Fall 1-1-2007

Regulation of Steroid Sulfatase by Glucocorticoids in Human Breast Cancer and Bone Cancer Cell Lines

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REGULATION OF STEROID SULFATASE BY GLUCOCORTICOIDS IN HUMAN BREAST CANCER AND BONE CANCER CELL LINES

A Thesis

Submitted to the Bayer School

of Natural and Environmental Sciences

Duquesne University

In partial fulfillment of the requirements for

the Degree of Master of Science

in Biological Sciences

By

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December 2007

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Approved November 30, 2007

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ABSTRACT

REGULATION OF STEROID SULFATASE BY GLUCOCORTICOIDS IN HUMAN BREAST CANCER AND BONE CANCER CELL LINES

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Thesis Supervised by Dr. Kyle W. Selcer

Steroid sulfatase converts inactive precursors steroids to active forms. It is suspected to have important physiological functions. The purpose of my research was to study regulation of steroid sulfatase by glucocorticoids in the human cell lines MDA-MB-231 (breast cancer) and MG-63 (osteosarcoma). MDA-MB-231 cells treated for 72 hours with 10 or 1.0μM cortisol showed decreased steroid sulfatase activity. Similarly, intact MDA-MB-231 cells treated with 10, 1.0 or 0.1μM dexamethasone showed a decline in steroid sulfatase activity. The decrease was not due to competitive inhibition by glucocorticoids. Qualitative analysis of steroid sulfatase mRNA using RT-PCR suggests levels are not affected by dexamethasone. MG-63 bone precursor cells showed

no change in steroid sulfatase activity after exposure to dexamethasone for 24 or 96 hours. However, MG-63 cell lysates demonstrated increased steroid sulfatase activity at the 96h time period. These data indicate that glucocorticoids can influence steroid sulfatase activity in human breast and bone cells.

ACKNOWLEDGMENTS

I would first like to thank Dr. Pollock and Dr. Davis for being on my committee. I would especially like to thank Dr. Pollock for his guidance and influence at my time at Duquesne. Our brief but poignant conversations have changed the way I look at the world. I wish to thank Dr. Selcer for his unfailing support, patience and faith throughout this entire process. I am grateful for the opportunity to have worked with you at Duquesne. I have truly gained wisdom from you not only about biology, but life in general. I would also like to thank the various members of the Selcer lab who were supportive lab mates and friends who aided me in this endeavor. Thank you to Dr. Woodley for letting me work with you, gaining experience in other areas of endocrinology, and for your friendship. Thanks must also go to Mary Ann Quinn, without whom my thesis probably would not have ever been completed. Last but not least, I appreciate the support of my family and friends who have kept a watchful eye on me by making sure I complete my thesis while maintaining a reasonable semblance of sanity.

TABLE OF CONTENTS

LIST OF FIGURES

Page

Chapter 1

1. INTRODUCTION

1.1 Overview

Steroid sulfatase (STS) is an enzyme involved in the regulation and production of estrogens and androgens from systemic precursors in various tissues, such as dehydroepiandrosterone sulfate (DHEAS) or estrone sulfate (E_1S). A great deal of emphasis has been placed on this enzyme because of its apparent role in stimulation of estrogen-dependent breast tumors (Nussbaumer and Billich, 2004). This endeavor has produced several capable STS inhibitors, which may have promise for use as clinical agents. Indeed, one of these inhibitors – STX 64 (667 Coumate) - has entered phase I clinical trials to test its effectiveness in postmenopausal women with breast cancer, yielding initially promising results (Stanway et al., 2006). Despite the steadily improving knowledge regarding the inhibition of steroid sulfatase, relatively little is known about the actual regulation of the enzyme. Steroid sulfatase is found almost ubiquitously throughout the human body, and has been implicated in a number of various physiological processes, specifically the regulation of the immune system, certain dermatological conditions, bone formation, and cognitive reasoning (Pasqualini and Chetrite, 2005). The therapeutic implications of understanding the regulation of steroid sulfatase are readily apparent, making further clarification of the role and function of this enzyme within the human body of paramount importance.

1.2 Background on Steroid Sulfatase (STS)

Steroid sulfatase (STS) (E.C. 3.1.6.2, arylsulfatase C) is a member of a superfamily of 12 different mammalian sulfatases (Reed, 2005), and is characterized by its ability to hydrolyze inactive aryl and alkyl sulfates to their active, unconjugated forms. STS is found almost ubiquitously throughout the human body, but is especially abundant in the microsomal fraction of the human placenta (Ghosh, 2003). Specifically, it is a membrane bound enzyme, localized primarily in the rough endoplasmic reticulum. The gene for STS has been mapped to the distal portion of the short arm of the X-chromosome, containing 10 exons and is about 146 kb in length, and the encoded protein has a molecular weight of 62 kDa (Pasqualini, 2005). Interestingly, the STS gene is also found on the Y-chromosome, but is nonfunctional since the promoter region and several exons have been transcriptionally inactivated. There is also evidence that two isoforms of the gene exist in humans (fast and slow), but it is possible that these are a result of post-translational modifications (Reed et al., 2005). The structure of STS has been determined via x-ray crystallography, revealing a "mushroom-like" appearance for the enzyme. These helices traverse the lipid membrane, anchoring the functional domain of the enzyme in a position which is amenable to interacting with the active site of the molecule (Ghosh, 2003). A recent study has also demonstrated the importance of both the N and C terminal domains in enzymatic function, which could lead to more developments in anti-STS cancer therapies (Sugakawara, 2006).

The information regarding the molecular regulation of STS is rather sparse, especially when compared to studies on STS gene structure. The promoter region of the gene has been characterized, revealing a promoter lacking a TATA box and low GC nucleotide content (Li et al, 1996). It is known that both tumor necrosis factor alpha (TNF-a) and interleukin-6 (IL-6) upregulate STS enzyme activity in MCF-7 breast cancer cells either via post-translational modifications or increasing substrate availability (Newman, 2000). Steroids have also been shown to have some effect upon the regulation of steroid hormones. Retinoic acid and carotenoids stimulate STS activity in MCF-7 breast cancer cell, but not in MDA-MB-231 cells (Ng, et al., 2000). Synthetic progestins such as medrogesterone, promegestone, normegestrol acetate, and tibolone (Chetrite et al.,1996, Chetrite et al., 1997, Chetrite et al., 1999) have been shown to inhibit STS activity in breast cancer cells. Retinoids and 1,25-dihydroxy vitamin D3 have also been shown to induce STS activity in HL-60 promyelocytic cells (Hughes et al, 2001). The underlying mechanism by which these steroids affect the expression of STS is not well understood, and further investigation of this matter would be prudent.

1.3 Overview of Steroid hormones

Before delving into more of the particulars of steroid sulfatase metabolism, it is necessary to discuss some background on steroid hormones to better understand the global context within which steroid sulfatase operates. Steroid hormones are rather simple molecules with similar shapes, but those seemingly minor differences elicit complex and specific responses within the body. All steroids are derived from cholesterol and possess a characteristic cyclopentanophenanthrene ring structure. It is the substituent

which varies from hormone to hormone that creates the unique properties of a given steroid.

Hormones are grouped into six different classes based on their structural and biochemical basis: androgens, estrogens, glucocorticoids, mineralocorticoids, progestagens, and vitamin D (Norman and Litwack, 1997). Androgens are synthesized in the adrenal cortex and gonads, and primarily responsible for male sexual maturation and function. Estrogens are produced in the ovarian follicle and are responsible for female sexual maturation and function. Glucocorticoids are synthesized in the *zona fasciculata* (adrenal cortex), affecting carbohydrate metabolism, the inflammation response, and resistance to stress. Mineralocorticoids are produced in the *zona glomerulosa* (adrenal cortex) and maintain salt and water homeostasis in the body. Progestagens originate in the corpus luteum of the ovaries and help in mediating the menstrual cycle and maintaining pregnancy. Finally, vitamin D is derived from cholecalciferol and is important in maintaining calcium metabolism.

All steroid hormones are converted from cholesterol to pregnenolone, and then to one of the various steroid hormone classes via the P450 cholesterol side chain cleavage (scc) enzyme. Once pregnenolone is produced, it can undergo one of two conversions: 17α – hydroxylation, which leads to the production of cortisol in the adrenals, or to progesterone, which can be further converted to aldosterone in the adrenals. The transformaton of pregnenolone to progesterone can also be catalyzed by 3B – hydroxysteroid dehydrogenase (3B-HSD) and Δ5-Δ4 isomerase. From here, further conversions can occur through the pregnenolone or progesterone pathway into a wide variety of hormones (Norman and Litwack, 1997).

Steroid hormones, regardless of type, exert their effects through similar mechanisms. Steroid hormones are present in blood as either bound (active) or unbound (inactive) in relation to transport proteins - roughly 95% of steroid hormones are bound to proteins (O'Malley et al., 1991). Because proteins are composed of cholesterol, they are readily able to diffuse through the lipid membrane, ultimately binding to a specific, inactive hormone receptor either in the nucleus or cytoplasm of the cell. The unoccupied steroid hormone receptor is thought to exist in the cell as a conjugate with a dimer of heat shock protein 90 to prevent the receptor from binding to DNA. The receptor becomes activated once it binds with its associated ligand, forms a homodimer with another steroid receptor and binds to the proper DNA sequence that will allow it to form a high-affinity complex with the receptor and the steroid response element (SRE), which precisely defines the acceptor sites for the steroid hormone receptors (O'Malley et al., 1991).

The production of specific steroid hormones is dependent upon their necessity at a given time in that tissue. The steroid hormones of greatest interest for the scope of this discourse involve the production of androgens and estrogens. Androgens (like testosterone or dehydroepandrosterone sulfate (DHEAS) are produced primarily in the testes in the male and in the ovary in females. Estrogens are actually formed from androgens in a reaction catalyzed by aromatase. Aromatase is a cytochrome P450 (CYP19) enzyme complex which can convert androstenedione to estrone (E1) and testosterone to estradiol (E2) (Selcer and Leavitt, 1991).

The enzyme steroid sulfatase (STS; EC 3.1.6.2)) is another pathway by which estrogens can be obtained in the body. STS cleaves sulfate groups from 3-hydroxysteroid sulfates, converting them into their biologically active forms (Purohit et al., 1997). STS

is involved in two pathways for estrogen production. In the first pathway, STS hydrolyzes inactive estrone-sulfate (E1S) to estrone (E1). Estrone can subsequently be converted to the more potent estradiol-17B via 17 B -HSD (Suzuki et al., 2003). In the second pathway, STS converts DHEAS to DHEA, which is converted to ADIOL via 17-ketoreductase. ADIOL is an androgen which mimics estradiol by binding to estrogen receptor, and thereby simulating the effects of estrogen. Some of these effects include increased estrogen and progesterone receptor synthesis (Adams, 1998), increasing proliferation of hormone-dependent breast cancer tissue (Poulin and Labrie, 1986; Pizzini et al., 1992).

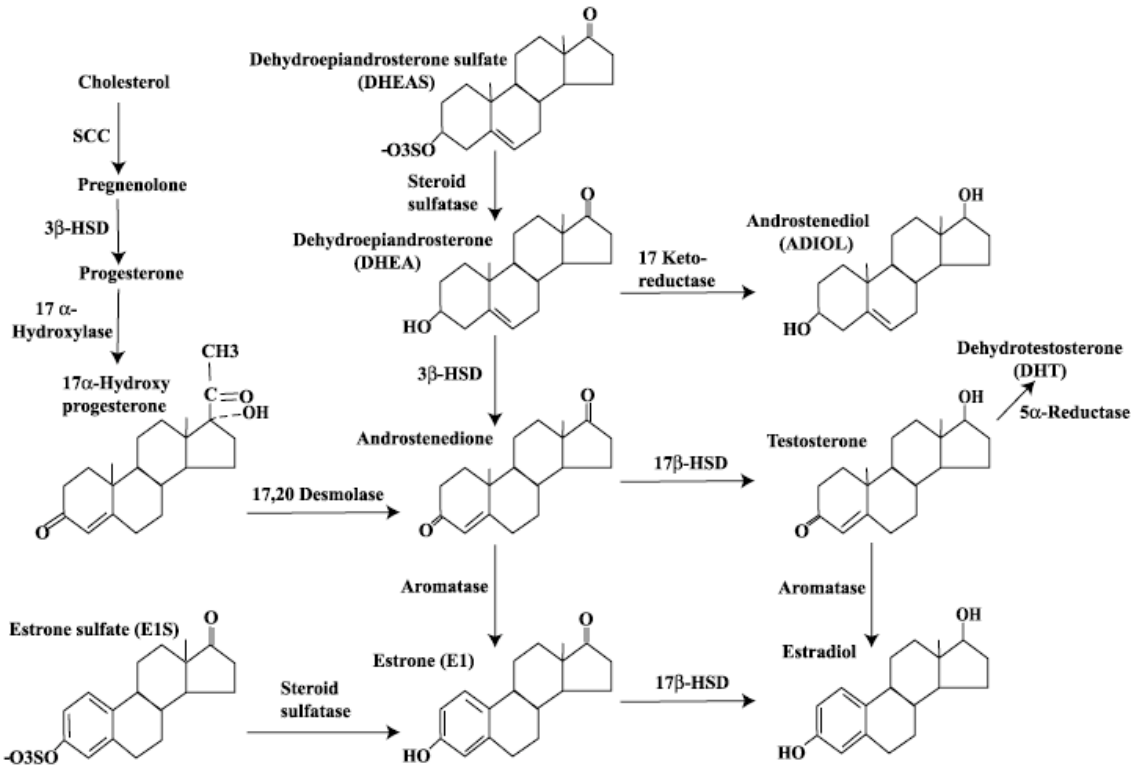

Figure 1. Synthesis of steroid hormones

1.4 Steroid Sulfatase in Breast Cancer

No discourse on steroid sulfatase could be accomplished without discussing the role of the enzyme in hormone-dependent breast cancer. Breast cancer is the most common cancer afflicting women today (with the exception of non-melanoma skin cancers), and is the second leading cause of death among women after lung cancer. One in seven women in the United States will develop invasive breast cancer in their lifetime (American Cancer Society, 2005). Although diagnostic and therapeutic procedures are continually improving, there is still much that needs to be learned in combating this devastating disease.

Breast cancer is generally categorized into two forms: hormone independent (estrogen receptor negative) and hormone dependent (estrogen receptor positive). Hormone independent breast cancers do not express estrogen receptor (ER) and therefore do not require hormones to proliferate. On the other hand, hormone dependent breast cancers (about 40% of all breast cancers) do express ER and require hormones to proliferate. The most important hormones in this regard are estradiol and androstenediol (Ahmed et al, 2002). These cancers can be treated via endocrine therapy (Budzar, 2001), and a number of effective treatment strategies have been developed.

The two principal methods of treating hormone-dependent breast cancers have been to either block the hormone receptor site by use of a specific receptor antagonist or directly inhibiting hormone synthesis by using anti-enzyme agents (Nakata et al, 2003). Tamoxifen, the most popular breast cancer drug treatment for the past 20 years, uses the former strategy (Dhingra, 2001). The drug is an anti-estrogen, meaning it competitively binds to the estrogen receptor site, effectively blocking the binding of estrogen to its

principal target. It has been used to treat both hormone-dependent and hormone-independent breast cancers, however, the success rate is much higher for hormone-dependent tumors (Jordan, 1997). Tamoxifen, while having proven itself an effective strategy for treating breast cancer tissues, is unfortunately not a panacea for treating breast cancer. There are varied adverse side effects associated with this drug, yet the most troubling involves an increased risk of developing endometrial cancer, which is a direct result of its estrogenic properties (Bentrem and Jordan, 1999). Moreover, it was seen that some hormone dependent breast cancer tumors became resistant to and/or fail to respond to antiestrogenic modulators (Pasqualini, 2005).

Another strategy for the treatment of hormone-dependent breast cancers involves the inhibition of steroid hormone synthesis by altering enzymatic activity. Biosynthesis of estrogens occurs via two main pathways. One is the aromatase pathway and the other is the steroid sulfatase pathway (Nakata et al, 2003). Much research has been done with these enzymes, with breakthroughs coming in the form of viable modulators for both the aromatase and steroid sulfatase.

Aromatase is the enzyme that forms estrogens from androgens. It is the major pathway for estrogen synthesis in the ovary via a cytochrome P_{450} aromatase enzyme complex (Brodie, 1994). The aromatase complex is also found in adipose tissue and normal and malignant breast tissues. Given its role in estrogen synthesis, a natural step for researchers was to investigate the development of aromatase inhibitors as a potential breast cancer therapy.

Numerous aromatase inhibitors have been developed with varying potency that have been found to effectively decrease high concentrations of estrogen in breast cancer

tissues (Geisler, 2003). However, there have been a number of concerns regarding the efficacy of these anti-aromatase compounds as treatments for clinical breast cancer. The highest incidence of breast cancer occurs in post-menopausal women, a time when ovarian estrogen production has ceased (Purohit et al, 2003). Studies have shown that there is no significant relationship between clinical response to aromatase inhibitors and circulating levels of estrogen achieved with these inhibitors (Pasqualini, 2005). In fact, the latest generation of aromatase inhibitors do not significantly improve the clinical response in breast cancer patients, which suggests that growth of breast cancer is not controlled by circulating levels of estrogens, but locally synthesized estrogens (Wong et al., 2004). As such, studies were undertaken to investigate the other source of estrogen formation in breast cancer tissues, namely the steroid sulfatase pathway.

Steroid sulfatase is an enzyme which converts various inactive hormones into their active forms by cleaving their sulfate group, and is a key player in estrogen formation. This enzyme has been implicated in the maintenance of hormone-dependent cancers by providing the estrogen locally for the growth of hormone-dependent breast cancer tissues (Santner et al., 1986; Reed and Purohit, 1997). Furthermore, evidence has suggested that steroid sulfatase may be more important to the pathogenesis of breast cancer than aromatase.

Steroid sulfatase is found in a greater percentage of breast cancers and its activity is nearly 10 times that of aromatase (Santner, 1984). In breast cancer tisues, steroid sulfatase mRNA levels are significantly increased (Utsumi et al., 2001). Quantitative determinations indicate that steroid sulfatase activity is 40-500 times higher than that of aromatase in breast cancer tumors (Pasqualini et al, 1996). STS mRNA levels have been

shown to be associated with poor prognosis in ER(+) breast cancer tissue, while aromatase showed no such association (Miyoshi et al., 2003). These studies, along with many others, show the important role steroid sulfatase plays in the growth of hormone-dependent breast cancer and its potential importance as a therapeutic target in addition to aromatase inhibitors.

For my experiments, I used the hormone-independent breast cancer cell line MDA-MB-231, meaning these cells do not express ER alpha. However, previous studies in the Selcer laboratory have determined steroid sulfatase activity levels to be much higher in this cell line than in the ER alpha positive breast cancer line MCF-7, a common cell line used in our lab. This characteristic makes the MDA-MB-231 cell line a more suitable model to study the general regulation of steroid sulfatase activity.

1.5 Steroid Sulfatase in Bone Formation

Estrogens play a key role in the production and maintenance of bone growth. Estrogen deficiency has been associated with decreased bone mineral density (BMD) and increased fracture risk. A study performed by Cummings et al showed that women older than 65 with serum estradiol levels of 5-9 pg/ml have ~60% lower risk of hip and vertebral fractures than women with undetectable levels of estradiol levels (Fujikawa et al., 1997). Furthermore, postmenopausal hormone replacement therapies help stop bone loss and actually restore BMD to premenopausal levels (Janssen et al, 1999). The major pathways of estradiol formation were examined (i.e., aromatase, 17β−hydroxysteroid dehydrogenase (17β−HSD), and steroid sulfatase) in three human osteoblast cell lines: HOS, MG-63, and U2 OS (Purohit et al., 1992). All enzymatic pathways were detected,

but in MG-63 osteoblast cells, STS activity was 1000-fold higher than that of aromatase (Reed et al., 2005). This clearly suggests that the formation of E1 from E1S as a prime source of estrogen for bone formation. A study performed with HOS and MG-63 osteoblasts also showed a high expression of STS mRNA and the ability to use both E1S and DHEAS as substrates for STS activity (Fujikawa et al, 1997). STS was also found to be active in osteoblast like cells derived from bone-fragments, showing that estrogen formation from E1S was nearly 20 times higher than that from androstenedione, and about 50 times higher than from testosterone (both precursors of estrogen in the aromatase pathway) (Muir et al., 2004). Given the importance of local estrogen formation in the formation of bone, it is conceivable that the use of STS inhibitors may actually promote bone loss in breast cancer patients. Fortunately, this is apparently not the case. Tibolone, a synthetic compound derived from 19-nortestosterone used for hormone replacement therapy, has a rtissue-specific effect on STS activity. Once ingested, tibolone is converted to metabolites which exhibit estrogenic effects on bone and the CNS, but exerts antiestrogenic effects on breast tissue, and actually inhibits STS activity in breast cancer cells (Raobaikady et al., 2005). However, more studies must be performed in order to better understand the role of STS in bone formation and the mechanism by which tibolone exerts its effects upon STS.

For my experiments, I used the human osteosarcoma cell line MG-63. This cell line has been used in prior studies in collaboration with the Selcer lab, and steroid sulfatase activity has been well characterized in this cell line, making MG-63 cells an ideal model system to study the regulation of steroid sulfatase in bone growth.

1.6 Steroid Sulfatase in Skin

Steroid sulfatase is found abundantly in the epidermis, and there is ample evidence that it plays a role in androgen production in this tissue. Perhaps one of the more interesting characteristics of steroid sulfatase in this regard occurs when the enzyme is defective or missing. X-linked ichthyosis (XLI) has long been understood as a disease caused by STS gene malfunction. XLI is a genetic skin disorder, which is characterized clinically by the appearance of large, brown scales on the skin along with an increased thickness of the stratum corneum (Reed et al., 2005; Sugawara et al., 1999). In about 90% of patients, the condition is caused by a complete deletion of the STS gene and flanking sequences (Hazan et al., 2005). However, seven point mutation have been identified in patients with complete STS deficiency. All of these mutations are located in the C-terminal region of the enzyme, suggesting its importance in enzymatic functioning (Ghosh, 2004; Sugawara et al.,1999). Luckily, XLI can be treated topically, as STS inhibitors may potentially further decrease STS activity in skin. Furthermore, local STS activity can increase DHEA production, which can be converted to 5α-diydrotestosterone (DHT), which activates the androgen receptor in axillary hair follicles. In women, it is known that an excess of DHT can cause androgenic alopecia or hirsutism (Pitts, 1987). Therefore, it is possible that STS inhibitors may be useful in treating skin or hair follicle conditions where androgen productions are elevated.

1.7 Steroid Sulfatase in the Immune System

Recent studies have shown that steroid sulfatase may also play a role in immune system functioning. STS has been suggested to play a role in the regulation of helper T-cell (Th) maturation (Daynes et al., 1990). Th cells progress to form either Th1 or Th2 cells, each of which subsequently produce an exclusive set of cytokines. Studies in aged mice demonstrated that DHEA suppressed the Th2 response, thereby enhancing the Th1 cytokine profile (Nussbaumer and Billich, 2005). It has been hypothesized that the STS present in macrophages plays a critical role in the immune response because the ability to regulate the conversion of DHEAS to DHEA (Reed et al., 2005). STS may also play a role in autoimmune disorders such as rheumatoid arthritis (RA). It has been shown that RA may result from an increased production of Th1 cytokines as well as decreased production of DHEA from DHEAS. Reasons for the latter are yet unclear, but studies have shown that TNF appears to have an inhibitory effect upon the steroid sulfatase pathway that converts DHEAS to its unconjugated form. This is an interesting finding because DHEA and other androgens exhibit anti-inflammatory properties (Weidler et al., 2005).

1.8 Steroid Sulfatase in the Central Nervous System

The steroid hormone dehydroepiandrosterone (DHEA) and its sulfated form (DHEAS) have been shown to play a vital role in the growth and development of the central nervous system (Hampl et al., 2006). In rats, DHEAS serum levels are higher in the brain than in the blood, a finding which led to the hypothesis that DHEAS might be synthesized de novo in the brain – giving rise to the term neurosteroid to describe any

steroid synthesized by the CNS. A recent study examining STS expression in the temporal lobe was performed to determine if this was also the case in humans, and it was ultimately found that STS mRNA expression was high throughout the temporal lobe and surrounding regions, suggesting that there is no de novo synthesis of DHEA in the brain, but more likely enters the temporal lobe from circulation as DHEAS and subsequently converted to its active form (Steckelbroeck et al, 2004). One of the more interesting theories about DHEA is that the decline in serum levels throughout the human body may have some impact on neurodegenerative diseases, such as Alzheimer's disease. In another study with rats, it was shown (via use of STS inhibitors) that increasing levels of DHEAS increased memory functioning. (Reed et al, 2005). This finding suggests that the inhibition of STS may possibly improve cognitive function, though experiments showing similar results in humans have yet to be performed.

1.9 Inhibition of Steroid Sulfatase

The majority of the research surrounding steroid sulfatase has revolved around the inhibition of the enzyme as a potential therapy for hormone-dependent breast cancer. However, it is becoming increasingly apparent that STS inhibitors could also be used as potential treatments for certain dermatological and immunological conditions (Purohit et al., 2003). The rationale behind the development of these inhibitors was the discovery that STS, perhaps more so than aromatase, is a key contributor to local estrogen production in breast cancer tissue (Li et al., 1996) in postmenopausal women. Another reason for the development of STS inhibitors is the fact that production of the estrogen

receptor binding androgen androstenediol (ADIOL) could also be blocked, thereby alleviating any estrogen like effects of this molecule (Reed et al., 2006).

The history of these inhibitors has seen a repetitive cycle of breakthroughs and drawbacks. Danazol was the one of the first inhibitors ever produced, however it was not very potent (Nguyen and Ferme, 1993). This led to the development of estron-3-methylthiophosphonate (3-MTP), which demonstrated steroid sulfatase inhibition b breast cancer cells, human placental and breast tumor tissues (Duncan et al., 1993). The next inhibitor synthesized was estron-3-O-sulfonate (EMATE), and unexpectedly proved to be a very potent compound. However, EMATE was found to be estrogenic, and could not be used clinically (Purohit, 2003). STX64 (667 COUMATE) was then later developed, and while it is a less potent inhibitor of steroid sulfatase than EMATE, it is nonestrogenic (Woo et al., 1998).

Recently, STX64 (667 COUMATE) was selected to enter Phase I clinical trials in postmenopausal women with breast cancer, ultimately showing that it is a potent inhibitor of STS in PBLs and tumor tissues, and has even been shown to decrease DHEAS levels by 86% (Reed, 2006). Other non-steroidal compounds which are proven steroid sulfatase inhibitors are (p-O-sulfamoyl)-N-alkanoyl tyramines, and chomenone sulfamates, with the latter proving to be as effective as EMATE without its estrogenic effects (Selcer et al., 1997; Billich et al., 2000). Several other inhibitors, widely recognized as second and third generation inhibitors, have been synthesized are currently being tested for clinical use. The main distinction between these two groups of inhibitors is that second generation inhibitors are primarily compounds that have had substituent groups altered so that they possess non-estrogenic properties. One of the more common second generation

inhibitors is 2-methoxyestradiol bis-sulfamate (2-MeOE2bisMATE). The primary attraction of this compound is that it has been shown to be effective in treating ER(-) breast tumors. Third generation inhibitors are interesting in that they can inhibit both aromatase and steroid sulfatase activity, otherwise known as dual aromatase-sulfatase inhibitors (DASIs) (Purohit, 2003). The continual advancements in steroid sulfatase inhibitor science make it seem likely that a therapy will become available which is truly effective for controlling breast cancer, as well as provide methods for treating other non-oncological illnesses that steroid sulfatase plays a role in.

Chapter 2

2. HYPOTHESIS, OBJECTIVES, AND EXPERIMENTAL DESIGN

2.1 Hypothesis

Previous studies in the Selcer laboratory have demonstrated that the glucocorticoid cortisol significantly decreases steroid sulfatase activity in MDA-MB-231 whole cells. In the present study, I sought to determine if this is a general effect of glucocorticoids on breast cancer cells, and to establish if this also occurs in bone cells. I hypothesized that glucocorticoids would decrease steroid sulfatase activity in other breast cancer cells and in bone cells.

2.2 Objectives

The primary objective of this study was to investigate the role glucocorticoids play in the regulation of steroid sulfatase, using human breast cancer cells and human bone cancer cells. More specifically, my goals were:

1. To determine the effect of glucocorticoids, particularly dexamethasone, a synthetic corticosteroid, on steroid sulfatase activity in the human breast cancer cell line MDA-MB-231 and in the human osteosarcoma cell line MG-63.
2. To determine if any effect found is due to alterations in steroid sulfatase protein activity or mRNA levels.

2.3 Experimental Design

These experiments were conducted using two different human carcinoma cell lines: 1) MDA-MB-231, which is a hormone-independent (ER-) breast cancer cell line, and 2) MG-63, which is an osteosarcoma cell line. Steroid sulfatase activity has been demonstrated previously in both cell lines.

A well-established, *in vitro* enzymatic assay was employed to determine the effect the synthetic steroid dexamethasone on steroid sulfatase enzymatic activity. Steroid sulfatase activity in each cell line was determined by measuring the conversion of $^{3}HE_1S$ to its unconjugated form $^{3}HE_1$ in estrogen-free/serum-free medium after steroid hormone treatment. The assay was performed on either whole cells or lysates prepared from these treated cells.

Presence and qualitative assessment of steroid sulfatase messenger RNA was examined using reverse transcriptase polymerase chain reaction. Primers designed specifically to amplify human steroid sulfatase cDNA were used in the assay. Total RNA was extracted from steroid-treated cells.

Chapter 3

3. MATERIALS AND METHODS

3.1 Chemicals and Reagents

^{3}H-estrone sulfate (ammonium salt, [6,7- ^{3}H(N)]-; 49 Ci/mmol) was purchased from DuPont/ New England Nuclear (Boston, MA). Unlabeled steroids were obtained from Sigma Chemical Co. (St. Louis, MO). Ultima Gold liquid scintillation cocktail was purchased from Packard Instrument Co. (Meriden, CT). EMATE was produced using the method designed by Purohit et al (1995). Toluene and Tris were acquired via Fisher Scientific (Pittsburgh, PA). Trypsin-EDTA,, Dulbecco's Phosphate Buffered Saline (PBS), MTT (3[4,5 Dimethylthiazol-2-yl]2,5diphenyltetrazliumbromide, Thiazol blue), and Cell Lytic Buffer were purchased from Sigma Chemical Company (St. Louis, MO). TRIzol reagent and SuperScriptTM One-Step RT-PCR with Platinum *Taq* kit were purchased from Invitrogen Life Technologies. Primers were designed and synthesized by Integrated DNA Technologies, Inc (IDT) (Coralville, IA).

3.2 Cell Culture and Media

MDA-MB-231 (hormone-independent) human breast cancer cells and MG-63 human osteosarcoma cells were acquired from the American Type Culture Collection (Manassas, VA). MDA-MB-231 cells were cultured in 100 x 20 mm tissue culture dishes

and medium was changed every other day. Growth medium used for routine cultures and cell growth assays was purchased from Sigma Chemical Co. (St. Louis, MO). Growth medium consisted of RPMI-1640 (pH 6.9) supplemented with L-Glutamine (0.5mM), 25mM HEPES (pH 7.5), sodium bicarbonate (0.2% v/v), 5% fetal calf serum (FCS), gentamicin (0.2% v/v) and antibiotic/antimycotic (Sigma A-7292) (1% v/v). Cells were routinely grown in 100mm tissue culture dishes in 10ml growth medium.

MG-63 cells were routinely cultured in 100 x 20 mm tissue culture dishes with media being changed every two days. Growth medium used for routine cultures and growth assays was purchased from Gibco/Invitrogen Life Technologies. Growth medium consisted of Mimimum essential medium, F-12 nutrient mixture, and 10% fetal bovine serum (FBS). Cells were grown in 100 x 20 mm tissue culture dishes in 10ml of growth medium.

3.3 Steroid Sulfatase Assay of Whole Cells After Treatment With Steroid Hormones

MDA-MB-231 cells grown in 100 x 20 mm tissue-culture dishes were washed with 10 ml PBS for 1 min. After removing the PBS, 0.5 ml trypsin-EDTA was added to the cells for 30-60 sec. The trypsin-EDTA was then removed and the dishes were tapped in order to dislodge the cells. Cells were resuspended in 6 ml growth media. Cells were counted using a standard hemocytometer. 500, 000 cells were plated in four separate six well plates and incubated overnight in whole growth media at 37° C. After the incubation, the medium was removed and the cells were washed with PBS three times. Next, the cells were incubated in the presence of 10μM, 1μM, and 0.1μM concentrations of cortisol or dexamethasone for 48 hours in estrogen-free RPMI-1640 medium in a six

well tissue culture plate from BD Falcon (Franklin Lakes, NJ). One well plated with cells was left untreated. One well in each dish was left unplated and used as a negative control for each dish. After two days (48 hours), the treatments were removed and the cells were washed three times with PBS. The cells were then incubated with estrogen-free RPMI-1640 medium containing not only the prescribed concentration of hormone, but also [^{3}H]E_1S at 37° C for a period of 24 hours, after which the experiment was terminated accordingly: 500µl medium from each well was pipetted into 13 x 100 mm borosilicate glass test tubes in duplicate. Three milliliters of toluene were then added to each tube for extraction of unconjugated steroids. The mixture was vortexed for 1 min and then centrifuged at 2500xg for 10 mins at 20°C to separate the aqueous and organic phases. Following the centrifugation, one milliliter of the organic phase was removed from the tubes and placed into scintillation vials in duplicate. Six milliliters of scintillation cocktail was added to each vial. The radioactivity was counted using a Packard Tri-carb scintillation counter at 50% efficiency for ^{3}H. The conversion values obtained for all treatments were adjusted for spontaneous product formation by substracting the value obtained for wells containing medium but no cells. Product formation for samples containing test compounds was compared to those of the control samples (no test compounds) and expressed as a percentage of the control. Cells remaining after sulfatase assay were counted by MTT assay.

MG-63 cells cultured in 100 x 20 mm tissue culture dishes were washed in 10mls of PBS for 10 minutes. After removing the PBS, 1.0mls of trypsin-EDTA was added to the cells for 1 minute. The trypsin-EDTA was removed and the dishes were tapped to dislodge the cells. The cells were then resuspended in 6mls of growth media, and counted

using a standard hemocytometer. Cells were plated at a density of 400,000 cells per well in a six well dish for an experiment lasting 24 hours, or at a density of 100,000 cells per well in a six well dish for experiments lasting 96 hours. Cells were incubated overnight in growth media at 37°C. After the incubation period, the medium was removed and the cells were washed with PBS three times. The cells were then incubated in the presence of 1μM dexamethasone for a period of either 24 or 96 hours. Two wells containing no cells were used as negative controls. An equivalent number of wells were left untreated as positive controls. Cells treated with 1μM dexamethasone for 24 hours were simultaneously treated and incubated with ^{3}H-E_1S at 37°C and the experiment was terminated. The aforementioned steroid sulfatase assay was performed to quantify the sulfatase activity (see above). Cells treated with 1μM dexamethasone for 96 hours had the media changed after 48 hours, and at the 72 hour time point, ^{3}H-E_1S was added along with the prescribed concentration of dexamethasone. The experiment was terminated at the 96-hour time point and the steroid sulfatase assay was performed.

3.4 Cell Microsome Preparation

Pre-confluent cultures of MDA-MB-231 and MG-63 cells were washed 3 times with PBS. Two mls of PBS were added to the dishes and a cell scraper was used to dislodge cells. Cells were then transferred to 15 ml polystyrene conical tubes and centrifuged at 1000xg for 10 min in order to pellet cells. The PBS was removed and the pellets were resuspended in 12 ml 0.25M sucrose, 50 mM Tris-HCl, pH 7.4. Cells were homogenized three times using a BioSpec Tissue Tearer homogenizer (BioSpec Products Inc., Bartlesville, OK). The homogenate was then centrifuged at 2500xg for 15 min at

4°C to pellet nuclei. Supernatant was transferred into Beckman Ultra-clear centrifuge tubes and centrifuged at 40,000xg for 1 h to obtain the microsomal pellet. Supernatant was discarded and the pellets representing the microsomes were combined and resuspended in 500 µl 50mM Tris-HCl, pH 7.4. Aliquots of microsomes were stored at -80°C.

3.5 BCA Protein Assay

The Pierce (Rockville, IL) BCA Protein Assay was used according to the manufacturer's instructions. A standard curve of bovine serum albumin was prepared with water as the diluent. Duplicate concentrations (in µg/tube) were 200, 150, 125, 100, 75, 50, 30, 20, 10 and 5. A blank of only water was prepared. Membranes were prepared as duplicates at differing amounts such that the volume of membrane and water equal 100 µl. Two milliliters of BCA working reagent was added to each tube, vortexed and incubated in a 37°C water bath for 30 min. Tubes were then allowed to be cooled to room temperature and absorbances were read at 562 nm using a spectrophotometer. Standards were plotted and the relationship between absorbance and concentration was determined by linear regression. Unknown protein concentration was calculated from the graph.

3.6 MTT Assay

Two hundred µl of MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) solution (5 mg/ml in RPMI-1640) was added to each well of a six-well plate containing cells (media removed) and incubated for three hr at 37°C. At end of the incubation period, the MTT medium was removed and the converted dye was

solubilized with one ml of 0.1% acidic isopropanol. Aliquots of 100 µl of the acidic isopropanol from a single well on the six-well plate, was then transferred to each of eight consecutive wells on a 96-well microtiter plate. Absorbance of converted dye was measured at a wavelength of 570 nm with background substraction at 690 nm. Absorbance of converted dye corresponded to the number of cells. To generate standard curves, MDA-MB-231 and MG-63 cells were plated into six-well tissue culture dishes with each well containing a different number of cells obtained by serial dilution. Cells were incubated in whole medium overnight prior to the MTT assay to allow them to adhere to the plates.

3.7 Cell Lysate Preparation

MDA-MB-231 and MG-63 cells were grown to confluence in 100 x 20mm tissue culture dishes in standard growth medium. Cells were rinsed once with PBS, letting the rinse stand for a period of 2 to 3 minutes. The PBS was removed and 1 ml of Cell Lytic Buffer (Sigma) was added to the cells and incubated for 15 minutes on a shaker. Cells were removed from the plate via a cell scraper. The lysed cells were collected and transferred into 1.5 ml eppendorf tubes and centrifuged for 15 minutes at 12,000 x g. The supernatant was transferred into new 1.5 ml eppendorf tubes and stored in a -80°C freezer.

3.8 RNA extraction

Total RNA was isolated using TRIzol Reagent (Invitrogen Life Technologies) following the manufacturer's protocol. MDA-MB-231 or MG-63 cells adhered in six

well tissue culture dishes were removed by adding 1 ml of TRIzol reagent to a single well. The resulting lysate was transferred into 1.5 ml eppendorf tubes and incubated at room temperature for 5 min. This allowed for complete dissociation of nucleoprotein complexes. 200 μl of chloroform was then added and the tubes were shaken vigorously by hand for 15 sec. Tubes were incubated for a period of 3 min at room temperature and centrifuged at 12,000 x g for 15 min at 4°C. The supernatant (RNA in aqueous phase), was removed, transferred to a fresh eppendorf tube and 500 μl of isopropyl alcohol was added and again centrifuged at 12,000 x g for 10 min at 4°C. The resulting RNA pellet was washed with 1000 μl of 75% ethanol, centrifuged at 7000 x g for 5 min at 4°C, air dried, and resuspended in 100 μl of diethylpyrocarbonate (DEPC) treated water. Following a 10 minute incubation in a 60°C water bath, RNA was quantified using a spectrophotometer (ThermoSpectronic, Genysis 8). The amount of total RNA extracted was determined by ultraviolet light absorption at 260 nm. RNA samples were stored at -80°C.

3.9 Reverse Transcriptase Polymerase Chain Reactions (RT-PCR)

RT-PCR was performed using the Superscript One-Step RT-PCR with Platinum *Taq* Kit (Invitrogen Life Technologies) following the manufacturers instructions. RT-PCR reactions consisted of: 1 μg template RNA, 1 μl of STS G (forward primer, 150 pmol), 1 μl STS K (reverse primer, 150 pmol), 1 μl RT/Platinum Taq Mix, 25 μl 2X reaction mix (a buffer containing 0.4 mM of each dNTP, 2.4 mM MgSoo4), and autoclaved water (up to 50 μl). The PCR reaction was run in a PTC-100 Programmable Thermal Controller MJ Reasearch Inc, Watertown, MA). The program for amplification

included the reverse transcription step, which consisted of a 30 min hold at 55°C and a 2 minute hold at 94°C. Next, the cDNA was amplified over thirty-five cycles composed of: 30 sec at 94°C, 1 min at 60°C, and 1 min at 72°C. The PCR reaction was terminated with a final extension of 10 min at 72°C. The PCR products were then separated in a 2% agarose gel containg 5 μl of 1mg/ml Ethidium bromide solution run in 1X TAE running buffer (40 mM Tris-acetate, 2mM Na-EDTA-2H20) for 1 hour at 80 volts. Ten μl of PCR product was mixed with 2 μl of 10X loading dye (41% w/v Bromophenol blue, 25% w/w Ficoll). A 100 bp DNA ladder (Invitrogen Life Technologies) was used as the standard.

3.10 Statistical Analysis

All statistical analysis were be performed using the computer program Prism (Graphpad Inc., San Diego, CA). Comparison of variables were made using One-way analysis of variance (ANOVA) and student's t-tests. Neuman-Keuls test was used for posteriori analysis of means. Probabilities of $p<0.05$ were considered significant.

Chapter 4

4. RESULTS

4.1 Steroid sulfatase activity in MDA-MB-231 whole cells after cortisol treatment

This experiment was performed as a follow up and confirmation of previous work in the Selcer laboratory which demonstrated that the glucocorticoid cortisol significantly decreased steroid sulfatase activity in MDA-MB-231 human breast cancer cells. Here, MDA-MB-231 cells were treated in a dose-dependent manner with 10μM, 1.0μM, 0.1μM and 0μM concentrations of cortisol, a steroid produced in the adrenal cortex over a 72 hour time period (Fig 2). Steroid sulfatase activity was assayed by analyzing the conversion of ^{3}H-E_1S to its unconjugated form. The data were adjusted for cell number and expressed in dpms (disintegrations per minute). One-way analysis of variance (ANOVA) indicated significant differences between different concentrations ($F=3.808$, df, 2,11 $p < 0.05$). The high and medium concentrations of cortisol showed a significant decrease steroid sulfatase activity when compared to control (Newman–Keuls test, $p<0.05$).

4.2 Steroid sulfatase activity MDA-MB-231 whole cells after dexamethasone treatment

The following set of experiments represent the next logical step in studying glucocorticoid regulation of steroid sulfatase in MDA-MB-231 cells. Since cortisol

decreased steroid sulfatase activity in these cells, we wanted to determine the effect on steroid sulfatase activity when treated with the more potent and clinically prescribed synthetic glucocortoid dexamethasone.

MDA-MB-231 whole cells were treated with 10μM, 1.0μM, 0.1μM and 0μM dexamethasone, a synthetic corticosteroid, in a dose dependent manner for 72 hours (Fig. 3). Steroid sulfatase activity was assayed by analyzing the conversion of ^{3}H-E_1S to its unconjugated form. Data was adjusted for cell number and expressed in dpms. One-way analysis of variance (ANOVA) showed statistically significant differences between concentrations ($F = 5.271$, df, 5,23, $p < 0.05$). Cells treated with dexamethasone showed a significant decrease in steroid sulfatase activity compared to control cells (Newman-Keuls test, $p < 0.05$).

MDA-MB-231 whole cells were treated in a dose dependent manner with dexamethasone. Cells were incubated for a 24 hour time period in the presence of dexamethasone at the following concentrations: 1μM, 0.1μM, 0.01μM, 0.001μM and 0μM (Fig 4). Steroid sulfatase activity was measured by analyzing the hydrolysis of ^{3}H-E_1S to its unconjugated form. Conversion counts for each concentration were as follows: 572.7 dpm (1 uM), 687.6 dpm (0.1 uM), 983.9 dpm (0,01 uM), 723 dpm (0.001 uM). Data was adjusted for cell number. One-way analysis of variance (ANOVA) revealed statistically significant differences between concentrations ($F = 13.71$, 3, 15 df, $p < 0.05$). Cells treated with higher doses of dexamethasone had a more significant decrease in steroid sulfatase activity as compared to cells treated with lower doses (Newman-Keuls, $p<0.05$).

4.3 Steroid sulfatase activity in MDA-MB-231 whole-cell lysates after dexamethasone treatment for various times

We used whole-cell lysate preparations for the next set of experiments because data in whole cells had been somewhat variable, and it was hoped that lysates would be more reliable for detection of steroid sulfatase activity. Whole cells were treated with dexamethasone at 1μM, 0.1μM, 0.01μM and 0μM concentrations for a 24 hour time period (Fig 5). Lysates were then prepared and steroid sulfatase activity was determined by analyzing conversion of ^{3}H-E_1S to its unconjugated form. Data were adjusted for protein concentration and expressed in disintegrations per minute (dpms). One-way analysis of variance (ANOVA) revealed no statistically significant differences between treatment groups ($F = 0.6818$, 3,15 df, $p > 0.05$).

MDA-MB-231 whole-cell lysates were also prepared from cells treated with dexamethasone in a dose dependent manner in 10μM, 1.0μM , 0.1μM, 0.01μM, and 0 μM concentrations for a period of 72 hours (Fig 6). Lysates were prepared and steroid sulfatase activity was determined by analyzing the conversion of ^{3}H-E_1S to its unconjugated form. Data were adjusted for protein concentration and expressed in dpms. One way analysis of variance (ANOVA) revealed no statistically significant differences between treatment groups ($F = 1.445$, 2,14 df, $p > 0.05$).

In a separate experiment, MDA-MB-231 whole-cell lysates, prepared from MDA-MB-231 cells plated in six-well tissue culture dishes, were treated with a 10μM dose of dexamethasone for a period of 24, 48 or 72 hours (Fig 7). Steroid sulfatase activity was analyzed, data were adjusted for protein concentrations and expressed in dpm/μg protein.

One-way analysis of variance (ANOVA) revealed no significant differences between treatment groups ($F = 1.008$, 5,17 df, $p > 0.05$).

4.4 Test for competitive inhibition of steroid sulfatase activity by glucocorticoids in human placental microsomes and MDA-MB-231 microsomes

The following experiments were performed to determine if effects on steroid sulfatase activity in MDA-MB-231 cells were due to competitive inhibition or some other mechanism. Microsomes were used because steroid sulfatase is found primarily in microsomes. Further, steroid sulfatase activity is known to be very high in the human placenta, making this tissue ideal for activity studies.

Placental microsomes were prepared from homogenization of frozen human placental tissue, which is known to be high in steroid sulfatase activity. Steroid sulfatase activity was assayed by analyzing the hydrolysis of ^{3}H]-E_1S to its unconjugated form in the presence of 10μM dexamethasone, 10μM cortisol, or 10μM vehicle control (ethanol) (Fig 8). Conversion dpms averaged 45,624 for dexamethasone, 40,982 for cortisol, and 40,730 for control microsomes. Analysis of variance revealed no significant differences between treatment groups ($F=1.044$, 2,8 df, $p > 0.05$).

Microsomes were then prepared from MDA-MB-231 cells cultured in growth medium using a standard protocol. Steroid sulfatase activity was assayed by analyzing the hydrolysis of [^{3}H]E1S to its unconjugated form in the presence of 10μM dexamethasone, 10μM cortisol, or 10μM vehicle (ethanol) (Fig 9). Conversion dpms averaged 594.6 for dexamethasone, 683.6 for cortisol, and 694.2 for control group

microsomes. Analysis of variance revealed no significant difference between treatment groups ($F = 2.981$, 3,11 df, $p > 0.05$).

In another experiment, whole-cell lysates were prepared from MDA-MB-231 cells and steroid sulfatase activity was measured in the presence of 10μM concentrations of dexamethasone and cortisol to also determine if changes in steroid sulfatase activity were due to competitive inhibition (Fig 10). Lysates were used because the microsomal preparation process may have altered steroid sulfatase activity such that the enzyme would not be responsive to glucocorticoid treatment. Data were adjusted for protein concentration and expressed in dpms. One-way analysis of variance (ANOVA) revealed statistically significant differences between the treatment groups ($F = 10.05$, 2,14 df, $p < 0.05$). Lysates from cells treated with cortisol showed a significant decrease in steroid sulfatase activity when compared to lysates from cells treated with vehicle alone (Neuman-Keuls, $p<0.05$).

4.5 Effect of dexamethasone on steroid sulfatase mRNA expression in MDA-MB-231 cells

MDA-MB-231 cell monolayers were plated in 35 mm tissue culture dishes (x 7) overnight in whole growth medium. Growth medium was then removed and estrogen/serum-free medium was added containing 10μM concentration of dexamethasone and incubated for a period of 4, 12, and 24 hours respectively. One dish was processed before addition of steroid and represented the 0 hour control. RNA was extracting using the TRIzol method, and steroid sulfatase mRNA expression was determined via RT-PCR. PCR products were run on a 2% agarose gel containing

ethidium bromide. All lanes contained bands of the correct size for the expected cDNA product. There were no apparent differences in PCR products between dexamethasone-treated and control cells (Fig 11). However, a more quantitative experiment will need to performed to truly assess the effect dexamethasone might have on steroid sulfatase expression.

4.6 Effect of dexamethasone on steroid sulfatase activity in MG-63 whole cells

Steroid sulfatase activity has also been demonstrated in bone cells, and it has been suggested that this enzyme may play an active role in bone growth physiology. With this in mind, the human osteosarcoma cell line MG-63 was used to determine the effect of glucocorticoids on steroid sulfatase activity within bone cells.

An initial experiment was conducted using intact MG-63 cells treated with 1μM dexamethasone for 24 hours. Cells were plated in six-well tissue culture plates overnight in growth medium (Fig 12). Medium was removed and the cells were incubated in estrogen/serum-free growth medium with either 1μM Dexamethasone or vehicle (ethanol) along with ^{3}H-E_1S for a period of 24 hours. Two wells were left unplated with cells and contained only medium with ^{3}H-E_1S to serve as controls for spontaneous hydrolysis. Steroid sulfatase activity was determined by analyzing the conversion of ^{3}H-E_1S to its unconjugated form. Data were adjusted for cell number and expressed in dpms. Paired t-test analysis revealed no significant effect of dexamethasone on steroid sulfatase activity after 24 hours ($t = 0.1225$, $p > 0.05$).

A second experiment was performed on intact MG-63 whole cells that were treated with 1μM dexamethasone for 96 hours (Fig 13). Cells were plated overnight in

whole growth medium, removed and replaced with estrogen/serum-free growth medium. After 72 hours, serum/estrogen-free medium was replaced with medium containing 1μM dexamethasone and vehicle (ethanol), as well as ^{3}H-E_1S for another 24 hours. Two wells were left unplated of cells and contained medium to serve as controls for spontaneous hydrolysis. Steroid sulfatase activity was determined by analyzing the conversion of ^{3}H-E_1S to its unconjugated form. Data were adjusted for cell number and expressed in dpms. A paired t-test analysis again revealed no significant effect of dexamethasone on steroid sulfatase activity after 96 hours ($t = 0.9218$, $p > 0.05$).

4.7 Steroid sulfatase activity in MG-63 lysates prepared from dexamethasone-treated cells

Whole cell lysates were prepared from MG-63 cells after plating them overnight in growth medium in six-well tissue culture dishes and incubating the cells in 1μM dexamethasone and vehicle (ethanol) for a period of 24 hours (Fig 14). Steroid sulfatase activity was analyzed by radiolabeled conversion assay and the resulting dpm data were adjusted for protein concentration. Data are expressed in dpms/ μg protein. A paired t-test analysis revealed no significant difference between treatment groups ($t = 1.828$, $p > 0.05$).

Another experiment was performed on MG-63 whole cell lysates prepared after having been plated in the presence of 1μM dexamethasone and vehicle (ethanol) for a period of 96 hours (Fig 15). Steroid sulfatase activity was analyzed by radiolabeled conversion assay and the resulting dpm data were adjusted for protein concentration. A paired t-test analysis revealed significant difference between treatment groups, with cells

treated with dexamethasone showing an increase in steroid sulfatase activity after 96 hours ($t = 2.579$, $p < 0.05$).

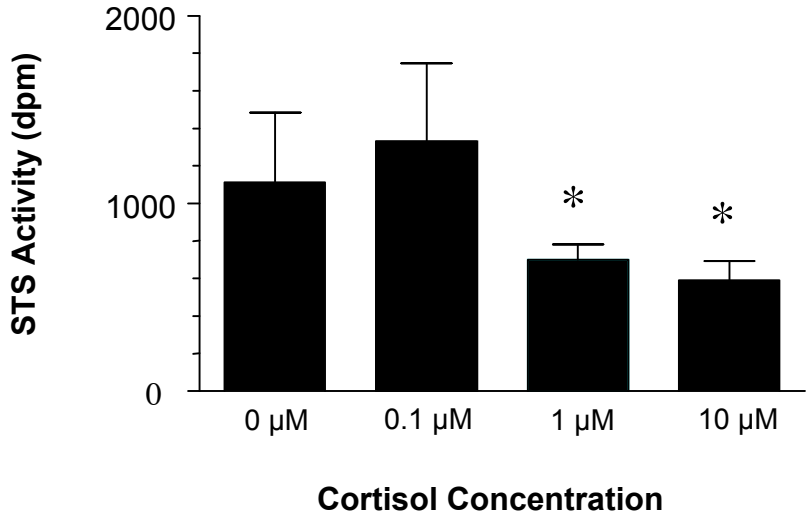

Figure 2. Steroid sulfatase activity in MDA-MB-231 cells treated with varying concentrations of cortisol (F). Cells were plated (~ 0.4-0.5 million cells) in six-well tissue culture dishes (x4) overnight in growth medium. The growth medium was then removed, cells were rinsed twice in PBS, and incubated for 72 hours in estrogen/serum-free medium with either 10μM, 1μM, 0.1μM or 0μM concentrations of cortisol (F) in each of four dishes. One well in each dish was unplated with cells, and contained only estrogen/serum-free medium with vehicle (ethanol). After 48 hours, medium was replaced with fresh estrogen/serum-free medium containing both drug treatments and ^{3}H-E_1S for remaining 24 hours. Steroid sulfatase activity was determined and normalized for cell number. Data are average of three experiments, the composite of 15 wells for each treatment group. Bars are +/- 1 standard error. Asterisks denote treatment group significantly different compared to control.

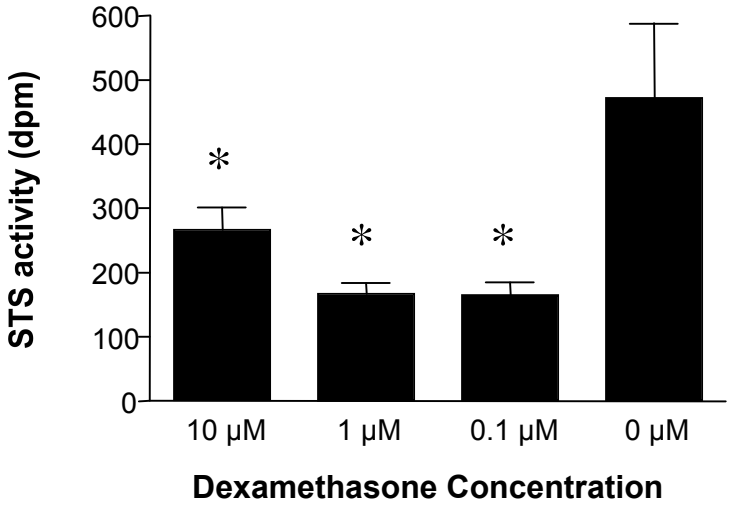

Figure 3. Steroid sulfatase activity in MDA-MB-231 cells treated with varying concentrations of dexamethasone. Cells were plated (~ 0.4-0.5 million cells) in six-well tissue culture dishes (x4) overnight in growth medium. The growth medium was then removed, cells were rinsed twice in PBS, and incubated for 72 hours in estrogen/serum-free medium with either 10μM, 1μM, 0.1μM or 0μM concentrations of dexamethasone in each of four dishes. One well in each dish was unplated with cells, and contained only estrogen/serum-free medium with vehicle (ethanol). After 48 hours, medium was replaced with fresh estrogen/serum-free medium containing both drug treatments and ^{3}H-E_1S for remaining 24 hours. Steroid sulfatase activity was determined and normalized for cell number. Data are the average of three experiments, the composite of 15 wells for each treatment group. Bars are +/- 1 standard error. Asterisks denote significant difference from control.

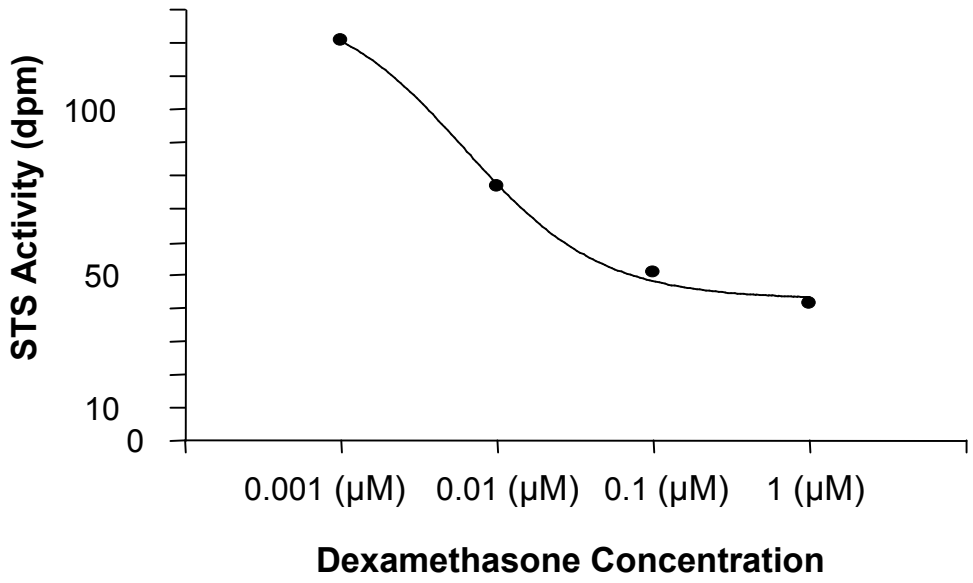

Figure 4. Steroid sulfatase activity in MDA-MB-231 cells treated with varying concentrations of dexamethasone. Cells were plated (~ 0.4-0.5 million cells) in six-well tissue culture dishes (x2) overnight in growth medium. The growth medium was then removed, cells were rinsed twice in PBS, and incubated for 24 hours in estrogen/serum-free medium containing both ^{3}H-E_1S and 1μM, 0.1μM, 0.01μM or 0.001μM concentrations of cortisol in each of four dishes. Two wells were unplated with cells, and contained only estrogen/serum-free medium with vehicle (ethanol). Steroid sulfatase activity was determined and normalized for cell number. Data are the average of four experiments.

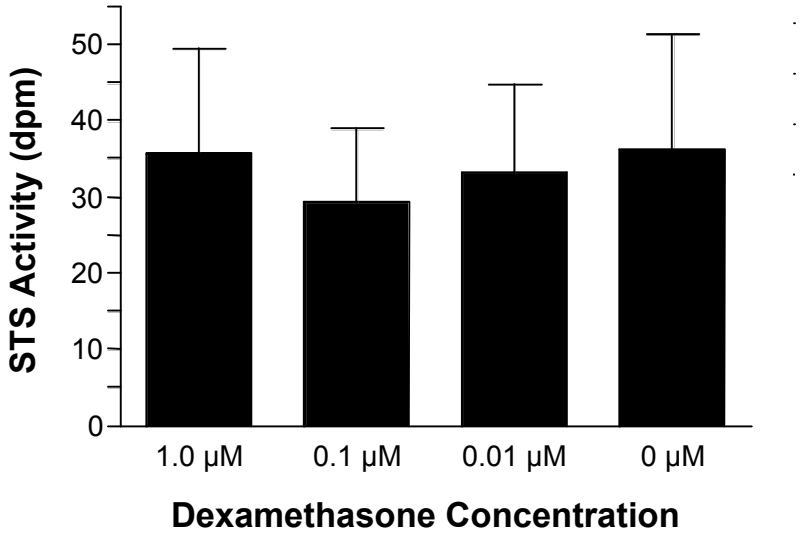

Figure 5. Steroid sulfatase activity in MDA-MB-231 whole cell lysates treated with 1µM, 0.1µM, 0.01µM and 0µM concentrations of dexamethasone. Cells were plated (~ 0.4-0.5 million cells) in six-well tissue culture dishes (x3) overnight. The growth medium was then removed, cells were rinsed twice in PBS, and incubated for 24 hours in descending concentrations of dexamethasone in estrogen-free, serum-free medium in triplicate. Three wells were treated with only the vehicle (ethanol) as a control. Cells were then rinsed and incubated for 2-3 minutes in PBS. 0.4 ml of cell-lytic buffer was then added to dish after removal of PBS and incubated on a rotator for 15 minutes. Cells were then harvested with a cell scraper, transferred to a 1.5mL eppendorf tube, and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was collected into new 1.5 mL eppendorf tubes and stored in a -80°C freezer until ready to use. Lysates were thawed slowly on ice prior to use. Conditions were set up by first preparing a mixture of, Tris-HCl pH 7.4, ^{3}H-E_1S, and E_1S. After all treatments were prepared

correctly in 13x100 mm test tubes and placed into a 37°C water bath. Lysates were added, and samples were incubated for 2 hours. The experiment was terminated with the addition of 3mL of toluene to each sample, and the standard sulfatase assay was performed. Steroid sulfatase activity was normalized for amount of protein. Data are the average of four experiments, each run in triplicate. Bars are +/- 1 standard error.

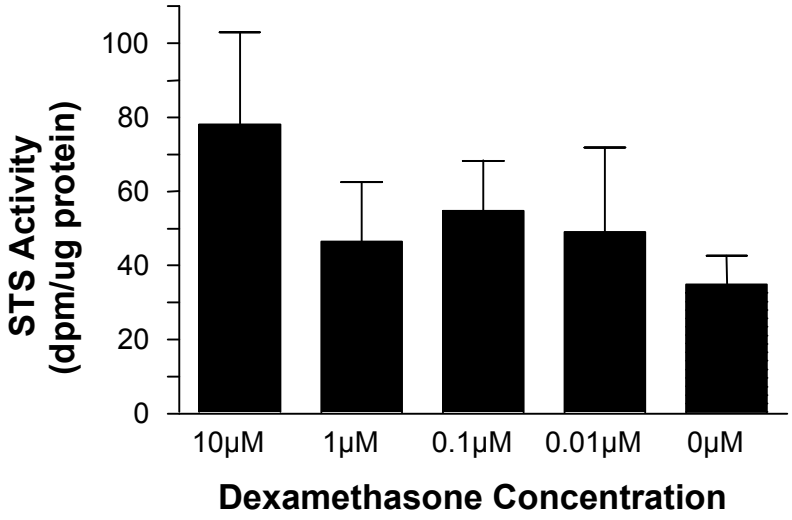

Figure 6. Steroid sulfatase activity in MDA-MB-231 whole cell lysates treated with 10μM, 1μM, 0.1μM, 0.01μM and 0μM concentrations of dexamethasone. Cells were plated (~ 0.4-0.5 million cells) in six-well tissue culture dishes (x3) overnight. The growth medium was then removed, cells were rinsed twice in PBS, and incubated for 72 hours in estrogen-free, serum-free medium with descending concentrations of dexamethasone in triplicate. Three wells were treated with only the vehicle (ethanol) as a control. Cells were then rinsed and incubated for 2-3 minutes in PBS. 0.4 ml of cell-lytic buffer was then added to dish after removal of PBS and incubated on a rotator for 15 minutes. Cells were then harvested with a cell scraper, transferred to a 1.5mL eppendorf tube, and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was collected into new 1.5 mL eppendorf tubes and stored in a -80°C freezer until ready to use. Lysates were thawed slowly on ice prior to use. Conditions were set up by first

preparing a mixture of, Tris-HCl pH 7.4, ^{3}H-E_1S, and E_1S. After all treatments were prepared correctly in 13x100 mm test tubes and placed into a 37°C water bath. Lysates were added, and samples were incubated for 2 hours. The experiment was terminated with the addition of 3mL of toluene to each sample, and the standard sulfatase assay was performed. Steroid sulfatase activity was normalized for amount of protein. Data are the average of three experiments, each run in triplicate. Bars are +/- 1 standard error.

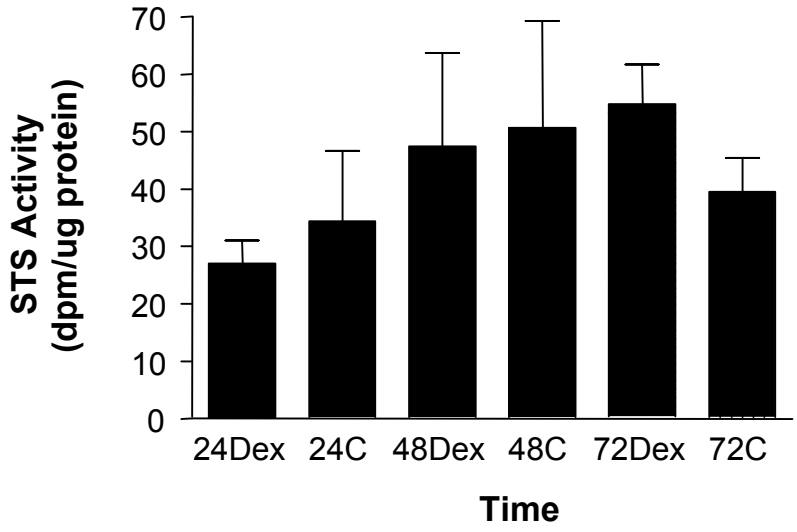

Figure 7. Time course assay with MDA-MB-231 whole cell lysates treated with 10µM dexamethasone. Cells (0.2-0.3 million) were plated in six-well tissue culture dishes in growth medium overnight. Growth medium was then removed and rinsed twice in PBS. Serum/estrogen free medium was then added to each well containing cells containing 10µM dexamethasone for a period of 24 hours, 48 hours, and 72 hours respectively. At the end of the different time periods, cells were then rinsed and incubated for 2-3 minutes in PBS. One mL of cell-lytic buffer was then added to dish after removal of PBS and incubated on a rotator for 15 minutes. Cells were then harvested with a cell scraper, transferred to a 1.5mL eppendorf tube, and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was collected into new 1.5 mL eppendorf tubes and stored in a -80°C freezer until ready to use. Lysates were thawed slowly on ice prior to use. Conditions were set up by first preparing a mixture of, Tris-HCl pH 7.4, ^{3}H-E_1S, E_1S, and either 10µM dexamethasone, cortisol, or vehicle control

(ethanol) in 13x100 mm test tubes and placed into a 37°C water bath. A 1:3 dilution of lysates were added, and samples were incubated for 2 hours. The experiment was terminated with the addition of 3mL of toluene to each sample, and the standard sulfatase assay was performed. Steroid sulfatase activity was normalized for amount of protein. Data are the average of five experiments. Bars are +/- 1 standard error.

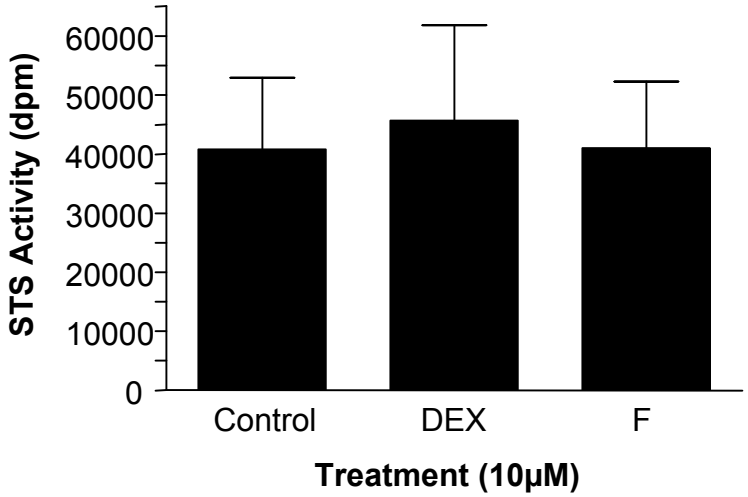

Figure 8. Steroid sulfatase activity in human placental microsomes treated with 10µM dexamethasone and 10µM cortisol (F). Placental microsomes were obtained from homogenization 1g of frozen placental tissue (human). Microsomes were prepared using standard preparation protocols. Aliquots of microsomes were stored at -80°C. Microsomes were thawed slowly on ice prior to use. Samples were prepared in 13X100mm test tubes by preparing mixtures of Tris-HCl, ^{3}H-E_1S, E_1S, and 10µM solutions of dexamethasone, cortisol, or vehicle control (ethanol). All tubes were placed in a 37°C water bath and membrane was added and incubated for two hours. Two tubes contained only Tris as a control for spontaneous hydrolysis. Reaction was terminated by adding 3mL of toluene to each tube, and standard steroid sulfatase assay was performed. Steroid sulfatase activity in dpm, normalized as a percent of control. Data are the average of three experiments. Bars are +/- 1 standard error.

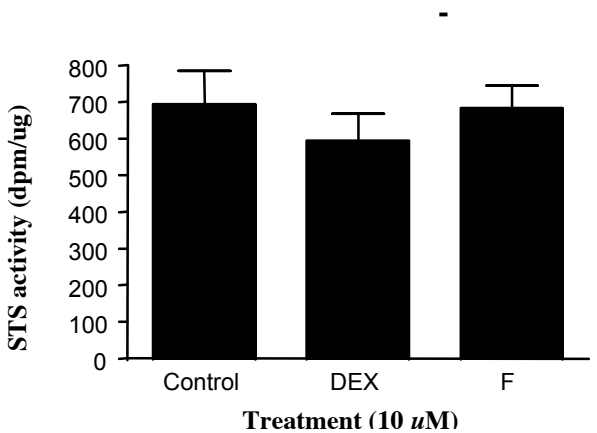

Figure 9. Steroid sulfatase activity in MDA-MB-231 microsomes treated with 10μM dexamethasone or 10μM cortisol (F). Microsomes were prepared by culturing cells in 100 x 20mm tissue culture dishes in growth medium until confluent. Cells were then harvested and microsomes were prepared using standard preparation protocols. Aliquots of microsomes were stored at -80°C. Samples were prepared in 13X100mm test tubes by preparing mixtures of Tris-HCl, ^{3}H-E_1S, E_1S, and 10μM solutions of dexamethasone, cortisol, or vehicle control (ethanol). All tubes were placed in a 37°C water bath and membrane was added and incubated for thirty minutes. Two tubes contained only Tris as a control for spontaneous hydrolysis. Reaction was terminated by adding 3mL of toluene to each tube, and standard steroid sulfatase assay was performed. Steroid sulfatase activity is in dpm, normalized as a percent of control. Data are the average of four experiments. Bars are +/- 1 standard error.

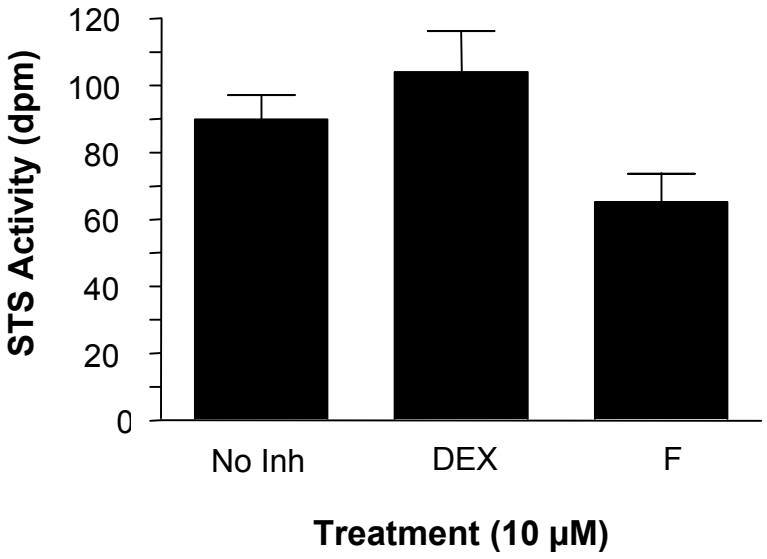

Figure 10. Competitive inhibition study performed using MDA-MB-231 whole cell lysates. Cells were plated in 100x20mm tissue culture dishes in growth medium. Once confluent, growth medium was removed. Cells were then rinsed and incubated for 2-3 minutes in PBS. 1 mL of cell-lytic buffer was then added to dish after removal of PBS and incubated on a rotator for 15 minutes. Cells were then harvested with a cell scraper, transferred to a 1.5mL eppendorf tube, and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was collected into new 1.5 mL eppendorf tubes and stored in a -80°C freezer until ready to use. Lysates were thawed slowly on ice prior to use. Conditions were set up by first preparing a mixture of, Tris-HCl pH 7.4, ^{3}H-E_1S, E_1S, and either 10µM dexamethasone, cortisol, or vehicle control (ethanol) in 13x100 mm test tubes and placed into a 37°C water bath. A 1:3 dilution of lysates was added, and samples were incubated for 2 hours. The experiment was terminated with the addition of

3mL of toluene to each sample, and the standard sulfatase assay was performed. Steroid sulfatase activity was normalized for amount of protein. Data are the average of five experiments. Bars are +/- 1 standard error.

Time	L	0	4	4	12	12	24	24
Dex		-	-	+	-	+	-	+

Fig 11. Steroid sulfatase activity in MDA-MB-231 cells after short time course treatment with dexamethasone. MDA-MB-231 cells were plated (~0.4-0.5 million) in 35 mm tissue culture dishes (x7) overnight in whole growth medium. Growth medium was removed after overnight growth and rinsed twice with PBS. Cells were then incubated in serum/estrogen-free medium for up to 24 hours in the presence of either 10μM dexamethasone (Dex) or vehicle (ethanol). At the differing time intervals (0h, 4h, 12h, and 24 h respectively), RNA was purified from cell monolayers, RNA levels were quantified, and the solution was frozen in a -80°C freezer until ready to be analyzed. One μg of template RNA was used for each sample condition.

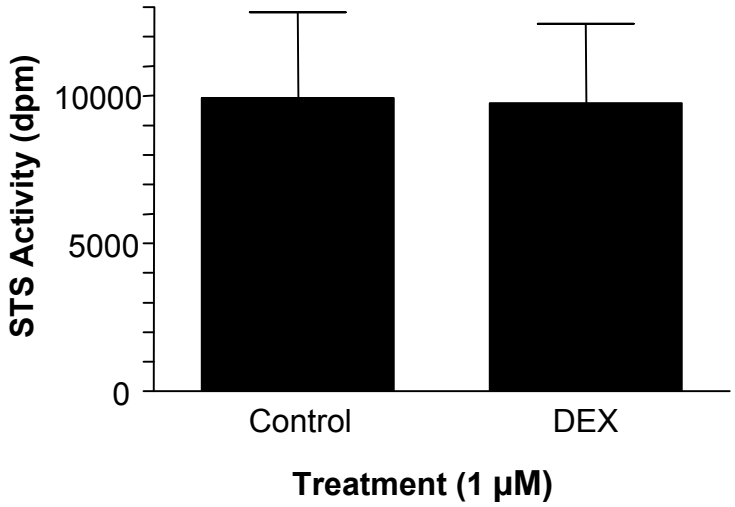

Figure 12. Steroid sulfatase activity in MG-63 cells treated with 1uM concentration of dexamethasone and vehicle control. Cells were plated (~ 0.2-0.3 million cells) in six-well tissue culture dishes (x2) overnight in growth medium. The growth medium was then removed, cells were rinsed twice in PBS, and incubated for 24 hours in estrogen/serum-free medium with dexamethasone and $^{3}H\text{-}E_1S$ in triplicate. Three wells were treated with only the vehicle (ethanol) as a control. Two wells were plated with only medium as a control for spontaneous hydrolysis. Steroid sulfatase activity was determined and normalized for cell number. Data are the average of three experiments, each run in triplicate. Bars are +/- 1 standard error.

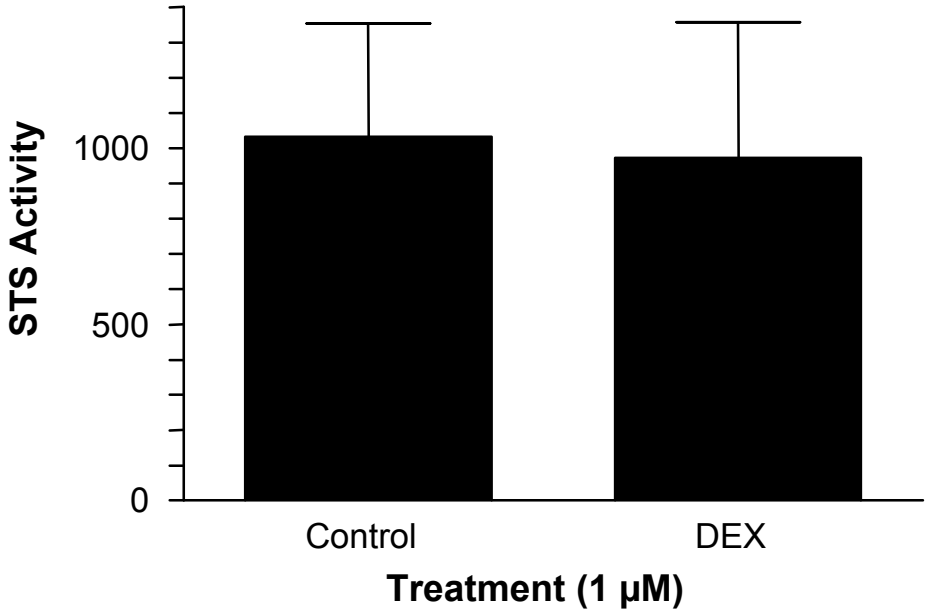

Figure 13. Steroid sulfatase activity in MG-63 whole cells treated with 1uM concentration of dexamethasone and vehicle control. Cells were plated (~ 0.2-0.3 million cells) in six-well tissue culture dishes (x2) overnight in growth medium. The growth medium was then removed, cells were rinsed twice in PBS, and incubated for 96 hours in estrogen/serum-free medium with dexamethasone in triplicate. Three wells were treated with only the vehicle (ethanol) as a control. Two wells were plated with only medium as a control for spontaneous hydrolysis. After 72 hours, medium was replaced with fresh estrogen/serum-free medium containing both drug treatment and ^{3}H-E_1S for 24 hours. Steroid sulfatase activity was determined and normalized for cell number. Data are the average of three experiments, each run in triplicate. Bars are +/- 1 standard error.

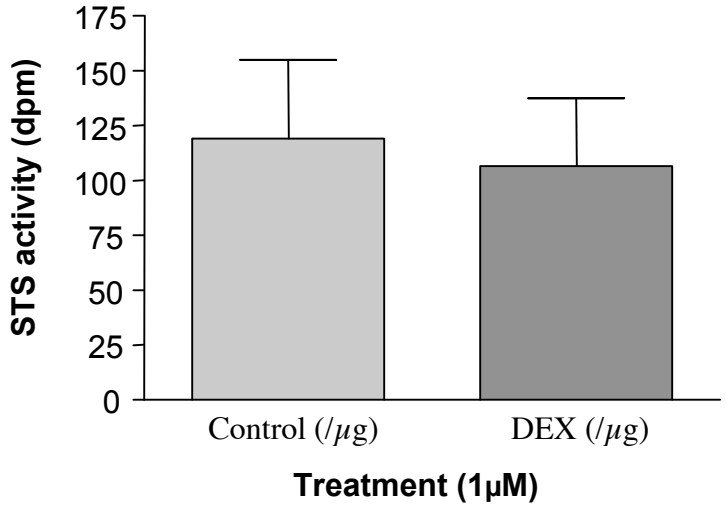

Figure 14. Steroid sulfatase activity in MG-63 whole cell lysates treated with 1μM concentration of dexamethasone or vehicle control. Cells were plated (~ 0.2-0.3 million cells) in six-well tissue culture dishes overnight in growth medium. The growth medium was then removed, cells were rinsed twice in PBS, and incubated for 24 hours in estrogen-free, serum-free medium with either 1μM of dexamethasone or vehicle (ethanol) in triplicate. Cells were then rinsed and incubated for 2-3 minutes in PBS. 0.4 ml of cell-lytic buffer was then added to dish after removal of PBS and incubated on a rotator for 15 minutes. Cells were then harvested with a cell scraper, transferred to a 1.5mL eppendorf tube, and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was collected into new 1.5 mL eppendorf tubes and stored in a -80°C freezer. Data are the average of three experiments, each run in triplicate. Bars are +/- 1 standard error.

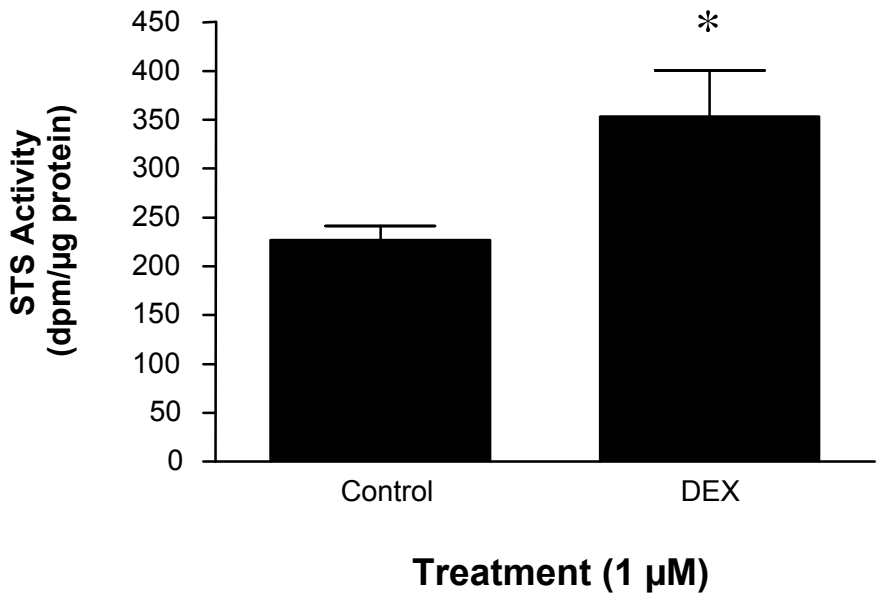

Figure 15. Steroid sulfatase activity in MG-63 whole cell lysates treated with 1µM concentration of dexamethasone and vehicle control. Cells were plated (~ 0.2-0.3 million cells) in six-well tissue culture dishes overnight in growth medium. The growth medium was then removed, cells were rinsed twice in PBS, and incubated for 96 hours in estrogen-free, serum-free medium with either 1µM dexamethasone or vehicle (ethanol) in triplicate. Cells were then rinsed and incubated for 2-3 minutes in PBS. 0.4 ml of cell-lytic buffer was then added to dish after removal of PBS and incubated on a rotator for 15 minutes. Cells were then harvested with a cell scraper, transferred to a 1.5mL eppendorf tube, and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was collected into new 1.5 mL eppendorf tubes and stored in a -80°C freezer until ready to use. Lysates were thawed slowly on ice prior to use. Conditions were set up by first

preparing a mixture of, Tris-HCl pH 7.4, ^{3}H-E_1S, and E_1S. After all treatments were prepared correctly in 13x100 mm test tubes and placed into a 37°C water bath. Lysates were added, and samples were incubated for 2 hours. The experiment was terminated with the addition of 3mL of toluene to each sample, and the standard sulfatase assay was performed. Steroid sulfatase activity was normalized for amount of protein. Data are the average of three experiments, each run in triplicate. Bars are +/- 1 standard error. Asterisk denotes significant difference from control group.

Chapter 5

5. DISCUSSION

The steroid sulfatase pathway has long been understood to be a major pathway of estrogen production in breast tumors (Santner, 1986). A substantial amount of research has been devoted towards understanding the inhibition of the enzyme steroid sulfatase, an endeavor which has yielded some promising results towards providing novel therapies for breast cancer patients. As the search for effective inhibitors of STS progressed, an interesting phenomenon became apparent: these inhibitors also exhibited effects in other parts of the body, suggesting a more comprehensive role of STS in human physiological systems. Although it is well established that steroid sulfatase is found almost ubiquitously throughout the body, little work had been performed on understanding its actual regulation. Previous work in the Selcer lab has shown that glucocorticoids exhibit an effect upon steroid sulfatase. With this in mind, it has been the focus of my research to further elucidate this effect on steroid sulfatase activity, particularly concentrating on the potent, synthetic glucocorticoid dexamethasone as a test compound.

Two different cell lines were used in my study: the hormone-independent breast cancer cell line MDA-MB-231 and the human osteosarcoma cell line MG-63. Our lab has had extensive experience with the MDA-MB-231 cell line and has determined it has a high level of steroid sulfatase activity (Selcer et al., 1997). Steroid sulfatase activity in the MG-63 cell line had previously been established in a study demonstrating steroid

hormone activity in several bone cell lines (Purohit et al, 1992). Also, it has been documented that glucocorticoids enhance osteoporosis in bones when expressed at higher than normal physiological levels (Canalis, 2002). Thus, understanding the role of steroid sulfatase in bone growth and formation could be very significant when considering diseases like osteoporosis or arthritis.

The first experiment I performed was essentially a follow-up to prior work done in our lab which would serve as a spring board to my particular study. MDA-MB-231 cells were treated with cortisol at 10μM, 1μM, 0.1μM and 0μM concentrations respectively, and steroid sufatase activity was determined using a steroid sulfatase assay well established in our lab (Selcer et al, 2002). Steroid sulfatase activity in MDA-MB-231 cells decreased at high and medium concentrations of cortisol treatment, while there was an insignificant difference between cells treated with low doses and no cortisol respectively. This result confirms previous unpublished work performed in the Selcer lab that cortisol decreases steroid sulfatase activity in general in a dose-dependent manner.

The next set of experiments involved using intact MDA-MB-231 cells and observing the effects of dexamethasone, which is a more potent glucocorticoid than cortisol. In one experiment, cells were treated with 10μM, 1μM, 0.1μM, and 0μM for a period of 72 hours and steroid sulfatase activity was assessed. Cells treated with all concentrations of dexamethasone showed a significant decrease in steroid sulfatase activity. Thus, dexamethasone was effective at a lower concentration than cortisol, as might be predicted.

Another set of experiments were performed to determine if the effect of dexamethasone was dose-responsive. This experiment used lower doses than the previous one. MDA-MB-231 cells were treated with 1μM, 0.1μM, 0.01μM, and 0.001μM concentrations of dexamethasone for a period of 24 hours. Twenty-four hours was thought to be enough time to see any effect of glucocorticoids, since steroid hormone action usually requires about four hours. The reduction in steroid sulfatase activity due to dexamethasone was shown to be dose responsive, and reduction was seen down to 0.01 uM steroid. These results support earlier findings that the synthetic glucocorticoid dexamethasone decreases steroid sulfatase activity in a rat granulose cell line and osteoprogenitor cel l line OPC1 (Clemens, J.W., Selcer, K.W., Doctor, J.S., Olshansky, A., Sero, J., unpublished data).

All of the aforementioned experiments measured steroid sulfatase activity in intact whole cells, but whole cell steroid sulfatase assays often show considerable variation. We thought that an assay based on lysates prepared from treated cells might provide more consistent results. Furthermore, we thought that a lysate-based assay would help more easily determine if any reduction in steroid sulfatase activity was due to competitive inhibition by the steroid treatments. Moreover, lysate-based assays are make it easier to correlate changes in activity with changes in protein levels or mRNA levels, since proteins and RNA can be extracted during the lysate preparation process.

Cell lysates were prepared from MDA-MB-231 cells after treatment with dexamethasone, and steroid sulfatase of the lysates was determined in vitro. In the first set of experiments, lysates were prepared from MDA-MB-231 cells that had been treated with dexamethasone for 24 hours at 1.0μM, 0.1μM, 0.01μM and 0μM concentrations. In

contrast to the results found for the intact cell assay, there were no significant differences in steroid sulfatase activity after treatment with dexamethasone. However, it should be noted that although the average values of steroid sulfatase activity were reduced in all of the dexamethasone treatment groups, there was a large amount of variation that precluded detection of any statistical significance.

In another similar experiment, whole-cell lysates were prepared from MDA-MB-231 cells that had been treated with dexamethasone for 72 hours at 10μM, 1.0μM, 0.1μM, 0.01μM and 0μM concentrations. Again, there was substantial variation and there were no significant differences. However, the trend was different than in the previous experiment in that the average steroid sulfatase activity values for all of the dexamethasone-treated lysate groups was higher than the control group.

In a final experiment, lysates were prepared from MDA-MB-231 cells after treatment with 10μM dexamethasone for a period of 24, 48 or 72 hours. Again there were no significant differences due to the variability; however, the trend for the 24 hour and 72 hour treatments matched the trend observed in the earlier experiments; i.e., in the presence of dexamethasone, average steroid sulfatase values were reduced over control values at 24 hours and increased over control at 72 hours.

Ultimately, one can conclude that the results for the lysate experiments are mixed. One observation is that the steroid sulfatase activity in lysates was extremely low when compared to whole cell homogenates or microsomes. This suggests that the reagents in the lysates might be interfering with the steroid sulfatase assay or might be degrading the steroid sulfatase enzyme. None of the experiments with lysates yielded any significant statistical differences between treatment groups; however, it appears that an overall trend

can be extrapolated from the results. At 24 hours, the data shows a slight decrease in steroid sulfatase activity, while at the 72 hour time point and at high concentrations, there is an upregulation of steroid sulfatase activity in these cells after treatment with dexamethasone. Clearly, further analysis of glucocorticoid regulation of steroid sulfatase in MDA-MB-231 lysates must be performed.

One consistent result occurred with the MDA-MB-231 whole cells: glucocorticoids decreased steroid sulfatase activity. There are several possibilities that could explain this decrease, one of which is that the glucocorticoids might be acting as competitive inhibitors of the substrate (estrone sulfate) used in the conversion assay. The next set of experiments performed involved using microsomal preparations from both placental tissue and MDA-MB-231 cells and cell lysates prepared from MDA-MB-231 cells in order to test if glucocorticoids are competitive inhibitors of steroid sulfatase. The rationale for including placenta stems from the fact that steroid sulfatase is found abundantly in human placental tissue, and has high activity in placental tissue (Salido et al, 1990). These factors make placenta an attractive candidate for many types of steroid sulfatase studies. Microsomal preparations were treated with 10μM dexamethasone or cortisol in the presence of radiolabeled substrate to determine their effects upon steroid sulfatase activity. There were no apparent differences in conversion of substrate between either steroid treatment and the control group, suggesting that neither dexamethasone nor cortisol are competitive inhibitors of steroid sulfatase. In contrast to the placental microsomal results, when this experiment was performed on MDA-MB-231 cell lysates, a significant decrease in steroid sulfatase activity was seen for cortisol but not dexamethasone. It is unclear why the lysate results for cortisol differ from those from the

microsomes. However, it is clear that dexamethasone, the more potent glucocorticoid, is not decreasing steroid sulfatase activity by competitive inhibition.

Another explanation for the decrease in steroid sulfatase activity due to glucocorticoids is a change in the rate of transcription of the steroid sulfatase gene, resulting in less steroid sulfatase mRNA, and consequently steroid sulfatase protein. One way to assess this is by examining steady-state levels of steroid sulfatase mRNA using RT-PCR. I performed an experiment to assess the effect of dexamethasone on steroid sulfatase mRNA expression in MDA-MB-231 cells using this technique. Several different studies have established the efficacy of RT-PCR analysis for measuring steroid sulfatase message. Previous work in our lab has established successful methods to amplify steroid sulfatase message, and it was determined that I would use the STS G/K primer pair previously developed in the lab for my analysis. I chose the MDA-MB-231 cell line for this study based on the high steroid sulfatase levels present in these cells. The particular primer set (G/K) ultimately produced a single, easily identifiable band of 502kb. Cells were treated with dexamethasone for a period of 24 hours and total RNA was extracted at 0 hour, 4 hour, 12 hour, and 24 hour time intervals, after which RT-PCR was performed. The results suggest that there is no discernable effect of dexamethasone on steroid sulfatase mRNA levels after exposure for those time periods. If correct, this suggests that the alterations which affect steroid sulfatase activity via glucocorticoid treatment are not occurring at the mRNA level. Perhaps the changes in steroid sulfatase activity are modulated some other way, such as through post-translational regulation, or through conformational changes in the enzyme. Alternatively, it is possible the qualitiative technique we used is not appropriate for detecting changes that are occurring.

The easiest way to remedy this would be to develop a real-time RT-PCR assay, which would provide a more quantitative method to determine actual mRNA levels.

There are several other avenues that can be explored from here to further understand the mechanism by which glucocorticoids are decreasing steroid sulfatase activity. Specific assays for the steroid sulfatase protein could be employed to test if the observed change is due to an allosteric change in the steroid sulfatase protein or a change in protein levels. ELISAs and/or Western blots can be performed to measure the level of the steroid sulfatase protein and those data can be compared with data on steroid sulfatase activity levels. The Selcer lab has already developed an antibody that specifically recognizes steroid sulfatase that could be employed in ELISA and Western blotting experiments.

Similar experiments were performed with MG-63 human osteosarcoma cells to ascertain how synthetic glucocorticoids like dexamethasone affect the regulation of steroid sulfatase in bone cells. In one set of experiments, intact MG-63 cells were treated with 1μM dexamethasone for a period of 24 hours. There was no significant alteration in steroid sulfatase activity for cells treated for this time period. This supports prior work performed in our lab which demonstrated that MG-63 cells showed no difference in steroid sulfatase activity after 2 day treatment of cells with either cortisol or dexamethasone (Selcer, K., and Doctor, J., unpublished data).

A 24-hour experiment performed with cell-free lysates yielded similar results to those performed on whole cells, with no difference in steroid sulfatase activity. However, a 96-hour lysate experiment resulted in a statistically significant increase in steroid sulfatase activity. This result is intriguing because this agrees with the result for

the MDA-MB-231 cells. Again, it is possible that increased steroid sulfatase is a long term effect of glucocorticoid exposure. Future experiments should focus on both long-term and short-term changes in steroid sulfatase activity, protein and mRNA after glucocorticoid exposure.

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