Regulation of levels and localization of thimet oligopeptidase in prostate cancer cells by β-estradiol

By: Christa C. DeFries
Advisor: Adele J. Wolfson

Submitted in Partial Fulfillment of the Prerequisite for Honors in Biological Chemistry

Wellesley College
April 2013

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Abstract

The enzyme thimet oligopeptidase (TOP) catalyzes the hydrolysis of gonadotropin-releasing hormone (GnRH) *in vitro*, and evidence suggests that TOP is the primary enzyme responsible for GnRH hydrolysis *in vivo*, as well. If this is true, TOP could play an important role in regulating steroid hormone production via the hypothalamic-pituitary-gonadal (HPG) axis. In addition to its function at the level of the hypothalamus, TOP may also have a direct effect at the target tissue level. In order to determine if TOP has a direct modulating effect androgen and estrogen functions, prostate cancer cells were treated with estradiol, an end product of the HPG axis. If TOP and steroid hormones comprise a feedback loop, treatment with estradiol could regulate TOP levels, localization or activity. After estradiol treatment, TOP staining was imaged confocally in prostate cancer cells. TOP was also detected and quantified in cell lysates by immunoblot. Quantification of TOP staining in prostate cancer cell images suggested that estradiol treatment increased cellular TOP levels and also increased nuclear TOP levels in particular. However, Western analysis showed that overall TOP levels varied very little with estradiol treatment. Although not definitive, these results indicate that TOP responds differently with direct treatment of prostate cancer cells by estradiol as compared to estradiol treatment within the context of the HPG axis.
Acknowledgements

Professor Wolfson, thank you for giving me the opportunity to work in your lab for the past three years. With your guidance I have learned so much and gained confidence in and outside the lab.

Louise Darling and Marc Tetel, thank you for your willingness to join the committee of a student you initially hardly knew, and your guidance and support as my thesis committee members in spite of that.

Rutendo Gambe, Yu Liu, Lisa Bruce, and all members of the Wolfson Lab, thank you for your positive attitudes, constant willingness to help, and shining personalities.

Zihan Dong and Young Hsu, thank you for sticking with me throughout our four years at Wellesley.

To my family, thank you for helping me get here.

Thanks also to the Roberta Day Staley and Karl A. Staley Fund for Cancer-Related Research Awards.
## Nomenclature

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
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<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
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<tr>
<td>HPG axis</td>
<td>hypothalamic-pituitary-gonadal axis</td>
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<td>ICC</td>
<td>immunocytochemistry</td>
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<td>JA-2</td>
<td>N-[1-(R, S)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-p-aminobenzoate</td>
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<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<tr>
<td>PEP</td>
<td>prolyl endopeptidase</td>
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<tr>
<td>TOP</td>
<td>thimet oligopeptidase</td>
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**Introduction**

The enzyme thimet oligopeptidase (TOP, EC 3.4.24.15, EP24.15) is present in most tissues of the human body. It catalyzes the hydrolysis of many short peptides, as long as they fulfill certain general restrictions. Many of the peptides it cleaves happen to be integral to important biological processes. One of its many substrates is gonadotropin-releasing hormone (GnRH), levels of which control the amounts of estrogens and androgens the body produces. Although the circumstances under which TOP degrades GnRH in the body are not well known, it certainly seems likely that this degradation could directly affect systemic estrogen and androgen levels. Androgens and estrogens have enormous influence on human reproductive function and behavior, and abnormal production of these hormones contributes to many diseases. If it is true that TOP has influence in the production of androgens and estrogens, TOP could be an important component in the process of human reproductive regulation.

**Thimet Oligopeptidase (TOP)**

TOP is a soluble, 75kDa enzyme first isolated from rat brain, and is part of a family of zinc metalloendopeptidases that also includes angiotensin-converting enzyme, nephrilysin, neurolysin, and thermolysin\(^1\)\(^-\)\(^3\). Human TOP was purified from erythrocytes in 1993, and has properties very similar to rat and chicken TOP\(^4\). Highest TOP activity is found in the brain, testes, and anterior and posterior pituitary, with lower activity in most other tissues, including spleen, liver, kidney, lung, adrenal glands, and thyroid\(^5\). In rat brain, TOP is found predominantly in the nuclei of neural and glial cells\(^6\)\(^,\)\(^7\). However, in a study that examined localization in the mouse pituitary tumor cell line ATt20, TOP was found at high levels in the
cytoplasm, and was also secreted\textsuperscript{8}. TOP is also present in plasma membranes of ATt20 cells, including on the extracellular surface\textsuperscript{9}. Overall, TOP is found in most tissues of the body, and on a cellular level, within the nucleus, cytoplasm, cell membrane and extracellular space.

TOP cleaves peptides that range in size from 8 to 17 amino acids long, preferentially at a bond on the carboxyl side of hydrophobic residues\textsuperscript{10}. Its substrates include GnRH, neurotensin, somatostatin, endothelin, bradykinin, enkephalin precursors and nociceptin\textsuperscript{10,11}. Thus TOP has been studied in relation to physiological pathways that incorporate these neuropeptides, including reproduction, blood pressure regulation and nociception\textsuperscript{12}. TOP also cleaves amyloid precursor protein similarly to the action of beta-secretase, creating amyloid fragments\textsuperscript{13,14}. TOP cleaves GnRH, a decapeptide, at the Tyr\textsuperscript{5}-Gly\textsuperscript{6} bond\textsuperscript{10} (Fig. 1). TOP can be phosphorylated by cAMP-dependent protein kinase (PKA) at Serine 644, which alters its kinetic parameters towards GnRH, but not towards those of most of its other substrates. Phosphorylated TOP has both a greater $K_M$ and $k_{cat}$, indicating that GnRH is less likely to move into the active site, but that when it does it is turned over more quickly. Tullai et al. suggest that this may help TOP handle sudden increases in GnRH concentration during pulsatile release\textsuperscript{15}.

![Figure 1. TOP and prolyl endopeptidase cleavage sites in GnRH.](image)

**Figure 1.** TOP and prolyl endopeptidase cleavage sites in GnRH. Prolyl endopeptidase enhances TOP degradation of GnRH by preliminarily removing the C-terminal glycaminamide of GnRH.
TOP in the Hypothalamic-Pituitary-Gonadal (HPG) Axis

The HPG axis controls development and reproduction through the production of androgens and estrogens. The median eminence of the hypothalamus releases GnRH in a pulsatile manner, which is transported through the hypophyseal portal system to the pituitary gland\textsuperscript{16,17}. The binding of GnRH to receptors on the secretory cells of the anterior pituitary signals them to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH)\textsuperscript{18}. LH and FSH travel through the bloodstream to the gonads. In females LH and FSH signal the ovaries to release estrogen, and stimulate follicle maturation and ovulation. In males, LH signals the testes to produce testosterone, and FSH initiates spermatogenesis. The final products of this system, estrogen, testosterone, and dihydrotestosterone (the activated form of testosterone), all act at the levels of both the pituitary and hypothalamus in a negative feedback loop to reduce production of LH, FSH and GnRH\textsuperscript{19}.

TOP certainly cleaves GnRH \textit{in vitro}, but this alone does not guarantee that TOP has any effect on the HPG axis or on steroid hormone regulation. TOP itself hydrolyzes GnRH too slowly to effectively degrade GnRH \textit{in vivo}, but Lew et al. found that the enzyme prolyl endopeptidase (prolyl oligopeptidase, POP, PEP, EC3.4.21.26) enhances TOP’s degradation of GnRH by removing the C-terminal glycinamide, thus increasing its affinity for TOP (Fig. 1)\textsuperscript{20}. Prolyl endopeptidase is found in all tissues, with especially high levels in the brain, available for use in GnRH degradation\textsuperscript{21}, and PEP and TOP are coexpressed in cells of the ventromedial nucleus of the hypothalamus\textsuperscript{22}. PEP levels in the region are also down-regulated in parallel with TOP, which suggests that TOP, with the help of PEP, could indeed be acting on GnRH in the hypothalamus\textsuperscript{22}. However, in order to cleave GnRH and have an effect on the HPG axis, TOP must be present in the extracellular space. TOP is both secreted and located on the extracellular
surface of cells, which should allow it access to secreted GnRH\textsuperscript{23}. In support of the theory that TOP cleaves GnRH in the brain, Lasdun et al. found that intracerebroventricular injection of GnRH and a TOP inhibitor into rat brain resulted in high recovery of GnRH – indicating less GnRH was degraded than if only GnRH were injected. In contrast, injection of inhibitors of two other endopeptidases suspected of cleaving GnRH did not change the amount of GnRH degraded\textsuperscript{24}. Wu et al. found TOP present in the region of the median eminence of rat hypothalamus where GnRH axons terminate near the hypophyseal portal vessels, further suggesting that TOP is in a position to cleave GnRH. They also measured TOP activity to be three times greater in the hypophyseal portal blood than in peripheral blood. They induced an LH surge in female rats, and found that when TOP was inhibited, LH serum concentration was higher than in rats with no inhibitor, which could imply more GnRH was being produced as a result of TOP’s inhibition\textsuperscript{25}.

Pierotti et al. studied TOP levels in the tissues of the HPG axis with respect to reproductive development. They found that TOP levels increased in the hypothalamus, anterior pituitary, ovaries and testes during the development of male and female rats. After puberty, TOP activity increased to a maximum in the preoptic area of the hypothalamus of males and females, and declined gradually afterward. In the anterior pituitary, TOP activity decreased after puberty and plateau-ed. In the ovaries, TOP activity rose during puberty, but returned to pre-pubertal levels afterward. However in the testes, TOP activity steadily rose during development to reach an activity at adulthood at least three times greater than in any other tissue\textsuperscript{26}. These fluctuations in TOP activity suggest that it may be involved in reproductive regulation.
Estradiol regulation of TOP

One way to determine whether TOP might be involved in regulation of steroid hormones through the HPG axis is to test whether it is involved in a feedback mechanism. If TOP plays an important role in the HPG axis, TOP levels would likely be subject to changes in levels of androgens and estrogens, the final products of the HPG axis. Our laboratory has found that TOP and estrogen receptor α (ERα) are coexpressed in reproductively-relevant brain regions including the medial preoptic area (mPOA), arcuate nucleus (ARC), ventrolateral portion of the ventromedial hypothalamic nucleus (VMNvl), and the midbrain central grey (MCG)27. Estradiol treatment decreases TOP levels in the VMNvl, with no differences in the other tested regions27. TOP activity also decreases in the VMN with estradiol treatment22. Ovariectomized rats have higher levels of TOP in the anterior pituitary, and medial and lateral preoptic nuclei compared to non-ovariectomized rats. Treatment with estrogen lowers activity in these regions26.

Other studies indicate that subcutaneous injection of male mice with N-[1-(R, S)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-p-aminobenzoate (JA-2), an inhibitor of TOP, results in heightened levels of testosterone in the blood (unpublished data, Tania Dhawan)28. Because JA-2 cannot cross the blood-brain barrier, this indicates that TOP might act locally at the level of the gonads, in addition to its hypothesized role in the HPG axis. In my thesis I focus on variation in TOP levels in relation to estradiol concentration in prostate cancer cells. Any effects observed, therefore, will not have occurred through the HPG axis, but through direct interactions between estradiol and its receptors at the level of the target tissue.
TOP in Prostate Cancer

Prostate cancer is the most common cancer in men, and the second leading cause of death due to cancer in the U.S. Every year, about 240,000 men in the U.S. are diagnosed with prostate cancer, and about 30,000 die from it. The overall five-year survival rate is 98%, but for men who are diagnosed with late-stage prostate cancer and those who have aggressive forms of the disease, the survival rate is only about 30%.

Most prostate cancers are androgen-sensitive. In this case, androgens act as growth factors for the cancer cells; in the presence of higher levels of androgens, the cells proliferate more rapidly, while in the absence of androgens, they cannot easily proliferate. Because of its characteristic androgen sensitivity, this type of prostate cancer can be treated with androgen ablation therapy, in conjunction with other treatment methods, such as surgery, radiation therapy, or chemotherapy. As the overall survival rate indicates, these methods are usually very effective in eradicating the cancer. However, if the cancer is not cured, yet remains exposed to the deprivation therapy for extended time periods, the therapy itself can lead to the cancer becoming androgen-insensitive. Androgen-insensitive prostate cancer can no longer be treated with androgen ablation therapy, grows more quickly, and has higher metastatic potential than androgen-sensitive cancers. This more aggressive form of prostate cancer is more difficult to treat, and leads to many more deaths than its androgen-sensitive counterpart.

The prostate epithelium consists of neuroendocrine cells that contain all the elements of a neural system, including neuropeptides, their receptors, and neuropeptidases. These cells likely regulate growth and secretion in normal prostate. Prostatic adenocarcinomas form in the presence of the epithelial neuroendocrine cells of the prostate, but most likely do not develop from them directly, as they are post-mitotic in normal prostate. However, it has been suggested that neuropeptides produced by these neuroendocrine prostate cells may act as growth factors in
later-stage prostate cancer in place of androgens. Neuropeptides including bombesin, calcitonin, neurotensin, endothelin-1, and parathyroid hormone-related peptide have been shown to increase prostate cancer cell culture proliferation in both androgen-sensitive and androgen-insensitive lines.

In 2004, Swanson et al. found that both TOP levels and activity were higher in androgen-sensitive prostate cancer cells compared to those of androgen-insensitive cells. Similarly, the media in which the androgen-insensitive cells were grown contained lower levels and showed lower activity of secreted TOP. Neprilysin (neutral endopeptidase, NEP, EC3.4.24.11, EP24.11), a cell-surface enzyme in the same family as TOP, also cleaves a variety of bioactive peptides including neurotensin, endothelin-1, and bombesin. Papandreou et al. found high levels of NEP in an androgen-sensitive prostate cancer cell line, and barely detectable levels of NEP in three androgen-insensitive lines. NEP activity was also greatly decreased in the three insensitive lines. Results from in vivo studies supported these observations. The downregulation of TOP and NEP in androgen-insensitive prostate cancer lines may be a mechanism by which the cells increase their rate of proliferation. By decreasing levels of the enzymes that degrade certain neuropeptides, the cancer cells may be providing themselves with access to greater concentrations of growth factors.

PC3 and DU145 Prostate Cancer Cell Lines

The PC3 cell line is an epithelial cell line derived from androgen-independent human prostatic adenocarcinoma metastasized to bone. Although the original PC3 cell line is androgen-independent, the line I use expresses AR in order to model androgen-dependent prostate cancer. PC3 cells contain both Estrogen Receptor α (ERα) and ERβ, although normal
prostate and prostate cancer cell lines LnCAP and DU145 contain primarily ERβ\textsuperscript{45}. The DU145 prostate cancer cell line is derived from brain metastasis and is androgen-independent. One strain, like PC3-AR has been transfected with AR and responds to testosterone treatment\textsuperscript{46}. This androgen-sensitive strain is called DUAR, and the original androgen-insensitive strain I call DUT.

**Research Goals and Hypotheses**

We previously found that TOP immunoreactivity and activity decrease in the mouse ventromedial hypothalamic nucleus in response to estradiol treatment\textsuperscript{22,27}. In order to investigate further how TOP levels change with estradiol treatment, and thus how TOP might fit in to the overall regulation of estrogens and androgens, we are studying changes in TOP levels that take place in the prostate cancer cell lines PC3 and DU145 in response to estradiol treatment. If TOP were involved in a negative feedback mechanism as part of the HPG axis, increased exposure to estradiol might cause TOP to be upregulated in the cells of the hypothalamus, thus allowing lower levels of GnRH to be released to the pituitary, resulting in a decrease in estrogen production. However, TOP was downregulated in mouse hypothalamus in response to estradiol treatment. Estradiol treatment of PC3 cells may help reveal the mechanisms by which TOP levels change. This study could also have implications regarding the role of TOP and other neuropeptidases in prostate cancer.
Materials and Methods

Cell culture

DU145 cells (DUAR and DUT) and PC3-AR cells were cultured in RPMI 1640 (Sigma S8761) with 10% FBS, and 0.002% sodium bicarbonate at 37°C and 5% CO₂. Cells were passaged at confluency, about every three to four days.

Immunocytochemistry

DU145 cells (DUAR and DUT) or PC3-AR cells were grown on Lab-Tek II CC²-treated 8-well glass slides in RPMI 1640 (Sigma S8761) with 10% FBS, and 0.002% sodium bicarbonate for 24 hours. Cells were incubated in DHT, beta-estradiol, or GnRH (10nm, 100nm, or 1.0µM) for 1 hour, then washed with 1x PBS (phosphate-buffered saline) buffer and fixed with 4% paraformaldehyde for 15 minutes. Cells were washed with 0.05M TBS (Tris-buffered saline) (pH 7.6), then incubated in 0.25% Triton-X for 10 minutes to improve antibody penetration. The cells were blocked in 20% normal goat serum (NGS) and 1% BSA in 0.05M TBS, then incubated in the primary antibody to TOP, monoclonal Rabbit-anti-TOP from Marc Glucksman’s Lab (Rosalind Franklin University) at 1:5000 with 1% NGS and 0.5% Triton-X in 0.05M TBS with 0.02% sodium azide, 0.1% gelatin, and 10% Triton-X (pH 7.6) for 24 hours. The cells were washed with 0.05M TBS with 0.02% sodium azide, 0.1% gelatin, and 10% Triton-X, then incubated in the secondary Alexa Fluor® 594 Donkey-anti-Rabbit IgG (Invitrogen cat# A21207) at 1:100 with 1.5% NGS in 0.05M TBS with 0.02% sodium azide, 0.1% gelatin, and 10% Triton-X for 90 minutes, then washed with the same buffer. The cells were incubated in nuclear stain DAPI at 1:10⁶ with 1.5% NGS in 0.05M TBS with 0.02% sodium azide, 0.1% gelatin, and 10%
Triton-X for 30 minutes, then washed with 0.05M TBS with 0.02% sodium azide, 0.1% gelatin, and 10% Triton-X, then washed with 0.05 TBS. The slides were coverslipped with Gel/Mount (biomeda, cat# M01). The cells were imaged for TOP and DAPI using a Leica TCS SP5 II confocal microscope with a 40x PLAN-APO oil objective (NA=1.25). Alexa Fluor 594 was excited with a HeNe 594nm laser at 71% transmission, and emission was accepted from 605-630nm. DAPI was excited with a 405nm UV diode laser at 20% transmission and emission was accepted from 430-457nm. Images were taken confocally at a z-depth less than 1um and collected at a speed of 400 pixels/sec. Gain and offset were kept constant for each series of cells.

**Image Quantification**

Confocal images were quantified using Nikon NIS-Elements AR 2.30, SP4 (Build 387). Cells not touching/overlapping with any neighboring cells were chosen for quantification. All red intensity was recorded (no set threshold), and minimum area of a cell was set as 75µM².

**Cell Lysis (Whole Cell Lysis)**

Cells were harvested and washed in 1X Phosphate Buffered Saline (PBS) solution. Cell pellets were immersed in 12 times their volume of RIPA lysis buffer (50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Nonidet-P40, 0.5% deoxycholic acid and 0.1% sodium dodecyl sulfate). Phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, dissolved in isopropanol and a general protease inhibitor cocktail (Sigma, cat. #P2714-1BTL) were freshly added in ratios of 1:100 to the total volume of buffer. The cells incubated in the lysis buffer for 1 hour. To remove DNA and insoluble material, cell samples were centrifuged at 13,000 rpm and 4°C for 30 minutes. The supernatant was then harvested and stored at -80°C.
**Cell Lysis (Cytoplasmic Fraction Separation)**

Cells were harvested and washed in 1X Phosphate Buffered Saline (PBS) solution. Cell pellets were immersed in 12 times their volume of Kinetic Assay Buffer (25mM TRIS Hydrochloric acid, 125mM KCl, 1µM zinc chloride, 10% glycerol, pH 7.8 and conductivity 12.0mS/cm²). Phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, dissolved in isoproponal and a general protease inhibitor cocktail (Sigma, cat. #P2714-1BTL) were freshly added in ratios of 1:100 to the total volume of buffer. Cell pellets were kept in 70% ethyl alcohol ice slurry throughout the sonication process to prevent protein denaturation. Settings for sonication apparatus were: duty cycle set at 15 and the output control set to 1.5. Individual pulse duration was one second and each pellet was pulsed approximately 6 times. Sonicated cell samples were then centrifuged at 13,000 rpm and 4°C for 30 minutes. The supernatant (cytoplasmic fraction) was then harvested and stored at -80°C.

**Assessment of Total Protein Concentration**

Spectrophotometric analysis was used to determine the total protein concentration per sample. Absorbance was set at 260 nm and 280 nm for the spectrophotometer. Each cell lysate sample was tested at a concentration of 1:25 in kinetic assay buffer (25mM TRIS Hydrochloric acid, 125mM KCl, 1µM zinc chloride, 10% glycerol, pH 7.8 and conductivity 12.0mS/cm²). Concentration of total protein for each sample was then calculated using the following formula: Protein concentration (mg/ml) = (1.55 x A280) - 0.76 x A260).
Western Blotting for TOP

(Adapted from 27) 1-20 micrograms total protein of each cell lysate sample was gel electrophoresed. Samples were prepared by combining the appropriate volume of each cell lysate sample (calculated based on total protein concentration), 2.5 µl of 4X NuPAGE LDS sample buffer (Invitrogen) and 1 µl of 10X NuPAGE sample reducing agent (Invitrogen), then brought up to a final volume of 10 µl with water. Samples were then heated at 70°C for 5 minutes. Proteins were separated by size on NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen, cat. # NP0321BOX) and run at 200 V for ~35 minutes according to the manufacturer’s instructions. Separated proteins were then transferred onto a PVDF Immobilon-P membrane (Millipore), with voltage settings of 100 V for 1.5 hours. Membranes were rinsed in 0.1 M Tris-buffered saline (TBS) with 0.05% Tween-20 (TBS-T), then blocked in TBS-T and 5% nonfat milk at room temperature for 1 h. Blots were cut at the 50-kDa marker. The upper blot was probed for TOP with a rabbit polyclonal antibody directed against rat TOP at two epitopes KPPAACAGD and LSKGLQVEGC 7 (dilution 1 : 20 000; generously provided by M. Glucksman, Rosalind Franklin University of Medicine & Science, Chicago, IL, USA), and the lower blot was probed for actin with a monoclonal antibody directed against the N-terminal peptide, Ac-Asp-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Lys of mouse actin (dilution 1 : 25 000; Chemicon International, Inc., Temecula, CA, USA) in TBS-T overnight at 4 °C. The next day, the membranes were incubated in a horseradish peroxidase-linked donkey anti-rabbit secondary immunoglobulin (IgG) (dilution 1 : 10 000; GE Healthcare UK Limited, Chalfont St Giles, UK) or sheep anti-mouse secondary IgG (dilution 1 : 10 000; Amersham Biosciences, Little Chalfont, UK) for 1 h at room temperature, and proteins were detected using an enhanced chemiluminescence kit (ECL Plus Western Blotting Detection System; GE
Healthcare, Amersham Biosciences, Little Chalfont, UK - discontinued). Purified recombinant rat TOP protein (a gift from M. Glucksman) was run as a positive control for each Western blot. Immunoreactive bands were visualised using a PhosphorImager (STORM Scanner 860; Molecular Dynamics, Sunnyvale, CA, USA) at an excitation wavelength of 450 nm. Images of each gel were taken and saved as 16-bit grayscale Tiff files and the area of each immunoreactive band was analysed using IMAGEQUANT, version 5.0 (GE Healthcare). TOP levels were normalized to actin.

**Quenched Fluorescence Assay**

TOP activity assays were conducted with a SpectraMax M3 Microplate Reader with SoftMax Pro microplate data acquisition software. Total volume for each sample was 200 µL in kinetic assay buffer (25mM TRIS Hydrochloric acid, 125mM KCl, 1µM zinc chloride, 10% glycerol, pH 7.8 and conductivity 12.0mS/cm²) including 5ug total protein and 1uL of substrate. Reaction was monitored for 20 min at room temperature. The substrate for TOP was 7-methoxycoumarin-4-acetylPro-Leu-Gly-Pro-Lys-dinitrophenol (MCA), 1.4 mM stock solution. Settings for the TOP assay were: emission wavelength, 405 nm; excitation wavelength, 320 nm. Reaction was carried out in the presence or absence of a specific inhibitor of TOP, cFPAF-pAB (N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (cFP), 16.5 M. The slope of the graph (fluorescence/second) was then used to determine the activity of TOP. The activity from samples with cFP was subtracted from samples without cFP added in order to account for background fluorescence/activity.
Statistical Analysis

JMP Pro 9.0 (SAS Institute Inc.) was used for all statistical analyses. All pairs of means were compared using a Tukey HSD test. P< 0.05 was considered statistically significant.
Results

In order to determine if TOP plays a role in steroid hormone regulation, we investigated the effects of hormone treatments on TOP levels and localization in prostate cancer cells. Initially, I explored the effects of hormone treatment through immunocytochemistry (ICC) and imaging of DU145 cells in culture. DU145 cells are obtained from brain metastasis and are androgen-insensitive. However, I used a strain of DU145 transfected with functional androgen receptor (designated DUAR), as well as the original insensitive strain (DUT).

Verification of Staining Specificity

To confirm that the antibody against TOP is specific for TOP, the antibody was incubated with 80x purified TOP protein for 24 hours before incubating with the cells during ICC. With the antibody already bound to TOP, it should not bind to cellular TOP, but instead remain in solution to be removed during washing steps. This leaves no binding site for the secondary antibody, and it too is removed. In the absence of the fluorophore on the secondary antibody, no fluorescence should be detected during imaging. If the TOP antibody binds only TOP, there should be no staining of the cells incubated with preabsorbed TOP antibody.

The DU145 cells incubated with the preabsorbed antibody showed highly reduced fluorescence in comparison to the cells treated with non-preabsorbed TOP antibody (Fig. 2). This result indicates that the antibody primarily binds TOP. The residual staining could be due to TOP antibody binding with lower affinity to a TOP-like protein in the cells, or to non-specific binding of the secondary antibody.
Figure 2. **TOP antibody preabsorption with wildtype TOP results in highly reduced TOP staining in androgen-sensitive DU145 cells.** TOP antibody was incubated in 80x wildtype TOP for 24 hours before being incubated with the prostate cancer cells. The cells were then incubated with a secondary antibody, which produces the red staining in the images above. When imaged, the cells incubated with the preabsorbed antibody showed highly reduced fluorescence in comparison to the cells treated with non-preabsorbed TOP antibody. This result indicates that the antibody primarily binds TOP. (Representative cells shown.)

**Immunocytochemical Analysis of Hormone Treatment on TOP Levels**

In order to investigate the effects of hormone treatment on TOP levels, both DUAR and DUT cells were incubated in 10nM DHT, estradiol, and GnRH for 1 hour, immunostained for TOP and imaged. TOP staining per cell was quantified (Fig. 3a,b). Estradiol and GnRH appeared to cause a rise in overall TOP levels in both androgen-sensitive and insensitive cells, while there was no change with DHT treatment in androgen-insensitive cells, and a slight rise due to DHT in androgen-sensitive cells. Repetition of this experiment in androgen-sensitive...
cells yielded a similar relationship between levels of TOP due to treatment with the three hormones, but the control cells had a much higher relative TOP intensity than in the initial experiment (Fig. 3c). However, the sample sizes in this repeated experiment were much smaller than the initial experiment due to difficulty quantifying the cells, and differences in TOP staining between treatment groups were not significant.

**Figure 3.** Estradiol and GnRH treatment raise levels of TOP in androgen-sensitive and androgen-insensitive prostate cancer cells. Androgen-insensitive (DUT) and androgen-sensitive (DUAR) DU145 cells were incubated in 10nM DHT, estradiol, or GnRH for one hour, then immunostained for TOP and imaged. (a) Quantification of TOP staining in androgen-insensitive (DUT) cells after hormone treatment. *P<0.05 ethanol control, n=13; DHT, n=60; estradiol, n=35; GnRH, n=14 (b) Quantification of TOP staining in androgen-sensitive (DUAR) cells after hormone treatment. * and **P<0.05 compared to ethanol control and DHT. Ethanol control, n=68; DHT, n=63; estradiol, n=40; GnRH, n=13. (c) Repetition of experiment in (b); Quantification of TOP staining in androgen-sensitive (DUAR) cells after hormone treatment. No significant differences in TOP staining. Ethanol control, n=5; DHT, n=5; estradiol, n=4; GnRH, n=5. Error bars indicate standard error.
DHT and estradiol appear to have different effects on TOP levels in DU145 cells, with DHT causing little change and estradiol producing an increase in total TOP levels. In order to further explore the effects of these steroid hormones on TOP levels, DUT and DUAR cells were incubated with DHT, estradiol, and a combination of estradiol and DHT (Fig. 4a). As before, DUT cells treated with DHT did not show a change in TOP levels, while cells treated with estradiol showed increased TOP levels. However, combined estradiol and DHT treatment resulted in TOP dropping to levels similar to the control and DHT treatment group. Treated DUAR cells all show increased TOP levels compared to the ethanol control cells (the control cells were not detectable), but unlike the DUT cells, TOP levels are just as high when cells are treated with DHT or a combination of estradiol and DHT as when treated with estradiol alone (Fig. 4b).

**Figure 4.** While estradiol treatment increases TOP levels, levels after combined estradiol and DHT treatment are similar to DHT-only treatment in DUT cells. Androgen-insensitive (DUT) and androgen-sensitive (DUAR) DU145 cells were incubated in 1.0uM DHT, estradiol, or 1.0uM DHT and 1.0uM estradiol for one hour, then immunostained for TOP and imaged confocally. (a) Quantification of TOP staining in androgen-insensitive cells after hormone treatment. *P<0.05 ethanol control, n=8; DHT, n=9; estradiol, n=8; estradiol and DHT, n=7. (b) Quantification of TOP staining in androgen-sensitive cells after hormone treatment. Staining of control cells was
Dose-Dependent Estradiol Response

Estradiol incubation appears to have a significant effect on TOP levels in DU145 cells. In order to further explore these effects, DUAR and DUT cells were incubated in increasing concentrations of estradiol to determine if there is a dose-dependent effect on TOP levels and localization. Although total TOP levels still rise with estradiol incubation, in DUT cells nuclear TOP levels in particular appeared to increase with increasing estradiol concentration (Fig. 5a). Quantification of nuclear TOP corroborates this observation (Fig. 5b).
Figure 5. TOP localizes to nuclei in androgen-insensitive (DUT) prostate cancer cells with estradiol incubation. DUT cells were incubated with increasing concentrations of estradiol for one hour, then immunostained for TOP and imaged confocally. (a) Representative cells shown. Scale bars are 10um. (b) Quantification of nuclear TOP staining. Differences not significant. Ethanol control, n=5; 0.01uM estradiol, n=3; 0.1uM estradiol, n=6; 1.0uM estradiol, n=5.
Western Analysis of Estradiol Treatment on TOP levels

Quantifying TOP levels through imaging is not ideal since it can be subjective: the demands of using the imaging software make it difficult to quantify large numbers of cells - they must not touch other cells in order to be correctly analyzed- and many variables such as application of the coverslip can affect the intensity of the image. We therefore moved to quantifying TOP levels using Western blot. We also were unsure if the DUT and DUAR cell lines were clearly androgen-insensitive and androgen-sensitive, respectively, so we began using the PC3 prostate cancer line. PC3 cells are derived from bone metastasis and are originally androgen-insensitive, although we use a line of PC3 cells that has been transfected with androgen receptor, and should thus be androgen-sensitive. We verified that the PC3-AR line does proliferate more rapidly when incubated in DHT than do PC3 cells (Michelle Brann, unpublished data).

PC3 cells now were incubated in estradiol at different concentrations, either for 1 or 24 hours. Cells were lysed, and lysates were separated by electrophoresis, then blotted and immunostained for TOP and actin. TOP bands were quantified and normalized to actin bands (Fig. 6). TOP band intensities fluctuated slightly, but generally remained at a constant intensity and showed no clear trend in relation to estradiol incubation. Data collection was made difficult by the apparent non-specificity of the TOP antibody. Multiple bands were detected by the TOP antibody across a range of molecular weights, yet background remained low (Fig. 7).
Figure 6. Western analysis reveals no change in intracellular TOP levels with estradiol incubation. Cells were incubated with 0M, 100pM, 1.0nM, and 100nM estradiol for either 1 or 24 hours, then lysates were separated by electrophoresis and immunostained for TOP and actin. (a)(c) Resulting Western blots. (b)(d) Quantification of TOP normalized to actin.
Figure 7. TOP detection in Western analysis. TOP bands shown in red box. Actin bands shown in yellow box. (a) and (b) correspond to Fig. 6 (a) and (c).

TOP Activity Analysis after Estradiol Treatment

Cytoplasmic TOP Activity in PC3-AR cells was measured after treatment with estradiol for 1 or 24 hours. Cells treated with estradiol for 1 or 24 hours showed decreased TOP activity in cytoplasm compared to cells treated with no estradiol (Fig. 8). Although TOP activity does not necessarily correlate with TOP levels, this result may make sense if TOP is moving to nuclei, causing a decrease in cytoplasmic TOP levels (Fig. 5).
Figure 8. TOP activity in prostate cancer cell cytoplasm decreases with estradiol treatment. PC3-AR cells were treated with three concentrations of estradiol (100pM, 1.0nM, 100nM) for 1 (yellow) or 24 (green) hours. TOP activity in the cytoplasmic fraction of each treatment group was evaluated using a quenched fluorescence activity assay. The buffer the samples were diluted in served as a control and showed almost no activity. Purified TOP and cells treated with no estradiol had significant TOP activity. Cells treated with all three concentrations of estradiol for both 1 and 24 hours had lower TOP activity than the untreated cells.

Overall, imaging studies suggested that estradiol incubation increases TOP levels in both androgen-sensitive and androgen-insensitive prostate cancer cells and increased localization to nuclei, while Western blot suggested that estradiol incubation has little effect on total TOP levels.
Discussion

In order to determine whether or not, and in what way, hormone treatment affects TOP levels and localization, prostate cancer cells were incubated in DHT, estradiol and GnRH, immunostained for TOP and imaged. Image quantification suggested that estradiol incubation increased TOP levels in prostate cancer cells. Although image quantification can be highly variable and sample sizes were generally very small, the jump in TOP levels with estradiol treatment compared to untreated cells was fairly consistent (Fig. 3a-b, 4). Although this relationship between control and estradiol-treated cells was not observed in a repeat of the initial hormone incubation experiment (Fig. 3c), these results were not significantly different and consisted of very small sample sizes. Interestingly, the relationship among TOP levels in cells treated with the three hormones remained similar. DHT-treated cells generally display TOP levels similar to untreated cells (Fig. 3a-b, 4), and always lower than estradiol-treated cells (Fig. 3, 4). Although we believed the DUT cell line was androgen-insensitive and the DUAR line was androgen-sensitive, DHT-treated cells had similar levels of TOP in both cell lines, compared to the control. GnRH treatment produced TOP levels similar to estradiol treatment in both cell lines. Imaging results also indicated that TOP levels increased in nuclei in DUT (androgen-insensitive cells) with estradiol treatment.

Since estradiol treatment affects TOP levels in certain regions of the mouse brain\textsuperscript{27}, we focused on TOP level changes due to estradiol treatment. To further study the effects of estradiol, we measured TOP levels in estradiol-treated prostate cancer cells by Western immunoblot. This method should be more reliable than image quantification since it involves less variation in detection and measures the TOP contents of thousands of cells rather than the
few whose TOP staining can be measured. However, TOP detection proved to be more complicated than expected as immunostained elecrophoresis-separated whole cell lysates displayed multiple bands detected by the antibody to TOP (Fig. 7). The bands quantified were closest in molecular weight to the purified TOP band. The results of these experiments indicated that TOP levels change very little, or fluctuate slightly in PC3-AR (androgen-sensitive) prostate cancer cells treated with estradiol (Fig. 6).

Overall, the quantified imaging results indicated that estradiol treatment may cause total TOP levels to increase in both androgen-sensitive and androgen-insensitive prostate cancer cells, and nuclear TOP levels to increase, but Western results suggested that estradiol treatment has little effect on cellular TOP levels in androgen-sensitive cells. It is possible that the conflicting results are a consequence of using two different cell lines for the imaging and Western experiments. Although the two lines are both prostate cancer cells, they do originate from different individuals and the DU145 cells contain primarily ERβ while the PC3 line contains both ERα and ERβ. DU145 cells might therefore respond to estradiol treatment differently than PC3 cells.

Males produce estrogens in the testis and brain. Estrogens and both ERα and ERβ play roles in the development and function of the male reproductive system. The two estrogen receptors bind estradiol with equal affinity, but have different functions and different tissue distribution. ER knockout mice have been used to study the roles of ERα and ERβ in the male reproductive system: knockout of ERα results in reduced male fertility through dilution of sperm and disrupted sperm morphology, and knockout of ERβ does not reduce fertility, but does result in increased aggressive behavior. At the level of prostate cancer, ERβ is thought to protect against uncontrolled cell proliferation and to be downregulated as prostate cancer
progresses. In contrast, ERα promotes proliferation, so estrogens can have both beneficial and harmful effects in prostate cancer. Thus presence of different levels of the two estrogen receptors may contribute to differences in behavior of the PC3 and DU145 cell lines.

Previous studies found that estradiol treatment of ovariectomized female mice lowers TOP levels and activity in reproductively relevant regions of the brain. This effect is opposite to the one we found from imaging results, in which estradiol treatment raised TOP levels. However, our result was obtained in cell culture with human prostate cancer cells while TOP level lowering was observed in female mice. Aside from responsiveness to steroid hormones, the two systems have little in common. It is possible that estradiol has a different, or no local effect on TOP levels while still having this TOP lowering effect in vivo. The fact that nuclear TOP appears to increase with estradiol treatment, whether or not total cellular TOP increases, indicates that estradiol can have a direct effect on the cells.

Nuclear TOP appears to increase with 1 hour estradiol incubation. Although TOP’s purpose in the nucleus is not known – most of its substrates are extracellular – localization to the nucleus has been observed multiple times, especially in the brain, where it is primarily nuclear. TOP contains a possible nuclear localization sequence (PETRRKAV) at residues 234-240, although its presence in a helix makes the sequence less likely to be involved in nuclear transport. While TOP is localized mostly to the nuclei in neurons, neurolysin, a closely related metallopeptidase with similar structure, is localized almost exclusively to the cytoplasm, and more often found on the extracellular surface of the plasma membrane than TOP. This seems to suggest that TOP has an important role in the nucleus, while neurolysin is more suited to processing signaling peptides. However, unlike in brain, the current results show that TOP is often more concentrated in the cytoplasm than in the nucleus in prostate cancer cells (Fig. 2, 5).
If the imaging results are correct, and estradiol treatment does cause a rise in TOP levels, this might be detrimental to the prostate cancer cells. As Swanson et. al hypothesized, TOP may degrade growth factors of prostate cancer, and it is downregulated in androgen-insensitive prostate cancer cells, which rely solely on these growth factors\textsuperscript{39}. If TOP is upregulated by estradiol treatment, prostate cancer cells may grow less rapidly due to decreased access to growth factors. Castagnetta et. al found that estradiol treatment causes increased proliferation of LNCaP (androgen-sensitive) prostate cancer cells\textsuperscript{59}. However, estradiol treatment of androgen-insensitive PC3 cells and castrate resistant prostate cancer in mice by Carruba et. al and Montgomery et. al respectively, inhibited growth\textsuperscript{60,61}. Administration of estrogens to prostate cancer patients also inhibits cancer growth, and estrogenic drugs were used to treat prostate cancer for 25 years. However, this treatment relies on the presence of the HPG axis: estrogens or estrogen-like drugs inhibit the release of gonadotropins, preventing production of testosterone and therefore deprive androgen-sensitive prostate cancer of androgens\textsuperscript{62}. If estradiol treatment does upregulate TOP in androgen-insensitive prostate cancer cells, this mechanism could play a role in inhibiting prostate cancer growth.

Future studies should optimize use of the TOP antibody with PC3 cell lysates in Western blots to confirm if there is or is not an effect on TOP levels with estradiol incubation. If there is indeed a change, it would be useful to investigate the mechanism by which the change occurs: by an increase or decrease in rate of protein degradation/translation. Similar experiments could also be carried out with DHT incubation of prostate cancer cells. DHT treatment may cause similar changes in TOP levels to estradiol treatment, since both are end products of the HPG axis. However, results up to this point indicate that their effects may not be similar with direct incubation.
Although the current study was inconclusive, some results suggest that TOP levels may increase overall and particularly in the nucleus in estradiol-treated prostate cancer cells. This suggests that the products of the HPG axis affect TOP levels, which indicates TOP may play a role in this system. An increase in TOP levels in androgen-insensitive prostate cancer cells may also have implications as to how prostate cancer proliferation might be controlled.
References


