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Androgen Receptor-Dependent Effects of Resveratrol on Tnsin mRNA Levels in Prostate Cancer Cells

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ANDROGEN RECEPTOR-DEPENDENT EFFECTS OF
RESVERATROL ON TENSIN mRNA LEVELS IN
PROSTATE CANCER CELLS

Courtney Pisano

A Thesis Presented to Philadelphia College of Osteopathic Medicine
In Partial Fulfillment
For the Degree of

Master of Science

In

Biomedical Sciences

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This thesis has been presented to and accepted by the Associate Dean for Curriculum and Research Office of Philadelphia College of Osteopathic Medicine in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Sciences.

We the undersigned duly appointed committee have read and examined this manuscript and certify it is adequate in scope and quality as a thesis for this master's degree. We approve the content of the thesis to be submitted for processing and acceptance.

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A Listing of Abbreviations

AR	androgen receptor
ARE	androgen responsive element
DLC-1	deleted in liver cancer 1
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
FAB	focal adhesion binding
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
MAP	mitogen-activated protein kinase
MEKK1	MAP/ERK kinase kinase
PI3K	phosphoinositide 3-kinase
PP1 α	protein phosphatase-1 α
PTB	phosphotyrosine-binding domain
PTEN	phosphatase and tensin homolog
RSV	resveratrol
SH2	Src homology 2
SMRT	silencing mediator for retinoic acid and thyroid hormone receptor
TRAIL	tumor necrosis factor apoptosis ligand

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Abstract

The chemopreventive effects of resveratrol (RSV) on cancers, including prostate cancer, have been well documented; but the mechanisms are not well known. It has been reported recently that tensin, a matrix-adhesion protein, which is greatly down-regulated in prostate cancer, has been induced by RSV in several cancer cell lines.

In order to know if RSV up-regulates tensin in prostate cells, we first treated LNCaP cells with RSV and demonstrated that tensin mRNA levels were upregulated by RSV in a time and dose dependent manner. Since LNCaP cells are androgen receptor (AR) positive and previous findings have shown that RSV down-regulates AR protein levels as well as its transcriptional activity, we were interested to see whether RSV effects on tensin are AR dependent. When LNCaP cells were treated with androgen (R1881), tensin mRNA levels were down-regulated and this effect was counteracted by RSV. These results strongly suggest that RSV upregulation of tensin mRNA at least partially involves AR. To further confirm its AR dependency, we used a previously established stable AR(+) expressing HeLa cell line in a series of experiments; with the AR(-) HeLa cell line serving as a negative control. Similar to what we saw in LNCaP cells, tensin mRNA levels were upregulated in the AR(+) cells, and RSV counteracted androgen induced downregulation of tensin mRNA levels. However, this effect was not shown in the AR(-) cells.

In summary, we have demonstrated that RSV up-regulates tensin expression in prostate cancer cells and this effect is, at least in part, AR dependent. Together with our previous observations, we provided further evidence at both cellular and molecular levels that RSV may serve as a preventative and therapeutic agent for prostate cancer.

Introduction

Prostate cancer is one of the biggest threats to men's health in the Western world and is the most frequently diagnosed cancer among men in the United States (Jones, DePrimo, Whitfield, & Brooks, 2005). It also accounts for the second largest number of male cancer deaths in the U.S. (Jemal et al., 2004; Jemal et al., 2005). The mechanisms of this disease are not yet completely understood, although both genetic and environmental factors are known to be involved in its development. In addition to age, race, environmental factors, and even diet, family history also seems to play a large role in the development of prostate cancer. Men with a family history of prostate cancer have a significantly greater risk of developing prostate cancer than those with no such history (Johns & Houlston, 2003). Age seems to be an important risk factor for this cancer in particular. In fact, 90% of males develop some form of prostatic disease between the ages of 40 and 90 (Chatterjee, 2003). As people are living longer, more men will be afflicted with this disease. Because of this, it is becoming more important than ever to find better treatments and a potential cure for prostate cancer.

There are two types of prostate cancer; androgen-dependent and androgen-independent prostate cancer. The earlier stages of prostate cancer are usually androgen-dependent. At this stage, tumor growth is reliant on androgen. Later stages of the cancer are usually termed androgen-independent, and are not reliant on androgen to cause abnormal growth (Agoulnik & Weigel, 2006). Androgens are hormones that are important in many biological functions including fetal development and male development during puberty. They are hydrophobic and have the ability to readily diffuse

through cell membranes. The biological effects of androgens are mediated through a transcriptional factor called the androgen receptor (AR) (Heinlein & Chang, 2004).

Androgen Receptor (AR)

AR is a 110-kDa phosphoprotein, a member of the nuclear receptor family of ligand-activated transcriptional factors. Without androgen, AR is located in the cytoplasm, bound by heat shock proteins. When androgen binds to AR, the receptor disassociates from the heat shock proteins and translocates into the nucleus. Once in the nucleus, the receptor binds to specific DNA sequences called androgen response elements (AREs) on the promoters of the target genes and recruits a series of co-activators or co-repressors in order to enhance or repress the expression of certain genes. The activity of AR is dependent not only on levels of hormone and receptor, but also on the levels and activities of co-activators/co-repressors (Agoulnik & Weigel, 2006) (Fig1). Intact AR signaling appears to be required for the initiation and growth of prostate cancer. This signaling has been found in all types of prostate cancers, primary and metastatic, in both androgen-dependent and androgen-independent prostate cancers. In androgen-dependent prostate cancer, the binding of androgen to AR stimulates the cancerous growth. AR continues to be expressed in androgen-independent tumors and AR signaling remains functional, but androgen-independent prostate cancer does not depend on androgen for growth.

Androgen-independent prostate cancers have demonstrated a variety of AR alterations that include AR amplification, point mutation, and changes in expression of AR co-regulatory proteins. These changes result in a "super AR" that can respond to lower concentrations of androgens or to a wider variety of agonistic ligands (Taplin & Balk, 2004).

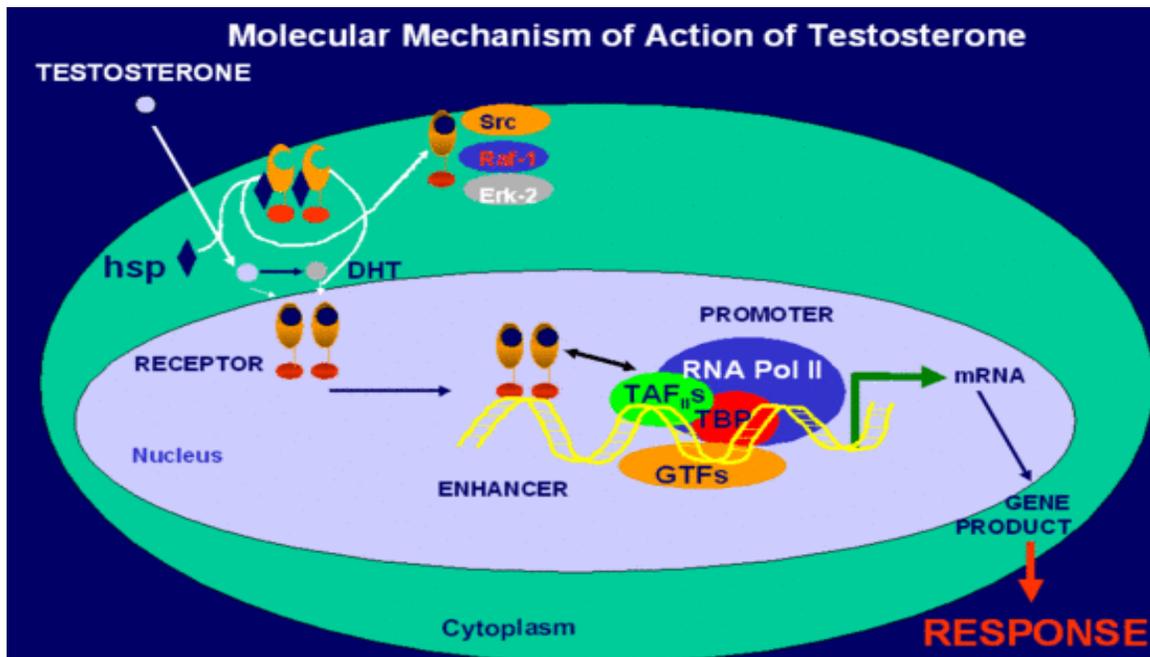


Figure 1: *Molecular Activation of androgen receptor (AR)*

AR is typically found in its inactive form, bound to heat shock proteins in the cytoplasm. When AR binds to androgen, such as, testosterone, the heat shock proteins dissociate, and AR translocates into the nucleus. AR then binds to AREs on the DNA and recruits co-activators (TAFs, TBP, GTFs). This enables androgen to have various biological effects (www.endotext.org/male/male3/index.html).

Co-activators and co-repressors of AR also play a role in the formation of prostate cancer. Co-activators of AR include a series of proteins which exhibit a variety of enzymatic activity including histone acetyl transferase activity, methyltransferase activity, kinase activity, and ubiquitin ligase activity (Smith & O'Malley, 2004). Although the relative contributions of most of these proteins to AR action have not yet been determined, there is one fairly consistent finding that has been identified in ARs relative contribution to prostate cancer. This is that artificial overexpression of a variety of co-activators induce AR activity in the presence of a broad range of metabolites including ligands for the other receptors (estradiol), adrenal androgens, androgen metabolites, and anti-androgens. These findings may be a reflection of the ability of a variety of ligands to bind to AR and to induce nuclear localization and DNA binding. Over-expression of co-activators is one of the causes for anti-androgen resistance (Agoulnik & Weigel, 2006; Edwards & Bartlett, 2005).

Another means of cell signaling that alters the activity of AR is through its effects on co-repressor activity. Co-repressors have characteristically been considered to be proteins that interact with antagonist-bound steroid receptors recruiting histone deacetylases. However, recent studies have indicated that co-repressors can reduce the activity of agonist-bound AR (Agoulnik et al., 2003). Elevated-MAP/ERK kinase kinase 1 (MEKK1) activity through growth factor signaling induces phosphorylation of one co-repressor, silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), dissociation from nuclear receptors, and relocalization of the co-repressor to the cytoplasm (Jonas & Privalsky, 2004). Thus, activation of this cell signaling pathway

would not only reduce the effectiveness of antagonists but also enhance the activity of agonist bound receptor (Agoulnik et al., 2003).

Although the AR is most frequently associated with increased expression of androgen-responsive genes, it is also known to repress expression of some genes. In a global gene expression study using a Genechip microarray, Wang et al found that 858 genes were differentially regulated in LNCaP cells treated with the androgen (R1881) for 16 hours. Of these, 784 genes were significantly up-regulated, while 74 were significantly down-regulated (G. Wang, Jones, Marra, & Sadar, 2006).

Due to the belief that the androgen receptor (AR) plays an important role in the development of prostate cancer, research has focused on AR in order to discover mechanisms for its actions in prostate cancer development (Agoulnik & Weigel, 2006). It is hoped that understanding the changes in AR signaling in prostate cancer will be a key to developing a more effective treatment, and perhaps even a cure for prostate cancer. Currently, there are limited ways to battle this disease. If diagnosed early enough, there are therapies that may improve prognosis. Surgery can be a successful but radical option. In this case, many men experience problems such as urinary incontinence after surgery which can result in a negative impact on the quality of life of patients (Seeni et al., 2008). Additionally hormone ablation therapy benefits about 80% of patients by retarding the progression of the disease, but may not be a solution (OH, 2002). It has also been reported that patients receiving this treatment may experience serious side effects including osteoporosis, anemia, fatigue, emotional distress, erectile dysfunction, and even the development of diabetes and cardiovascular disease (Herr & O'Sullivan, 2000; Holzbeierlein, McLaughlin, & Thrasher, 2004; Keating, O'Malley, & Smith, 2006;

Nelson, Lee, Gamboa, & Roth, 2008; Thompson, Shanafelt, & Loprinzi, 2003). In addition, almost all prostate cancers eventually develop into an aggressive, hormone-independent form, with little hope for further intervention. (OH, 2002). Therefore, the best approach for combating prostate cancer is deterring its onset. This makes chemoprevention an attractive approach (Shi et al., 2009).

Prostate cancer is a multifactorial disease and epidemiologic studies have suggested that nutrition plays an important role in carcinogenesis and that 30% of cancer morbidity and mortality can potentially be prevented with proper adjustment of diet (Hsieh & Wu, 1999). Several dietary factors such as lycopene, vitamin E, and resveratrol (RSV), have been considered as potential prostate cancer chemopreventive agents (Shi et al., 2009).

Resveratrol

RSV (3,5,4'-trihydroxystilbene) is a polyphenol transhydroxystilbene that is found at high levels in grapes and red wines. Resveratrol is a relatively simple molecule that has beneficial effects on human health. It plays a role in the prevention of human pathological processes such as inflammation, atherosclerosis and carcinogenesis (Harada et al., 2007). RSV exhibits several potential chemopreventive activities in animal and cell models. This includes inhibition of pathways responsible for cell signaling, cell growth, and tumorigenesis such as the phosphoinositide 3-kinase (PI3K)/AKT pathway. In addition, RSV promotes the transcription of pro-apoptotic molecules such as FasL and BiM. Finally, RSV up-regulates tumor suppressor pathways such as phosphatase and PTEN, a tensin homolog (Q. Chen, Ganapathy, Singh, Shankar, & Srivastava, 2010).

Although the mechanism is unknown, studies have indicated that it has an important role in prostate cancer chemoprevention (Careri, Corradini, Elviri, Nicoletti, & Zagnoni, 2003; Y. Wang, Catana, Yang, Roderick, & van Breemen, 2002).

Previously, it has been seen that RSV down-regulates the expressions of both AR and AR target genes (Jones et al., 2005; Seeni et al., 2008; T. T. Wang et al., 2008). *In vitro* studies with PC3, an androgen-independent prostate cancer cell line, have shown that the RSV effects on AR activity are concentration dependent; AR activity is enhanced at low concentrations (0.1-10 μ M) of RSV and repressed at high concentrations (100 μ M) (Gao, Liu, & Wang, 2004). Recently, it was also reported that RSV represses AR target gene expression, at least partially, by enhancing AR degradation in a time- and dose-dependent manner (Harada et al., 2007). Given RSV's repressive effects on AR and AR's significance in the development of prostate cancer, these studies support a role for RSV as a potential candidate for the treatment and prevention of prostate cancer.

In 2005, Rodrigue et al searched RSV affected genes in the human erythroleukemic K562 cell line. After 24 hr incubation of K562 cells in the presence of 50 μ m RV, a RFDD-PCR technique was used to identify 134 cDNA fragments of 100-750 nucleotides. Among them, 62 were up-regulated and 72 down-regulated (Rodrigue et al., 2005). One specific gene that was markedly induced by RSV was tensin. This effect was also seen in MCF7, a human breast cancer cell line (Rodrigue et al., 2005).

Tensin (Tensin 1)

Tensin 1 is a 220kDa protein whose integrin binding domain links it to a component of the extracellular matrix (ECM). It is a member of a family of tensin molecules that share similar structures. Tensin1 contains multiple actin binding domains and the interaction between tensin constitutes the site of anchorage of the actin filaments and is localized to integrin-mediated focal adhesions. Tensin1 also contains a phosphotyrosine-binding (PTB) domain. These interactions allow tensin to link actin filaments to integrin receptors. The N-terminal domain of tensin1 associates with protein phosphatase-1 α (PP1 α) and mediates PP1 α localization to adhesions (H. Chen, Ishii, Wong, Chen, & Lo, 2000; Hall, Daugherty, Choi, Horwitz, & Brautigan, 2009; Lo, 2004).

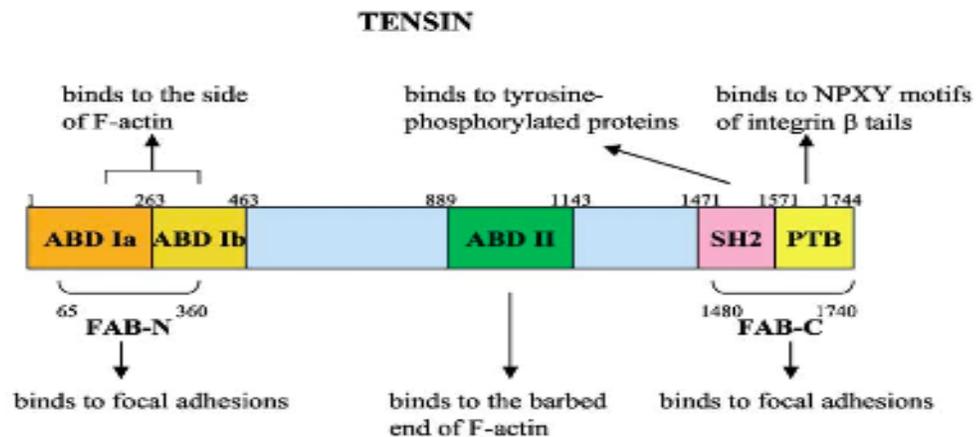


Figure 2: Schematic structure of *tensin*. Functional and Structural domains are shown.

Tensin is broadly expressed in human tissues including heart, skeletal muscle, kidney, lung, small intestine, liver, colon, prostate, testis, and ovary (Lo, Yu, Degenstein, Chen, & Fuchs, 1997). In 1997, Chen et al generated mice lacking tensin expression and studied the growth of the mice. It was found that even though tensin is expressed broadly in mouse embryos and a variety of postnatal tissues, the tensin-null mice developed normally and appeared healthy for several months. However, while most of the tissues seemed to be normal in older mice, multiple cysts were detected in the kidney, an organ that normally expresses high levels of tensin. The progressive cyst formation led to kidney degeneration and the mice subsequently died from renal failure. These results demonstrated that tensin is not necessary for mouse embryogenesis, but is required for the maintenance of normal renal function (Lo et al., 1997).

Knockout mice were also used to investigate the role of tensin in wound healing. In 2001, Ishii and Lo looked at tensin's role in the process of skeletal muscle regeneration. Cardiotoxin was used to induce regeneration in the anterior tibial muscles of tensin-knockout and wild-type mice. The histological analyses shown that the regeneration process lasted longer in knockout than in wild-type mice. This indicates that tensin play an important role in wound healing (Ishii & Lo, 2001). Tensin is also the first cytoskeleton-associated protein found to contain a Src Homology 2 (SH2) domain. The SH2 domain may allow tensin to translate tyrosine kinase or other regulatory signals in to a direct effect on the structure of the cytoskeleton. By binding to actin filaments and tyrosine phosphorylated proteins, tensin may serve as a scaffold around which signaling complexes can be assembled and hence, link the cytoskeleton with the signal transduction pathways (Davis et al., 1991)

Another study examined the mechanism of tensin-promoted cell migration. In 2003, Chen and Lo mapped the focal adhesion-binding (FAB) sites on tensin1 using deletion mutagenesis and transfection analysis. They then generated mutants that specifically inactivated the FAB sites without disrupting the binding to actin and phosphotyrosine (pTyr)-containing proteins. The mutants were introduced into cells in order to define the structural requirements for subcellular localization of the protein and to determine how it affected cell migration. Their results demonstrated that the localization of tensin1 to the focal adhesion site is a critical step in the regulatory process of cell migration. Also, because SH2 domains may mediate signals related to cell migration, they inactivated the SH2 domain and showed that this mutant abolished tensin-promoted cell migration, indicating that a functional SH2 domain is also required for tensin-mediated cell migration (H. Chen & Lo, 2003). These studies found that tensin played major roles in cell migration, wound healing, muscle regeneration and renal functions and that tensin and its downstream signaling molecules may be targets for therapeutic interventions in renal disease, wound healing and cancer (H. Chen et al., 2000; Lo, 2004).

Since these studies, there has been specific focus on tensin expression and cancer. Interestingly, it has been found that tensin expression is greatly suppressed in some cancer cells, especially prostate cancer cell lines (Lo, 2004). Recent discoveries have shown that an important function of tensin is its association with a RhoGAP protein, which is an evolutionary conserved protein domain of GTPase activating proteins towards Rho-like small GTPases. The specific RhoGAP protein that tensin associated with is known as Deleted in Liver Cancer-1 (DLC-1), which is a negative regulator of

tumor formation and plays a role in cell migration. This suggests that tensin may play an important role in tumor suppression and may serve as a checkpoint in cell growth. Furthermore, there have been studies done with various tensin mutants in hopes to understand its importance in cancer. In 2009, Hall et al transfected Human Embryonic Kidney cells (HEK293) to express DLC-1 alone or co-express DLC-1 with S-tag-tensin1 wild type, or F302A, which is the mutant tensin that showed reduced association with DLC-1. As studied by cell migration and morphology, compared to the wild type, the cells with the mutant tensin showed increased migration and invasion. These findings suggest that tensin may serve as a checkpoint of cell migration (Hall et al., 2009). Based on these previous findings, it may be beneficial to upregulate tensin levels in cancer cells in order to further study tensin's importance in cancer formation and its potential uses as a therapeutic agent.

Materials and Methods

Cells, Cell culture and Treatment:

LNCaP and K562 cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in Roswell Park Memorial Institute (RPMI, Cellgro, Mediatech, Inc, Herndon, VA) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotics/antimycotics (Abx/Amx) (Gibco, Invitrogen Corporation, Carlsbad, CA). Cells were trypsinized from tissue culture dishes (Cellgro, Mediatech, Inc., Herndon, VA) and resuspended in RPMI containing 1% Abx/Amx and 10% FBS for maintenance and subculturing. Cells were incubated at 37°C and 4.7% CO₂.

AR(+) cells were created by cloning the AR cDNA into the retroviral vector pOZ-N and integrating it into the genome of AR-negative HeLa cells from American Type Culture Collection (Manassas, VA) (Shi et al., 2009). The AR(-) cells were obtained from American Type Culture Collection (Manassas, VA). Both cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Cellgro, Mediatech, Inc, Herndon, VA) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotics/antimycotics (Abx/Amx) (Gibco, Invitrogen Corporation, Carlsbad, CA). Cells were trypsinized from tissue culture dishes (Cellgro, Mediatech, Inc., Herndon, VA) and resuspended in DMEM containing 1% Abx/Amx and 10% FBS for maintenance and subculturing. Cells were incubated at 37°C and 4.7% CO₂.

Cell Treatment:

Cells were treated with RSV from a 200mM stock solution (Sigma Aldrich, St. Louis MO), and R1881 from a 10 μ M stock solution (Sigma Life Sciences, St. Louis MO).

Analysis of tensin mRNA levels:

Total RNA was purified from LNCaP, K562, AR(+), and AR(-) cells using RNeasy Plus Micro Kit (Qiagen, Germantown MD). For cDNA synthesis, 5ug of total RNA was reverse transcribed using random primers (Stratagene, La Jolla, CA) and Superscript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA). Tensin and GAPDH were amplified by PCR using the following specifically designed primers (Invitrogen Corporation, Carlsbad, CA). Tensin primers: Forward, 5'-CGAATAGCTATCAAGAAAATC-3'; Reverse, 3'-CCTCTGAGGATCTGGTAGAG-5'; GAPDH primers: Forward, 5'-ACAGCCTCAAGATCATCAGCAA-3'; Reverse, 3'-ACCACTGACACGTTGGCAGT-5'.

The following PCR conditions were used routinely in a Genius thermal cycler (Techne Inc., Burlington, NJ): denatured at 95°C for 2 minutes followed by 30 cycles of 95°C, 1 minute, 60°C, 1 minute, 72°C, 1 minute; and enzyme was inactivated by incubation of 72°C for 5 minutes. PCR products were analyzed on a 1% agarose gel containing 1% ethidium bromide, and visualized under ultraviolet light.

Cell Lysis and Western Blotting:

Cells were collected and lysed with EBC buffer containing 20mM Tris HCl (pH 8.0), 125 mM EDTA, and 0.5% NP-40. Lysates were collected and centrifuged at maximum speed for 20 minutes, and the resulting supernatant was collected. Protein samples were quantified using Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Protein samples were separated by SDS-PAGE electrophoresis, using 10% resolving gel and a 4% stacking gel. Proteins were then transferred to a nitrocellulose membrane using iBLOT Dry Blotting System (Invitrogen Corporation, Carlsbad, CA). Proteins were stained with MemCode Reversible Stain Kit for Nitrocellulose Membranes (Pierce, Rockford IL). Immunoblotting was performed using Pierce Fast Western Blot Kit (Thermo Scientific USA) using Tensin (H-300) rabbit polyclonal IgG (5 μ g/mL) or AR (N-20) sc-816 rabbit polyclonal IgG (5 μ g/mL) (Santa Cruz, Santa Cruz CA). The blots were detected using Immuno-Star WesternC Chemiluminescence Kit (Bio Rad, Hercules CA).

Statistical Analysis:

To determine if effects were significant, Statview Software was used to conduct a Bonferroni/Dunn ANOVA. P-values less than 0.05 were considered significant.

Results

RSV Up-regulates Tensin mRNA Levels in LNCaP Cells

Based on previous reports that RSV upregulated tensin in different cancer cell lines, we tested whether RSV would have similar effects on tensin expression in prostate cancer cells. First, to serve as our positive control, we conducted an experiment similar to what was described by Rodrigue et al in 2005 (Rodrigue et al., 2005). Specifically, K562 cells were treated with 100 μ M of RSV for 24 hrs and RT-PCR was used to determine levels of tensin mRNA. As expected, the RSV treatment upregulated tensin mRNA levels in K562 cells; GAPDH mRNA levels were comparable between the treatments (Fig3A). When LNCaP cells were treated with RSV, in the same manner, tensin mRNA levels increased as compared to the control, while GAPDH levels remained unchanged (Fig3B). This PCR was conducted in triplicate. The data indicate that RSV increases tensin mRNA levels in prostate cancer cells.

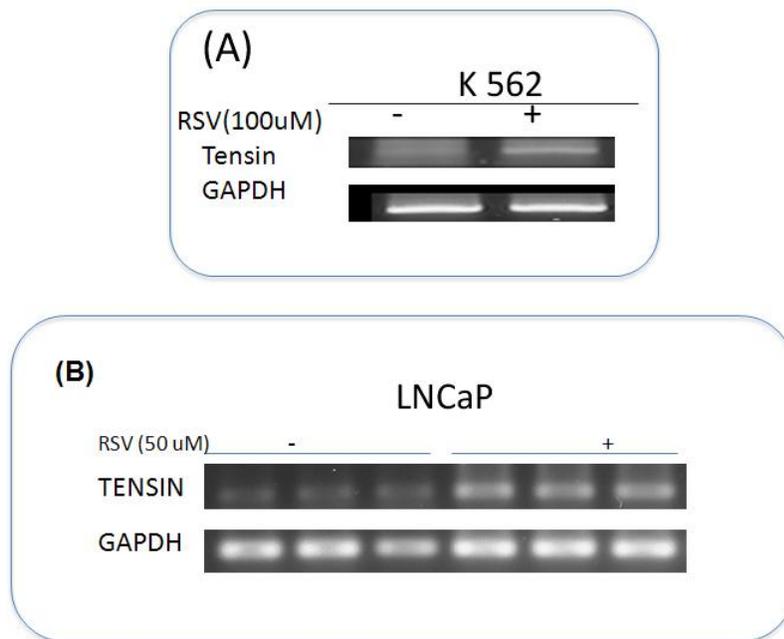


Figure 3. *Expression of tensin in LNCaP cells with RSV treatment.* Cells were incubated with indicated concentrations of RSV for 24hrs. RNA was extracted and RT PCR was performed. GAPDH was used as an endogenous control for RT PCR. PCR products were separated on 1% agarose gel and visualized under UV light. A) K562 cells were used as positive control for this experiment. They were treated with or without 100 μ M RSV. B) LNCaP cells were incubated with or without 50 μ M RSV. The PCR was conducted in triplicate.

RSV Affects Tensin mRNA Levels in LNCaP Cells Optimally at 50 μ M

To further confirm effects of RSV on tensin mRNA levels in LNCaP cells, we treated LNCaP cells with various concentrations of RSV. Total RNA was purified and RT-PCR was performed. PCR products were separated on 1% agarose gel and visualized under UV light. As shown in Fig 4A, when LNCaP cells were treated with 10 μ M RSV tensin expression was upregulated compared to the control. Stronger increases were seen at 25 μ M and 50 μ M. However, at higher concentrations (100 μ M), we saw a decrease in expression. In addition, we saw a significant increase in the number of dead cells when treated at such high concentrations. To further visualize these results, ImageJ software was used to quantitate the data from Fig4A and numbers are shown in graphical analysis (Fig4B). Although it appears that tensin mRNA levels are increased with 10 μ M treatment of RSV, the significant effect was seen when cells were treated with 50 μ M ($p < 0.05$). Therefore, we subsequently used 50 μ M for the rest of the experiments.

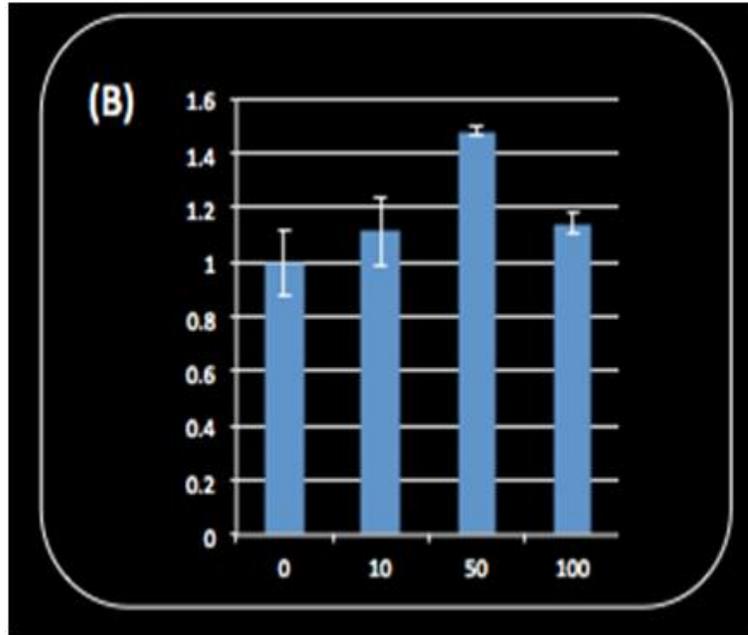
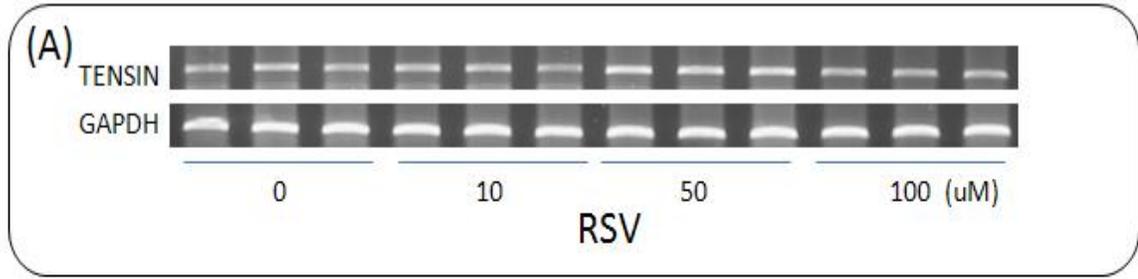


Figure 4: *Tensin* expression with various RSV concentrations. A) LNCaP cells were treated at various concentrations of RSV. RNA was extracted and RT PCR was performed in triplicate. GAPDH was used as an endogenous control for RT PCR. PCR products were separated on 1% agarose gel and visualized under UV light. B) Graphical presentation of Fig4A using ImageJ software. Same areas were measured for all bands and intensities of each area were quantified. GAPDH was used to normalize each band. The control was set as 1.

RSV Affects Tensin mRNA Levels in a Time-dependent Manner

LNCaP cells were treated with 50 μ M RSV and samples were collected at different time points. Total RNA was purified and RT-PCR was performed. PCR products were separated on 1% agarose gel and visualized under UV light. As shown in Fig 5, the RSV effects on tensin mRNA levels were first seen at 12hrs and stronger effects were observed with longer incubations. Thus, together with what we observed in Fig 4, RSV effects on tensin mRNA levels are dose- and time-dependent up to 48hrs.

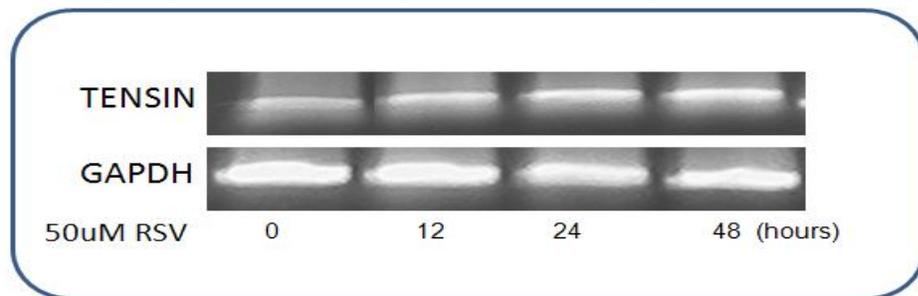


Figure 5: *Time dependent effects of RSV on tensin mRNA levels.* LNCaP cells were treated with 50 μ M RSV and collected at various time points. RNA was extracted and RT PCR was performed. GAPDH was used as an endogenous control for RT PCR. PCR products were separated on 1% agarose gel and visualized under UV light.

RSV Counteracts Androgen-induced Downregulation of Tensin in LNCaP Cells

It is well established that androgen functions through interaction with AR. The ligand-bound receptor translocates into the nucleus and up- or down-regulates target gene expression. We were interested in determining whether the effects of RSV on tensin expression is through the function of AR. LNCaP cells were treated with either 50 μ M of RSV, 10 nM of androgen (R1881), or a combination of RSV (50 μ M) and R1881 (10nM). Total RNA was purified and RT-PCR was performed. PCR products were separated on 1% agarose gel and visualized under UV light (Fig 6A). The results from Fig6A were then quantified using ImageJ software (Fig6B). Comparing to the control, when cells were treated with androgen, tensin mRNA levels were significantly down-regulated ($p < 0.05$). This androgen-induced downregulation was significantly counteracted by RSV ($p < 0.05$). It is well known that androgen functions through AR and tensin was down-regulated by androgen treatment. Because RSV counteracted this effect, it suggests that RSVs effects on tensin are mediated through the AR.

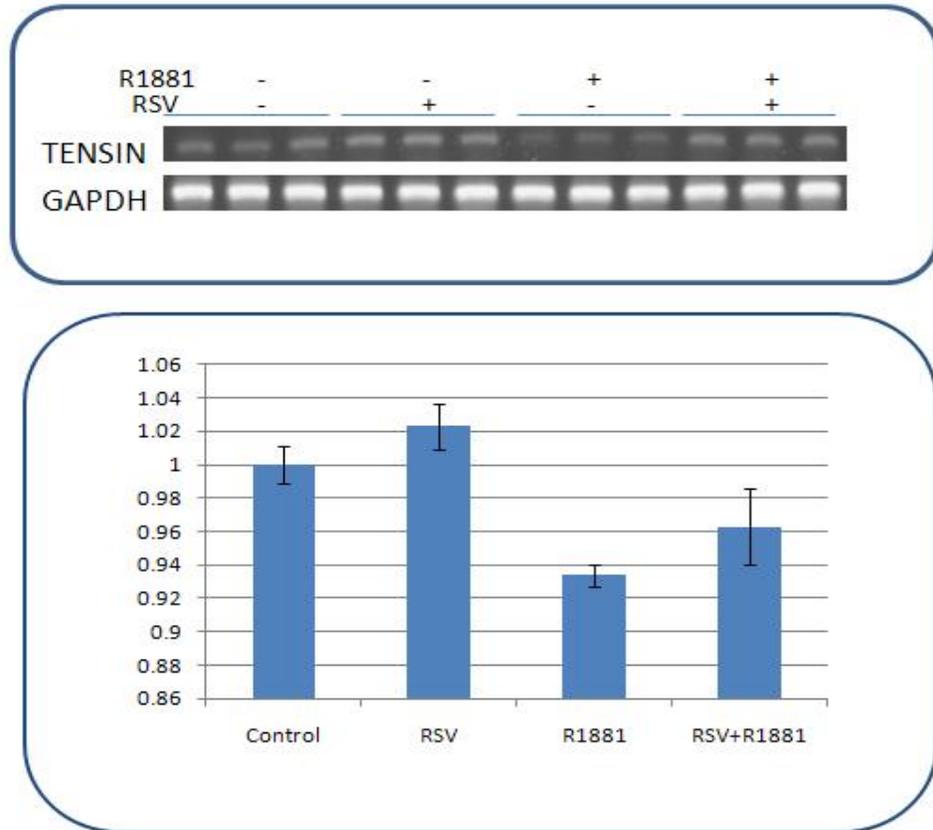


Figure 6: *Effects of RSV and Androgen on tensin in LNCaP cells.* A) LNCaP cells were treated with or without R1881 or RSV as well as in combination for 24 hours. RNA was extracted and RT PCR was performed in triplicate. B) Fig6A quantified using ImageJ software. The control was set as 1.

Confirmation of AR Expression in the AR(+) Cell Line

In order to test the hypothesis that RSV increased tensin mRNA levels by a mechanism that involves AR, experiments were conducted using a cell line which stably overexpression of AR (Shi WF, Leong M et. al, PloS One, 2009 Oct 9; 4(10):e7398) (Shi et al., 2009) in parallel with the AR(-) parent cell line as a negative control. First, the Western blot was conducted with anti-AR antibodies using lysate from AR(+), AR(-); and LNCaP cell lysate was used as a control. The molecular weight of AR is ~110kD. A band around 110kD was seen in both AR(+) and LNCaP cell lysates, but not in that of AR(-).

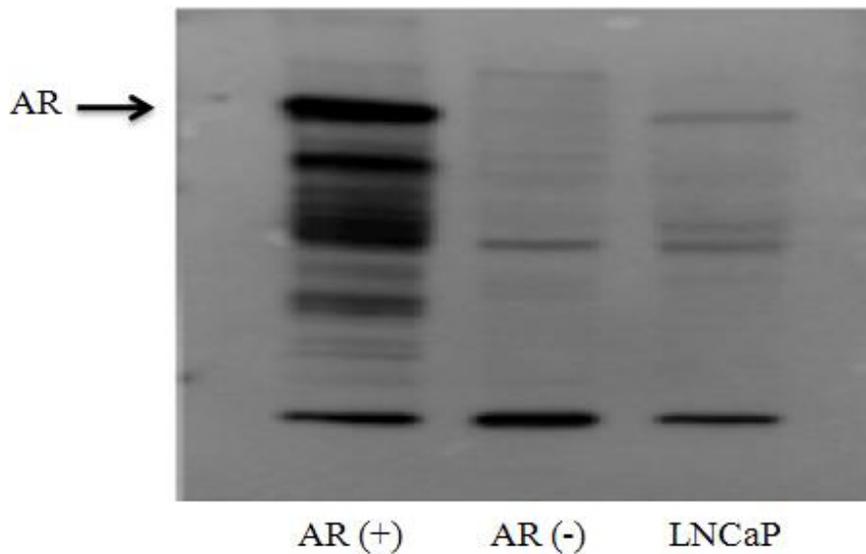


Figure 7: *Confirmation of AR expression in AR(+) cell line.* Western Blot for confirmation of AR expression in AR(+) cell lines. Proteins (50 μ g) in whole cell lysate were subjected to SDS-PAGE, followed by Western Blot analysis with anti-AR antibody.

RSV Effect on Tensin Upregulation is at least partially AR-dependent

It has been established previously that 100 μ M RSV was optimal for the AR(+) and AR(-) cell lines (Shi et al., 2009). To examine the RSV effects on AR(+) cell lines, we treated all three cells, AR(+), AR(-) and K562, with 100 μ M of RSV for 24hrs. RT-PCRs were conducted and GAPDH was used as an internal control and mRNA were comparable between the treatment and non-treatment. Same as demonstrated in previous experiments, the RSV treatment upregulated tensin mRNA levels significantly in K562 cells (Fig8). The RSV effects on tensin mRNA levels were seen in the AR(+) cells, but not in AR(-) cells (Fig8).

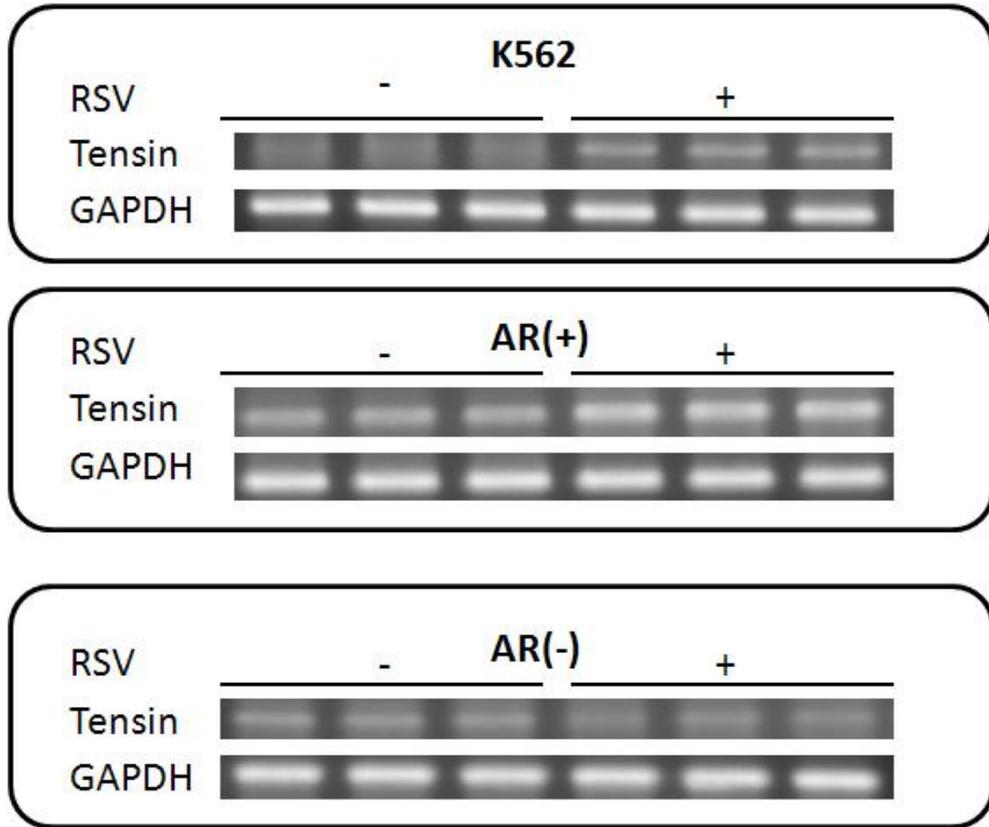


Figure 8: Effects on RSV on tensin mRNA levels in AR(+) cells. K562, AR(+), and AR(-) cells were treated with or without 100 μ M of RSV for 24 hours. RNA was extracted and RT PCR was performed in triplicate. GAPDH was used as an endogenous control for RT PCR. PCR products were separated on 1% agarose gel and visualized under UV light.

RSV Up-regulates Tensin mRNA Levels in a Time-dependent Manner up to 48hrs

In order to further study RSV effects on tensin mRNA levels, AR(+) cells were treated with 100 μ M of RSV and collected at different time points. Total RNA was purified and RT-PCR was performed. As shown in Fig 9, RSV effects on tensin expression were seen as early as 6hrs and increased with longer treatments up to 24hrs. However, tensin levels appeared to become lower with longer incubations.

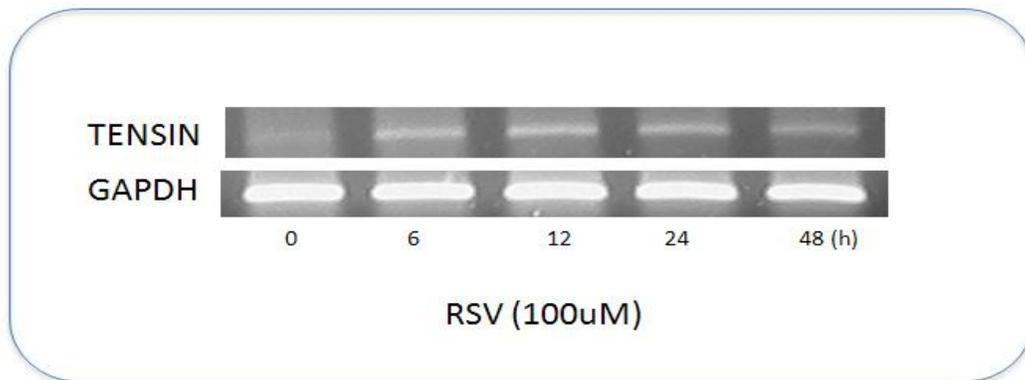


Figure 9: *Effects of RSV on tensin expression in a time dependent manner.* AR(+) cells were treated with 100 μ M RSV and collected at different time points. RNA was extracted and RT PCR was performed. GAPDH was used as an endogenous control for RT PCR. PCR products were separated on 1% agarose gel and visualized under UV light.

RSV Counteracts Androgen-induced Downregulation of Tensin in AR (+) Cells

In order to further demonstrate that the effects of RSV on tensin mRNA levels are AR-dependent, we conducted an experiment that was designed similarly to that in Figure 6. Total RNA was purified and the RSV effects on tensin were estimated by RT-PCR. As shown in Figure 10, when AR(+) cells were treated with 10nM of R1881, tensin mRNA levels were down-regulated as compared to the control. When AR(+) cells were treated with 100 μ M of RSV, tensin mRNA levels increased as compared to the control. More importantly, when AR(+) cells were treated with both RSV (100 μ M) and R1881 (10nM), the androgen-induced downregulation of tensin mRNA was counteracted by RSV. However, none of these effects were observed in the AR(-) cell line. Because the only difference between these cell lines is the presence or absence of the androgen receptor, we conclude that the observed RSV effects on tensin mRNA levels are AR dependent.

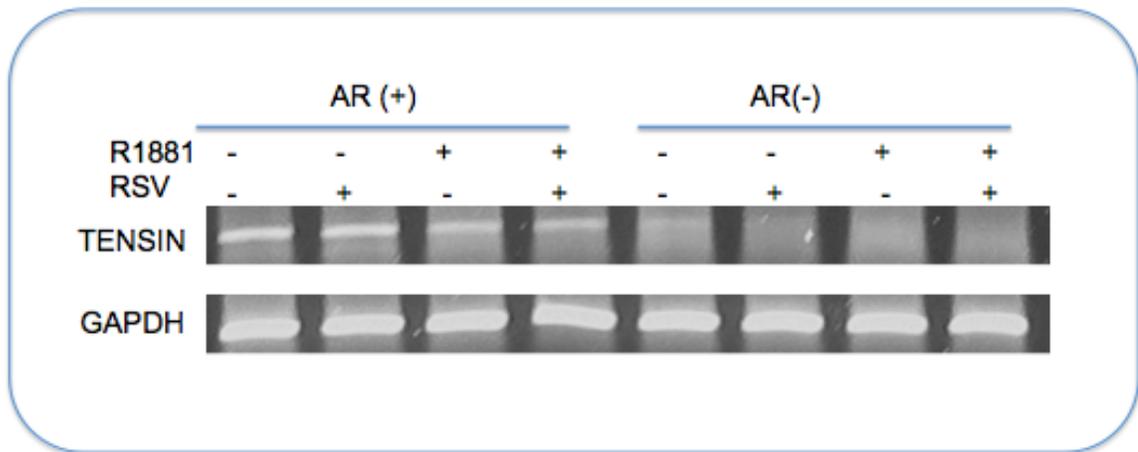


Figure 10: *RSV counteracts androgen-dependent downregulation of tensin in AR(+) cells.* AR(+), and AR(-) cells were treated with or without R1881 or RSV as well as in combination for 18 hours. RNA was extracted and RT PCR was performed. GAPDH was used as an endogenous control for RT PCR. PCR products were separated on 1% agarose gel and visualized under UV light.

Expression of Tensin Protein Levels

In order to examine the effects of RSV on tensin protein levels, LNCaP cells were treated with various concentrations of RSV for 24 hrs and whole cell lysates were separated on SDS-PAGE followed by western blot. A cell lysate containing tensin (Santa Cruz, Santa Cruz, CA) was used as a positive control. The bands in all treatments including the positive control were seen at the same molecular marker around 40kDa (Fig11A).

The intact tensin molecule has been reported as a 220kDa protein. However, it is a substrate of the calcium-dependent cysteine protease calpain II. Therefore, tensin is rapidly cleaved into several fragments, one of these cleavage products corresponds to the size of 40-45kDa. The bands we observed were around 40-45kD; therefore we used a calpain II inhibitor MDL in an attempt to preserve the intact tensin protein. LNCaP cells were treated with various concentrations of MDL and incubated for 16hrs. However, western blot shows that all bands were once again between 40-45kDa (Fig 11B).

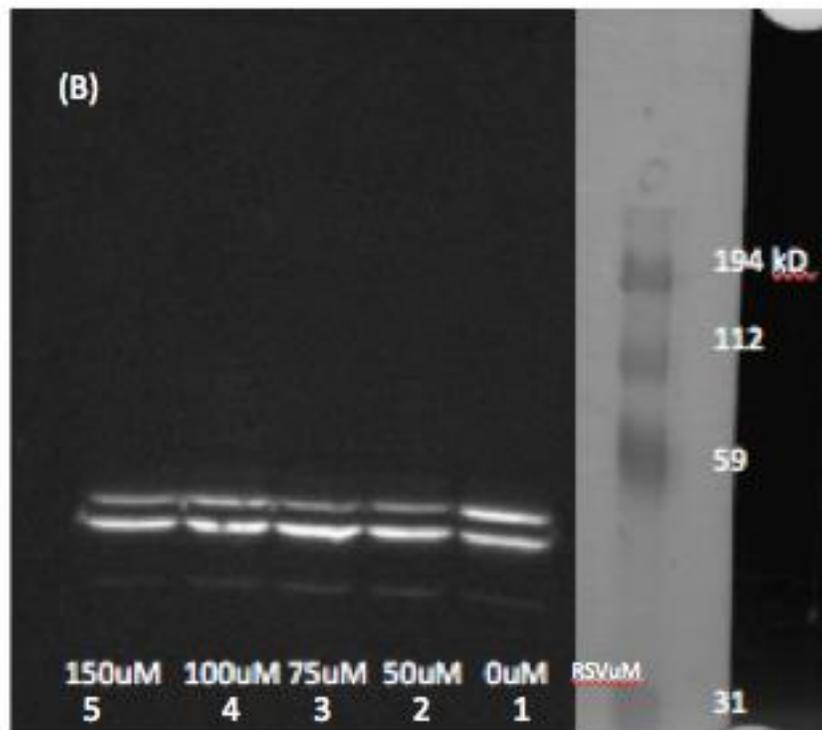
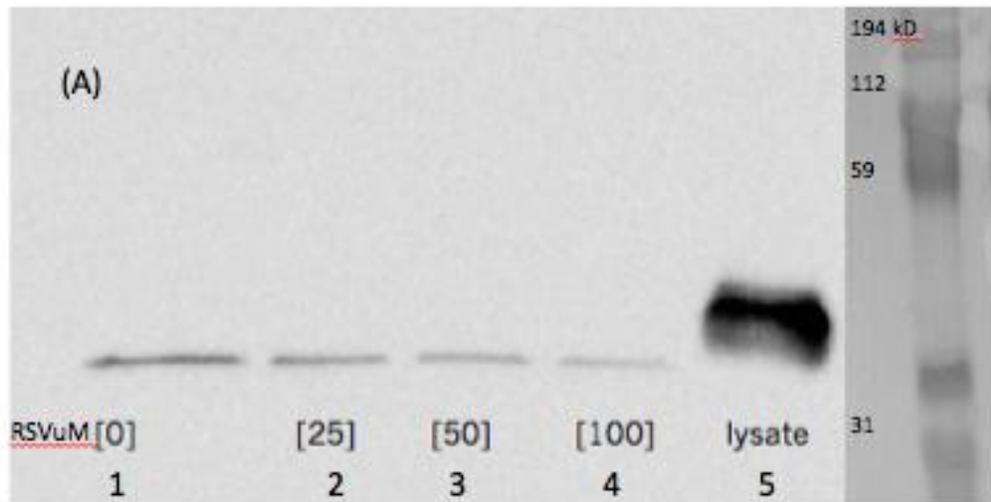


Figure 11: Protein expression of tensin. A) LNCaP cells were treated with indicated concentrations of RSV for 24hrs. Proteins (20 μ g) in whole cell lysate were subjected to SDS-PAGE, followed by Western Blot analysis with anti-Tensin antibody. B) LNCaP cells were treated with indicated concentrations of MDL for 16hrs. Proteins (20 μ g) in whole cell lysate were subjected to SDS-PAGE, followed by Western Blot analysis with anti-Tensin antibody.

Discussion

Despite advances in treatments and therapies, prostate cancer remains second only to lung cancer as the leading cause of cancer-related death in men (Seeni et al., 2008). Prostate cancers are unique in requiring androgens to maintain growth and avoid apoptosis. Therefore, androgen depletion combined with anti-androgenic agents is the initial recommended treatment for androgen-dependent prostate cancer (Shi et al., 2009). This treatment, while effective in about 80% of these cases, is not without its problems (OH, 2002). Hormone ablation treatments can have a negative impact on the quality of life in men. Several side effects include hot flashes, osteoporosis, anemia, fatigue, sarcopenia, gynecomastia and emotional distress as well as loss of libido and erectile dysfunction (Herr & O'Sullivan, 2000; Holzbeierlein et al., 2004; Nelson et al., 2008; Thompson et al., 2003). In addition there is increased risk of developing diabetes, cardiovascular disease and fatal cardiac events (Keating et al., 2006). There is also evidence suggesting that this treatment also impacts cognitive functioning. Cognitive deficits can impact a patient's ability to make informed treatment decisions, perform occupational or intellectual pursuits, and can have a negative impact on their overall quality of life (Herr & O'Sullivan, 2000; Holzbeierlein et al., 2004; Nelson et al., 2008; Thompson et al., 2003).

While it is important to develop improved treatments for established cancers, another approach is to focus on chemoprevention. Epidemiological studies indicate that RSV enriched beverage consumption can significantly lower prostate cancer instance (Schoonen, Salinas, Kiemeny, & Stanford, 2005). RSV has also been recognized as a novel potential cancer chemopreventive agent in both animal and cell models (Seeni et

al., 2008). In 1999, Hsieh et al demonstrated that RSV inhibited the growth of LNCaP cells; additionally they used flow cytometric analysis to show that RSV induced apoptosis of these cells (Hsieh & Wu, 1999). RSV has also been shown to inhibit the PI3K/AKT pathway, which is responsible for cell growth and tumorigenesis. RSV enhances the therapeutic potential of Tumor Necrosis Factor Apoptosis (TRAIL) by up-regulating death receptors, and engages in a mitochondrial pathway of apoptosis (Q. Chen et al., 2010). Additionally RSV has been found to induce the nuclear translocation of p53 and the expression of p53-responsive genes (Zheng et al., 2010).

There have also been multiple animal studies with RSV, showing a number of anti-cancer activities that were recently summarized by Namasivayam, N. A few of the most well documented effects of RSV in animal studies include antioxidant effects, modulation of carcinogen metabolism, anti-inflammatory potential, antioxidant properties, antiproliferative mechanisms by induction of apoptosis, and cell differentiation with vast research on RSV as an anti-cancer agent (Namasivayam, 2011). While there is no published data of these RSV effects in humans, there are currently a number of clinical trials going on which are examining RSV's effects on diseases such as diabetes, Alzheimer's disease, and a number of cancers (<http://clinicaltrials.gov/ct2/results?term=resveratrol>). Hopefully, these trials will one day provide further information about RSV and its mechanism.

It has been reported that RSV has inhibited the growth of androgen-responsive prostate cancer cells, in part by decreasing AR expression. In addition, there has been much research to show that down-regulation of AR and its target genes is not solely due to a decreased amount of AR, but RSV also decreases AR activity (Gao et al., 2004;

Jones et al., 2005). Even though the detailed mechanisms by which RSV inhibits AR function remains unclear, RSV seems to be a potentially important agent in the prevention of prostate cancer (Harada et al., 2011). Since RSV has proven to be an effective anti-cancer agent, researchers wanted to gain more knowledge about its target genes. In 2005, during a search to recognize such genes, it was found that tensin protein and gene expression levels were greatly increased by RSV in leukemia cells and breast cancer cells (Rodrigue et al., 2005).

Tensin plays a role in cell proliferation and migration as well as closely associates with DLC-1. Because DLC-1 itself is a negative regulator of tumor formation and plays a role in cell migration, it has been suggested that tensin may play a role in tumor suppression and perhaps serve as a checkpoint in cell growth. Tensin expression is down-regulated in several types of cancers, such as breast cancer and prostate cancer. (Lo, 2004; Martuszezwska et al., 2009). Due to tensin's normal function, a decrease in tensin expression might contribute to formation and/or progression of tumors. If RSV can maintain or restore normal levels of tensin, this may contribute to its chemopreventive and/or therapeutic effects.

We have demonstrated that tensin mRNA levels are upregulated by RSV in LNCaP cells, an androgen dependent cancer cell line (Fig 3), and that this upregulation is dose- and time-dependent. Maximal effects at 50 μ M in LNCaP cells (Fig 4) and mRNA increases were seen as early as 6 hrs (Fig 5). In LNCaP cells, higher doses of RSV were less effective, and were associated with increased cell death (Fig 4). Additionally, it appears that prolonged incubation up to 48hrs enhances RSVs effect on tensin mRNA levels (Fig 5). There have been several findings of RSV causing increased cell death at

high concentrations and prolonged treatment. For example, Hsieh et al saw cell death by apoptosis in LNCaP cells beginning with 25 μ M RSV with a 4day treatment (Hsieh & Wu, 1999). In addition, Wang et al saw apoptosis of LNCaP cells beginning with 25 μ M RSV after only 24 hours (T. T. Wang et al., 2008). Additionally there have not been published reports confirming that high levels of RSV cause necrosis in LNCaP cells. However, preliminary data from our lab suggest these findings in other types of cancer cells. Using Annexin 5 labeling, it has been shown that at concentrations of RSV over 100 μ M and up to 200 μ M, apoptosis that was seen at lower concentrations is no longer taking place in the oral cancer cell line Cal27 (personal communication). At these higher concentrations of RSV the cell membrane becomes leaky and is no longer intact, indicating that necrosis may be taking place. Due to these findings it is plausible to hypothesize that at higher concentrations of RSV the LNCaP cells may be undergoing necrosis. This being said, it was important during the course of this research to use RSV concentrations that were optimal for different cells lines. When working with LNCaP cells, 50 μ M RSV was used; AR(+), AR(-), and K562 cells were treated with 100 μ M. This ensured that the optimal results were achieved for each of the experiments that were carried out.

In most cases, protein levels mirror mRNA levels. After observing the upregulation of tensin mRNA levels by RSV, we attempted to show the effects on protein levels. When we tried to measure tensin protein levels in LNCaP cells, we were unable to show tensin at the expected molecular weight of 220kD. This is consistent with the previous report that tensin protein was not detected in LNCaP cells (H. Chen et al., 2000). However, bands were consistently seen between 40-45kD, even in a positive

control lysate (tensin) (Fig 11A). Chen et al reported that there are multiple cleavage sites on tensin by calpain II and one of the cleaved products is around 40kD. However, the biological significance of this cleavage is unknown.

MDL is a membrane-permeable calpain II inhibitor (H. Chen et al., 2000). We used different concentrations of MDL to treat LNCaP cells in order to show the undegraded tensin protein. However, this experiment was also inconclusive. We observed two bands, which appeared between 40kDa to 45kDa (Fig 11B). There are some possible explanations for this occurrence. First, calcium is needed for calpain II activity, and it is not known whether MDL can inhibit calpain II activity in the calcium levels present in the LNCaP cells. (H. Chen et al., 2000). While there most likely were not very high amounts of calcium present during our experiments, we did not measure calcium levels at the time of treatment. Some previous studies have hinted to this possible effect, however, we did not investigate this further. Second, tensin may be cleaved by other calcium-dependent cysteine proteases like calpain II that interact with focal adhesion molecules (H. Chen et al., 2000). It has been reported that proteins similar to tensin are substrates for these calcium-dependent cysteine proteases. So far, the only known protease of this family to cleave tensin is calpain II (H. Chen et al., 2000). It can be hypothesized that tensin may be a substrate for other calcium-dependent cysteine proteases and further experiments could be done using inhibitors of proteases in this family that may cleave tensin. Also, calcium levels should be monitored during all experiments, to ensure calpain II will not be active to cleave tensin.

Based on previous evidence that RSV down-regulates AR expression and activity (Harada et al., 2011; Shi et al., 2009), further work was done with AR during our

research. LNCaP cells are androgen-dependent prostate cancer cells, AR in these cells is responsive to androgen. When cells were treated with R1881, a synthesized androgen, tensin mRNA levels decreased significantly (Fig 6). When cells were treated with RSV tensin mRNA levels increased. When cells were treated with a combination of R1881 and RSV, RSV significantly counteracted androgen's effects on tensin. These results lead us to further examine a potential mechanism of RSV's effects on tensin through AR. To confirm that this effect is AR dependent, the AR(+) cell line was utilized in a series of experiments; and the AR(-) parent cell line serve as a negative control. Similar to what we saw in LNCaP cells, tensin mRNA levels were down-regulated in AR(+) cells when treated with androgen and this effect was counteracted by RSV. However, these effects were not seen in AR(-) cells. Since the only difference between the AR(+) and AR(-) cell lines is the presence or absence of the androgen receptor, these results demonstrate unambiguously that the effects of RSV on tensin mRNA levels is AR-dependent. However, whether tensin is directly or indirectly regulated by AR is unknown.

Data from previous reports indicate RSV's effects on AR were not by affecting AR/heat shock protein interaction, nuclear translocation, or binding of AREs (Harada et al., 2011; Shi et al., 2009). It is likely that RSV affects AR activity by fine-tuning the ratios of transcriptional activators and repressors recruited by AR. These ratios will affect the histone code on the promoter of target genes and ultimately determine the expression of the targets (Fig12). The tensin promoter has not yet been identified, so it is not known whether there is an AR binding site on its promoter. To determine if RSV is up-regulating tensin directly through AR, the tensin promoter needs to be identified and possibly a Chromatin Immunoprecipitation (Chip) assay performed to confirm AR interactions.

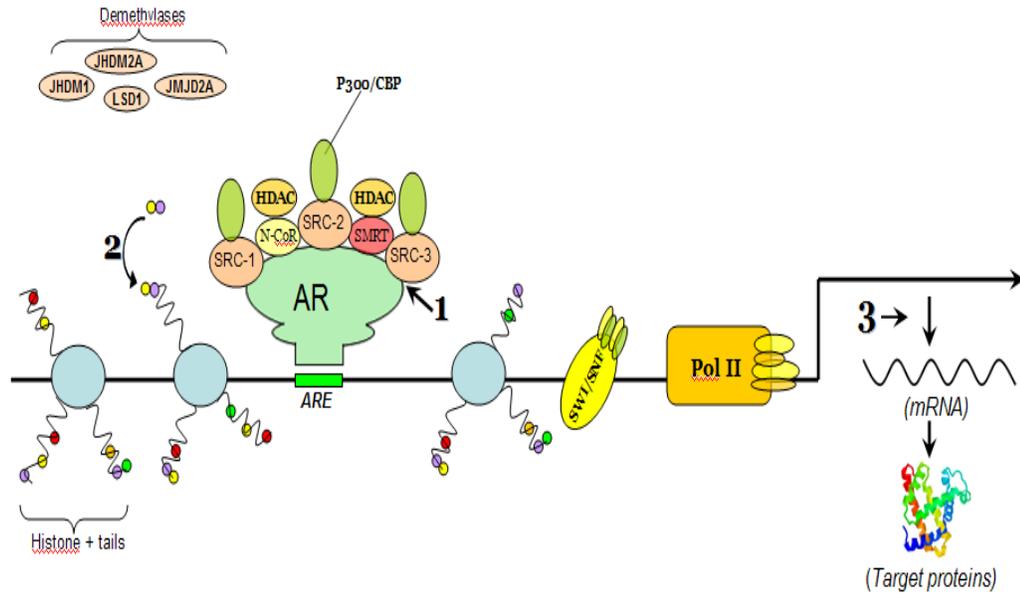


Figure 12: *Proposed model of RSV effects of AR target gene expression through modulating AR transcriptional activities. RSV may exert its effects on AR directly by affecting AR conformation, leading to subsequent alteration of the recruitment of one or more co-regulators. RSV may lead to alterations in (1) co-regulator recruitment, (2) histone code modification, and (3) transcriptional regulation.*

Prostate cancer is generally categorized into two stages. The early stage is referred to as androgen-dependent and the late stage is referred to as androgen-independent. However, AR is expressed and is functional in both stages. During the late stage, a variety of AR alterations include amplification, point mutation, and changes in expression of its co-regulatory proteins (Taplin & Balk, 2004). Since RSV affects AR activity, the results from our research indicate that RSV may be beneficial in both androgen-dependent and androgen-independent prostate cancers. It could be used as a chemopreventive agent to counteract androgens in the early stage, as well as a therapeutic agent in the late stage to interfere with AR directly.

Given the increasing incidence of prostate cancer in the US, it is imperative to find better means to combat this disease. Although many targets of RSV have been identified in the past, the identification of tensin as another target has added a new dimension in this research area. Since tensin is down-regulated in cancers including prostate cancers, up-regulating tensin by RSV could be one of the strategies in cancer prevention and therapy.

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