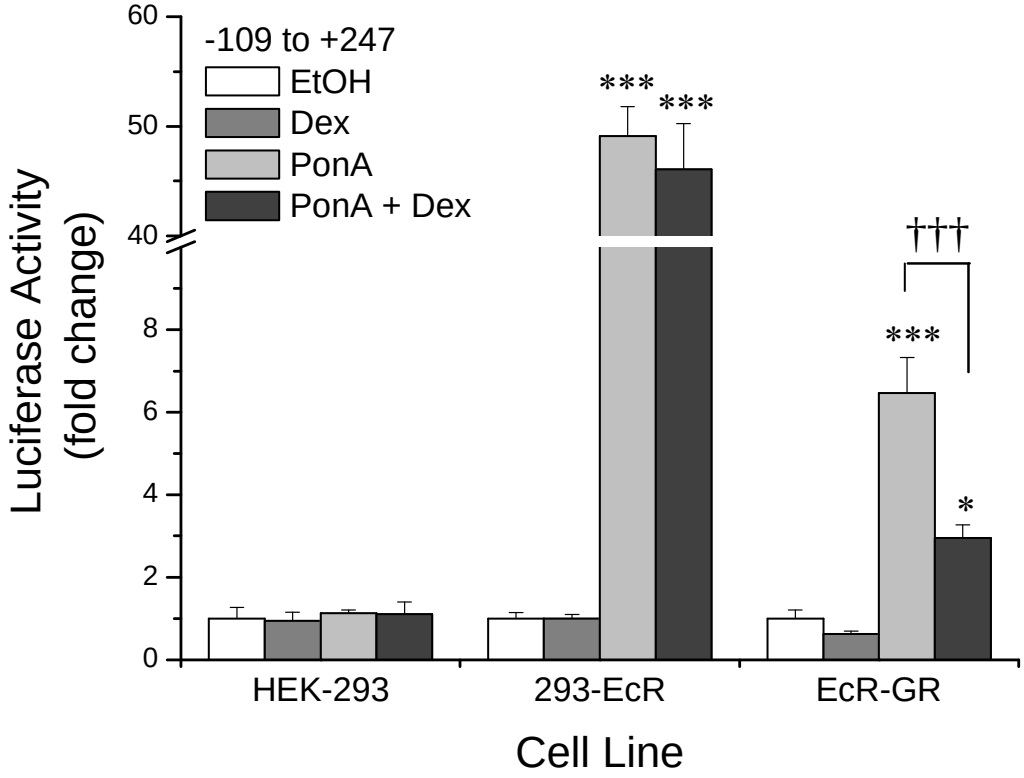
Figure 3.3 Glucocorticoid receptor luciferase reporter. Stable transfection of the 293-EcR cells with the expression vector, pDsRed-hGR produced the EcR-GR cell line. HEK-293, 293-EcR, and EcR-GR cells were transfected with the mouse mammary tumor virus promoter reporter construct pLTRluc. Twenty-four hours after transfection, medium was replaced and cells were treated with DMSO (white) or 10 nM Dex (grey) for an additional 24 hours. Cells were lysed and relative firefly luciferase activity was measured using a Dual Luciferase reporter assay. The data are presented as in previous figures of this chapter and represent triplicate experiments (***, $p<0.001$).

Figure 3.4 Glucocorticoid responsiveness of SEPP1 luciferase reporter constructs. HEK-293, 293-EcR, and EcR-GR cells were transfected with either **(A)** -1652 to +247 SEPP1 luciferase reporter or **(B)** -109 to +247 SEPP1 luciferase reporter. Twenty-four hours after transfection, medium was replaced and cells were treated with EtOH (white), 10 nM Dex (grey), 10 μM PonA (light grey), or a combination of 10 nM Dex and 10 μM PonA (dark grey) for an additional 24 hours. Cells were lysed and relative firefly luciferase activity was measured using a Dual Luciferase reporter assay. Triplicate samples were run in each experiment and data are presented as the mean ± standard error of relative activity changes observed over at least three biological replicates. ANOVA of each cell line revealed no significant differences among the treatments in the HEK-293 cells but highly significant, $p<0.0001$, differences in the 239-EcR and EcR-GR cells. Post hoc tests reveal differences from the vehicle control (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) or differences among select treatment subsets (†††, $p<0.001$).

A


-1652 to +247
EtOH
Dex
PonA
PonA + Dex
Luciferase Activity
(fold change)
10
9
8
7
6
5
4
3
2
1
0
***
**
*
***
†††
HEK-293
293-EcR
EcR-GR
Cell Line

B


-109 to +247
EtOH
Dex
PonA
PonA + Dex
Luciferase Activity
(fold change)
60
50
40
8
6
4
2
0
***
***
***
*
†††
HEK-293
293-EcR
EcR-GR
Cell Line

microscopy was used to visualize localization of the DsRed-labeled GR under the experimental treatment conditions. The GR remained in the cytosol following EtOH or ponasterone A treatment, but was observed in the nucleus when dexamethasone was present. Additionally, the GR remained localized to the cytosol following treatment with the nuclear export inhibitor leptomycin B (Sigma-Aldrich, St. Louis, MO), confirming that nuclear translocation of the receptor occurred only following ligand binding by dexamethasone (Appendix).

When the EcR-GR cells were treated with 10 nM dexamethasone, promoter activity was repressed by ~82% on the -1652 to +247 fragment, as compared to vehicle control (Figure 3.4A). Activation was repressed by ~37% on the -109 to +247 fragment under the same conditions (Figure 3.4B). Simultaneous treatment of the EcR-GR cells with ponasterone A and dexamethasone caused attenuation of ponasterone A activity, with an ~84% reduction in activation observed on the -1652 to +247 fragment as compared ponasterone A only treatment. An ~55% reduction was observed on the -109 to +247 fragment under the same conditions. In comparison, dexamethasone treatment was unable to exert a significant influence on ponasterone A activation in the 293-EcR cells, with only an ~26% reduction in activity observed on the -1652 to +247 fragment and an ~6% reduction observed on the -109 to +247 fragment. Treatment with dexamethasone alone did not cause repression of promoter activity in the 293-EcR cells. Neither ponasterone A nor dexamethasone exerted a significant effect on the SEPP1 promoter constructs in HEK-293 cells. Repression by dexamthasone did not appear to be dose-dependent,

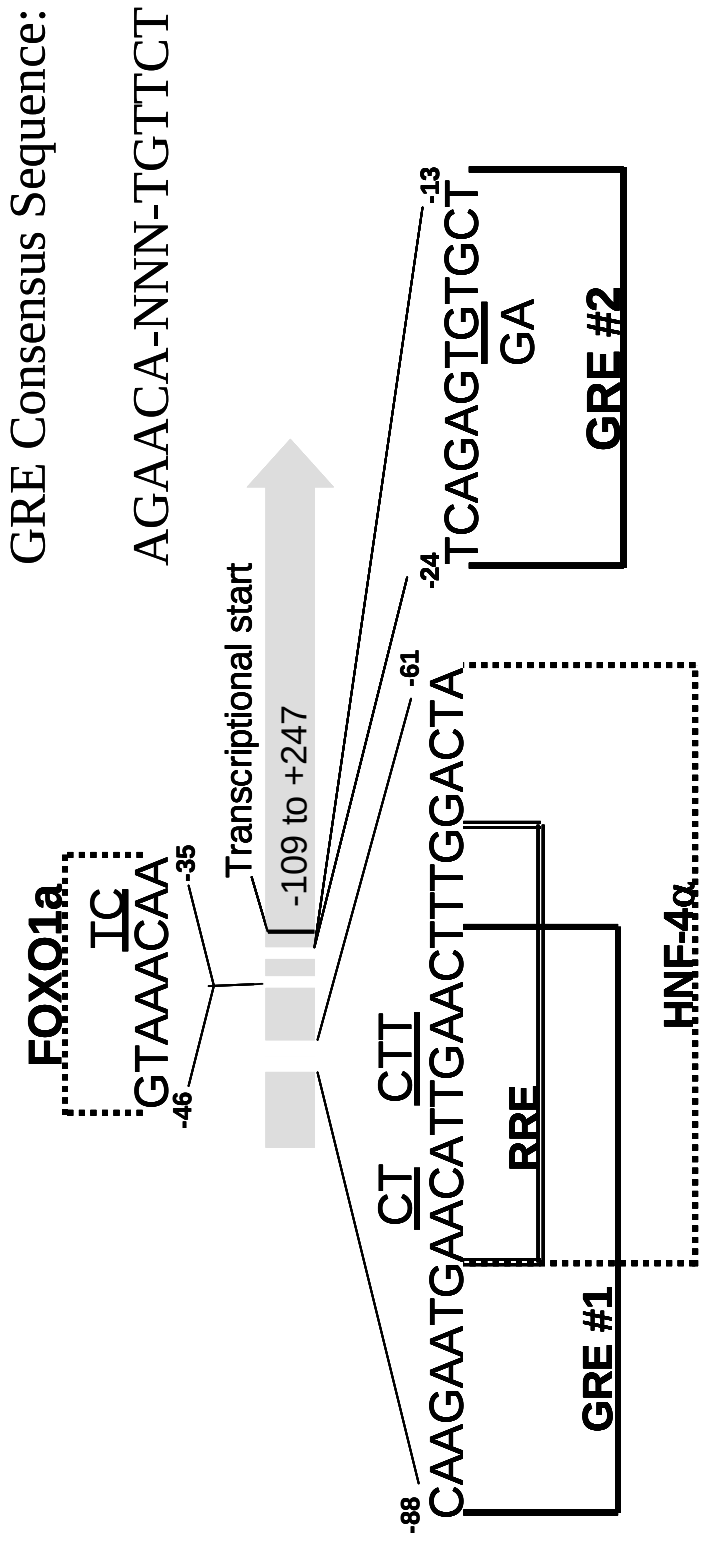
as a 1000 nM dose produced similar levels of activity in the assay (data not shown). Additionally, while the magnitude of transcriptional activity was decreased with a 4-hour, versus 24-hour treatment period, a similar pattern of activity was observed between cell lines and luciferase reporter constructs (data not shown).

Based on the luciferase reporter assay results observed in the -109 to +247 region, this region of the SEPP1 promoter was examined for evidence of response elements that could potentially serve as binding sites for GR or VgEcR, as well as RXR. Two putative GREs were identified using the Transcription Element Search System (Schug & Overton, 1997). These response elements are referred to as GRE #1 and GRE #2 and are found at position -87 and -24 of the SEPP1 promoter, respectively. The precise sequences suggest that these sites may not function as classical GREs but appeared to best define half-sites (Nordeen et al., 1990). In addition, a putative retinoid receptor binding site was identified at position -73 of the SEPP1 promoter, and is referred to as a putative RRE. GRE #1 and the RRE are sequential with one another and together could form a potential binding site for VgEcR-RXR. These sites also overlap with a previously characterized HNF-4α binding site in the SEPP1 promoter (Speckmann et al., 2008; Walter et al., 2008).

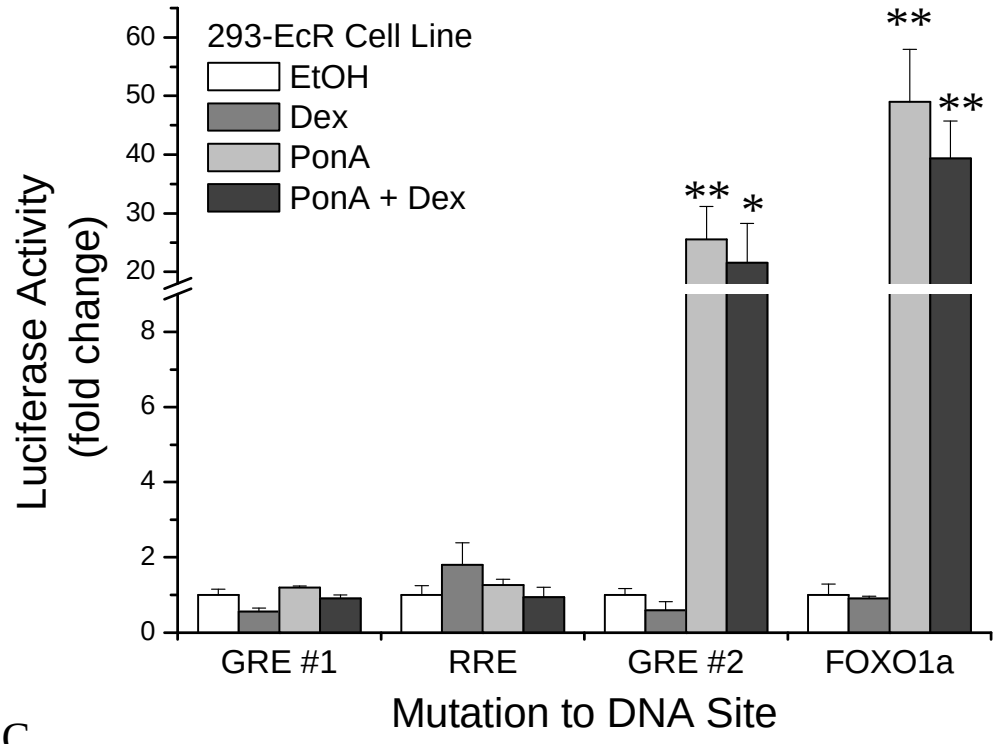
In order to determine if these binding site(s) were responsible for the VgEcR-RXR and GR mediated effects, the luciferase reporter assay was repeated with SEPP1 reporter constructs in which the two putative GREs or the RRE were mutated (Figure 3.5A). Despite the fact that GRE #2 was located within the -53 to +247 fragment that did not display any ponasterone A-induced luciferase activity in

Figure 3.5 Site-directed mutagenesis of GRE's identified within the SEPP1 promoter. (A) Schematic of the two putative GREs and RRE identified within the -109 to +247 SEPP1 promoter fragment along with previously identified sites in the same region (FOXO1a and HNF-4α). These response elements were mutagenized, as indicated by the bases identified with a bar, using a PCR-based strategy. **(B)** 293-EcR, and **(C)** EcR-GR cells were transfected with, appropriate mutant, -109 to +247 SEPP1 reporter constructs. Twenty-four hours after transfection, medium was replaced and cells were treated with EtOH (white), 10 nM Dex (grey), 10 μM PonA (light grey), or a combination of 10 nM Dex and10 μM PonA (dark grey) for an additional 24 hours. Cells were lysed and relative firefly luciferase activity was measured using a Dual Luciferase reporter assay. Triplicate samples were run in each experiment and data are presented as the mean ± standard error of relative activity changes observed over at least three biological replicates. ANOVA of each cell line revealed no significant differences when GRE #1 or the RRE is mutated, indicating that this is the important site for transactivation in 293-EcR and EcR-GR cells, but significant, $p<0.005$, differences in the 293-EcR and EcR-GR cells when evaluating a mutation of GRE #2 or the FOXO1a binding site. Post hoc tests reveal differences from the vehicle control (EtOH) (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) or differences among select treatment subsets (†, $p<0.05$; ††, $p<0.01$).

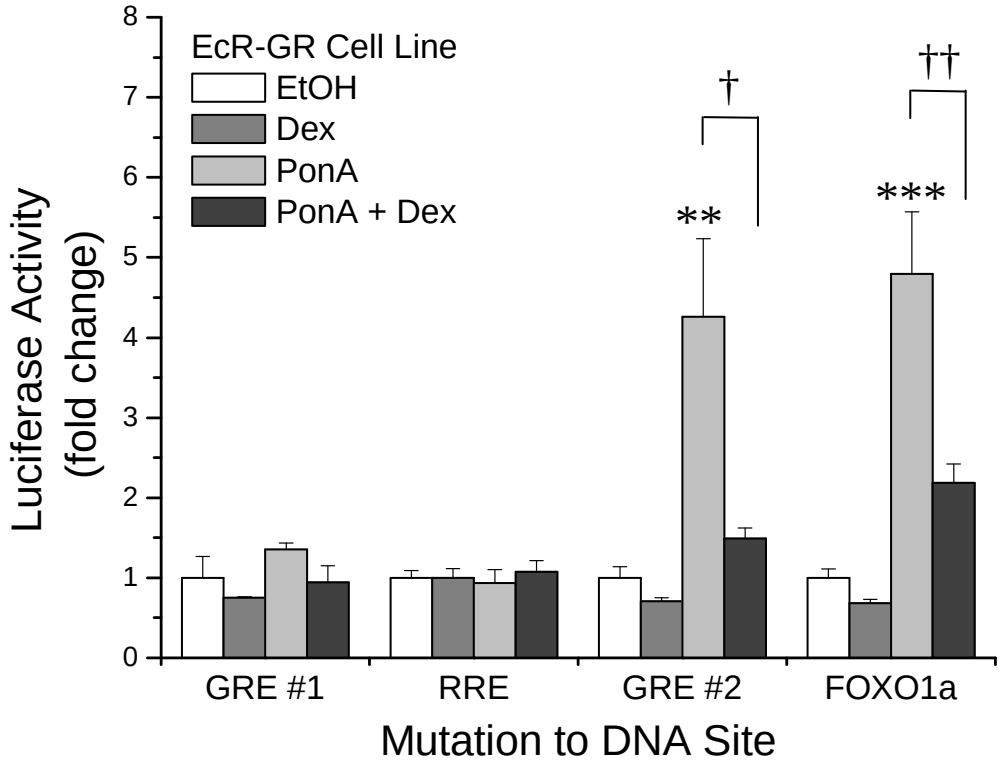
A


GRE Consensus Sequence:
AGAACA-NNN-TGTTCT
FOXO1a
TC
GTAAACAA
-35
-46
Transcriptional start
-109 to +247
-13
-24
-61
TCAGAGTGTGCT
GA
GRE #2
CT CTT
CAAGAATGAACATTGAACTTTGGACTA
RRE
GRE #1
-88
HNF-4α

B


293-EcR Cell Line
EtOH
Dex
PonA
PonA + Dex
Luciferase Activity
(fold change)
60
50
40
30
20
8
6
4
2
0
**
*
**
**
GRE #1
RRE
GRE #2
FOXO1a
Mutation to DNA Site

C


EcR-GR Cell Line
EtOH
Dex
PonA
PonA + Dex
Luciferase Activity
(fold change)
8
7
6
5
4
3
2
1
0
†
††
**
***
GRE #1
RRE
GRE #2
FOXO1a
Mutation to DNA Site

293-EcR cells (Figure 3.2B), a mutant form of this binding site was tested. This GRE more closely matched the consensus sequence, with an inverted repeat of the GR binding site that could accommodate a GR homodimer, and therefore, could be involved in GR-mediated repression.

In both 293-EcR and EcR-GR cells, ponasterone A-induced transactivation was completely lost upon mutation of GRE #1 or the RRE (Figure 3.5B and 3.5C), with the firefly:renilla luciferase ratio being decreased by ~10 fold on the RRE mutant construct compared to the mutant GRE#1 reporter (data not shown). These results suggest that both of these response elements serve as binding sites for the VgEcR-RXR transcriptional activation system. Transactivation was still observed with the mutated GRE #2 (Figure 3.5B and 3.5C) construct in both cell lines following ponasterone A treatment; however, it was slightly reduced compared to the non-mutated form. This indicates that this element may also be involved in activation of SEPP1 through VgEcR-RXR, although to a much lesser extent than GRE #1 or the RRE. The addition of dexamethasone plus ponasterone A resulted in attenuation of ponasterone A activity on the mutated GRE#2 luciferase reporter in the EcR-GR cells but not with the mutated GRE #1 reporter, indicating GRE #2 is not involved in the GR-mediated repression. FOXO1a has previously been shown to regulate SEPP1 transcription in hepatic cells through a binding site at position -46 of the promoter (Speckmann et al., 2008; Walter et al., 2008). As this regulatory mechanism involved coordination of FOXO1a with the dexamethasone-responsive cofactor PGC-1α, we also evaluated SEPP1 transcription following mutation to

the FOXO1a site. Neither a change in PonA-induced SEPP1 transactivation, nor repression by GR was observed in either 293-EcR or EcR-GR cells following mutation of the FOXO1a site (Figure 3.5B and 3.5C).

Quantitative PCR results further qualified the induction of SEPP1 by VgEcR-RXR, and its repression by the GR (Figure 3.6A). In 293-EcR, gene induction of ~5 fold was observed following 24 hours of ponasterone A treatment, and dexamethasone treatment had no effect on this induction. Similar to the responses observed with the luciferase activity assays, the ability of ponasterone A to activate SEPP1 was attenuated in EcR-GR cells. SEPP1 expression was reduced by ~80% in these cells, even in the absence of dexamethasone treatment. Treatment with dexamethasone for 8 or 16 hours eliminated the ability of ponasterone A to induce gene expression, and led to additional repression of SEPP1 in a time dependent manner. In addition, immunochemical analysis of SelP from a Ni-NTA bead pull-down of the media from EcR-GR cells demonstrated a similar pattern of protein expression (Figure 3.6B).

To determine if the GR directly binds the GRE #1 site, electrophoretic mobility shift assays were utilized (Figure 3.7). A protein:DNA complex bound to the GRE #1 was observed in both the 293-EcR and EcR-GR cells. There appears to be minimal modulation of the amount bound in the 293-EcR cells consistent with the expectation of binding by VgEcR-RXR with or without ligand present as is expected for the ecdysone-inducible system (Figure 3.7, lanes 12-15); however, a dexamethasone-dependent inhibition of binding was observed in the EcR-GR cells (Figure 3.7, lanes 2-5 and 9-11). The amount of protein:DNA complex observed

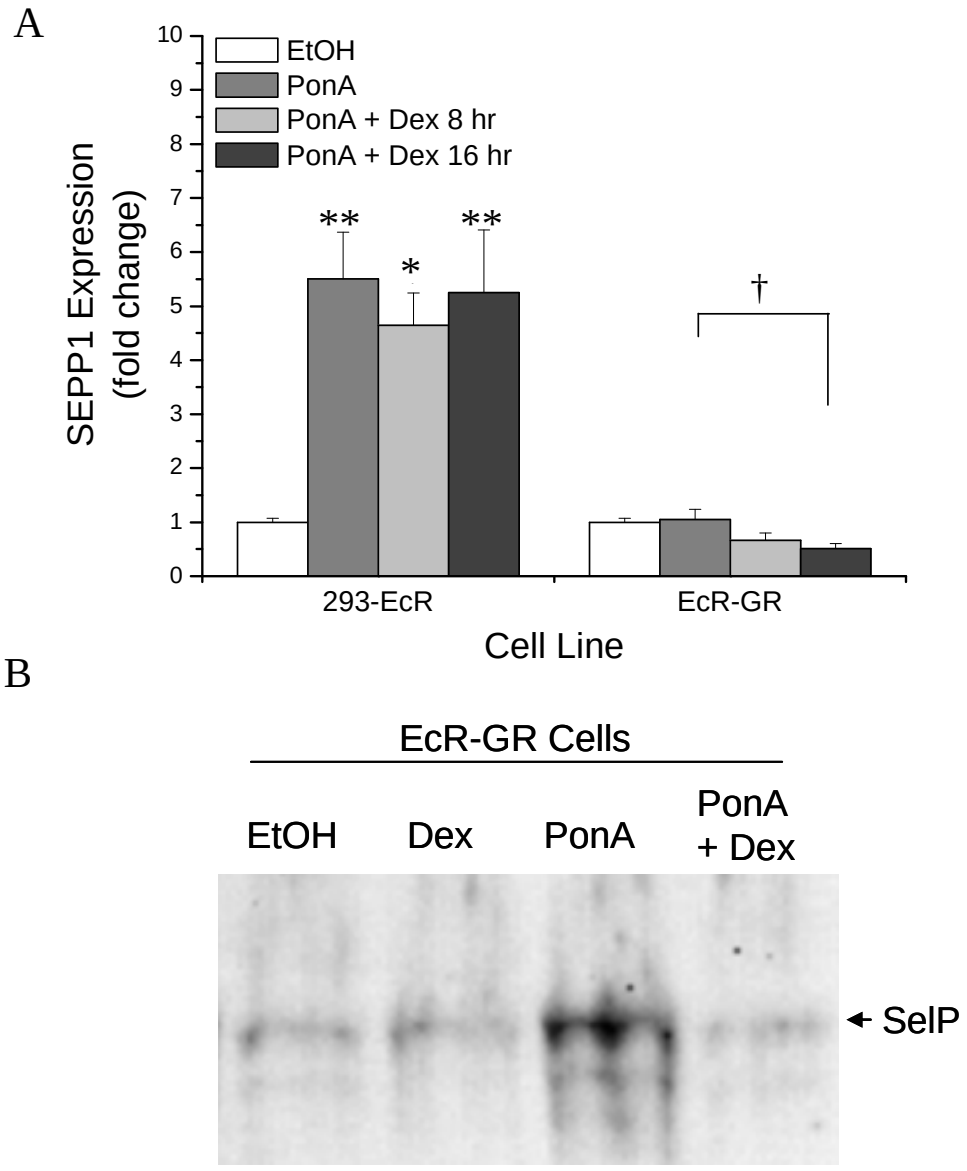


Figure 3.6 Analysis of SEPP1 expression in 293-EcR and EcR-GR cells. (A) 293-EcR and EcR-GR cells were treated with EtOH or 10 μM PonA 24 hrs prior to mRNA collection. Beginning 8 hours after PonA was added, cells were treated with 10 nM Dex for 8 or 16 hours prior to mRNA purification. SEPP1 expression was measured by quantitative PCR. Triplicate samples were run in each experiment and data are presented as the mean ± standard error of relative activity changes observed over at least five biological replicates. ANOVA of each cell line revealed significant differences of SEPP1 expression in 293-EcR and EcR-GR cells, $p<0.05$. Post hoc tests reveal differences from the vehicle control (*, $p<0.05$; **, $p<0.01$) or differences among select treatment subsets (†, $p<0.05$). **(B)** SelP protein from Ni-NTA bead pull-downs from culture media demonstrate expression increases in EcR-GR cells following 24 hrs treatment with PonA but Dex treatment attenuated the SelP expression.

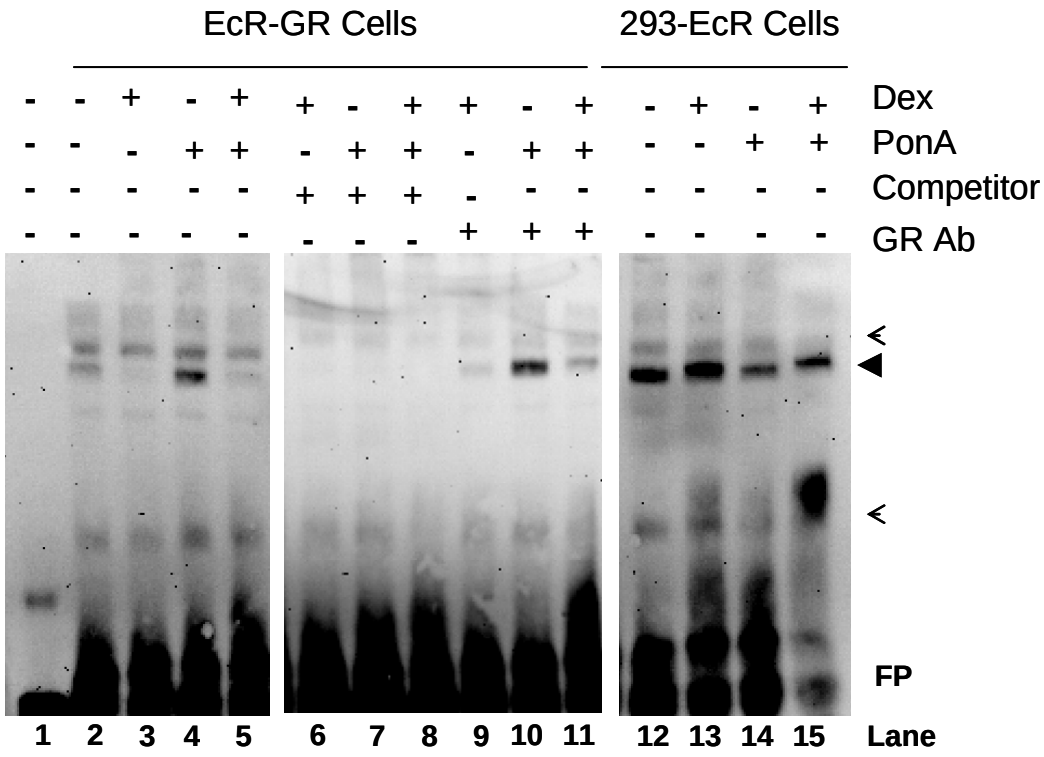


Figure 3.7 Electrophoretic mobility shift assays with GRE #1. Lane 1 contains the labeled GRE #1 fragment without an incubation with nuclear extract displaying the migration of the probe alone. Lanes 2-5 are the GRE #1 fragment with nuclear extract from EcR-GR cells that were treated with EtOH, Dex, PonA, or PonA + Dex, respectively. Lanes 6-8 show the same samples (without the EtOH control) but excess unlabeled probe is included to identify bands that represent specific protein:DNA complexes. In lanes 9-11 antibodies to GR are added to determine if the protein:DNA complex contains GR; a supershifted band was not observed. Lanes 12-15 show the four conditions with nuclear extract from the 293-EcR cells; all lanes display a strong protein:DNA complex. The specific complex is highlighted with the large arrow, the small arrows identify nonspecific bands that are in all lanes with nuclear extract, and FP stands for the free probe at the bottom of the gels.

appears to be consistent with the results from the heterologous reporter assays (Figure 3.4). However, there was a failure to demonstrate that the protein:DNA complex contains the GR as addition of the BuGR2 antibody (Calbiochem, Gibbstown, NJ) directed at residues 395-411 of the GR did not produce a supershift or substantially alter the relative levels of protein:DNA complex. Testing with a separate GR antibody (Affinity Bioreagents, Rockford IL) directed at residues 245-259 also failed to produce a supershift (data not shown).

Discussion

The effects of supplemental selenium intake have been evaluated in multiple chronic and acute diseases, including cancer, cardiovascular disease, and inflammatory conditions such as sepsis, trauma, and burns (Clark et al., 1996; Mark et al., 2000; Nomura et al., 2000; Brown & Arthur, 2001; Angstwurm & Gaertner, 2006; Angstwurm et al., 2007). In many studies, selenium has demonstrated beneficial properties but the results of the Selenium and Vitamin E Trial (SELECT) do not support the utility of supplemental selenomethionine in prostate cancer prevention (Lippman et al., 2009). The mechanism by which selenium exerts its effects during disease conditions is not completely understood; however, it has been hypothesized to be due to the antioxidant activity of selenoproteins (Diwadkar-Navsariwala & Diamond, 2004; Irons et al., 2006). These proteins contain selenium incorporated as the amino acid selenocysteine during translation of the protein (Tujebajeva et al., 2000; Small-Howard et al., 2006; Howard et al., 2007). Adequate selenium intake is important in maintaining proper translation and

function of the selenoproteins (Bermano et al., 1996; Wingler & Brigelius-Flohe, 1999). Therefore, maintenance of selenoprotein function may be the mechanism by which supplemental selenium intake exerts a beneficial health effect. In particular, the primary function of SelP is thought to be selenium distribution and the majority of the protein is synthesized in the liver for this purpose. However, most tissues can express SEPP1; suggesting alternative functions beyond selenium delivery may exist for SelP (Burk & Hill, 2005) as well as the possibility of tissue selective modulation of SEPP1 expression.

While the majority of SelP is expressed in the liver of adult mammals, SEPP1 orthologs in developing fish and mammals demonstrate broad tissue expression. Zebrafish, who have an extensive selenoproteome, includes two SEPP1 isoforms encoded by distinct genes; one (sepp1a) with a selenocysteine-rich C-terminus containing 16 selenocysteine residues, and a second isoform (sepp1b) that lacks the selenocysteine-rich C-terminus (Kryukov and Gladyshev, 2000). These genes demonstrate distinct spatiotemporal expression patterns throughout the development of the zebrafish with sepp1a displaying expression in multiple organs including the heart, brain and kidney, but only limited hepatic expression, while sepp1b demonstrates strong hepatic expression (Thisse et al., 2003). In addition, a recent study of the expression of the murine ortholog of SEPP1 in mouse embryos also highlights a potential role of SelP in growth and developmental processes. Spatiotemporal expression of Sepp was observed in the central nervous system, limb buds, blood cells, lung, liver, intestine, testis, and developing epithelia, as well as in extraembryonic tissues, during organogenesis. The authors suggest that this

increase in Sepp may provide antioxidant protection against the reactive oxygen species formed during embryogenesis, as well as provide a transplacental or intraembyronic selenium transport function (Lee et al., 2008). Additional evidence supporting a role for SelP in growth and development includes observations from the SelP knockout mouse, which displays a phenotype that includes growth retardation, neurological impairment, and male infertility (Hill et al., 2003; Schomburg et al., 2003; Renko et al., 2008). The regulatory signals responsible for modulating SEPP1 expression for the purpose of growth and development are currently under investigation.

Recently, hepatic SEPP1 expression was shown to be controlled through coordination of the transcription factors FOXO1a and HNF-4α by the coactivator PGC-1α (Speckmann et al., 2008; Walter et al., 2008). Discovery of this mechanism introduces the idea that SEPP1 can be regulated in response to hormonal stimuli and may be responsive to various nuclear receptors due to the versatility of PGC-1α.

Nuclear receptors are members of a large superfamily of proteins that function as ligand-inducible transcription factors (Germain et al., 2006; Teboul et al., 2008). This family contains steroid hormone receptors such as the glucocorticoid, estrogen, and androgen receptors, as well as receptors for thyroid hormones and retinoic acid. In addition, orphan nuclear receptors exist for which ligands have not been identified (Teboul et al., 2008). Examples of such orphan receptors include HNF-4α and chicken ovalbumin upstream promoter-transcription factors (Benoit et al., 2006). These receptors regulate gene transcription by binding to hormone response

elements in the promoter region of target genes. Most receptors bind as homo- or hetero-dimers to response elements composed of two core hexameric motifs. Consensus sequences for these motifs include AGAACA for steroid receptors and AG(G/T)TCA for the remaining nuclear receptors (Aranda and Pascual, 2001). Multiple nuclear receptor types can bind these sequences and mediate transcriptional activity, allowing for differential control of overlapping gene networks (Bedo et al., 1989; Umesono et al., 1991). Nuclear receptors have a well established role in growth, development and homeostasis as has been reviewed (Flamant et al., 2006).

The decrease in serum selenium observed during critical illness is believed to result from redistribution of the micronutrient to high priority organs (Angstwurm and Gaertner, 2006). The selenium distribution (Hill et al., 2003; Renko et al., 2008) and negative acute phase functions (Dreher et al., 1997) of SelP support a potential role for this protein in selenium changes observed during critical illness. Recently, a newly developed immunoassay was used to show a decrease in SelP in the serum of septic patients (Hollenbach et al., 2008). The exact mechanism responsible for this decreased protein expression is not known; however, the authors propose that it is due to proinflammatory cytokines that are induced as a result of the acute phase reaction occurring during sepsis, since several cytokines can repress SEPP1 expression (Dreher et al., 1997; Mostert et al., 2001). The evidence presented here also supports a potential role for the GR in regulating SEPP1 expression. Glucocorticoid responsiveness of SEPP1 could be of significance in critically ill patients, as these patients tend to have increased free

plasma cortisol levels (Hamrahian et al., 2004). Such regulation of SEPP1 by glucocorticoids could serve as an alternative explanation for the changes in SelP, and therefore the changes in serum selenium levels, observed during critical illness. However, a recent study demonstrates that the decrease in SelP in the acute-phase response appears to be a deficit in translation rather than a transcriptional response (Renko et al., 2009); therefore, the data herein may be more relevant for development or differentiation.

Here the VgEcR-RXR gene expression system was identified as a tool for studying the expression of SEPP1. The results indicate that once activated by ponasterone A, VgEcR-RXR is capable of inducing transcription of SEPP1 through a GRE located at position -87 or a RRE at position -73 of the promoter. In the EcR-GR cells, treatment with the GR agonist dexamethasone resulted in an attenuation of the ponasterone A-induced transcription of SEPP1 compared to ponasterone A treatment alone. This suggests that once activated by dexamethasone, the GR can travel to the nucleus and alter VgEcR-RXR binding at the site identified as GRE #1. While the EMSA failed to demonstrate GR binding through a supershift of the protein:DNA complex, nuclear extracts from the EcR-GR cells do display dexamethasone-dependent modulation of the protein:DNA complex that was consistent with the heterologous reporter expression assays. When a functional GR was stably integrated to make the EcR-GR cells, a generalized repression of SEPP1 was observed compared to the 293-EcR cells. This data supports the idea that the GR may indirectly regulate expression of this gene, and this effect was further validated by the evaluation of the protein levels of

SelP expressed in the EcR-GR cells; however, it should be noted that the conclusions drawn from the EMSA results are limited by the absence of a supershift under positive control conditions.

An indirect mechanism of GR modulation of transcription has been described previously through the interaction with CCAAT/enhancer-binding proteins (Rudiger et al., 2002). These proteins are involved in a broad spectrum of biological activities including development and differentiation (Ramji & Foka, 2002). Whether a GR interaction with a CCAAT/enhancer-binding protein might be involved in SEPP1 regulation will require further study, and the precise cause for the repression observed in this study is unknown; however, transfection of GRs has previously been shown to be sufficient for the repression of hormone-responsive genes (Gougat et al., 2002).

The GR usually binds DNA as a homodimer; however, it has been demonstrated that monomers can bind to 'half-sites' and modulate transactivation when the binding site is far as 37 base pairs from the TATA element. The maximum distance at which this activity is retained is unknown; however, it was shown to be lost when the binding site was inserted 350 base pairs upstream from the transcriptional start site (Strahle et al., 1988). The GRE #1 site identified here is 47 base pairs 5' to the TATA element and could potentially be mediating activation through this mechanism. Alternatively, a GR monomer could potentially be working synergistically with another transcription factor (Strahle et al., 1988) or perhaps another cryptic GRE may be present within this region that has not yet been identified. Another site for GR binding might explain the repression observed with

dexamethasone treatment as well as the reduction of the protein:DNA complex observed in the EMSA if GR binding would modulate the occupancy of other regulators of SEPP1 expression.

The local region identified as GRE #1 is within a region that has already demonstrated insulin-dependent attenuation of SEPP1 expression by modulation of HNF-4α activity (Speckmann et al., 2008), and therefore, this could be a critical region that determines the expression levels of SEPP1 based on the affinity and availability of transcriptional regulators in different cell types. Other genes have HNF-4α responsive elements that overlap with GR or RXR responsive elements and perhaps this allows for more intricate modulation of these genes in development (Crestani et al., 1998; Bailly et al., 2001). It is unlikely that the effects on transactivation observed here are related to interactions with HNF-4α since this transcription factor is not expressed in HEK-293 cells (Lucas et al., 2005), and it is unclear how HNF-4α-mediated SEPP1 regulation would account for alterations in serum selenium levels in critically ill patients since insulin sensitivity changes would allow for more SelP expression (Lazzeri et al., 2009).

In addition to GREs of the -109 to +247 fragment, it appears there are other dexamethasone-dependent repressive elements acting within the -1652 to +247 fragment. Ponasterone A-induced activation is reduced on this fragment as compared to the -109 to +247 fragment (Figures 3.2, 3.4 and 3.5). Plus, attenuation of the SEPP1 promoter was observed on the larger fragment in the EcR-GR cells following dexamethasone treatment, but was not observed on the smaller fragment (Figure 3.4). *In silico* evaluation of this region identified additional potential GREs,

but again, these sites are primarily half-sites and do not appear to be classical GREs. Furthermore, the region 5' to -109 in the SEPP1 promoter appears to have additional repressive elements (Figure 3.2). These elements are not well characterized and *in silico* evaluation did not reveal obvious potential repressive elements; however, one complex repeat region has demonstrated repression of SEPP1 expression with certain polymorphisms (Al-Taie et al., 2002). This region overlaps the 5' end of the promoter reporter construct -391 to +247 used in this study, and perhaps was responsible for the attenuated response observed compared to the -109 to +247 promoter construct.

Finally, despite the fact that the VgEcR-RXR system is not expected to transactivate host genes by itself, changes in endogenous gene levels have been previously observed in mammalian cells treated with ecdysone receptor ligands (Oehme et al., 2006; Panguluri et al., 2007). In the experiments described here, activation of the transcriptional machinery was shown to be sufficient for changes in expression of at least one host gene, SEPP1. Due to the complex nature of selenoprotein translation (Tujebajeva et al., 2000; Small-Howard et al., 2006; Howard et al., 2007), many cell lines that are commonly used express selenoproteins poorly; however, HEK-293 cells have been successfully used in other studies for the expression of selenoenzymes (Madeja et al., 2005; Squires et al., 2007). Therefore, this 293-EcR system may function as a particularly effective system for the study of SelP transcription and translation processes. While serving as a beneficial tool in the studies presented herein, the potential for this system to

transactivate host genes may be considered as a possible limitation to the use of this inducible gene expression system in other studies.

In conclusion, data are provided supporting alternative mechanisms for extrahepatic regulatory mechanisms of SEPP1 expression that may help explain SEPP1 expression in inflammation, development and differentiation. An engineered, fusion transcription factor that contains the DNA binding domain from GR, coupled with a strong transactivation domain, along with RXR, was used to identify the site responsible for the induction of SEPP1 expression. However, these studies revealed that the native GR inhibits the expression of SEPP1 through an indirect mechanism. Therefore, the ability of corticosteroids, and perhaps retinoids, to modulate SEPP1 expression may be a mechanism that could result in altered tissue selenium distribution since SelP is the major carrier of selenium.

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CHAPTER 4

SELENOPROTEIN P PROTECTS CELLS FROM LIPID HYDROPEROXIDES GENERATED BY 15-LOX-1

Introduction

A relationship between chronic inflammation and carcinogenesis has been noted in numerous malignancies, including colon cancer and hepatocellular carcinoma (Itzkowitz and Yio, 2004; Macarthur et al., 2004). It is believed that leukocytes and other phagocytic cells involved in the inflammatory process may lead to an induction of DNA damage in proliferating cells through the production of reactive oxygen and nitrogen species (Coussens and Werb, 2002). Additionally, enzymes expressed during the inflammatory process, including lipoxygenase (LOX) enzymes, have been shown to be upregulated in certain malignancies (Kelavkar et al., 2000; Gupta et al., 2001). Specifically, 15-LOX-1 expression is directly proportional to severity of prostate cancer, as measured by Gleason staging (Kelavkar et al., 2000; Kelavkar et al., 2001). Membrane lipids released and metabolized during inflammation, such as arachidonic acid (AA), have also been linked to various malignancies, including prostate cancer (Hursting et al., 1990). 15-LOX-1 can metabolize arachidonic acid to reactive hydroperoxy intermediates,

such as 15-hydroperoxyeicosatetraenoic acid (15-HpETE), the oxidative precursor of 15-hydroxyeicosatetraenoic acid (15-HETE) (Natarajan and Nadler, 2004; Sordillo et al., 2008). In addition, end products of lipid peroxidation have been implicated as being mutagenic (Ray and Husain, 2002), further contributing to evidence that inflammation may result in carcinogenesis through its ability to increase the oxidative tone of the cellular environment.

Cells possess several enzymes that can reduce lipid peroxides. Multiple selenoenzymes are specifically involved in the reduction of oxidized lipids. Glutathione peroxidase 4 (GPx4, also called phospholipid hydroperoxide GPx, or PHGPX) is an essential selenoenzyme that is associated with protection from lipid hydroperoxides (Yant et al., 2003). Thioredoxin reductase can also reduce some oxidized lipids (Bjornstedt et al., 1995), as well as indirectly modulate lipid peroxides through the reduction of peroxiredoxins (Mitsumoto et al., 2001). The role of selenoprotein P (SelP) as a lipid hydroperoxidase is still being elucidated. SelP is one of only two selenoproteins found in the extracellular environment, with GPx 3 being the other (Takahashi and Cohen, 1986; Akesson et al., 1994), and one function is in selenium distribution (Hill et al., 2003; Renko et al., 2008). As opposed to GPx4 and thioredoxin reductase, SEPP1 knockout mice are viable but they display altered selenium distribution (Hill et al., 2003; Burk et al., 2006). Besides the selenium distribution function, multiple pieces of evidence support an antioxidant function of SelP. This protein has been attributed to protecting rats against diquat-induced liver toxicity through a decrease in lipid peroxidation (Burk et al., 1995). Depletion of SelP from plasma enhances plasma protein oxidation

mediated by peroxynitrite-induced oxidation and nitration (Arteel et al., 1998). In addition, SelP protects low-density lipoproteins from peroxidation (Traulsen et al., 2004). In a cell-free in vitro system, SelP has been shown to reduce phospholipid hydroperoxide to a greater extent than other reactive oxygen species, including hydrogen peroxide and *tert*-butyl hydroperoxide (*t*-BHP) (Saito et al., 1999), and the N-terminal domain of the protein is beleived to be responsible for this effect (Saito et al., 2004).

These data suggest that an enzymatic activity of SelP may be specific for lipid-derived substrates, as opposed to other sources of reactive oxygen stress. The purpose of this study was to determine if SelP displayed lipid hydroperoxidase activity directed at 15-LOX-1-generated metabolites. In this chapter, the ability of SelP to reduce 15-HpETE and to protect human embryonic kidney (HEK-293) cells from oxidation is reported. Furthermore, SelP was capable of protecting a target cell population from oxidation produced by cells engineered with inducible 15-LOX-1 that were provided arachidonic acid substrate. The evidence presented suggests SelP may play a role in reducing lipid hydroperoxides following membrane lipid metabolism, which could serve to protect the cells from the toxic effects of chronic inflammation.

Methods

Materials

The HEK-293 cell line was purchased from American Type Culture Collection (Manassas, VA). Advanced DMEM, CD-293, and zeocin were purchased from Invitrogen (Carlsbad, CA). Ovalbumin was purchased from Fisher Scientific (Pittsburgh, PA). Ponasterone A was purchased from A.G. Scientific (San Diego, CA). Arachidonic acid was purchased from NuCheck Prep (Elysian, MN). *E. coli* thioredoxin, *E. coli* thioredoxin reductase, and *tert*-butyl hydroperoxide were purchased from Sigma-Aldrich (St. Louis, MO). Purified 15-HpETE and 15-HETE were purchased from Cayman (Ann Arbor, MI), as was the 15-HETE enzyme immunoassay. Rat Selenoprotein P was a gift from Drs. Kris Hill and Raymond Burk, Vanderbilt University. Diphenylpyrenylphosphin (DPPP) was purchased from Dojindo Molecular Technologies (Rockville, MD).

Cell Culture

Unless otherwise noted, the human embryonic kidney line HEK-293, as well as all subsequently engineered cells, were cultured in Advanced DMEM medium containing 2% fetal bovine serum and 2mM L-glutamine. Cells were maintained at 37°C in a humidified incubator with 5% CO_2. Advanced DMEM is supplied with 5 µg/l sodium selenite ($NaSeO_3$) and the serum contained 37 ng/ml selenium. Therefore, even with only 2% serum, the selenite content of this media results in selenium-sufficient media.

Conditional Expression of 15-LOX-1

pVgEcR (Invitrogen) encodes the fusion transcription factor used to generate ecdysone-inducible cells. HEK-293 were transfected with pVgEcR and selected for zeocin resistance to generate stable expression of the VgEcR gene product and are referred to as 293-EcR. 293-EcR cells that conditionally express 15-LOX-1 were used as previously described (Yu et al., 2004; Cordray et al., 2007).

Preparation and Purification of 1-Palmitoyl-2-(13-hydroperoxy-cis-9,trans-11-octdecadienoyl) Phosphatidylcholine (PLPC-OOH)

PLPC-OOH was prepared and quantified as previously described (Saito et al., 1999). Briefly, PLPC was oxidized with soybean lipoxidase and resulting PLPC-OOH was extracted with ethyl acetate. The ethyl acetate extract was evaporated, dissolved in methanol, and PLPC-OOH was purified by HPLC. PLPC-OOH was dissolved in methanol and stored at -20°C.

Biochemical Enzyme Assay

A NADPH-coupled reaction was used to assess the ability of SelP to reduce various lipid substrates. Lipid substrates tested in the assay included 10 μM 15-HETE, 10 μM 15-HpETE, 100 μM *tert*-butyl hydroperoxide, and 60 μM PLPC-OOH. The assay was run in a 384 well UV transparent clear bottom plate. Reaction mixtures contained 0.1M Tris-HCl, pH 7.4, 0.24 mM NADPH, 1mM EDTA, 0.025% Triton-X-100 / 0.3% sodium deoxycholate, ~0.1 Units *E. coli* thioredoxin reductase, 3.2 μg rat SelP, and appropriate lipid substrate aliquots.

After a 10 minute incubation at 25°C, the reaction was initiated by the addition of 6.66 µM *E. coli* thioredoxin to the sample wells. In control experiments, reactions mixtures without rat SelP or *E. coli* thioredoxin were used to evaluate the spontaneous reaction rates. The oxidation of NADPH was measured by monitoring the absorbance at 340 nm (A_{340}) for ~500 sec.

Enrichment of Selenoprotein P

Increased transcription and translation of SelP has previously been observed in 293-EcR cells treated with the ecydsone analog ponasterone A (PonA) (Rock and Moos, 2009). 293-EcR cells were maintained in serum-free CD-293 cell culture medium supplemented with 2mM L-glutamine, 1 µM sodium selenite and 10 µM PonA. After 3 days, supernatant was collected from the cells following centrifugation at 250 x g for 5 minutes. The supernatant was concentrated ~20 fold using a Centricon Plus-70 centrifugal filter (Millipore) with a 30-kDa cutoff membrane. This concentrated media retains SelP, and was used in experiments to evaluate antioxidant properties of SelP. As a control, supernatant was collected and concentrated from vehicle (EtOH) treated 293-EcR cells.

Inductively coupled plasma (ICP) spectrometry (Perkin Elmer Optima 3100 XL) was used to determine the selenium content in the concentrated cell culture media. The instrument was calibrated using SPEX CertiPrep Laboratory Performance Check Standard 1 (Metuchen, NJ). The results were collected in parts per million (ppm) using WinLab32 for ICP software (v. 3.4.0.0253) and then converted to Se concentration. Selenium content of the concentrated supernatants

was used to calculate SelP concentration of the supernatant based on the assumption that there are 10 selenium atoms per molecule of SelP. Protein expression was verified by immunoblotting. The supernatant collected from PonA-treated cells was referred to as (+) SelP, while that collected from EtOH-treated cells was referred to as (-) SelP.

Measurement of Lipid Hydroperoxides

Lipid hydroperoxides were measured using DPPP, a molecular probe that becomes fluorescent upon oxidation by lipid hydroperoxides (Takahashi et al., 2001). HEK-293 cells were plated on a 384-well tissue culture plate at a concentration of 18,000 cells/well. Cells were labeled with 100 µM DPPP or DMSO control and were incubated overnight. Cells were supplemented with (+) SelP supernatant at a concentration of 60 nM SelP. An equivalent amount of (-) SelP concentrated supernatant was also tested, as was 100 nM sodium selenite, and selenium-sufficient blank control medium containing 5 µg/l sodium selenite. Immediately following the addition of these supplements, cells were treated with 0-100 µM 15-HpETE, 30 µM 15-HETE or EtOH control. Because some variability in results were observed between batches of the hydroperoxy lipids purchased from Cayman, all reported results were tested from the same batch number of 15-HpETE (13250-6) or 15-HETE (156030-19). Fluorescent intensities following excitation at 351 nm were measured at the emission wavelength of 380 nm with a Perkin-Elmer Victor^3 V plate reader.

Enzyme Immunoassay

EcR-15-LOX cells were seeded on a 24-well plate at a concentration of 1×10^5 cells/well. After 24 hours, culture medium was changed to serum-free CD-293 supplemented with 2 mM L-glutamine and 0.1% ovalbumin. Cells were treated with 10 µM ponasterone A for 24 hours, followed by a 2-hour treatment with 60 µM arachidonic acid. Vehicle treatment with EtOH served as controls for both treatment conditions. Culture medium was collected and 15(S)-HETE levels were measured by enzyme immunoassay according to the manufacturer's instructions.

Immunoblotting

Following collection of culture medium for enzyme immunoassay evaluation, EcR-15-LOX cells were resuspended in lysis buffer, sonicated at 4°C, centrifuged at 14,000 rpm for 10 minutes, and supernatant was collected. For EcR-15-LOX samples, 5 µg of the supernatant protein were separated by SDS-PAGE. For enriched supernatant samples collected from 293-EcR cells, 15 µl of the sample were separated by SDS-PAGE. Following separation, proteins were transferred to a nitrocellulose membrane and membranes were probed for 15-LOX-1 (Cayman Chemical, Ann Arbor, MI) or SelP (gift from Kris Hill & Raymond Burk). A peroxidase conjugated secondary antibody was used to detect chemiluminescence indicative of protein expression.

Transcellular Assay

EcR-15-LOX cells were plated in a 384-well tissue culture plate at 6,000 cells/well. Cells were treated with 10 µM ponasterone A and incubated overnight. HEK-293 cells were grown in 25cm^2 flasks in serum-free CD-293 cell culture medium supplemented with 2 mM L-glutamine and 0.1% ovalbumin. These cells were labeled with 100 µM DPPP or DMSO control. After 24 hours, DPPP-labeled HEK-293 cells, or unlabeled controls, were added into the wells with the EcR-15-LOX cells at a concentration of 18,000 cells/well. Cells were allowed to recover for 1 hour prior to the addition of (+) SelP at 60 nM SelP or (-) SelP control supernatant. Immediately following the addition of the concentrated supernatant, cells were treated with 60 µM arachidonic acid. Vehicle treatment with EtOH served as controls for both ponasterone A and arachidonic acid treatments. Thirty minutes after arachidonic acid addition, fluorescent intensities were measured with a Perkin-Elmer Victor^3 V plate reader as described above.

Statistical Analysis

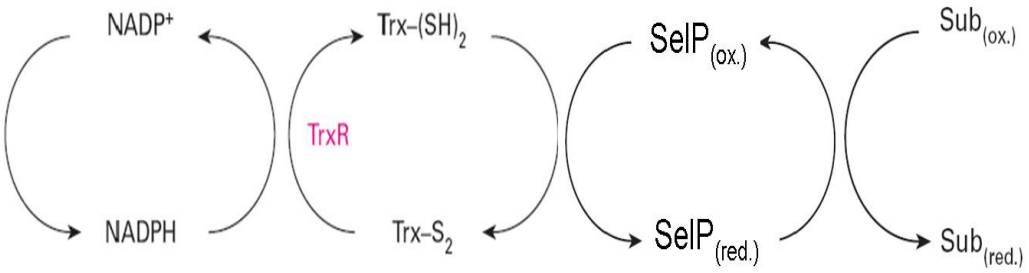
GraphPad Instat, version 3.06, was used to evaluate the statistical significance of the results. Statistical significance was determined by one-way ANOVA with Bonferroni multiple comparison post hoc tests, and differences were considered significant for $p<0.05$.

Results

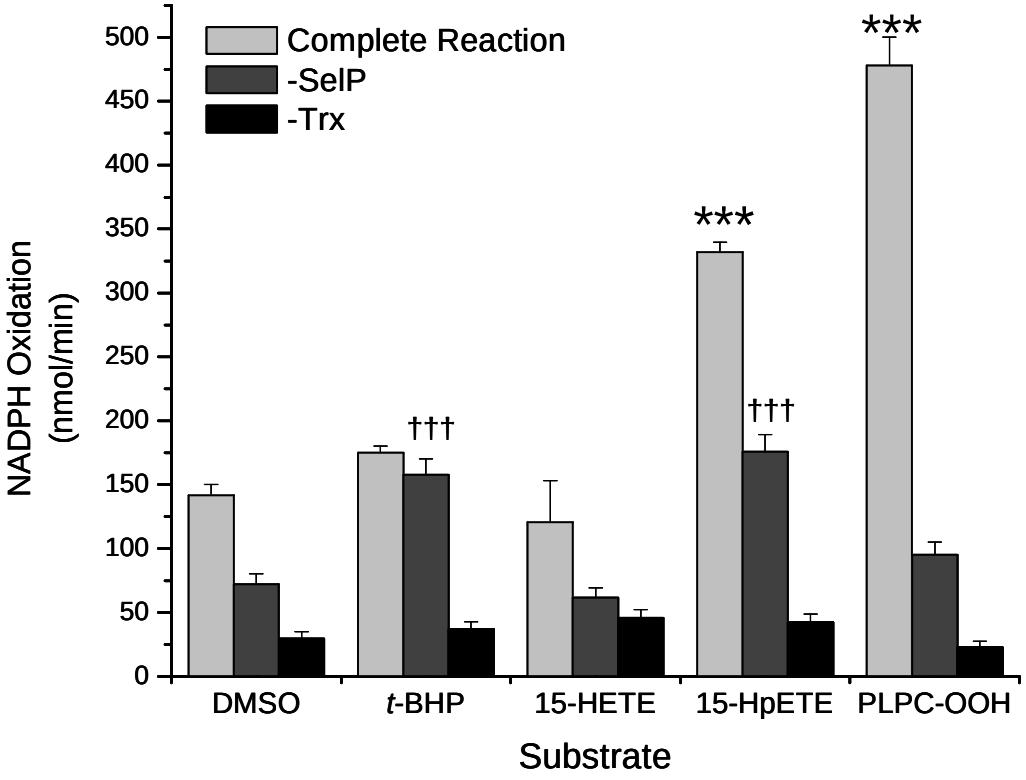
The ability of SelP to reduce PLPC-OOH through a NADPH-coupled biochemical assay has been described previously (Saito et al., 1999; Takebe et al., 2002). Similar methods were followed to test the ability of SelP to reduce 15-HpETE and 15-HETE. Because 15-HpETE was previously shown to inhibit the activity of mammalian thioredoxin reductase 1 (Yu et al., 2004), *E. coli*, rather than mammalian, thioredoxin reductase and thioredoxin were used in this assay. The *E. coli* form of this enzyme does not possess a C-terminal selenocysteine (Gromer et al., 2004) such as that found to be responsible for covalent binding of electrophilic lipids on the mammalian enzyme (Cassidy et al., 2006). An NADPH-coupled showed that activity of the E. coli enzyme was not inhibited by 15-HpETE (data not shown). NADPH oxidation was used as an indirect measure of the hydroperoxidase activity of SelP. Nonenzymatic NADPH oxidation rates were observed when thioredoxin was not added to the reaction mixtures and did not show substrate selectivity. NADPH oxidation when SelP was not added to the reaction mixtures reflects activity by the *E. coli* thioredoxin system and substrate preferences for *t*-BHP and 15-HpETE was observed. However, with complete reaction mixtures, the PLPC-OOH was the best substrate as measured by the most NADPH oxidation (Figure 4.1). 15-HpETE was the next best substrate, with ~70% of the activity observed with the PLPC-OOH substrate. However, ~50% of that activity may be contributed by the thioredoxin system coupled in this reaction. Essentially no selective SelP activity was observed for 15-HETE and *t*-BHP.

Figure 4.1 Lipid hydroperoxidase activity as measured in a NADPH-coupled reaction. (A) NADPH oxidation was used as an indirect measure of the hydroperoxidase activity of SelP against various lipid substrates (Sub). The *E. coli* thioredoxin reductase (TrxR) system was used to enzymatically reduce SelP as oxidized (ox) lipid substrates became reduced. (B) NADPH oxidation was measured in each reaction mixture base, described in the Methods, with *t*-BHP, 15-HETE, 15-HpETE, or PLPC-OOH as potential substrates as well as the vehicle control (DMSO). Each substrate was tested with a complete reaction mixture, or mixtures lacking either SelP or thioredoxin (Trx). The SelP activity in the complete reaction mixtures with the 15-HpETE and PLPC-OOH substrates were significantly different from the other conditions (***, $p<0.001$). The NADPH oxidation was increased with *t*-BHP and 15-HpETE substrates in the mixtures without SelP (-SelP) (†††, $p<0.001$) indicating that the *E. coli* thioredoxin system utilized had background activity on these substrates independent of SelP.

A


NADP+
Trx–(SH)2
SelP(ox.)
Sub(ox.)
TrxR
NADPH
Trx–S2
SelP(red.)
Sub(red.)

B


Complete Reaction
-SelP
-Trx
NADPH Oxidation (nmol/min)
0
50
100
150
200
250
300
350
400
450
500
***
***
†††
†††
DMSO
t-BHP
15-HETE
15-HpETE
PLPC-OOH
Substrate

Since SelP-mediated activity was observed, experiments were run to determine whether the activity of SelP observed in the biochemical assay could be translated to a cell-based system. SelP was derived from the supernatant of ponasterone A treated 293-EcR cells and selenium content of concentrated supernatant was determined by ICP spectrometry. Increased selenium content was observed in the (+) SelP supernatant versus (-) SelP (0.285 ppm vs. 0.066 ppm). The (-) SelP concentrated media selenium content was comparable to the background level of selenium measured in the CD-293 media when it was not concentrated (data not shown). The SelP content in these media were confirmed by immunoblotting, which showed considerable SelP expression in (+) SelP supernatant, but minimal SelP (generally <5% of the induced media) in the (-) SelP supernatant (Figure 4.2). Using the assumption of ten selenium atoms per molecule of SelP (Burk and Hill, 2005), the (+) SelP supernatant was calculated to have a concentration of ~360 nM SelP, while the (-) SelP was calculated as ~80 nM SelP; however, this media may have an even lower SelP content as this selenium content was similar to the defined CD-293 media selenium content.

A fluorescent-based assay (DPPP) was used to detect lipid hydroperoxide levels following exposure of HEK-293 cells to the reactive lipid metabolite 15-HpETE. With cells grown in selenium-sufficient medium, the cellular oxidiation was evaluated from 0-240 minutes. Under all treatment conditions, cellular oxidation reaches its maximum after approximately 25-30 minutes of 30 μM 15-HpETE exposure. SelP enriched media consistently demonstrated a reduction of cellular oxidation over this time course (Figure 4.3A). Addition of 15-HpETE

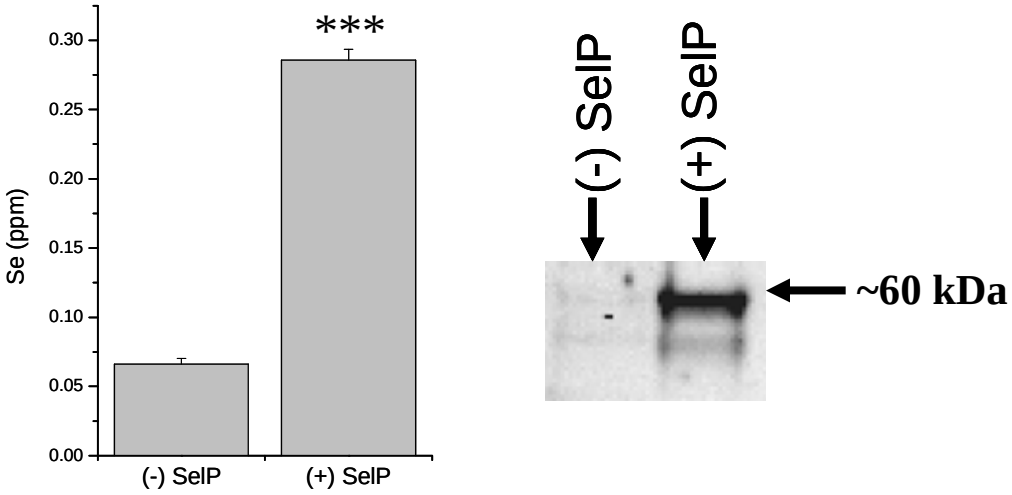
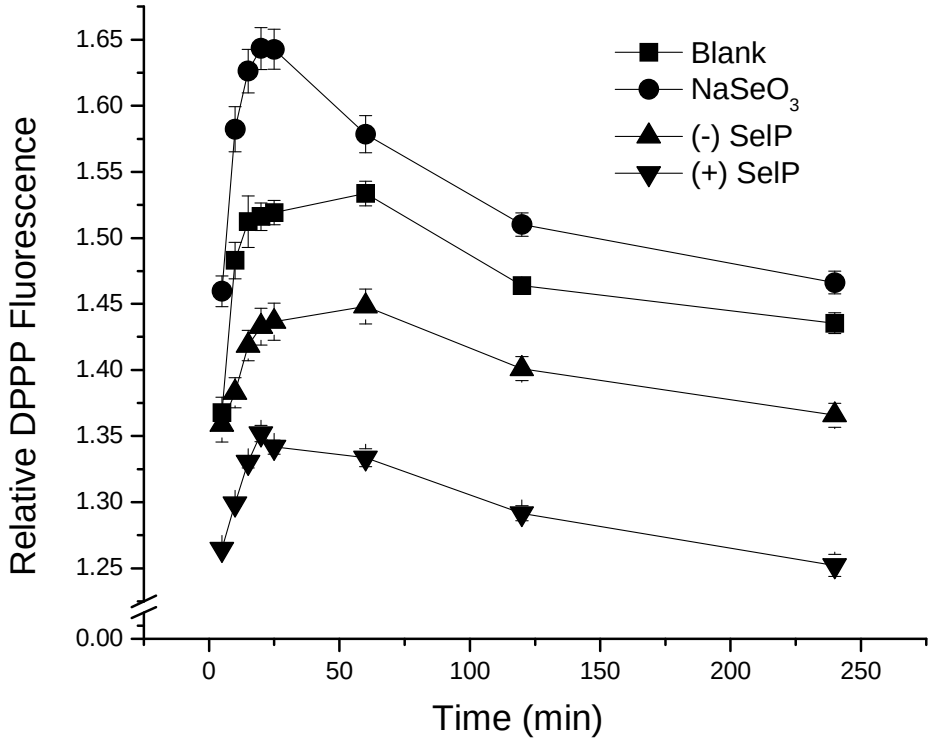


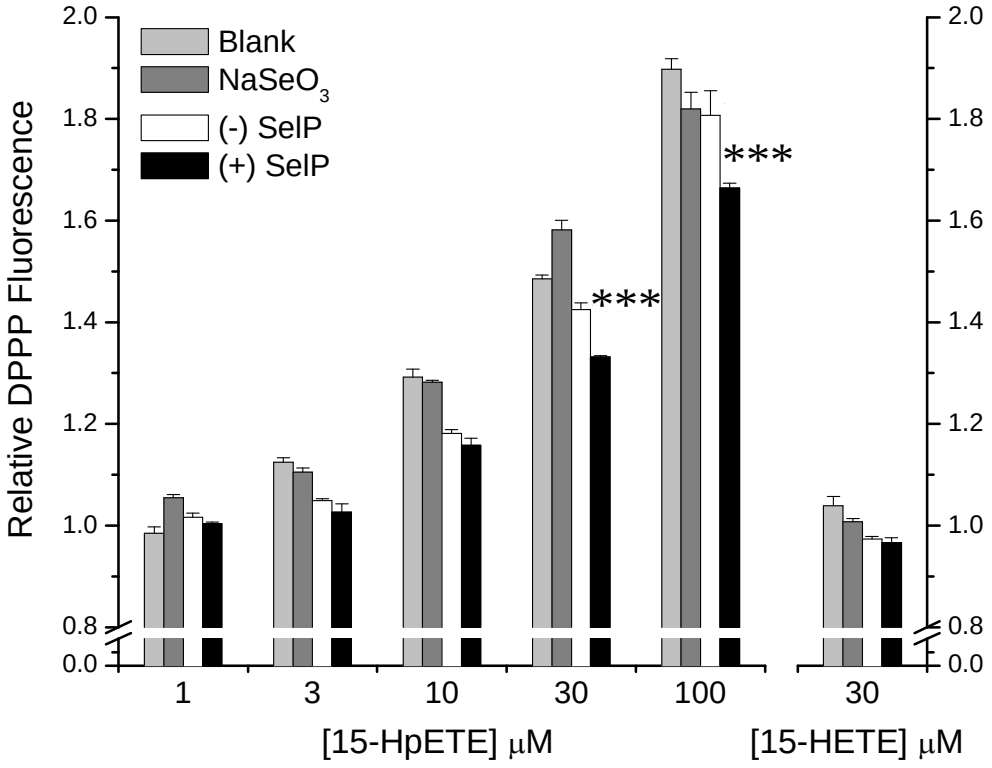
Figure 4.2 Assessment of selenium and SelP content in concentrated media. *Left*: ICP analysis of selenium (Se) content in media samples; (+) SelP represents media from cells induced to express SelP by ponasterone A while (-) SelP represents media from cells treated with EtOH (vehicle control). The selenium difference between these concentrated media is highly significant (***, $p<0.001$). *Right*: Immunoblot analysis for SelP in these media samples.

Figure 4.3 SelP protects HEK-293 cells from oxidation by the pharmacological addition of 15-HpETE. (A) Time course oxidative changes measured by DPPP fluorescence following 30 µM 15-HpETE addition in cells with standard media (blank, square), 100 nM sodium selenite ($NaSeO_3$, circle), concentrated control media ((-) SelP, up triangle), and SelP enriched media ((+) SelP, down triangle). The (+) SelP condition is statistically different at all time points ($p<0.001$). **(B)** Dose response of 15-HpETE oxidative changes measured by DPPP fluorescence (*left*), as well as 15-HETE at 30 µM (*right*). The (+) SelP condition is significantly different from the other conditions at 30 and 100 µM 15-HpETE (***, $p<0.001$).

A


Relative DPPP Fluorescence
1.65
1.60
1.55
1.50
1.45
1.40
1.35
1.30
1.25
0.00
0
50
100
150
200
250
Time (min)
Blank
NaSeO3
(-) SelP
(+) SelP

B


Relative DPPP Fluorescence
Blank
NaSeO3
(-) SelP
(+) SelP
***
***
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.0
1
3
10
30
100
30
[15-HpETE] μM
[15-HETE] μM

resulted in a dose-dependent increase in DPPP fluorescence and the cells with SelP enriched media demonstrated significant protection from oxidation at 30 and 100 µM 15-HpETE (Figure 4.3B). The addition of SelP reduced relative DPPP fluorescence compared to both standard (blank) medium and (-) SelP controls (~12% and ~7% reduction, respectively). Since some studies, with viability as an outcome, have shown that both SelP and 100 nM sodium selenite can improve viability following oxidative stress (Steinbrenner et al., 2006a; Steinbrenner et al., 2006b), the effect of selenium supplementation with 100 nM sodium selenite was also tested in this assay. However, in this case, the addition of sodium selenite exerted an oxidative effect, as evidenced by an increase in relative DPPP fluorescence as compared to standard (identified as blank) control medium following 15-HpETE addition. In addition, no increase in DPPP fluorescence was observed following treatment with 30 µM of the less oxidative 15-HETE lipid metabolite (Figure 4.3B).

To determine if SelP could protect cells from oxidation following 15-LOX-1 catalysis of arachidonate, overexpression of 15-LOX-1 was achieved using an ecdysone-inducible gene expression system (Figure 4.4A). Following addition of arachidonic acid, the enzymatic activity of 15-LOX-1 was confirmed through detection of 15-HETE by enzyme immunoassay. Production of the metabolite increased significantly following treatment of cells with the combination of ponasterone A and arachidonic acid, but was observed at only a minimal level under control conditions (Figure 4.4B). DPPP was used to detect lipid hydroperoxides following arachidonic acid metabolism by this system. A

A

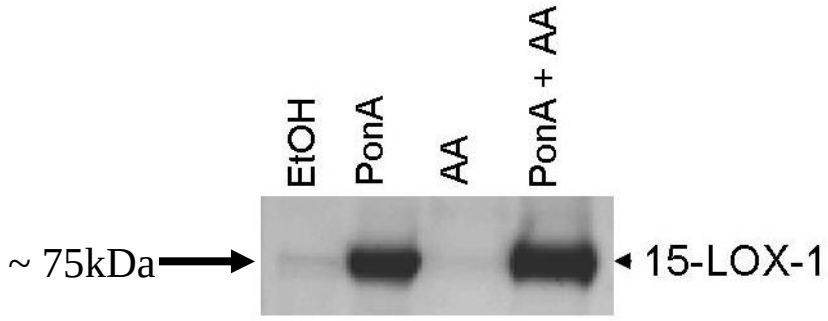
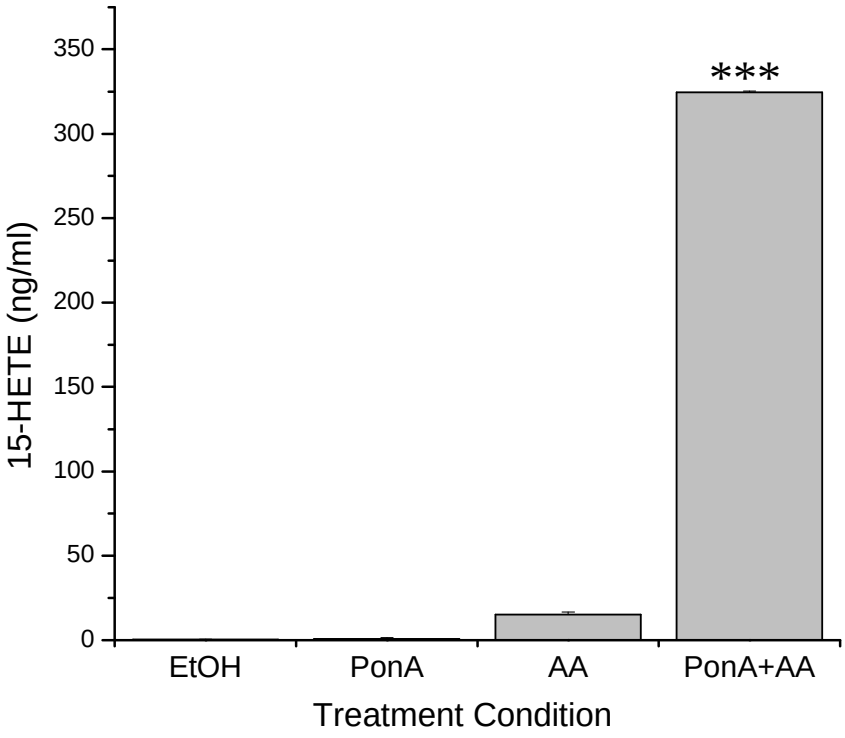
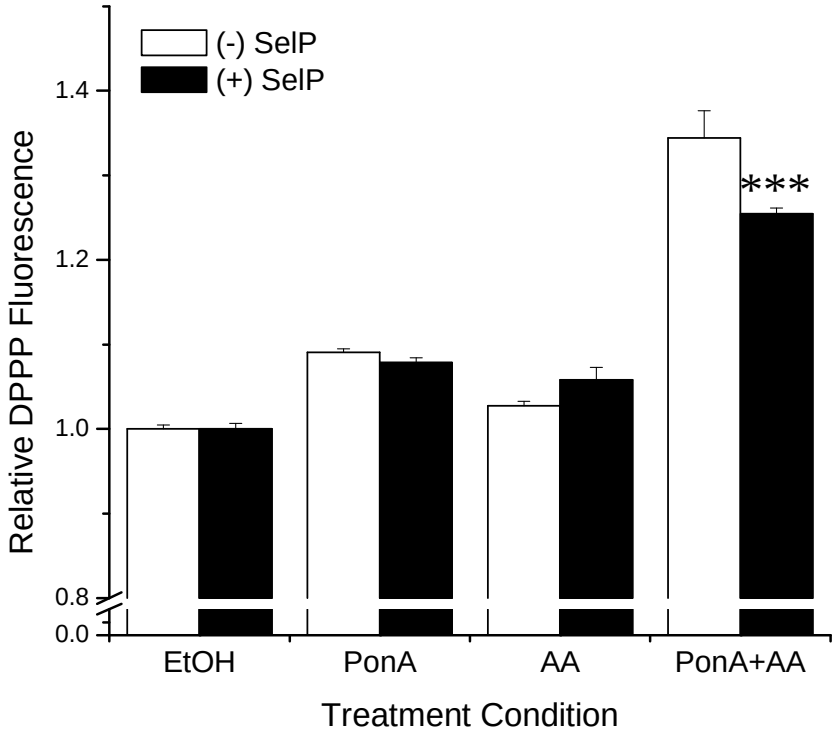


Figure 4.4 SelP can protect target cells from the oxidation by effector cells following 15-LOX-1 catalysis of arachidonate. **(A)** Inducible expression of 15-LOX-1 in 293-EcR cells with integrated 15-LOX-1. Four conditions are evaluated; vehicle control (EtOH), ponasterone A (PonA), arachidonic acid (AA), and the combination ponasterone A and arachidonate (PonA+AA). **(B)** Production of the 15-LOX-1 metabolite 15-HETE under the four conditions just described. **(C)** Protection from oxidation, as measured by DPPP fluorescence, with the addition of SelP enriched media, (+) SelP, compared to the control concentrated media, (-) SelP (***, $p < 0.001$).

B


350
300
250
200
150
100
50
0
15-HETE (ng/ml)
***
EtOH
PonA
AA
PonA+AA
Treatment Condition

C


(-) SelP
(+) SelP
1.4
1.2
1.0
0.8
0.0
Relative DPPP Fluorescence
***
EtOH
PonA
AA
PonA+AA
Treatment Condition

transcellular assay in which DPPP-labeled HEK-293 cells were added onto EcR-15-LOX cells allowed for the measurement of lipid hydroperoxides in cells distant from those that were responsible for metabolizing arachidonic acid. In having lipid metabolites move through the extracellular environment prior to acting on DPPP-labeled cells, the ability of the predominantly extracellular SelP to reduce the reactivity of these metabolites was able to be evaluated. DPPP fluorescence of the HEK-293 cells increased following treatment of EcR-15-LOX cells with the combination of ponasterone A and arachidonic acid as compared to control conditions (Figure 4.4C). The addition of 60 nM SelP attenuated this increase in fluorescence as compared to (-) SelP control.

Discussion

The health effects of selenium have been studied in multiple disease states, including cancer, cardiovascular disease, and inflammatory conditions (Clark et al., 1996; Mark et al., 2000; Nomura et al., 2000; Brown and Arthur, 2001; Angstwurm and Gaertner, 2006; Angstwurm et al., 2007). Benefits of supplemental selenium intake are believed to be due to antioxidant activity of selenoenzymes (Diwadkar-Navsariwala and Diamond, 2004; Diwadkar-Navsariwala et al., 2006; Irons et al., 2006); proteins capable of redox reactions through selenium atoms incorporated as the amino acid selenocysteine (Tujebajeva et al., 2000; Small-Howard et al., 2006; Howard et al., 2007). Recent clinical trials have provided conflicting results regarding many of the selenium-based health claims. Specifically, the anti-cancer benefit from selenium appears to exist only for individuals with low serum

selenium levels (Bleys et al., 2008). Earlier dietary supplementation studies demonstrated a decrease of cancer incidence that was most pronounced in individuals with lower serum selenium levels *(Clark et al.,* 1996). Additionally, serum selenium levels have been shown to be inversely correlated to the incidence of certain cancers (Clark et al., 1993; Mark et al., 2000; Nomura et al., 2000). Recent results of the Selenium and Vitamin E Trial (SELECT) did not support the utility of supplemental selenomethionine in prostate cancer prevention in selenium sufficient individuals (Lippman et al., 2009). Still, the antioxidant activity of selenoenzymes are likely important in human health.

While protection against oxidative injury by the glutathione peroxidases have been extensively characterized (Arthur, 2000; Steinbrenner and Sies, 2009), the antioxidant activity of SelP is less well characterized. Biochemical data have supported a role for SelP as a phospolipid hydroperoxidase (Saito et al., 1999; Takebe et al., 2002). However, the reducing capacity of SelP in this assay was measured to be two orders of magnitude lower than activity observed by phospholipid hydroperoxide GPx (Ursini et al., 1985), suggesting that the contribution of SelP as an antioxidant protein might be minimal as compared to other selenoproteins.

This study attempted to directly link SelP and the reduction of lipid hydroperoxides derived from 15-LOX-1 catalysis. This study extends work that demonstrated that selenium supplementation of endothelial cells produce significantly higher 15-HETE to 15-HpETE ratios, while selenium deficiency increased oxidation of arachidonic acid to 15-HpETE (Weaver et al., 2001). This

activity appears to be distinct from GPx4 modulation of lipoxygenase pathways involved in cell death (Seiler et al., 2008). Further evidence supporting a lipid hydroperoxidase function of SelP in a cell-based system includes a report that lipid hydroperoxides are increased in myofibroblasts when SelP expression is knocked down (Kabuyama et al., 2007). Loss of SelP also led to apoptosis and decreased cell viability through activation of c-Jun N-terminal kinases in this model. In endothelial cells and astrocytes, SelP has been shown to protect against *t*-BHP-induced cytotoxicity when cells were maintained in selenium deficient medium (Steinbrenner et al., 2006a; Steinbrenner et al., 2006b). SelP protected against cell death to the same extent as selenium supplementation with 100 nM sodium selenite and this effect was attributed to increased expression and activity of cytosolic GPx. Both SelP and sodium selenite increased this antioxidant protein, and the use of a GPx specific inhibitor, counteracted SelP-mediated cytoprotection.

Here it is shown that when HEK-293 cells were maintained in selenium-sufficient medium, 60 nM SelP reduced lipid hydroperoxides following exposure of the cells to 15-HpETE. This SelP concentration is considerably higher than that required to protect endothelial cells (0.6 nM) or astrocytes (2 nM) from the oxidative damage of *t*-BHP (Steinbrenner et al., 2006a; Steinbrenner et al., 2006b). The normal physiological concentration of SelP in selenium-replete human serum is estimated at 50 nM (Mostert, 2000). A decrease to less than 5% of selenium-replete values has been observed in animals with severe selenium deficiency (Yang et al., 1989; Nakayama et al., 2007). This suggests that the discrepancy in SelP

concentration required to exert antioxidant effects may be related to whether cells are maintained under selenium -sufficient or -deficient conditions.

The results presented here also show that reduction of lipid hydroperoxides in HEK-293 cells was achieved when SelP and 15-HpETE were added concurrently. In addition, short-term treatment with sodium selenite leads to increased oxidative tone in the cells, as reflected by an increase in lipid hydroperoxides following simultaneous addition of sodium selenite and 15-HpETE. Protection of endothelial cells and astrocytes against *t*-BHP-induced cytotoxicity required pre-incubation with SelP or sodium selenite and no protection was observed in endothelial cells if SelP and *t*-BHP were added simultaneously (Steinbrenner et al., 2006a; Steinbrenner et al., 2006b). This delayed effect would account for the time required to synthesize cytosolic GPx, the enzyme ultimately responsible for SelP-mediated protection in this model. The reduction of lipid hydroperoxides that was observed following short-term treatment with SelP likely represents direct enzymatic activity of the protein, rather than a genomic effect requiring the transcription and translation of secondary genes such as glutathione peroxidase.

The cellular protection from oxidation by lipid hydroperoxides afforded by SelP observed in this study, while significant, was modest (only a 7-12% reduction as compared to control conditions). This level of antioxidant activity by SelP could be a consequence of the extracellular localization of this protein. Intracellular reduced glutathione protects endothelial cells against 15-HpETE-induced cell injury and stimulates the conversion of 15-HpETE to 15-HETE (Ochi et al., 1992). Specifically, phospholipid hydroperoxide GPx has been shown to reduce the

hydroperoxy ester lipids formed by 15-LOX-1 metabolism (Schnurr et al., 1996) and is capable of inhibiting the activity of lipoxygenase enzymes (Huang et al., 1999). If intracellular selenoproteins including glutathione peroxidases are the primary source of antioxidant defense against the products of lipid metabolism, it is possible that the reactivity of these metabolites is minimized prior to reaching the extracellular environment, therefore reducing the need for SelP to act as a detoxifying protein.

In conclusion, SelP has been shown to reduce lipid hydroperoxides in HEK-293 cells after exposure to 15-HpETE. This was observed following pharmacological treatment with the metabolite, as well as endogenous production through ecdysone-inducible expression of 15-LOX-1. These results provide evidence that the lipid hydroperoxidase activity of SelP initially observed in biochemical assays also occurs in a cell-based model of 15-LOX-1 catalyzed arachidonic acid metabolism. By reducing lipid hydroperoxides following cell membrane metabolism, SelP may serve to decrease oxidative tone of tissues under inflammatory conditions. This could provide protection against the toxic effects of lipid peroxidation, leading to a decrease in DNA damage and mutations and potentially contributing to any anti-carcinogenic effects of selenium supplementation.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Introduction

Selenium has been linked to potential beneficial health effects in multiple disease states, including cancer, cardiovascular disease, and inflammatory conditions (Clark et al., 1996; Mark et al., 2000; Nomura et al., 2000; Brown & Arthur, 2001; Angstwurm & Gaertner, 2006; Angstwurm et al., 2007); however, recent clinical trials have provided conflicting results that have brought into question the safety and efficacy of selenium supplementation (Bleys et al., 2008; Lippman et al., 2009; Stranges et al., 2007). Beneficial effects of supplemental selenium intake have been attributed to antioxidant activity of selenoenzymes (Diwadkar-Navsariwala and Diamond, 2004; Diwadkar-Navsariwala et al., 2006; Irons et al., 2006); proteins capable of electron transfer through selenium atoms incorporated as the amino acid selenocysteine (Tujebajeva et al., 2000; Small-Howard et al., 2006; Howard et al., 2007). SelP is an extracellular glycoprotein that plays a vital role in delivering selenium to extrahepatic tissues (Akesson et al., 1994; Hill et al., 2003; Renko et al., 2008). An antioxidant function has also been observed for this protein (Burk et al., 1995; Arteel et al., 1998; Saito et al., 1999;

Traulsen et al., 2004; Steinbrenner et al., 2006a; Steinbrenner et al., 2006b). Through an increased understanding of the mechanisms regulating selenoprotein P expression and activity, it could be possible to gain insight into the way in which selenium exerts its physiological effects.

For this reason, the experiments presented in this dissertation were designed to test the hypothesis that mechanisms regulating the expression of selenoprotein P provide for modulation of this protein so it may function to provide antioxidant protection in extrahepatic tissues. These studies characterized the regulation of selenoprotein P through glucocorticoid response elements and determined the role of selenoprotein P in regulating the cellular oxidative stress induced by reactive hydroperoxylipid intermediates. This chapter will summarize the major findings of these experiments. A brief discussion of the findings, as well as suggested future research directions will also be included.

Summary

Chapters 2 and 3 of this dissertation focused on characterizing the regulation of SEPP1 through both VgEcR and GR. First, multiple electronic databases were utilized in order to identify putative TFBS within the SEPP1 promoter. Identification of putative glucocorticoid and retinoid responsive elements supported the usefulness of these bioinformatic tools, as transcriptional activation by VgEcR involves binding of a heterodimer formed between GR and RXR (Saez et al., 2000). Both a putative GRE at position -87 and a putative RRE at position -73 of the SEPP1 promoter were found to be necessary for transactivation of the SEPP1

promoter by VgEcR. These sites overlap with an active HNF-4α site involved in modulating SEPP1 expression, suggesting this may be an important regulatory region of the promoter potentially capable of promiscuous binding by nuclear receptors. A direct binding site for GR was not identified on the SEPP1 promoter and it appears that SEPP1 repression may be mediated through an indirect mechanism of the GR at GRE #1.

Antioxidant activity of SelP against reactive hydroperoxylipid intermediates was evaluated through the experiments presented in Chapter 4. Enzymatic reduction of 15-HpETE, but not 15-HETE, was observed in a NADPH-coupled biochemical assay. SelP was also effective at reducing lipid hydroperoxides following exposure of cells to 15-HpETE. This was observed both when the protein and metabolite were added simultaneously and in a transcellular assay where 15-LOX-1 was actively metabolizing arachidonic acid. While the effects were modest, these results directly link SelP and the reduction of lipid hydroperoxides derived from 15-LOX-1 catalysis. This provides further evidence of the antioxidant activity of SelP and suggests that the toxic effects of ROS formed during inflammation could be reduced when SelP is present at physiological concentrations.

Discussion

Despite the beneficial effects that have been noted, it appears that the data regarding selenium supplementation is conflicting and inconclusive. This highlights the difficulty in translating results from *in vitro* and animal models to

clinical effects in humans. In addition, variability in the nutritional status and predisposing disease factors among different human populations makes it difficult to establish correlations in the available clinical data. Therefore, further studies are required in order to fully elucidate the mechanism and extent of selenium's health effects.

Because the benefits of supplemental selenium intake are believed to be due to antioxidant activity of selenoproteins, it is thought that optimal health outcomes are achieved when the trace element is available in a supply adequate to prevent it from becoming the limiting factor in selenoprotein synthesis. As the major selenium supply protein of the body, SelP plays an important role in ensuring that adequate tissue selenium levels are available for proper Sec translation and selenoprotein synthesis. Understanding the regulatory and functional mechanisms of SelP can therefore provide evidence of the way in which selenium is distributed and utilized within the body.

Despite the fact that the VgEcR system is not expected to transactivate host genes by itself, induction of SEPP1 was observed when this system was activated by PonA in HEK-293 cells. In an attempt to determine the mechanism responsible for this unexpected effect, electronic database analyses were used to identify putative TFBS in the SEPP1 promoter. While these methods identified binding sites that appeared likely candidates for mediating activation by VgEcR, in the end, these sites were approximately 13 base pairs downstream from the GRE and RRE shown to be responsible for transactivation. However, the TESS-identified GRE within Motif 6 of the MEME output did in fact correspond to a previously

characterized FOXO1a site (Speckmann et al. 2008; Walter et al. 2008), lending support to a role for bioinformatics in identifying biologically relevant gene features. While these *in silico* techniques can identify candidate binding sites, it should be recognized that their use is just one step in the process of characterizing protein binding sites in genes. The inability of these tools to identify the actual sites responsible for transcriptional activity observed here highlights the importance of using experimentation to verify outcomes of electronic database analyses.

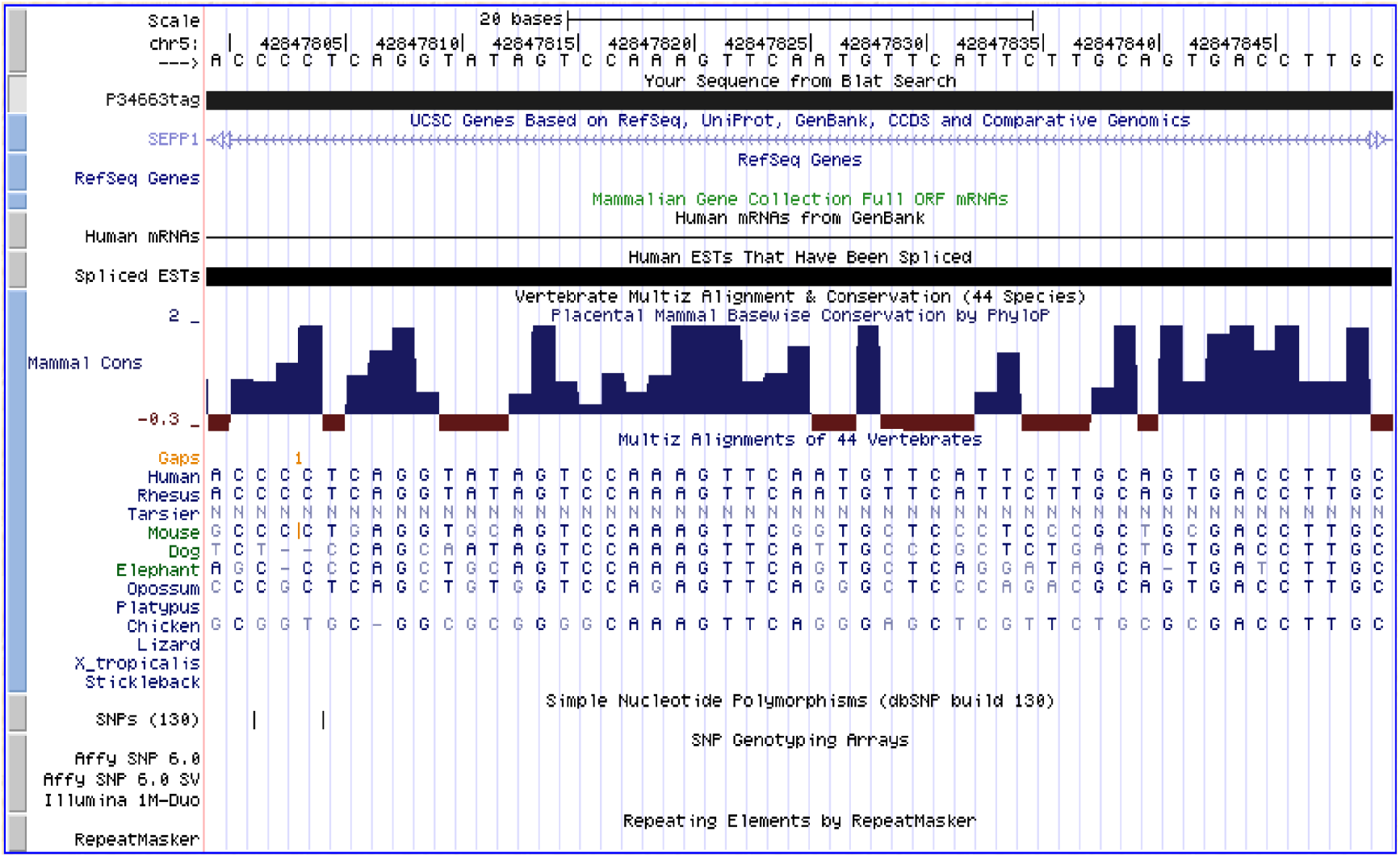
In addition to HNF-4α, the GR has now also been shown to play a role in regulating SEPP1 expression. Regulation of SEPP1 by nuclear receptors (Speckmann et al. 2008; Rock and Moos 2009), as well as spatiotemporal expression during embryogenesis (Thisse et al. 2003; Lee et al. 2008), supports a role for this protein in developmental processes. It is unclear whether such a function is newly evolved or has been conserved across species.

Due to the proximity of GRE #1 and RRE to the candidate GRE and RARγ binding sites identified in Chapter 2, these active sites fell within the evolutionarily conserved region of SEPP1 identified by ConSite analysis. In phylogenetic footprinting of human sequences, rodents are the most common species used for comparison as studies have revealed only a small portion of the non-coding regions are conserved at this evolutionary distance. Additionally, these two species show high similarity in distinguishable segments of the genome, while being flanked by apparently random sequence (Lenhard et al., 2003; Miziara et al., 2004). For these reasons, the ConSite analysis described in Chapter 2 evaluated evolutionary conservation between the human and murine SEPP1 genes. Once GRE #1 and

RRE were shown to be necessary for transcriptional activation of SEPP1 by VgEcR, conservation of the sequence was evaluated across a greater number of species ranging in evolutionary distance from humans. This was accomplished using the University of California, Santa Cruz (UCSC) Genome Browser. This database is a collection of genome assembly sequence data and integrated annotations for a large number of organisms (Kuhn et al., 2009). The browser represents annotations as a series of horizontal tracks laid out over the genome (Kent et al., 2002). The Conservation track displays the results of a Multiz alignment, providing a view of the evolutionary relatedness of sequences across a wide range of animals (Kuhn et al., 2009). Sequence conservation across species as divergent as the primate, mouse, dog, elephant, opossum, and chicken was observed when the reverse compliment of the SEPP1 promoter region corresponding to GRE #1 and RRE was queried (Figure 5.1). In particular, bases identified in Chapter 3 as being responsible for VgEcR transactivation were highly conserved. These results support the idea that this region is an important regulatory region of the promoter; however, it should be noted that with the exception of the chicken, all species identified within this phylogenetic analysis are mammals, so it is unknown whether this regulatory region is conserved in lower vertebrates.

Zebrafish express two SelP isoforms, each encoded by a distinct gene. One isoform shares sequence and structural similarities with the full length human protein, while the other lacks a Sec-rich C-terminus (Kryukov & Gladyshev 2000). These two isoforms are also observed in pufferfish and correspond to the rat isoform that terminates at the second UGA of SelP. This conservation across

Figure 5.1 Phylogenetic footprinting of GRE #1 and RRE binding sites. The University of California, Santa Cruz (UCSC) Genome Browser was used to evaluate evolutionary conservation of the GRE #1 and RRE sequences responsible for VgEcR transactivation of the SEPP1 promoter. The browser represents annotations as a series of horizontal tracks and the Conservation track displays the results of a Multiz alignment. Sequence conservation was observed across multiple mammalian species, as well as the chicken, for the reverse compliment of the SEPP1 promoter sequence queried.


Scale
20 bases
chr5:
42847805
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42847845
---> A C C C C T C A G G T A T A G T C C A A A G T T C A A T G T T C A T T C T T G C A G T G A C C T T G C
Your Sequence from Blat Search
P3466Stag
UCSC Genes Based on RefSeq, UniProt, GenBank, CCDS and Comparative Genomics
SEPP1
RefSeq Genes
RefSeq Genes
Mammalian Gene Collection Full ORF mRNAs
Human mRNAs from GenBank
Human mRNAs
Human ESTs That Have Been Spliced
Spliced ESTs
Vertebrate Multiz Alignment & Conservation (44 Species)
Placental Mammal Basewise Conservation by PhyloP
2
Mammal Cons
-0.3
Multiz Alignments of 44 Vertebrates
Gaps
Human A C C C C T C A G G T A T A G T C C A A A G T T C A A T G T T C A T T C T T G C A G T G A C C T T G C
Rhesus A C C C C T C A G G T A T A G T C C A A A G T T C A A T G T T C A T T C T T G C A G T G A C C T T G C
Tarsier N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
Mouse G C C C C T G A G G T G C A G T C C A A A G T T C G G T G C T C C C T C C C G C T G C G A C C T T G C
Dog T C T - - C C A G C A A T A G T C C A A A G T T C A T T G C C C G C T C T G A C T G T G A C C T T G C
Elephant A G C - C C C A G C T G C A G T C C A A A G T T C A G T G C T C A G G A T A G C A - T G A T C T T G C
Opossum C C C G C T C A G C T G T G G T C C A G A G T T C A G G G C T C C C A G A C G C A G T G A C C T T G C
Platypus
Chicken G C G G T G C - G G C G C G G G G C A A A G T T C A G G G A G C T C G T T C T G C G C G A C C T T G C
Lizard
X_tropicalis
Stickleback
Simple Nucleotide Polymorphisms (dbSNP build 130)
SNPs (130)
SNP Genotyping Arrays
Affy SNP 6.0
Affy SNP 6.0 SV
Illumina 1M-Duo
Repeating Elements by RepeatMasker
RepeatMasker

species supports the hypothesis of differential functions between the two domains of human SelP.

The N-terminal domain of SelP is thought to be responsible for the antioxidant activity of this protein, while data support a selenium distribution function for the C-terminal domain (Saito et al., 2004). The C-terminal domain is critical in preventing the developmental defects observed in SEPP1 knockout mice, including neurological and fertility impairments (Hill et al., 2007). Additionally, transgene expression of hepatic SEPP1 resolves the knockout phenotype (Renko et al., 2008). Therefore, while evidence of the antioxidant activity of SelP continues to accumulate, it is clear that the selenium distribution effect of this protein is of paramount importance in preventing developmental defects.

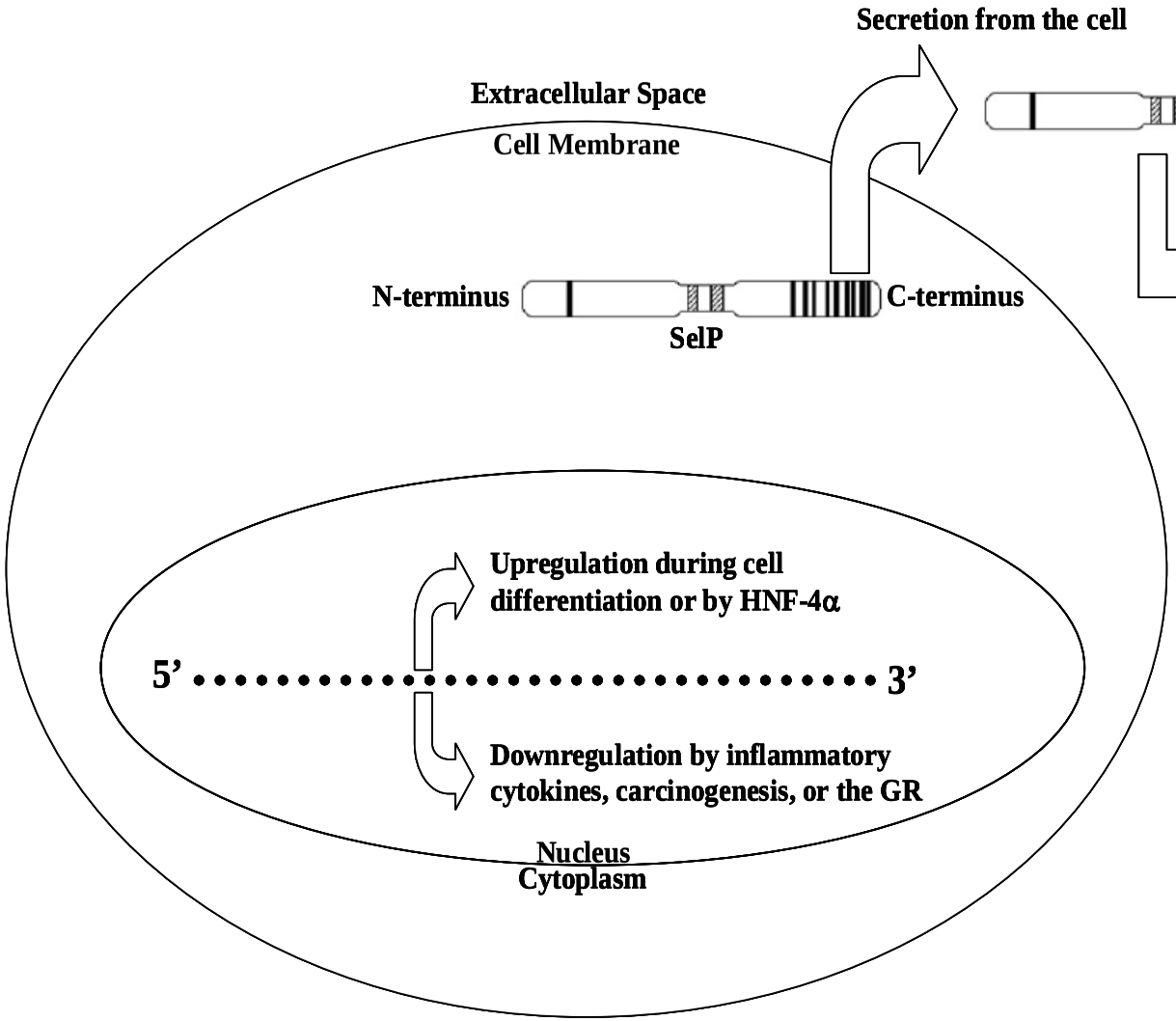
It is unclear whether there is interplay between the antioxidant activity of SelP and the proposed role of this protein in development. Evidence suggests that selenium is necessary for mammalian embryonic development (Bedwal and Bahuguna, 1994), a process that generates ROS through aerobic and anaerobic metabolic pathways (Ornoy, 2007). While antioxidant systems are still developing at this stage of growth, expression of cytosloic GPx and superoxide dismutase mRNA has been noted in mouse embryos (El-Hage and Singh, 1990; Baek et al., 2005; Yon et al., 2008). Developmental studies have also shown spatiotemporal expression of a SEPP1 ortholog in both zebrafish (Thisse et al., 2003) and murine model systems (Lee et al., 2008). It was suggested that these expression patterns may correspond to a function for SelP in protecting against embryonic oxidative damage (Lee et al., 2008).

Figure 5.2 outlines a proposed mechanism by which SelP is regulated and subsequently exerts its physiological effects. In considering this model, it is important to recall that the majority of SelP is synthesized in the liver for the purpose of delivering selenium throughout the body (Burk and Hill, 2005). Studies in knockout animals have shown that delivery to the brain and testes are of particular importance (Hill et al., 2003; Burk et al., 2006), and ApoER2 appears to be responsible for SelP uptake into these tissues (Olson et al., 2007; Burk et al., 2007). As the C-terminal domain is necessary to prevent neurological and fertility impairments under selenium-deficient conditions (Hill et al. 2007), it appears that this region of the protein is responsible for supplying selenium to the tissues once uptake has occurred. It seems likely that SelP derived from the liver is degraded in these tissues for the purpose of increasing the supply of selenium available for the synthesis of other selenoproteins. This supply could also facilitate local de novo synthesis of SelP, providing a mechanism for tissue-specific selenium retention, such as is observed in the brain and testes during selenium-deficient conditions (Burk et al., 1972; Behne et al., 1988).

This proposed mechanism of selenium delivery by SelP to the brain and testes is in contrast to distribution in the kidney, where the C-terminal domain of SelP is not required (Hill et al., 2007). In this case, it seems the N-terminal domain is sufficient, introducing the idea that the various SelP isoforms could function differentially in tissues throughout the body.

Under selenium-sufficient conditions, where selenoprotein synthesis is not limited, locally expressed SelP may primarily function in antioxidant defense. It is

Figure 5.2 Proposed mechanism of selenoprotein P regulation and activity. SEPP1 is upregulated in cells undergoing differentiation. Additionally, the transcription factor hepatocyte nuclear factor 4α (HNF-4α) has been shown to increase SEPP1. Multiple cytokines repress SEPP1 and expression of SelP is decreased in sepsis, leading to the suggestion that SelP functions as an acute phase protein during inflammation. Once synthesized, SelP is excreted into the extracellular space. SelP produced in the liver primarily functions in selenium delivery to extrahepatic tissues, where the protein supplies selenium for the production of additional selenoproteins. Additionally, de novo synthesis of SelP can occur in extrahepatic tissues, providing a mechanism for selenium retention during deficiency. In selenium-sufficient conditions, where selenoprotein synthesis is not limited, SelP may function as an antioxidant protein.


Secretion from the cell
Extracellular Space
Cell Membrane
N-terminus
C-terminus
SelP
Functions:
1. Selenium supply for selenoprotein synthesis
2. Tissue selenium retention
3. Antioxidant activity
Upregulation during cell differentiation or by HNF-4α
5'
3'
Downregulation by inflammatory cytokines, carcinogenesis, or the GR
Nucleus
Cytoplasm

unclear whether this might be a tissue-specific effect. While the magnitude of SelP's antioxidant activity was nominal in HEK-293 cells, a more pronounced effect could potentially be observed in cells of neuronal or testicular origin, tissues where the protein seems to be of vital importance. The Sec in the N-terminal region of the protein is proposed to be responsible for the antioxidant activity of SelP; however, catalytic reduction of this Sec would be required in order to maintain this activity. The enzymatic system reponsible for catalyzing this reduction has yet to be identified, but could potentially involve the selenoprotein enzyme thioredoxin reductase. Alternatively, it is speculated that there could be a role for the nine C-terminal Sec residues in cycling electrons on the N-terminal Sec.

Future Directions

The GR was shown to modulate SEPP1 expression, but direct binding of this protein to the promoter was not observed in the studies presented here. Therefore, further studies are required in order to fully characterize the mechanism responsible for GR regulation of SEPP1. Greater GR-mediated repression of ponasterone A-induced activation was observed on the -1652 to +247 fragment of the SEPP1 promoter as compared to the -109 to +247 fragment (Figures 3.2, 3.4 and 3.5), suggesting additional repressive elements exist within the full length promoter. While an attempt to identify potential putative binding sites responsible for this activity revealed additional GRE half-sites, no site corresponding to the classical GRE consensus sequence was found. Based the activity of half-sites observed on the -109 to +247 fragment, it seems prudent to test the activity of the additional

half-sites in the full promoter through similar methods, including site-directed mutagenesis. Additionally, a complex DNA-repeat region within the SEPP1 promoter has demonstrated repression of SEPP1 expression with certain polymorphisms (Al-Taie et al., 2002). These simple sequence repeats can function as binding sites for regulatory proteins (Kashi et al., 1997), and thus, mutant forms of this site should be evaluated to determine its effect on GR-mediated SEPP1 expression.

While conclusions are limited by the absence of a positive control in the EMSA, data presented here seem to support an indirect role of the GR in regulating expression of SEPP1. An indirect mechanism of GR modulation of transcription has been described previously through an interaction with CCAAT/enhancer-binding proteins (Rudiger et al., 2002) and subsequent *in silico* analysis of the SEPP1 promoter revealed the presence of a CCAAT/enhancer-binding protein site immediately downstream from GRE #1. Studies aimed at evaluating the interplay between these two transcription factors may provide further evidence of the way in which SEPP1 is regulated. Because CCAAT/enhancer-binding proteins are involved in development and differentiation (Ramji & Foka, 2002) these studies could also provide insight into the function of SelP during development.

There are multiple ways in which to build upon the evidence of SelP's antioxidant activity. Data presented here are specific in terms of characterizing activity against a particular oxidative metabolite (15-HpETE). However, the ability to consider this data in a biological context is limited by the fact that it does not account for the complex nature of the inflammatory process in which multiple

reactive metabolites are formed. In the future, studies could be run which consider this complexity. One approach would be to test alternative eicosanoids formed during inflammation as substrates for SelP, using assays similar to those described here. Alternatively, the role of SelP on downstream effects of eicosanoid signaling could be evaluated. For example, metabolites of LOX- and COX-catalysis have been shown to oxidize peroxiredoxins (Cordray et al., 2007). These proteins play a role in regulating ROS signaling through their reduction of hydrogen peroxide in cells (Wood et al., 2003). SelP was found to be ineffective at preventing the oxidation of peroxiredoxins in metabolically active EcR-15-LOX cells (data not shown); however, SelP protection could potentially be observed in a transcellular system, where oxidation is measured in cells distant from those forming the reactive metabolites.

Other general markers of oxidative stress, such as DNA adduct formation or apoptosis could also be assessed. Specifically, SEPP1 knockdown has been shown to induce apoptosis through activation of c-Jun N-terminal kinases (Kabuyama et al., 2007), so further characterizing the role of SelP in this pathway would be a logical approach for future studies. The nuclear factor kappa beta pathway is another pathway under ROS-mediated control that could be evaluated in such studies (Bubici et al., 2006).

Immunohistochemistry has previously been used to visualize SelP tissue expression (Burk et al. 1997). This method could potentially be useful in determining whether there is tissue-specific expression of SelP isoforms. Antibodies specific for each of the isoforms would need to be developed in order to

run such a study; however, the outcomes could provide new insight into the tissue-specific functions of SelP. In addition to providing information on the function of extrahepatic SelP, this study could also work to further characterize differential functions of the SelP isoforms.

Finally, using animal models would be an extremely effective method for translating the results of *in vitro* experiments to a more complete biological context. The development of SEPP1 knockout mice has provided a very useful tool for running such experiments. Application of an *in vivo* model of inflammation in these animals could provide a wider context in which to consider the antioxidant activity of SelP.

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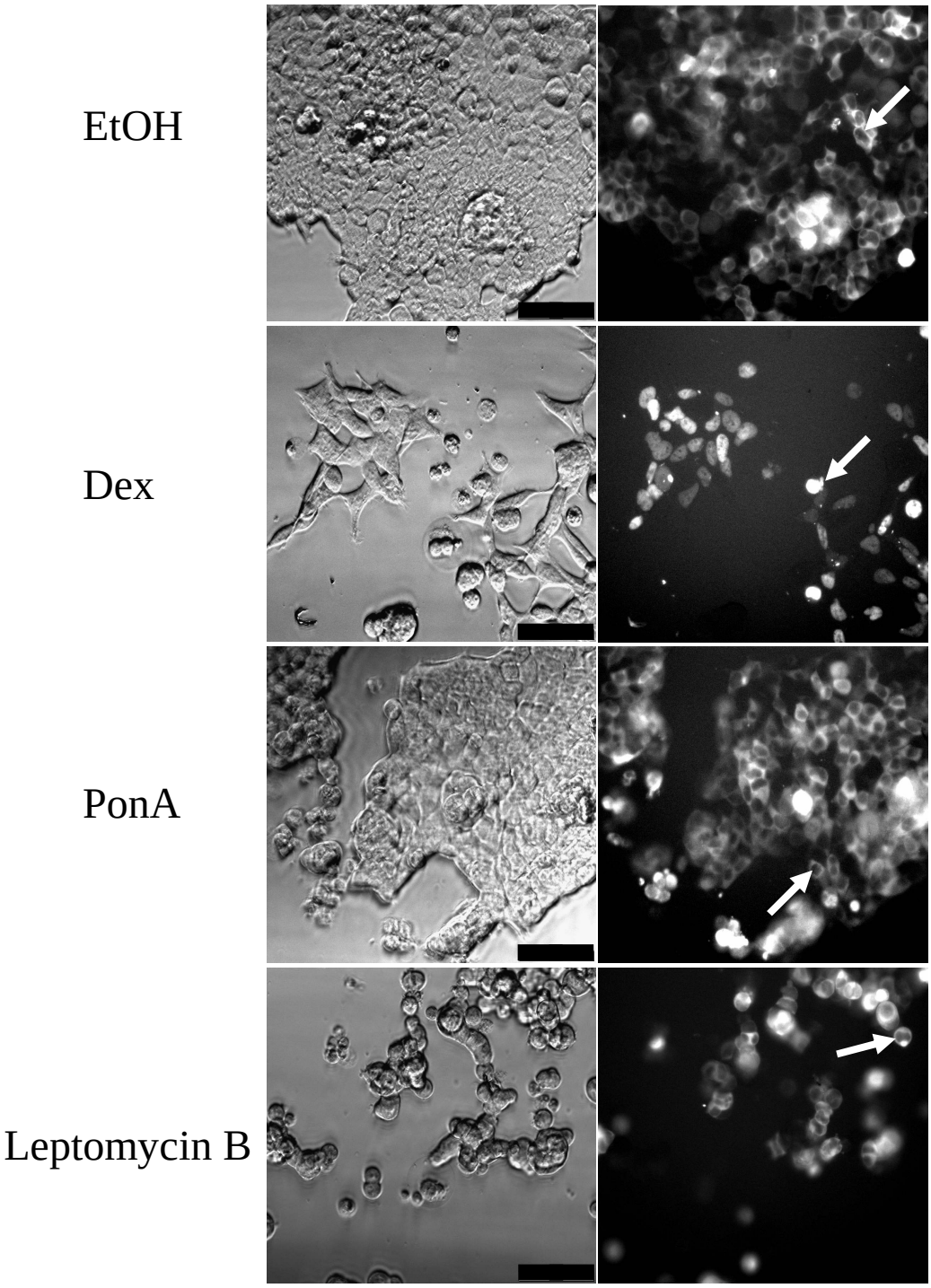
APPENDIX

NUCLEAR LOCALIZATION OF THE GLUCOCORTICOID RECEPTOR

Localization of GR following treatment of EcR-GR cells. EcR-GR cells were treated with 10nM Dex, 10μM PonA, or vehicle control for 24 hours in order to evaluate GR localization under experimental treatment conditions. Treatment with 10nM of the nuclear export inhibitor leptomycin B was also tested. Phase contrast images appear on the left and fluorescent images appear on the right. Black bars are equal to 1 millimeter. Fluorescently-labeled GR is shown in white and nuclei are indicated by arrows. Cytoplasmic localization of GR is observed with vehicle control, PonA, and leptomycin B treatments. Nuclear localization was observed only following the addition of Dex, confirming that the GR was not translocating to the nucleus in the absence of ligand.


EtOH
Dex
PonA
Leptomycin B