ABSTRACT

Title of Document: THE EFFECT OF RESVERATROL ON PROSTATE CANCER PREVENTION

Tien-Chung Wang, Master of Science, 2006

Directed By: Thomas T.Y. Wang, Ph. D., Research Chemist, Phytonutrients Laboratory, BHNRC, ARS, USDA
Bernadene A. Magnuson, Ph.D., Assistance Professor, Department of Nutrition and Food Science

Epidemiological as well as *in vitro* studies generally support a beneficial effect for consumption of fruits and vegetables in prevention of prostate cancer. This leads to the hypothesis that phytochemicals in fruits and vegetables may contribute to cancer preventive effects. Resveratrol, a phytoalexin, has been proposed as a prostate cancer preventive candidate. However, relatively little is known of the molecular targets in prostate cell as well as mechanisms that may contribute to the prevention of prostate cancer. This thesis attempted to elucidate the molecular effects of resveratrol and the mechanisms underlying the prostate cancer preventive effect of this compound.

Our results indicated that resveratrol exerts inhibitory effects on the growth of androgen responsive human prostate cancer cell, LNCaP. The effects of resveratrol

correlate with the activation of the p53-dependent pathway and the inhibitory effect on hormonal-mediated effects. At the molecular level, we found resveratrol inhibited androgen-responsive genes (ARGs) through both androgen- and estrogen-mediated events. Moreover, resveratrol also regulates the IGF-1 pathway through regulation of androgen- and estrogen-mediated events. These results provide the molecular basis to support the potential prostate cancer preventive effect of resveratrol.

THE EFFECT OF RESVERATROL ON PROSTATE CANCER PREVENTION

By

Tien-Chung Wang

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2006

Advisory Committee:
Thomas T.Y. Wang, Ph.D., Research Chemist, Co-Chair
Bernadene A. Magnuson, Ph.D., Assistant Professor, Co-Chair
Mark A. Kantor, , Ph.D., Associate Professor

Acknowledgements

I would like to express my sincere gratitude to my advisor, Dr. Bernadene A. Magnuson, for her guidance, nurturing, encouragement and support of my graduate study. I am also grateful to my thesis advisor, Dr. Thomas Wang, for advising my entire thesis. I also want to thank to my advisory committee, Dr. Mark A. Kantor for his invaluable academic offerings and encouragement.

I also want to thank for Dr. Yoko Takahashi whom I have learned the experimental skills. She also donated her data to complete my thesis. I also want to mention Samina Shami, from USDA. Without her encouragement and assistance, this thesis can't be finished.

Special thanks are given to my dear sister, mother, and fiancé. My education would not be accomplished without their endless support. Each single piece of my progress should be honorably attributed to their sacrifice and love to me. This thesis is dedicated to them.

Table of Contents

List of Abbreviations

AR	Androgen Receptor
ARE	Androgen Response Element
ARG	Androgen Responsive Gene
B2M	β-2 microglobulin
CDK	Cyclin Dependent Kinases
CDS	Charcoal Dextran-treated FBS
COX	Cyclooxygenase
CYP	Cytochrome p450
E2	17β-estradiol
ER	Estrogen Receptor
ERα	Alpha form of ER
ERβ	Beta form of ER
ERK	Extracellular signal Regulated-protein Kinase
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
IGF-1	Insulin-like Growth Factor 1
IGF-1R	Insulin-like Growth Factor 1 Receptor
iNOS	Inducible Nitric Oxide Synthase
JNK	c-Jun-NH_2-terminal Kinase
MAPK	Mitogen-Activated Protein Kinases
NF-κB	Nuclear Factor-kappa B
NMBA	*N*-nitrosomethylbenzylamine
PBS	Phosphate Buffered Saline
PKB	Protein Kinase B
PSA	Prostate-Specific Antigen
ROS	Reactive Oxygen Spices
SEPP1	Selenoprotein P
SPAK	Ste20-related Proline-Alanine rich Kinase
SRB	Sulforhodamine B
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAMP	Transgenic Adenocarcinoma Mouse Prostate

Chapter 1: Introduction

According to American Cancer Society statistics for 2006, prostate cancer is the most frequently diagnosed cancer, and is a leading cause of cancer death in men in the US (1). Surgery and external beam radiation or radioactive seed implants may be used to treat early-stage disease. Androgen ablation therapy, chemotherapy, and radiation are used for metastatic disease (1). Hormonal therapy can control prostate cancer for long periods by shrinking the size of the tumor, but tumors often become androgen-independent, leading to a relapse within 2 years (2). Because there is no complete cure for prostate cancer, many current research efforts are focused on preventive approaches that reduce disease incidence. Bioactive food components are increasingly being evaluated as potential prostate chemopreventive agents (3, 4). One such agent is resveratrol, a phytochemical which has been considered as a chemopreventive for human prostate cancer (5, 6).

Since Pezzuto et al. (7) published a paper of cancer chemopreventive activity of resveratrol in Science in 1997, interest in the plant-derived chemical, resveratrol (Figure 1), has exploded over the last few years (Figure 2) and continues to increase due to a wealth of data suggesting that resveratrol possesses as potent and wide-range of biological effects (8-10). Its beneficial effects include prevention of breast and prostate cancers, as well as lowering the risk of cardiovascular disease (11). However, the molecular mechanisms underlay the beneficial effects of resveratrol on prevention of cancer, such as prostate cancer, remain largely unknown. Hence, to fully realize the potential beneficial effects of resveratrol on prostate cancer prevention and to

elucidate the molecular effect and mechanism of action of resveratrol on cells would be the critical first step. This thesis seeks to address this question.

Base on published literatures, this thesis tested the hypothesis that resveratrol can modulate multiple pathways that are important in prostate cancer development. In the following chapters, experiments were designed to study effects of resveratrol through three possible mechanisms: androgen-mediated pathway, estrogen-mediated pathway, and IGF-1 mediated pathway.

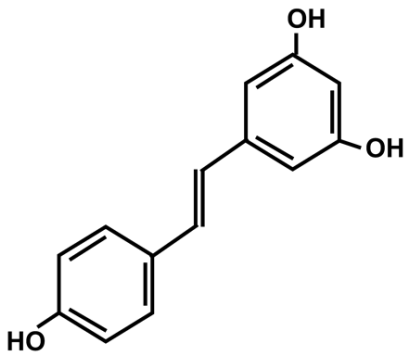

Figure 1 Structure of resveratrol

Figure 2

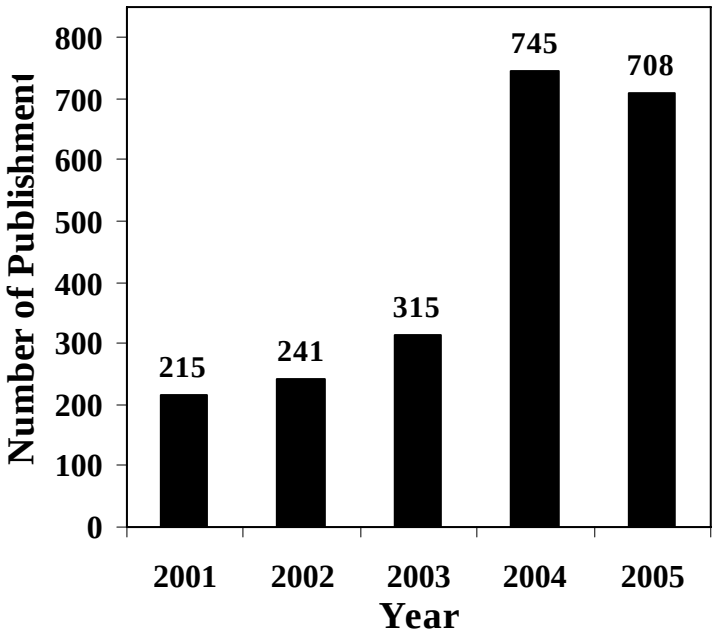

Figure 2. Number of research articles published from 2001 to 2005, relative to resveratrol. A search was conducted using key words “resveratrol” and year using Pubmed data base.

In chapter three, we explored the effect on androgen receptor (AR) as one possible mechanism for resveratrol on prostate cancer prevention. Previous studies have shown that resveratrol decreases expression of prostate-specific antigen (PSA) (12). Androgen is known to act through AR (13). This suggests that the effects of resveratrol may be mediated by the AR pathway. In this study, we demonstrated that resveratrol has an effect on multiple genes belonging to the AR-dependent pathway. Moreover, we also provide data to support that resveratrol modulates the ARG expression not only through the AR but also through the ER-mediated pathway. These results provide support for our hypothesis that resveratrol modulates multiple pathways that impact prostate cancer development, and the prostate cancer preventive effects of resveratrol may partially mediate through androgen and estrogen dependent signaling.

In addition to the androgen and estrogen pathways, there are other possible pathways influenced by resveratrol in which may contribute to prostate cancer prevention. In chapter four, we demonstrated that resveratrol can inhibit the expressions of the IGF-1 and IGF-1R at the mRNA level. IGF-1 exerts its effects through IGF-1R and is known to stimulate prostate cancer cells and is associated with an increased risk of prostate cancer (14). Thus, modulation of IGF-1 pathway may also contribute to resveratrol's prostate cancer preventive effects.

Based on the results present in this thesis, we concluded that the inhibitory effects of resveratrol on the growth of prostate cancer cell maybe mediated through modulation of AR, ER, and IGF-1 pathways. Future study may include: 1) effect of

resveratrol on normal prostate cells; 2) effect of resveratrol on AR-independent cells; and 3) an animal study to demonstrate AR, ER, and IGF pathways of resveratrol in prostate cancer carcinogenesis.

Chapter 2: Literature Review

2.1 Resveratrol

2.1.1 General Information about Resveratrol

Trans-resveratrol was first detected in grapevines (*Vitis viniferu*) in 1976 by Langcake and Pryce (15). These investigators using GLC-MS method found that resveratrol was synthesized by leaf tissues in response to fungal infection (mainly *Botrytis cinereu*) or exposure to ultraviolet light.

It is now well documented that trans-resveratrol (3, 4', 5 trihydroxystilbene) (Figure 1) is a phytoalexin produced by spermatophytes, such as grapevines, in response to injury (11). For example, in response to an invading fungus, resveratrol is synthesized by plants from p-coumaroyl CoA and malonyl CoA to repel the invading fungus (16).

2.1.2 Sources

Resveratrol is commonly found in Polygonum cuspidatum roots, but it is also presented in other plants such as eucalyptus, spruces, and lilies, as well as in common foods such as mulberries and peanuts (11). The most abundant natural sources of resveratrol are *Vitis vinifera*, *Vitis labrusca*, and *Vitis rotundifolia* grapes, which are used to make wines (11). Resveratrol exists in the grapevines, roots, seeds, and stalks, but the highest concentration is in the skin of grapes (17), which contains 50-100 μg per gram (7, 18).

The resveratrol content of wine is related to the length of time during the fermentation process with grape skin. Because the skins are removed during white wine production, the concentration of resveratrol in red wine is significantly higher than the concentration of resveratrol in white wine (17). A fluid ounce of red wine averages 160 μg of resveratrol. Other foods contain less resveratrol. For example, edible peanuts only contain an average of 73 μg resveratrol per ounce (19).

Table 1. The content of resveratrol in foods

Food	Red grapes	Red grape juice	White wines	Red wines	Peanuts (Boiled)
Resveratrol	1.5 –7.8 μg/g	1.14-8.69 mg/L	0.05-1.8 mg/L	1.98-7.13 mg/L	1.7 –7.1 μg/g

2.1.3 Resveratrol Bioavailability

Resveratrol can be absorbed and metabolized by humans and 75% of resveratrol is excreted via feces and urine (20). In human serum, the major form of resveratrol is trans-resveratrol glucuronides, rather than the free form of the compound (21). After administering a dose of 360 μg/kg body weight resveratrol either dissolved in grape juice, vegetable juice, or white wine, the plasma peak concentration of resveratrol can reach 20nM free form of resveratrol and 2μM total resveratrol (22).

Studies in mice, rats, and dogs suggest that resveratrol is well absorbed and rapidly glucuronidated and sulfated both in the liver and intestinal epithelial cells (23). Most importantly, peak plasma level of 6.6 μM unmetabolized resveratrol in the rat can be reached after a high oral dose of 50 mg/kg (24). However, it is eliminated rather rapidly (24). In contrast, glucuronidated resveratrol seems to reach much higher plasma levels than the unmetabolized resveratrol (24).

2.1.4 Biological Activities of Resveratrol

Resveratrol appeared to possess various biological activities. As mentioned above, resveratrol is synthesized in Polygonum cuspidatum roots. Polygonum cuspidatum roots have been used in traditional Asia oriental medicine (18). As a phenolic compound, resveratrol appears to contribute to the antioxidant potential of red wine and thereby may play a role in the prevention of human cardiovascular diseases (25). Resveratrol has been shown to modulate the metabolism of lipids, and to inhibit the oxidation of low-density lipoproteins and the aggregation of platelets (9). Hence, resveratrol may provide cardiovascular protection (26). Resveratrol also appears to possess anti-inflammatory and anticancer properties (8, 27). The biological activity of resveratrol related to cancer will be discussed in detail as follow.

2.2 Resveratrol and Cancer Prevention

Cancer development can be divided roughly into three stages: initiation, promotion, and progression (27). Initiation, the first stage, is thought to be a

mutagenic event that produces a cell with altered genetic code with advanced growth characteristic. The second stage, tumor promotion, is a slow and reversible, epigenetically controlled clonal expansion of the initiated cell that produces a benign tumor. Tumor progression is the irreversible conversion of the benign tumor to a malignant phenotype.

The effects of resveratrol on cancer have been explored (10). Resveratrol appeared to affect initiation, promotion as well as progression of cancer. These effects are described as follow.

2.2.1 Antioxidant Effects of Resveratrol

Tumor initiation is considered due to DNA damage (28, 29).One possible route is the control of reactive oxygen spices (ROS) in cells (30). Several studies have been conducted to examine the effect of resveratrol on initiation of cancer (31). At the level of tumor initiation, resveratrol appeared to act as an antioxidant by inhibiting free radical formation, and thus as an anti-mutagen (7).

The anti-tumor initiating activity of resveratrol may be attributed its ability to control ROS (32). Sharma et al. (33) reported that resveratrol inhibited, in a dose-dependent manner, free-radical formation (ED_{50} = 27 μM) when human promyelocytic leukemia (HL-60) cells were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA). Stojanovic et al. (34) found resveratrol acts as a free radical scavenger in the radical liposome oxidation(34). Jang et al. (35) showed resveratrol

inhibited the production of H_2O_2 and myeloperoxidase activity while it restored the cellular glutathione levels and the activity of superoxide dismutase (35). Cadenas and Barja (36) demonstrated that resveratrol effectively prevented oxidative damage in the renal DNA of rats treated with the kidney-specific carcinogen $KBrO_3$ (36).

2.2.2 Stimulation of Detoxification Enzymes by Resveratrol

In addition to scavenging reactive oxygen species, resveratrol may also turn-on de novo synthesis of genes encoding detoxification (37). Incubation of H9C2 cells, mouse hepatoma cells, with 25–100 μM resveratrol resulted in a marked enhancement in the activity of a phase II metabolizing enzyme NAD(P)H:quinone oxidoreductase in a concentration- and time-dependent manner, thereby stimulating detoxification of carcinogens (7, 38, 39). Likewise, resveratrol caused induction of NAD(P)H:quinone oxidoreductase, 10 μM resveratrol completely inhibited, in a concentration-dependent manner, the activity of the carcinogen activating enzymes cytochrome P450 (CYP)1A1/CYP1A2 induced by benzo[*a*]pyrene, an environmental aryl hydrocarbon, in microsomes and intact HepG2 cells. (40). CYP450 isozymes are a large family of detoxifying enzymes that play important roles in the metabolism of carcinogens (41). The metabolites from CYP catalyzed reaction generally activate the pro-carcinogens resulting in carcinogens that interact with the DNA of target cells (42). Resveratrol's inhibitions of CYP 1A1 and CYP1B1 are consistent with decreased pro-carcinogen action.

2.2.3 Anti-inflammatory and Anti-tumor Promoting Effects of Resveratrol

The promotional stage of carcinogenesis has been proposed as a reversible process that involves changes in cellular signal transduction cascades. This leads to abnormal expression of genes involve in the regulation of cellular proliferation and growth (32). Because of the causal relationship between inflammation and tumor promotion, enzymes that mediate the proinflammatory process, such as cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS), have been implicated in the pathophysiology of certain types of human cancers (43). Thus, substances with potent anti-inflammatory activities are anticipated to exert chemopreventive effects, particularly in the promotion stage.

Resveratrol has been reported to inhibit TPA-induced COX-2 when treated mouse with 25 μmol resveratrol in 200 μl acetone per mouse on dorsal skin in transcription level (44). In F344 rat study, *N*-nitrosomethylbenzylamine (NMBA)-induced up-regulated both COX-1 and COX-2 mRNA expression were decreased by administering resveratrol at 1–2 mg/kg in NMBA-induced esophageal tumor tissues (45). Resveratrol, in a dose-dependent manner, inhibited phorbol ester-mediated induction of COX-2 activity significantly from 2.5 μM in human mammary and oral epithelial cells (46). Moreover, resveratrol was reported to significantly inhibit the expression of COX-2 without changing the COX-1 level in the rat colonic inflammation induced by trinitrobenzenesulfonic acid (47). Inhibition of COX-2 may account for resveratrol's anti-tumor promoting effect. Topically applied resveratrol pretreatment inhibited TPA-induced COX-2 expression in female ICR mouse skin,

further supported the anti-tumor promoting potential of resveratrol (48). In addition, the anti-tumor promotional effects of resveratrol may also be related, in part, to its ability to enhance or restore gap-junctional intracellular communication in cells exposed to tumor promoting agents (49).

2.2.4 Induction of Apoptosis by Resveratrol

Apoptosis represents an important cellular protective mechanism against neoplastic transformation, eliminating a damaged cell or repressing the outgrowth of transformed cells (50). Since disruption of apoptosis promotes survival and outgrowth of damaged or initiated cells, suppression of this physiologic phenomenon may result in non-genotoxic carcinogenesis (51). Therefore, induction of apoptosis in precancerous or malignant cells is considered as another promising strategy for chemoprevention (52).

Resveratrol has been shown to trigger apoptosis in various transformed or malignant cell types by activating tumor suppressor protein p53 and specific caspases, stimulating cytochrome *c* release, up-regulating pro-apoptotic Bax or down-regulating anti-apoptotic Bcl-2 (53). A p53-dependent mechanism for resveratrol-induced apoptosis has been demonstrated. According to Huang et al. (54), resveratrol induced apoptosis of human embryonic fibroblasts that express wild type p53 in a dose-dependent manner in the range 2.5–40 μM , but not in p53 deficient cells (54). A p53-dependent pathway involving an increased expression of Bax and up-regulation

of p21 was suggested as a mechanism by which resveratrol induced apoptosis in Hep G2 cells at 10 μg/ml (~44 μM; 55). Zhou et al. (56) demonstrated that 10 μM resveratrol induced apoptosis in human esophageal cancer cells through down-regulation of Bcl-2 and up-regulation of Bax. Surh et al. (57) also have found that resveratrol-induced apoptosis in human promyelocytic leukemia cells by internucleosomal DNA fragmentation and decreased expression of Bcl-2. The mechanisms by which resveratrol induces apoptosis in various cancer cells appear to be cell type-specific only in p53-dependent cells but not in p53-independent cells (55).

2.2.5 Regulation of Cell Cycle Progression by Resveratrol

Besides induction of apoptosis, cell cycle arrest is another feature of many effective chemopreventive agents (58). Cell cycle is controlled by cyclin dependent kinases (CDKs) which are composed of two proteins, a cyclin and a kinase (59). Cell cycle progression can be halted at several points by the tumor suppressor genes through modulation of these CDKs as a result of activation in response to checkpoints sensing DNA or chromosome damage (60). If loss of cell cycle regulation occurred in an organism, it can lead to cancers (61). Any mutation that removes or otherwise modifies a checkpoint inhibitor (such as p53) or removes or modifies a transcription inhibitor (such as Rb) will lead to a loss of cell cycle regulation and lead to cancer (62). The roles of the G1 to S and the G2 to M transition and the corresponding checkpoints in the cancer development have been well established (63).

Accumulating data from various *in vitro* studies indicate that resveratrol possesses strong anti-proliferative properties. Resveratrol prevented proliferation of tumor cells by inhibiting DNA synthesis with a IC_{50} value of 8–10 μM in both murine mastocytoma P-815 cells and human myelogenous leukemia K-562 cells (64) and interfering with various stages of cell cycle progression (58). A number of studies reported that a variety of different human cancer cell lines, treated with resveratrol, arrested cell cycle in the G1/S boundary (65), in the S phase (66, 67),or in the G2/M phase (68, 69) . By using FACS, Joe et al. (70) have found resveratrol (300 μM) can induce S phase-arrest in breast cancer, liver cancer, colon cancer, esophageal cancer cell lines(70).

2.2.6 Resveratrol as Phytoestrogen and Antiestrogen

Resveratrol appears to possess estrogen-like activity and is often referred to as phytoestrogen. Estrogens, produced in ovaries and testis, have many biological effects in the body beyond the reproductive system (71) . Estrogen acts through the estrogen receptors (ERs) to exert its biological activity (71) . The estrogen receptors are localized in the nucleus and formed dimer when bound to an estrogen (71). The dimer then interacts with the Estrogen Response Element (ERE), which regulates transcription of estrogen responsive genes (71). Resveratrol was shown to bind to ER in cytosolic extracts from MCF-7 and rat uteri (72). It appeared to have a greater affinity for the beta form of ER (ERβ) than the alpha form of ER (ERα) (73). Moreover, resveratrol also exhibits antiestrogenic activity and appears to inhibit the growth of human breast cancer cells (74) .

2.2.7 Resveratrol and Prostate Cancer

Prostate cancer remains one of the most common malignancies in Western countries and is also increasing in Asian countries (75). It is the second leading cause of cancer deaths among American men (76). There are three established risk factors for prostate cancer: age, ethnic group, and family history (77). In addition, exposure to androgen is also a well documented risk factor for prostate cancer. Other hormone may also influence prostate cancer development, such as estrogen and IGF-1. It was also shown that 10 nM R1881, a synthesized androgen, can up regulate the insulin-like growth factor 1 receptor (IGF-1R) expression on prostate cancer, both LNCaP and PC3 (78), and it has been shown consistently that high levels of the circulating insulin-like growth factor 1 (IGF-1) are associated with an increased risk of prostate cancer (79). Thus, IGF-1 pathway may be involved in prostate carcinogenesis. Estrogen is another steroid hormone which has evidence in prostatic carcinogenesis (80). Estrogen has been shown to possess the ability to influence androgen-response gene expression (81).

Diet is one of possible factors that may influence the incidence of prostate cancer (4, 82). Various phytochemicals, such as genistein, lycopene, and sulforaphane, have been proposed to contribute to the prevention of prostate cancer (83). Among the phytochemicals, resveratrol has been proposed to be a candidate compound for prostate cancer prevention (5). The following sections review the effect of resveratrol on prostate cancer prevention.

2.2.7.1 Inhibition of Multistage Carcinogenesis

For the initiation, the first approached stage for the cancer prevention, of prostate cancer, resveratrol has been found to act as an antioxidant and prevent oxidative DNA damage (84). Many studies have found resveratrol can inhibit the prostate cancer cell growth by inhibition of cell cycle (85) and induction of apoptosis (86). For the cell cycle, Morris et al. (87) have found that resveratrol can inhibit both LNCaP cell, an androgen responsive prostate cancer cell, and DU145, an androgen-unresponsive prostate cancer cell growth, by induction of apoptosis when treated cells with 100 μM of resveratrol for 72 hours (87). Kumajerwala et al. (88) also found that resveratrol induced the prostate LNCaP cancer cells to enter the S phase and inhibited the DNA synthesis at concentrations above 15 μM (88). For the induction of apoptosis in prostate cancer cell, DU 145 cells were treated with resveratrol and apoptosis was measured by determining nucleosome content, and resveratrol has been found to induce serine phosphorylation of p53 and activate apoptosis in a mutant p53 prostate cancer cell line (89).

2.2.7.2 Inhibition of Androgen-Dependent Signaling by Resveratrol

Androgens stimulate proliferation and impede apoptosis of normal prostate epithelial cells as well as androgen-responsive prostate cancer cells (90). The efficiency of androgen ablation in the treatment of hormone-dependent metastatic prostate cancer is largely resulted from the induction of apoptosis of neoplastic prostate epithelial cells, with incidental apoptosis of the normal prostate epithelium

(91). The neoplastic cells are susceptible to apoptosis upon androgen withdrawal whether dormant or proliferating; this is highly significant in light of the low proliferative index characteristic of neoplastic prostate disease (91). Thus, compounds that modulate androgen-mediated pathway may be potentially effective chemopreventive agents.

Mitchell et al. (92) showed that resveratrol has anti-androgenic effects at 100 μM in the androgen-dependent human prostate cancer cell line, LNCaP (92). They found repression of AR expression, the AR-specific co-activator ARA70 and PSA. These effects of resveratrol on AR-dependent pathway correlated with cell-growth suppression and induction of apoptosis by resveratrol (92). In a related study, Hsieh & Wu (12) also found that 25 μM resveratrol can suppress PSA expression in LNCaP cells. However, they did not detectably alteration in AR expression (12). Together, these studies provide evidence that resveratrol may antagonize androgen action in prostate cancer cells but the precise mechanism remains unclear. These findings also offer preliminary evidence that cancer preventive properties of resveratrol may include modulation of androgen-dependent prostate cancer cells through androgen antagonistic activities.

2.2.7.3 Inhibition of Androgen-Independent Proliferative Signaling by Resveratrol

It is almost without exception that after the androgen ablation therapy of hormone-refractory metastatic prostate cancer, a more aggressive, hormone-unresponsive prostate cancer will appear (91).

As mentioned before, prostate cancer cell's hormone responsive status may also influence the response to resveratrol (93). The growth of DU-145, androgen-insensitive human prostate cancer cell, is inhibited by resveratrol through phosphorylation of p53 at serine 15 residue. This effect, accompanied by the activation of MAPKs, leads to enhanced p53 DNA binding (94). By using flow cytometric analysis, Hsieh et al. (86) showed that resveratrol (50-100 μM) partially disrupts the G1/S transition in three androgen-nonresponsive prostate cancer cell lines. Moreover, Hsieh et al. (86) also found that resveratrol-induced apoptosis of DU-145 cells was mediated by blocking the expression of the heat shock protein 70 at 50-100 μM.

2.2.8 Hypothesis of Thesis

The documented effects of resveratrol on multistage carcinogenesis as well as *in vitro* effects on prostate cancer cell have prompted proposal of resveratrol as a candidate for prostate chemoprevention. However, the precise mechanisms of

resveratrol action in prostate cancer cells remain unclear. The current thesis seeks to address this question. Based on available literatures, we hypothesized that

1) Resveratrol modulates multiple pathways in prostate cancer cells.

2) The growth inhibitory effects of resveratrol are mediated in part through modulation of AR-mediated pathway.

3) In addition to androgen, modulation of ER- and IGF-1- mediated events by resveratrol may also contribute to the prostate cancer preventive effects of resveratrol.

Chapter 3: Resveratrol Modulates Androgen Responsive Genes through Both the Androgen Receptor and the Estrogen Receptor Pathways in the Androgen Responsive Cell LNCaP

3.1 Abstract

Resveratrol is a phytochemical that has been considered for use as a prostate cancer chemopreventive agent. This study examines the mechanism by which resveratrol modulates gene expression in LNCaP prostate cancer cells. Expression of androgen- and estrogen-regulated genes was measured in LNCaP cells cultured in the presence or absence of hormonal stimulation and the presence or absence of resveratrol. The results show that resveratrol suppresses expression of androgen-responsive genes including PSA and Ste20-related proline-alanine rich kinase (SPAK) in LNCaP cells grown in 10% FBS. However, resveratrol has little or no effect on basal expression of β-2 microglobulin (B2M) and selenoprotein P (SEPP1) under same cell growth conditions. LNCaP cells cultured in the presence of R1881, a synthesized androgen, expressed high levels of PSA, SPAK, B2M and SEPP1. The R1881-induced expression of these genes is uniformly blocked by resveratrol. For PSA and SPAK, resveratrol also blocks or down-regulates E2-induced transcription. These results indicate that resveratrol selectively alters the expression of ARGs in LNCaP cells, by modulating both androgen- and estrogen-induced signaling pathways in these cells.

3.2 Introduction

Prostate cancer is the second leading cause of cancer death in American men (95). Conventional therapy and surgical approaches have not significantly reduced the incidence, morbidity or mortality associated with this cancer. Androgen deprivation therapy for metastatic prostate cancer has been used to delay disease progression, but tumors often become androgen-independent, leading to a relapse within 2 years. Because there is no effective cure for prostate cancer, many current research efforts are focused on preventive approaches that reduce disease incidence. Bioactive food components are increasingly being evaluated as potential chemopreventive agents (96, 97) . One such agent is resveratrol, a phytochemical which has been considered as a chemopreventive compound for human prostate cancer (5).

Resveratrol is a polyphenol (3, 4, 5-trihydroxystilbene), categorized as phytoalexin, it is produced in plants exposed to environmental stress or pathogenic attack (98), and it is found principally in the skin of grapes, peanuts and other plant species (99). Red wine contains 1-10 mg of resveratrol/L (4-40 μM; 100). Published studies suggest that resveratrol may inhibit all three major stages of carcinogenesis: initiation, promotion, and progression (7, 99). At the molecular level, resveratrol was shown to possess antioxidant property (101) and anti-inflammatory property (8), and is weakly estrogenic (100).

Additional studies are needed to assess the potential usefulness of resveratrol in treating or preventing human prostate cancer. In particular, its mechanism of action remains unclear. Initial studies suggest that resveratrol inhibits cell growth in culture

(85, 102), inhibits DNA synthesis (88), and increases apoptosis in LNCaP cells (89). It has been suggested that resveratrol may increase expression and serine phosphorylation of p53 (103), thus activating p53-dependent signaling. Resveratrol also decreases expression of PSA (85). This result suggests that the effects of resveratrol may be mediated by the AR. AR is a member of the nuclear receptor super family that mediates the cells response to androgenic hormones (104). The androgen/AR complex binds to AREs in the promoter region of target genes and cooperates with coactivators to facilitate transcription of ARGs. ARGs such as PSA play important cellular functions including cell cycle regulation, transcription, cell proliferation, differentiation, and metabolism (105, 106). The overall effect of androgens is proliferative and anti-apoptotic, and exposure to androgens is considered as a risk factor for prostate cancer (107).

The present study tests the hypothesis that resveratrol affects multiple pathways to impact prostate cancer development, and the aspects of many resveratrol effects are mediated partly by AR- or ER- dependent signaling. Our experimental results support this hypothesis. Furthermore, we showed that resveratrol has selective effects on ARGs in androgen-responsive human LNCaP prostate cancer cells.

3.3 Materials and Methods

3.3.1 Chemicals

Resveratrol, 17β-estradiol, and dimethylsulfoxide (DMSO) were from Sigma Chemical Co. (St. Louis, MO). The R1881, a synthesized androgen, was from NEN Life Science Products (Boston, MA). The pure antiestrogen ICI 182,780 was from Tocris Cockson, Inc. (Ellisville, MO).

3.3.2 Cells and Cell Culture

LNCaP human prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Media A (RPMI 1640 medium (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Sigma), 100 U/mL penicillin and 100 μg/mL streptomycin (BioSource International, Camarillo, CA)) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Cells were incubated in the presence of 5% CO_2 in air at 37 ˚C. LNCaP cells were cultured at $1x10^6$ cells/well in 6-well plates for gene expression studies and at $5x10^4$ cells/well in 24-well plates for sulforhodamine B (SRB) assay.

3.3.3 Cell Growth Assay

Cell growth was analyzed using the SRB assay as described previously (108). LNCaP cells ($5x10^4$ cells/well) were treated with 0 (vehicle, DMSO), 1, 5, 10, 25, 50 μM resveratrol for 0-96 hours in 24-well plates (Costar). The medium was replaced every 24 hours. The cell number was determined using the SRB method. For

experiments with R1881 or E2, cells were switched to media B (RPMI 1640 medium without phenol red, 2 mM L-glutamine (Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin) with 10% CDS (Charcoal Dextran-treated FBS; Hyclone, Logan, UT) 24 hours after plating to minimize the effect of serum hormones.

3.3.4 ARG Expression in Cultured Cells

LNCaP cells were plated in 6-well plates (1.0×10^6 cells/well) and switched to media B with 10% CDS 24 hours after plating cells to minimize the effect of serum hormones. Twenty-four hours later, we exchanged fresh media containing 1 nM R1881 or E2 with or without 25 µM resveratrol or 10 µM ICI 182,780. Fresh media with test compounds were added after 24 hours and cells were harvested after 48 hours in the presence of test compounds. For time course experiments, samples were taken up to 72 hours and RNA was harvested every 24 hours using the Trizol method (Invitrogen). Messenger RNA of ARGs was quantified by TaqMan real-time PCR as described previously (109). Briefly, 1 microgram of total RNA was reverse-transcribed using the Stratascript First Strand cDNA Synthesis Kit (Stratagene, La Jolla, CA). RT-PCR was then performed using the TaqMan universal PCR Master mix (Applied Biosystems, Foster City, CA) on an ABI Prism 7000 thermocycler (Applied Biosystems) following the manufacturer's protocol. Each 25 µl PCR reaction contained 2.5 µl of cDNA. Results were calculated relative to untreated control using the Ct method as described in the manufacturer's protocol. All experiments were repeated three times and each PCR reaction was run in triplicate.

3.3.5 Western Blot Analysis

LNCaP cells were plated on a 100 mm Falcon tissue culture plate ($2x10^6$cells/plate) in media A containing 10% FBS. After 24 hours, the media were changed to media B with 10% CDS and the cells were incubated for an additional 24 hours. After the incubation, the cells were then treated with either 1 nM R1881 or 1 nM E2 in the presence or absence of 25 μM resveratrol. Media containing the test compounds were replaced every 24 hours. Cells were treated with the test compounds for 96 hours. After treatment the cells were washed once with Phosphate Buffered Saline (PBS, pH 7.4) and harvested into a 1.5-mL micro-tube with a RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA) following the manufacturer's protocol. The protein concentration of cell lysate was determined by DC® protein assay (BioRad, Richmond, CA). Lysate protein (60 μg) was separated on Ready Gel® precasted gels (10 well, 15 % resolving Tris-HCl, Bio-Rad, Hercules, CA) and transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA) according to Bio-Rad's protocol. For immunodetection, the membrane was blocked in blocking buffer (1xPBS/Casein Blocker containing 0.1% Tween 20, Bio-Rad, Hercules, CA) for 2 hours at room temperature. The membrane was then washed four times (5 min each) with washing buffer (1x PBS containing 0.1% Tween 20). It was then incubated at 4 ^oC overnight with primary rabbit antibody for PSA (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:10,000 dilution in blocking buffer. After primary antibody incubation, the membrane was washed four times (3 times at 5 min each and one time for 15 min) with washing buffer. Subsequently, the membrane was incubated for 2 hours at room temperature with anti-rabbit horseradish peroxidase secondary antibody (Santa Cruz Biotechnology, Santa cruz, CA) at 1:20,000 dilution in blocking

buffer. After secondary antibody incubation, the membrane was washed with washing buffer for four times (5, 5, 10 and 15 min each) and visualized using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, lL) following the manufacturer's instructions. The image detection is used the Sensicam CCD camera (Cooke Cooperation, Eugene, OR) with LabWorks software and integrated into the Epi Chemi II imaging workstation from UVP (UVP, Upland, CA).

3.3.6 Statistics

StatView (SAS Institute Inc., Cary, NC) software was used for statistical analysis. Multiple group data were analyzed using ANOVA followed by post-hoc analysis with Fisher's PLSD test. The unpaired Student's t-test was used to compare experiments within two groups. Values were considered significant when $p<0.05$.

3.4 Result

3.4.1 Effects of Resveratrol on LNCaP Cell Growth

This study examines the effects of resveratrol on growth and gene expression in human LNCaP prostate cancer cells *in vitro.* LNCaP cells were cultured in the presence of 10% FBS and 0, 1, 5, 25 or 50 μM resveratrol for 0-96 hours. Cell number was defined as the description in Materials and Methods. Growth inhibition was concentration- and time-dependent at concentrations below 50 μM or time points shorter than 96 hours and growth was significantly inhibited at concentrations as low as 10 μM resveratrol (Figure 3B). Resveratrol can inhibit LNCaP cell growth after 24

hours at 50 μM. When treated the LNCaP with 25 μM resveratrol, we can see the inhibition after 72 hours.

3.4.2 Effects of Resveratrol on p53-dependent Pathway

Previous studies suggested that the effects of resveratrol may be mediated by p53 or p53 –dependent processes (89). Here, this idea was tested by examining expression of p21 mRNA, which is tightly regulated by p53 (103). Figure 3 shows time and dose-dependent measurements of p21 mRNA in LNCaP cells exposed to resveratrol. The results show that p21 mRNA increases significantly after 24 hours exposure to 25 μM resveratrol, but not at lower resveratrol concentrations (Figure 3C). At 25 μM, resveratrol significantly induces p21 mRNA by 24 hours and the level of p21 mRNA remains elevated for at least 48 hours (Figure 3D).

Figure 3

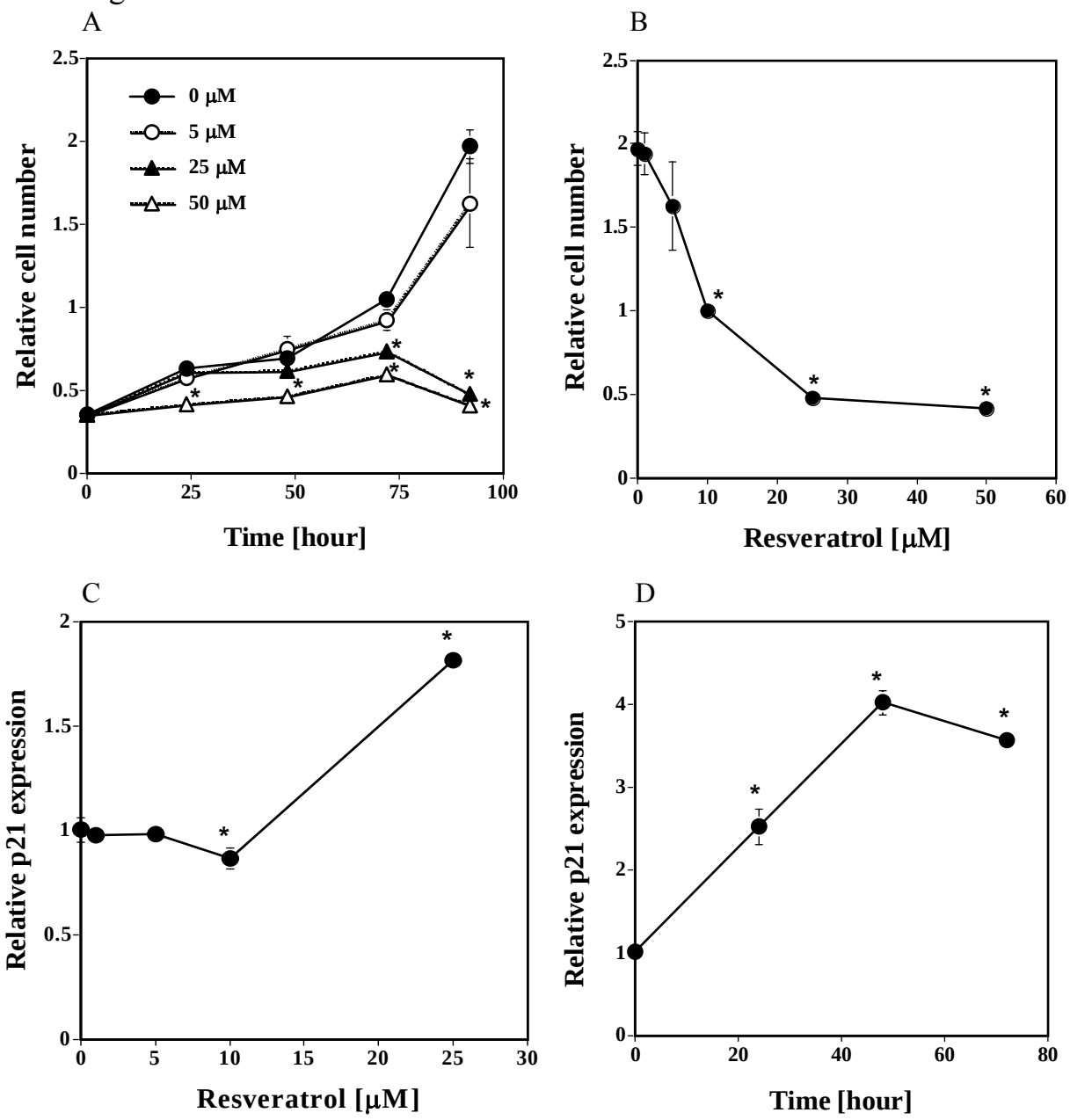

Figure 3. Effect of resveratrol on cell growth. LNCaP cells were treated with a vehicle (DMSO) or resveratrol for 0-96 hours in a medium containing 10% FBS. Cell growth was measured by the SRB assay. Values are expressed as mean ±SD. Asterisks indicate statistical significance using ANOVA followed by post-hoc analysis ($p<0.05$). (A) Cells were grown in the presence of the indicated concentration of resveratrol and aliquots were taken at 24 hours intervals from 0 to 96 hours for measurement of A_{530}. (B) Cells were grown in the presence of 0, 1, 5, 10, 25 or 50 μM resveratrol and incubated for 96 hours. (C) LNCaP cells were treated with 0 (vehicle control), 1, 5, or 25 μM resveratrol for 0-72 hours, total RNA isolated and analyzed for p21 mRNA by real-time PCR. Values are expressed as mean ±SD. Letters indicate statistical significance using ANOVA followed by post-hoc analysis ($p<0.05$). Cells were grown in the presence of the indicated concentration of resveratrol for 72 hours. (D) Cells were grown in the presence of 25 μM resveratrol and aliquots were taken at 24 hour intervals from 0 to 72 hours.

3.4.3 Differential Effects of Resveratrol on Expression of Androgen-Responsive Genes

Because resveratrol inhibits growth of LNCaP cells at 10 μM, which is lower than the apoptosis inducing concentration, additional mechanism maybe involved in order to explaining this phenomenon. Given growth of the prostate cancer cells required androgen (110), we hypothesized that resveratrol may act through modulation of androgen-mediated events to inhibit the LNCaP cell growth when treated the cells in 10% FBS. Furthermore, resveratrol might act as an anti-androgenic compound to inhibit the androgen-responsive effects. This idea was tested by measuring an expression of four androgen-responsive genes (ARGs): prostate specific antigen (PSA), Ste20-related Proline-Alanine rich Kinase (SPAK), β-2 microglobulin (B2M) and Selenoprotein P (SEPP1; 105) in LNCaP cells exposed to resveratrol. Cells were grown in 10% FBS and treated with 25 μM resveratrol for 48 hours. Figure 4A shows that resveratrol inhibits expression of PSA and SPAK under these conditions. More detailed analysis shows that resveratrol inhibits expression of SPAK in a dose- and time-dependent manner (Figure 4B) and at concentrations as low as 5 μM. In contrast, resveratrol has no significant effect on the expression of B2M and SEPP1 (Figure 4C). However, different results were observed when cells were cultured in 10% CDS, which is depleted of serum factors including steroid hormones. Under these conditions, moderate or no reduction of PSA (Figure 5A) and SPAK mRNA (Figure 5B) is observed after incubation in the presence of 25 μM resveratrol for 48 hours. This result suggests that the effect of resveratrol on ARG

expression may be modulated by the presence or absence of hormonal factors other than androgen in serum.

Figure 4

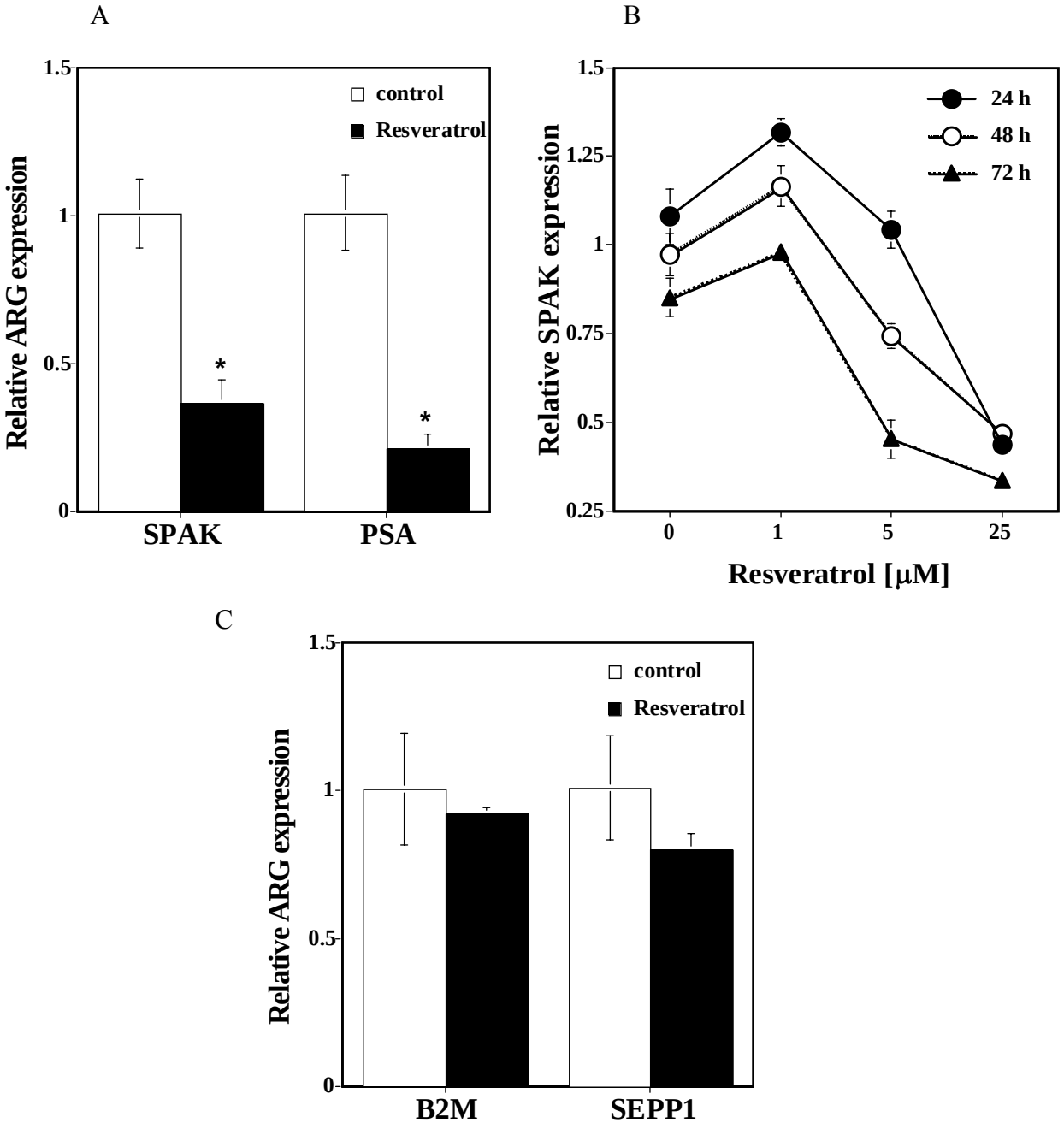

Figure 4. Effects of resveratrol on ARGs in LNCaP cells cultured in media containing 10% FBS. LNCaP cells were treated with 25 μM resveratrol for 48 hours, total RNA was isolated and relative mRNA expression of ARGs was measured by real-time PCR. Values are normalized to control cells and expressed as mean ±SD. (A) Expression of PSA and SPAK. (B) Cells were incubated in the presence of 0, 1, 5, or 25μM resveratrol and aliquots were taken at 24 hours intervals from 0 to 72 hours. SPAK mRNA was quantified by PCR. (C) Expression of B2M and SEPP1. Asterisk indicates values that are significantly different from control using ANOVA followed by post-hoc analysis ($p<0.05$).

Figure 5

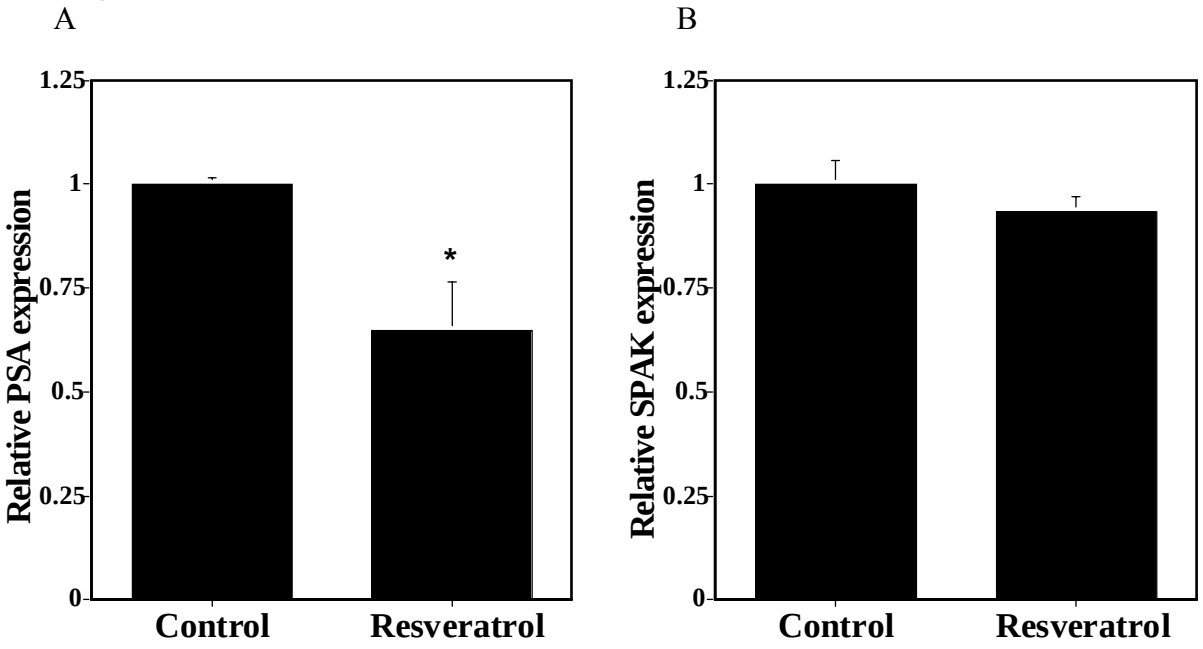

Figure 5. Effects of resveratrol on ARGs in LNCaP cells cultured in media containing 10% CDS. LNCaP cells were treated with 25 μM resveratrol for 48 hours, total RNA isolated and relative mRNA expression of PSA or SPAK was measured by real-time PCR. Values are normalized to control cells and expressed as mean ±SD. (A) PSA; (B) SPAK. Asterisk indicates that values are significantly different from control using ANOVA followed by post-hoc analysis ($p<0.05$).

3.4.4 Resveratrol Inhibits Synthetic Androgen R1881-induced Gene Expression

If the effect of resveratrol on ARGs is modulated by hormones, then it may act through the androgen-response pathway, which is critical for prostate cancer cell growth. This possibility was examined by growing cells in 10% CDS in the presence of R1881, induces ARG expression, and then determining the impact of exposure to resveratrol. Figure 6A shows that the mRNA expression all four ARGs, PSA, SPAK, B2M, and SEPP1, are significantly induced by R1881, but PSA and SPAK are induced more strongly than B2M and SEPP1 at mRNA level. Under these conditions, 25 μM resveratrol inhibits mRNA expression of PSA, SPAK, B2M, and SEPP1. To correlate the transcriptional effect with protein expression, we examine the effect of resveratrol on PSA expression, as illustrated in Figure 6B, resveratrol inhibited the R1881-induced PSA expression as what we found in the result of mRNA.

3.4.5 Effects of Resveratrol on R1881-induced Cell Growth in LNCaP Cells

To correlate the effects on ARG mRNA and protein to cell growth end point, we also examine if resveratrol also inhibits androgen-stimulated cell growth. LNCaP cells were plated in 10% CDS with 1 nM R1881 and 0, 1, 25 or 50 μM resveratrol. The results show that resveratrol inhibits cell growth induced by R1881 in a concentration dependent manner and at as low as 1μM (Figure 6C).

Figure 6

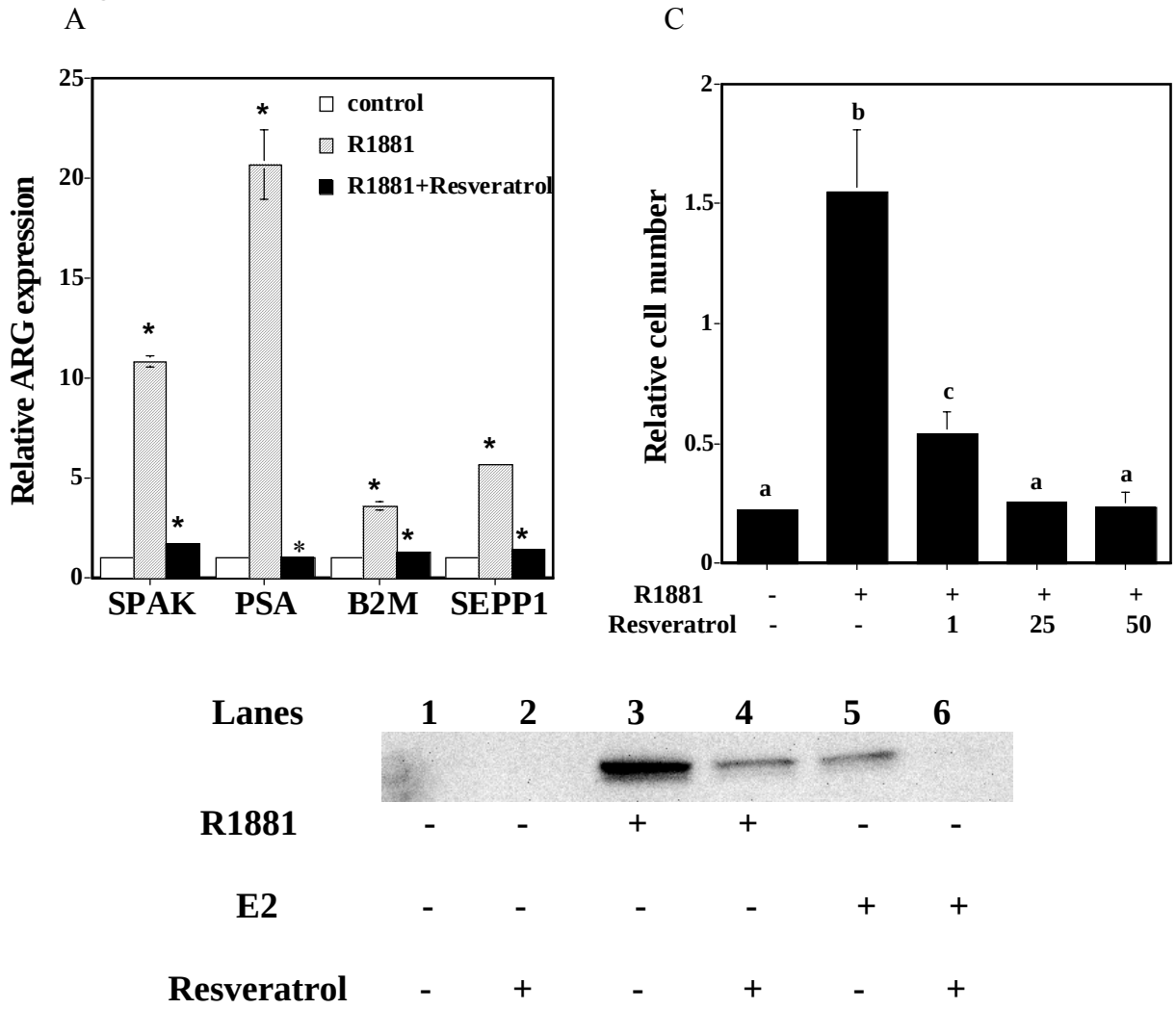

Figure 6. (A) Effects of resveratrol on R1881 inducted mRNA expression of ARGs in LNCaP cells. LNCaP cells were treated with R1881 in presence or absence of 25 μM resveratrol for 48 hours in media containing 10%CDS, total RNA isolated and relative mRNA expression of ARGs was measured by real-time PCR. Values are normalized to control cells and expressed as mean ±SD. Letters indicate values that are significantly different from control using ANOVA followed by post-hoc analysis ($p<0.05$). (B) Western blot result of inhibitory effects of resveratrol on R1881 and E2 induction of PSA in LNCaP cells. Lane 1: control, treated with DMSO; Lane 2: 25 μM resveratrol; Lane3: 1 nM R1881; Lane 4: 1 nM R1881+25 μM resveratrol; Lane 5: 1 nM E2; Lane 6: 1 nM E2+25 μM resveratrol. LNCaP cells were treated for 96 hours. (C) Inhibitory effects of resveratrol on R1881 induced LNCaP cell grwoth. LNCaP cells were treated with/without R1881 and 0 (vehicle), 1, 25, 50 μM resveratrol for 96 hours in 10% CDS medium. Cell growth was measured by the SRB assay. Values are expressed as mean ±SD. Letters indicate values that are significantly different using ANOVA followed by post-hoc analysis ($p<0.05$).

3.4.6 Resveratrol Inhibits ARGs mRNA Level Induced by E2 in 10% CDS

Previous studies showed that E2 stimulates expression of PSA and that the phytoestrogen, genistein, also influences ARG expression in LNCaP cells (91). This suggested that estrogen-mediated events might also play a role in the effects of resveratrol on ARGs in LNCaP cells treated in 10% FBS. This idea was examined by growing LNCaP cells in 10% CDS with or without E2 in the presence or absence of 25 μM resveratrol. Figure 7 shows that E2 induces expression of PSA (A), but not SEPP1 (B), and this effect of E2 can be blocked by resveratrol or the anti-estrogen ICI 182,780. Consistent with mRNA results, at the protein level PSA protein expression was also induced by E2 and inhibited by resveratrol (Figure 7B). Similar to R1881, resveratrol also inhibits estradiol-stimulated growth of LNCaP cells. Figure 7C shows that 1 nM E2 stimulates LNCaP cell growth by approximately 2-fold, but 5 μM or higher resveratrol inhibits this effect. Resveratrol inhibits E2-stimulated cell growth in a dose-dependent manner from 1 to 50 μM. A significant effect can be observed at 5 μM.

Figure 7

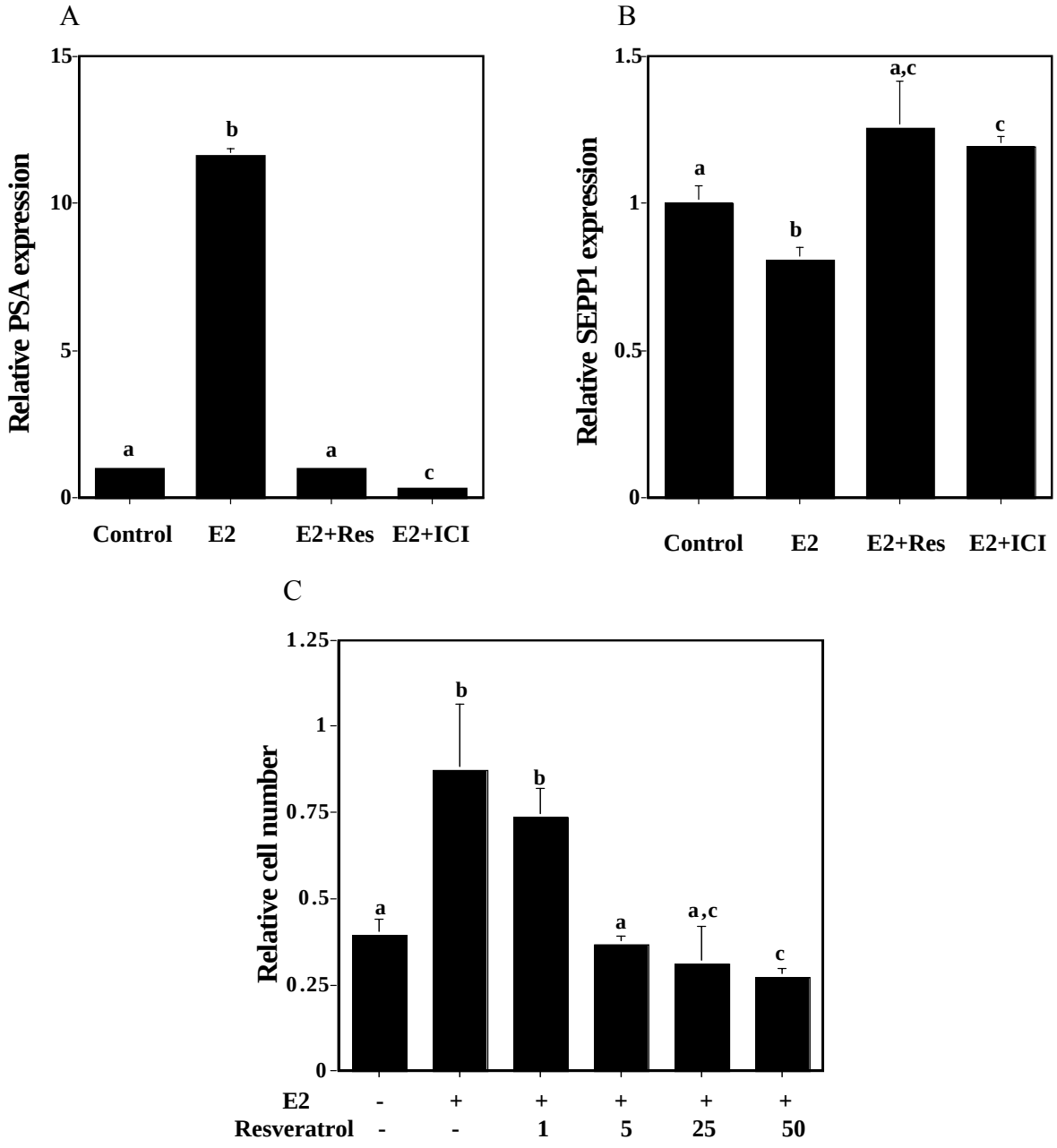

Figure 7. Inhibitory effects of resveratrol and ICI 182,780 on estrogen induction of ARGs in LNCaP cells cultured in media containing 10% CDS. LNCaP cells were treated with E2 (1nM) in presence or absence of 25 μM resveratrol or 10 μM ICI 182,780 for 48 hours, total RNA isolated and relative mRNA expression of ARGs was measured by real-time PCR. Values are normalized to control cells and expressed as mean ±SD. (A) PSA. (B) SPAK. Letters indicate values that are statistically significant using ANOVA followed by post-hoc analysis ($p<0.05$). (C) Inhibitory effects of resveratrol on estrogen- induced LNCaP cell growth. LNCaP cells were treated with vehicle or with E2 in presence of 0 (vehicle), 1, 25, 50 μM resveratrol for 96 hours in media containing 10% CDS. Cell growth was measured by the SRB assay as described in materials and methods. Values are expressed as mean ±SD. Letters indicate values that are significantly different from control using ANOVA followed by post-hoc analysis ($p<0.05$).

3.5 Discussion

This study examines the effects of resveratrol on growth and gene expression in the androgen-responsive prostate cancer cell, LNCaP. The results suggest that resveratrol, a phytochemical with the potential as a chemopreventive agent, modulates hormonally-regulated cell growth and gene expression.

Data presented here show a novel effect of resveratrol on the expression of androgen-regulated genes. In particular, when grown in the presence of 10% FBS, resveratrol inhibits the expression of PSA and SPAK, but not B2M and SEPP1 (Figure 4). To our knowledge, this is the first demonstration that resveratrol differentially alters the expression of ARGs in LNCaP cells. This effect seems to be due to the differential responses of these genes to the steroid hormones, androgen and estrogen. In steroid hormone-deprived condition, R1881 induces the expression of PSA, SPAK, B2M and SEPP1 in LNCaP cells, and resveratrol blocks this androgen-dependent expression in all cases. By contrast, E2 can stimulate the expression of PSA in 10% CDS (Figure 7A), but not the expression of SEPP1 (Figure 7B). Thus, the lack of inhibitory effects in cell culture in 10% FBS may be due in part to the presence of estradiol. These results support that the culturing condition may greatly affect molecular end points of resveratrol and interpret of the biological activity of resveratrol. Resveratrol may be a general inhibitor of androgen receptor-mediated events. For example, resveratrol strongly down regulates PSA, a well-known ARG and a marker for prostate cancer in clinical diagnosis (111). Although regulation of PSA by resveratrol has been documented (12, 92), little is known of the regulation of other ARGs, such as SPAK, B2M, and SEPP1. Using SPAK, a novel androgen-

regulated gene whose function is not well known, we demonstrated that it is down regulated by resveratrol in a dose- and time- dependent manner (Figure 4B). In addition, we also showed the effect of resveratrol on ARG mRNA is correlated with protein levels. Our western blotting data showed that R1881-induced PSA protein level can be inhibited by resveratrol (Figure 6B), consistent with Hsieh et al. (12). The antibodies of SPAK, B2M, and SEPP1 can not find in the market or can not get the western blot concentrations, so we assume there is a linear relationship between protein and mRNA level. Our research demonstrated resveratrol's inhibition of androgen-stimulated growth of LNCaP cells (Figure 6C). In addition to an effect on an AR-mediated event, modulation of AR-mediated, lends further support to the event by resveratrol as a potential mechanism. We found resveratrol significantly inhibited R1881-induced the growth of LNCaP cell started at 1 μM. One microM is reachable in human's body after oral intake 360 μg/ kg body weight of resveratrol. Other researches focused on the function of higher concentration resvertrol, but *in vivo* study showed 75% of resveratrol is excreted via feces and urine (20), and very less resveratrol shows as free form (21). In human serum, the major form of resveratrol is trans-resveratrol glucuronides (21). For the future study, it will be necessary to know whether trans-resveratrol glucuronides also has the effect as free resveratrol does.

Another novel observation was finding that resveratrol modulates estrogen-stimulated events in LNCaP cells, as described above (Figure 7C). This is consistent with the fact that E2 may play an important role in normal prostate physiology and in the development of prostate cancer (112). Both PSA and SPAK are induced by E2

and this effect, as well as the E2 induced cell proliferation, is inhibited by resveratrol (Figure 7B, C). Resveratrol is considered as a phytoestrogen and a mixed agonist/antagonist for estrogen receptor α and β (72, 113). Interestingly, at a concentration of 1 μM, resveratrol slightly induces expression of SPAK and may be acting as an ER agonist, while at 5 μM or higher, resveratrol acts as an antagonist and suppresses E2 induced transcription of SPAK (Figure 4B). This is same as what Matsumura et al. (114) found that resveratrol acts as an ER agonist at low concentration (~1 μM), but becomes an ER antagonist at higher concentration.

In addition to AR and ER-mediated event, the inhibitory effect of resveratrol on prostate cancer cell growth may in part be mediated by p53 or p53-dependent events (103). This is consistent with our observation that 25 μM resveratrol induces p21 expression in LNCaP cells. There appears to be a threshold for resveratrol-dependent activation of p21, because no activation was observed at lower doses. This may be relevant when considering pharmacological uses of resveratrol. In particular, it was reported that oral dosing of rats with 50 mg/kg resveratrol can lead to a plasma concentration of ≥10 μM resveratrol glycogen within 8 hours (101).

Figure 8 presents a summary of our findings regarding the effects of resveratrol on prostate cancer growth. We propose that resveratrol exerts concentration-dependent effects on multiple cellular pathways. At low (~5 μM) concentration, resveratrol can modulate steroid hormone-mediated events including hormone-dependent gene expression. At higher concentration, additional responses may occur, such as activation of the p53/p21 pathways. These events allow

resveratrol to inhibit cell growth and may explain the chemopreventive effects of resveratrol on prostate cancer.

Figure 8

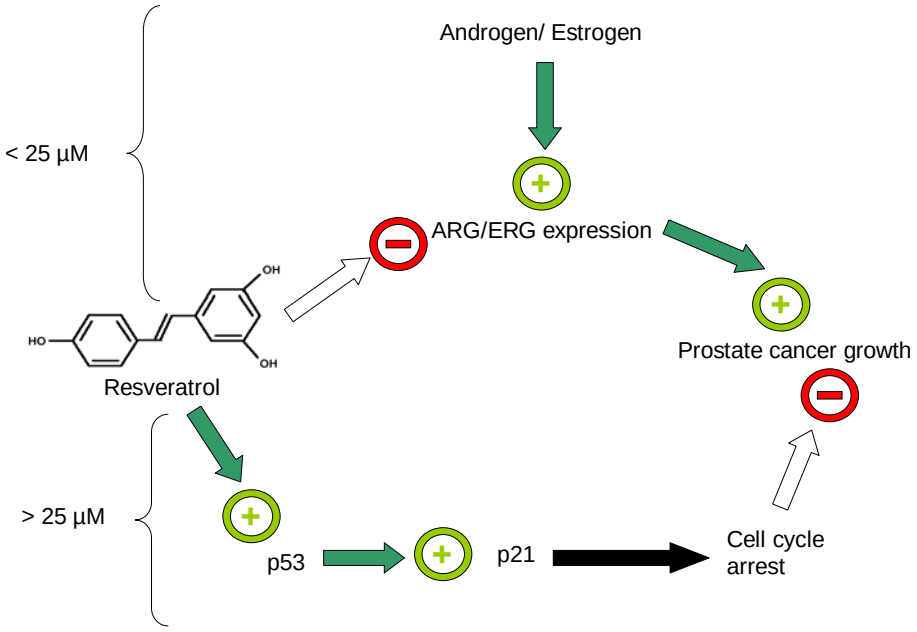

Figure 8. Summary of effects of resveratrol on LNCaP cell growth and gene expression. ARG: androgen-responsive gene; ERG: estrogen-responsive gene.

Chapter 4: Modulation of IGF-1 Pathway by Resveratrol in Human Prostate Cancer LNCaP Cells

4.1 Abstract

Resveratrol is a potential prostate cancer chemopreventive agent. This study examines modulation of IGF-1 pathway as a mechanism by which resveratrol may exert its molecular effect in LNCaP prostate cancer cells. Resveratrol suppressed the expression of IGF-1 and IGF-1R mRNA in LNCaP cells cultured in 10% FBS at 5 μM. LNCaP cells cultured in the presence of R1881, a synthetic androgen, demonstrated induction of both IGF-1 and IGF-1R mRNA levels. This R1881-induced expression of IGF-1 and IGF-1R mRNA were completely blocked by treatment of cells with 25 μM resveratrol. The estrogen, 17β-estradiol (E2), also induced transcription of IGF-1 and IGF-1R mRNA, but has less potent than the R1881. The effect of E2 on IGF-1 and IGF-1R mRNA was also partially inhibited by treatment of cells with 25 μM resveratrol. These results support the hypothesis that resveratrol may exert a effect on IGF-1 and IGF-1R mRNA in LNCaP cells through the modulation of both androgen- and estrogen-induced signaling pathways.

4.2 Introduction

The cytokine, insulin-like growth factor 1 (IGF-1), is required for normal growth of prostate (115). IGF-1 exerts its biological effect through receptor-mediated events that involve signal transduction through protein phosphorylation (14). The

major receptor that interacts with IGF-1 in prostate cancer cells is the IGF-1R. IGF-1R is a transmembrane tyrosine kinase that is widely expressed in many cell types. More importantly, IGF-1R is often over expressed in cancer cells (116). Recent epidemiological and experimental studies showed that IGF-1 also plays an important role in prostate carcinogenesis (117). High levels of IGF-1 in serums of humans were found to be associated with increased risk of prostate cancer (118). In addition, several groups reported that IGF-1 treatment can lead to enhancement of the growth of prostate cancer cells regardless of their androgen dependency (117, 119). Calorie restriction diet, which reduced serum IGF-1 level, appears to be associated with decreasing the risk of prostate cancer in humans (118), further supporting a role of IGF-1 in prostate carcinogenesis. Therefore, available literatures consistently support that IGF-1 may increase prostate cancer risk through increasing growth of cancer cells (79). However, the mechanism by which IGF-1 promotes prostate cancer remains still largely unknown.

The male sex hormone, androgen, is required for normal prostate cell growth and development (106). However, androgen is also known to be associated with prostate carcinogenesis (106). Androgen has been showed to up-regulate IGF-1R protein expression and sensitize prostate cancer cells to the biological effects of IGF-1 (78). Moreover, IGF-1 is also known to modulate the AR-dependent process in normal and cancer human prostate cell lines (120, 121). Thus, the interaction between AR and IGF-1 pathways exists. In addition to androgen, female steroid hormone, estrogen, also stimulated the proliferation of the prostate (112). Previous study showed that 1 μM estradiol regulates the IGF-1 gene transcription in HepG2 cell, a

liver carcinoma cell line (122). IGF-1 has been shown to modulate the ER-dependent process (120) and activate ER-mediated gene transcription (123). Thus, similar to androgen, the interaction between ER and IGF-1 pathways also exists. However, the effect of estrogen on the IGF-1 pathway in prostate cells is not known. To test this, LNCaP cells were treated with 1 nM E2 in the presences or absence of 10 μM ICI 182,780, a pure anti-estrogen. ICI 182,780 acts through competing with E2 binds with ER.

Dietary factors are recognized as determinants of circulating IGF-1. For example, energy and/or protein deprivation markedly decrease serum IGF-1 concentrations in human body (124). In our laboratory, previous data showed that the phytoestrogen, genistein, down-regulated IGF-1 and IGF-1R mRNA levels in microarray experiments (109). Both genistein and resveratrol are phytoestrogens, and share some similar effects on AR and ER mediated pathways as reported in chapter 3. Resveratrol has been showed to influence the IGF-1 expression in the human breast cancer by regulating IGF-1R mRNA (125). Hence, we hypothesized that the inhibitory effect of resveratrol on prostate cancer cell growth may in part occur through modulating the components of IGF-1 pathway. Furthermore, we proposed that resveratrol may exert its effect on IGF-1 and IGF-1R through both AR- and ER-dependent pathways.

4.3 Materials and Methods

4.3.1 Chemicals

Resveratrol, E2, and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). The R1881, a synthesized androgen, was purchased from NEN Life Science Products (Boston, MA). The pure antiestrogen ICI 182,780 was from Tocris Cockson, Inc. (Ellisville, MO).

4.3.2 Cell and Cell Culture

The LNCaP human prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained in a RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Invitrogen), 2 mM L-glutamine (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (BioSource International, Camarillo, CA). Cells were incubated in the presence of 5% CO_2 in air at 37 ˚C.

4.3.3 Cell Culture for the Detection of IGF-1 and IGF-1R Gene Expression

To assess the effect of steroid hormones and resveratrol on IGF-1 and IGF-1R gene expression, LNCaP cells were plated in six-well plates (0.5×10^6 cells/well). For the resveratrol-only experiment, the cells were cultured in 10% FBS in RPMI1640 medium. After 24 hours, cells were treated with 0, 1, 5, or 10 μM resveratrol for 72 hours.

For androgen and estrogen experiments using R1881 and E2, respectively, the cells were switched to a RPMI 1640 medium without phenol red, with 10% Charcoal Dextran-treated FBS (CDS; Hyclone, Logan, UT) 24 hours after plating cells, to minimize the effect of serum hormones. The cells were incubated with 1 nM of R1881 or E2 either in the presence or absence of 25 μM resveratrol or 10 μM ICI 182,780, and media were replaced with test compounds every 24 hours. After 48 hours following addition of test compounds, the cells were harvested. RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instruction. Quantization of mRNA expression was performed using real-time PCR as described below.

4.3.4 Semi-quantitative Determination of mRNA Levels.

The TaqMan real-time RT-PCR method was used to quantify gene expression. Briefly, one microgram of total RNA was reverse-transcribed using the Stratascript First Strand cDNA Synthesis Kit (Stratagene, La Jolla, CA). RT-PCR was then performed using the TaqMan universal PCR Master mix (Applied Biosystems, Foster City, CA) on an ABI Prism 7000 thermocycler (Applied Biosystems) following the manufacturer's protocol. Each 25 μl PCR reaction contained 2.5 μl of cDNA. Results were calculated relative to untreated control using the Ct method as described in the manufacturer's protocol. All experiments were repeated three times and each PCR reaction was run in triplicate. Real-time PCR primers and probes for IGF-1 (GenBank accession No. NM_000618) and IGF-1R (GenBank accession No. NM_000875) were obtained commercially (Assays-on-Demand, Applied Biosystems).

4.3.5 Statistical Methods

For statistical analysis, StatView (SAS Institute Inc., Cary, NC) software was used. Multiple group data were analyzed using ANOVA followed by post-hoc analysis with Fisher's PLSD test. The unpaired Student's t-test was used to compare experiments within two groups. The significance level was set at $p < 0.05$ in all cases.

4.4 Results

4.4.1 Resveratrol Inhibits the Expression of IGF-1 and IGF-1R mRNA in LNCaP Cells

To test our hypothesis that resveratrol can modulate IGF-1 mediated pathways, we first asked whether resveratrol can affect the mRNA expression of IGF-1 and IGF-1R. We found treatment of LNCaP cells with resveratrol leads to inhibition of IGF-1 mRNA level (Figure 9). The inhibition of the IGF-1 mRNA level occurred at a does-dependent manner, with a significant effect of resveratrol on IGF-1 mRNA observed at 5 μM (Figure 9A). Resveratrol also exerts an effect on IGF-1R mRNA expression, but the effect of resveratrol on IGF-1R is different from that of IGF-1 (Figure 9B). Exposure of LNCaP cells to 1 μM resveratrol leads to a significant increase in the expression of IGF-1R mRNA. However, at concentrations higher than 5 μM, we found that resveratrol inhibited the expression of IGF-1R mRNA.

Figure 9

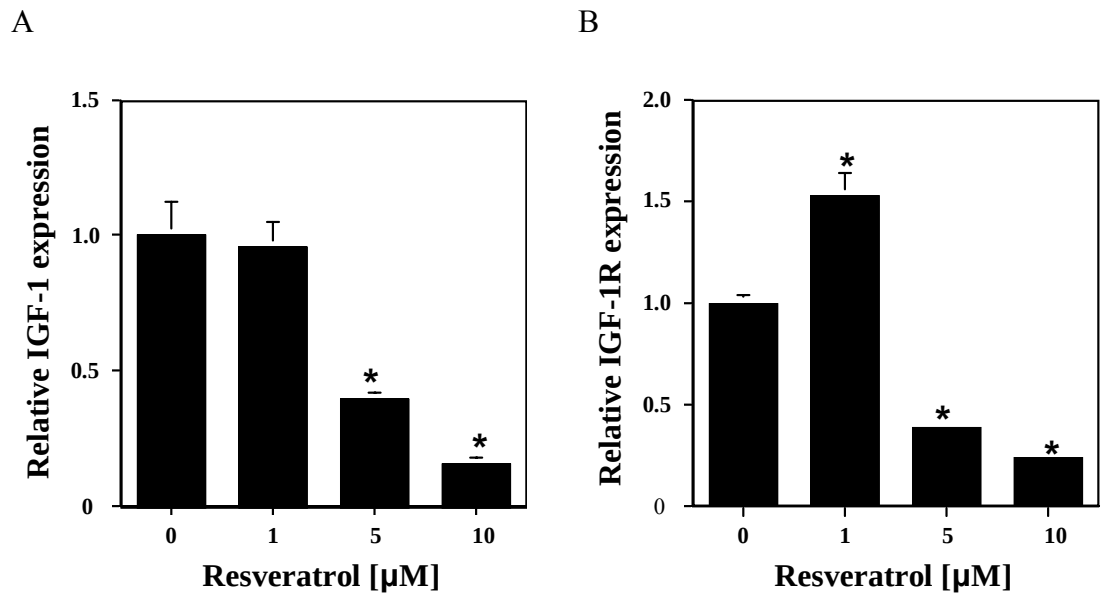

Figure 9. Resveratrol inhibited the expression of IGF-1 (A) and IGF-1R (B) at the mRNA level. LNCaP cells were treated with 0, 1, 5, and 10 μM resveratrol for 72 hours; total RNAs were isolated and relative mRNA expressions of IGF-1 or IGF-1R were measured by real-time PCR as described in materials and methods. Values are normalized to control cells and expressed as mean ±SD. Asterisks indicate that values are significantly different from control using ANOVA followed by post-hoc analysis ($p<0.05$).

4.4.2 Resveratrol Inhibited Androgen Induced IGF-1 and IGF-1R mRNA Level

As mention in Introduction, androgen is known to induce IGF-1R mRNA expression in prostate cancer. Consistent with others finding (71), we also observed induction of IGF-1R mRNA by 1 nM R1881, a synthetic androgen (Figure 10). Moreover, we also observed induction of IGF-1 mRNA by R1881. To test our hypothesis whether resveratrol can affect IGF-1 and IGF-1R mRNA expression through modulation of androgen-dependent pathway, the effects of resveratrol on R1881-induced expressions of IGF-1 and IGF-1R mRNA were examined. LNCaP cells were exposed to 1 nM R1881 in presence or absence of 25 μM resveratrol for 48 hours as described in Materials and Methods. As shown in Figure 10A, treatment with 25 μM resveratrol completely abolished the induction of IGF-1 mRNA by R1881. In addition, treatment of LNCaP cells with 25 μM resveratrol also completely inhibited the induction of IGF-1R mRNA by R1881 (Figure 10B).

Figure 10

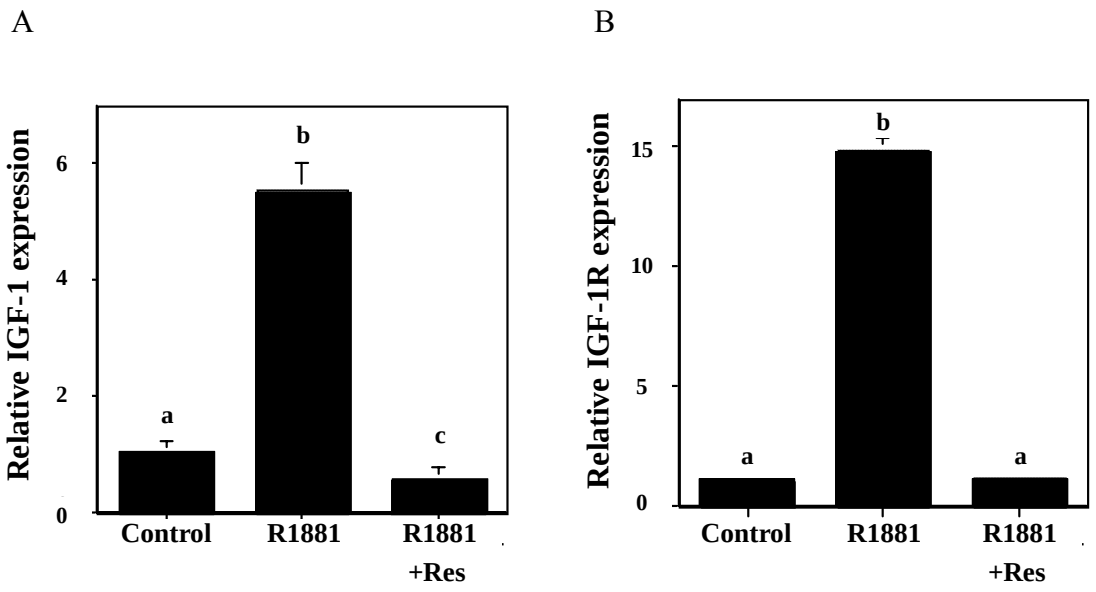

Figure 10. Effect of Resveratrol (Res) on R1881 induction of IGF-1 (A) and IGF-1R (B) mRNA levels. LNCaP cells were treated with R1881 in the presence or absence of 25 μM resveratrol for 48 hours in media containing 10%CDS; total RNA isolated and relative mRNA expression of IGF-1 and IGF-1R were measured by real-time PCR as described in Materials and Methods. Values are normalized to control cells and expressed as mean ±SD. Letters indicate values that are significantly different from each other using ANOVA followed by post-hoc analysis ($p<0.05$).

4.4.3 E2 Induced IGF-1 and IGF-1R mRNA Level in LNCaP

In addition to androgen, estrogen has been showed to induce the mRNA expression of IGF-1 in liver cancer cells (105). We first tested whether IGF-1 and IGF-1R mRNA are modulated by estrogen in prostate cancer cells. As showed in Figure 11A, treatment of LNCaP cells with E2 (1nM) leads to induction of IGF-1 mRNA. Moreover, exposure to E2, also leads to increase in IGF-1R mRNA (Figure 11B). To determine if the effect of E2 is through an ER-mediated event, the effects of the pure anti-estrogen ICI 182,780 on E2-induction of IGF-1 and IGF-1R were examined. As shown in figure 11A, the E2 induced the expression of IGF-1 mRNA in LNCaP cells, was completely inhibited by ICI 182,780 (Figure 11A). Similarly, the E2 induced the expression of IGF-1R mRNA was also inhibited by treatment with 10 μM ICI 182,780 (Figure 11B).

4.4.4 Resveratrol Inhibited Estrogen Induced IGF-1 and IGF-1R mRNA Level

Since IGF-1 and IGF-1R both modulate by E2, we also considered whether the effect of resveratrol observed in Figure 9 may be due in part to an effect on the E2-mediated pathway. The cells were exposed to 1 nM E2 in the presence or absence of 25 μM resveratrol for 48 hours, and the expression of IGF-1 and IGF-1R mRNA were determined as described in Materials and Methods. As showed in Figure 12A, resveratrol significantly inhibited the E2-induced increase in IGF-1 mRNA. Similarly, as shown in Figure 12B, treatment with resveratrol also significantly inhibited the E2-induced increase in IGF-1R mRNA.

Figure 12

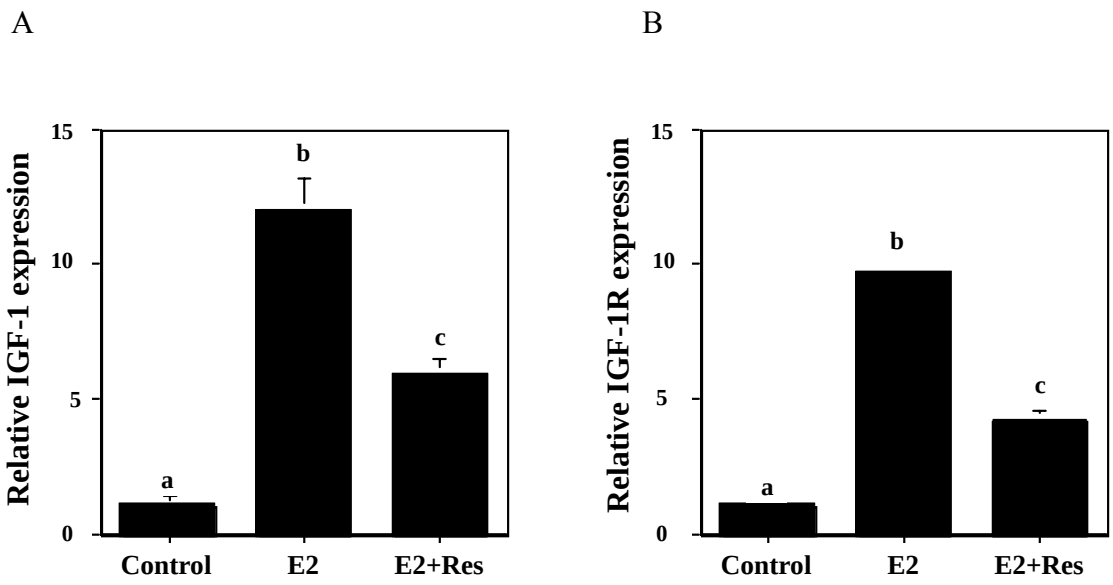

Figure 12. Inhibitory effects of resveratrol on E2 induction of (A) IGF-1 and (B) IGF-1R mRNA in LNCaP cells. LNCaP cells were treated with E2 (1nM) in the presence or absence of 25 μM resveratrol for 48 hours; total RNA isolated and relative mRNA expression of ARGs was measured by real-time PCR. Values are normalized to control cells and expressed as mean ±SD. Letters indicate values that are significantly different using ANOVA followed by post-hoc analysis ($p<0.05$).

4.5 Discussion

The present study provided experimental data to support the hypothesis that resveratrol's inhibition of prostate cancer growth may involve modulation of the IGF-1-mediated pathway. As shown in Figure 9, treatment of LNCaP cells with resveratrol leads to inhibition of the mRNA expression of both IGF-1 and IGF-1R started at 5 μM. Given the growth promotional effect of IGF-1 on LNCaP cells (126), modulation of IGF-1 and IGF-1R may contribute to the resveratrol growth inhibitory effect on LNCaP. The effects of resveratrol on IGF-1 and IGF-1R appeared to be due to inhibition of both androgen, as well as estrogen -mediated event. This is supported by inhibition of androgen and estrogen induction of IGF-1 and IGF-1R by resveratrol.

The mechanism, resveratrol acts to inhibit the androgen-induced increase of IGF-1R, may involve signal transduction pathway mediated by Src/ERK (MAP Kinase cascades). Consistent with our results, Pandini et al. (78) have also reported that the synthetic androgen R1881 induced the IGF-IR expression in AR-positive prostate cancer cells LNCaP. In accordance with IGF-IR up-regulation by androgen, LNCaP cells were sensitized to the mitogenic effect of IGF-I, but this did not occur in AR-negative prostate cancer cells PC3. Pandini et al. (78) further demonstrated that androgen-induced IGF-IR up-regulation involves the activation of the Src-extracellular signal-regulated kinase pathway, because it was inhibited by both the Src inhibitor and the MEK-1 inhibitor. Hence, it is possible that our observed inhibitory effect of resveratrol on androgen induced-IGF-1R may involve modulation of Src/ERK-mediate events. Further studies are necessary to elucidate this effect.

In addition to confirm the effect of androgen on IGF-1R, we also observed a role for E2 on regulation of IGF-1R, a novel observation. Umayahara et al. (122) have found that estrogen induced IGF-1 transcription in HepG2 cell, but there is no research found that estrogen can regulate the expression of IGF-1R. As shown in Figure 11, E2 also can up-regulate the mRNA expression of IGF-1 and IGF-1R in prostate LNCaP cells. The effects of E2 on IGF-1 and IGF-1R were completely inhibited by the pure antiestrogen, ICI 182,780, supporting the effects of E2 are mediated through ER-dependent events. Given that IGF-1 promotes LNCaP growth, induction of IGF-1 and IGF-1R by E2 may in part contribute to E2 growth promotive effect on LNCaP cells.

As shown in Figure 12, we found resveratrol partially inhibited the mRNA expression of IGF-1 and IGF-1R induced by E2. This effect of resveratrol may be through modulation of ER-dependent events. In a study of phytoestrogen, Mueller et al. (127) found resveratrol is a weak agonist of both ERα and ERβ, but at high dose, 100 μM, resveratrol becomes the antagonist to both ERα and ERβ. This is consistent with what we have found as illustrated in Figure 9, resveratrol induced the mRNA expression of IGF-1R at low the concentration (1μM), but inhibit the mRNA expression of IGF-1R occurred with increasing concentration of resveratrol.

IGF-1 is known to stimulate proliferation, induce the metastasis, and decrease the apoptosis in cells (128). Based on this, IGF-1 pathway in prostate cancer cell appeared to subject to the regulation by androgen and E2. As mentioned above, resveratrol appeared to exact its effect through these pathways supporting that the

growth inhibitory effect of resveratrol may be mediated through this mechanism. We further reason that resveratrol may inhibit the prostate cancer growth and metastasis *in vivo* by decreasing the expression of IGF-1 and IGF-1R. Additional study is necessary to support this hypothesis. After feeding 50 mg/Kg body weight in rats, the concentration of resveratrol can reach 6.6 μM. The physiological concentration of resveratrol seems can inhibit the LNCaP cell growth through inhibition of IGF-1 and IGF-1R.

In summary, in this study, we found that in addition to androgen, estrogen also modulates the IGF-1 and IGF-1R mRNA expression through an ER-dependent pathway. We also found that resveratrol can inhibit the expression of IGF-1 and IGF-1R mRNA. These effects on IGF-1 pathways appeared to be through modulation of both androgen- and estrogen-mediated pathways. These results provide a potential mechanism by which resveratrol can act as a prostate cancer preventive agent.

Chapter 5: Future Study

The precise mechanism underlying the cancer preventive effect of phytochemicals, in general, remains largely unknown. This thesis attempted to address this question and focused on the effect of resveratrol, a candidate prostate cancer preventive phytochemical. The results presented in this thesis show that resveratrol can inhibit the growth of prostate LNCaP cancer cells. The effect of resveratrol appeared to be correlated with modulation of multiple hormone/ cytokine pathways. These included androgen, estrogen, and IGF-1- mediated pathways. Our results provided information on the molecular targets (i.e. ARGs, IGF-1R) for resveratrol in prostate cancer cells and established the foundation that allows for further testing of resveratrol's prostate cancer preventive potential. Further studies at *in vivo* level can be designed to test whether the pathway affected by resveratrol *in vitro* can lead to the prevention of tumor progression. These experiments can be conducted by using xenograft prostate cancer cell model or animal models to develop spontaneous prostate cancer, such as the transgenic adenocarcinoma mouse prostate (TRAMP) model (129). Future studies may also involve elucidation of the mechanisms by which resveratrol affects the AR pathway. One potential approach could be to examine if resveratrol affects SRC/ERK-mediated events. This may shed light on how resveratrol's inhibition of androgen induction of ARGs, IGF-1 and IGF-1R may occur.

References

1. American Cancer Society. Cancer Facts & Figure 2006. 2006.
2. Silvestris, N., Leone, B., Numico, G., Lorusso, V., and De Lena, M. Present status and perspectives in the treatment of hormone-refractory prostate cancer. Oncology, *69:* 273-282, 2005.
3. Chong, P. and Rashid, P. Can we prevent prostate cancer? Aust Fam Physician, *34:* 265-267, 2005.
4. Sonn, G. A., Aronson, W., Litwin, M. S., Chong, P., and Rashid, P. Impact of diet on prostate cancer: a review
   Can we prevent prostate cancer? Prostate Cancer Prostatic Dis, *8:* 304-310, 2005.
5. Stewart, J. R., Artime, M. C., and O'Brian, C. A. Resveratrol: a candidate nutritional substance for prostate cancer prevention. J Nutr, *133:* 2440S-2443S, 2003.
6. Ratan, H. L., Steward, W. P., Gescher, A. J., and Mellon, J. K. Resveratrol--a prostate cancer chemopreventive agent? Urol Oncol, *7:* 223-227, 2002.
7. Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., Fong, H. H., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C., and Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science, *275:* 218-220, 1997.
8. de la Lastra, C. A., Villegas, I., Ulrich, S., Wolter, F., Stein, J. M., Bradamante, S., Barenghi, L., and Villa, A. Resveratrol as an anti-inflammatory and anti-aging agent: mechanisms and clinical implications
   Molecular mechanisms of the chemopreventive effects of resveratrol and its analogs in carcinogenesis
   Cardiovascular protective effects of resveratrol. Mol Nutr Food Res, *49:* 405-430, 2005.
9. Bradamante, S., Barenghi, L., and Villa, A. Cardiovascular protective effects of resveratrol. Cardiovasc Drug Rev, *22:* 169-188, 2004.
10. Ulrich, S., Wolter, F., and Stein, J. M. Molecular mechanisms of the chemopreventive effects of resveratrol and its analogs in carcinogenesis. Mol Nutr Food Res, *49:* 452-461, 2005.
11. Fremont, L. Biological effects of resveratrol. Life Sci, *66:* 663-673, 2000.
12. Hsieh, T. C. and Wu, J. M. Grape-derived chemopreventive agent resveratrol decreases prostate-specific antigen (PSA) expression in LNCaP cells by an androgen receptor (AR)-independent mechanism. Anticancer Res, *20:* 225-228, 2000.
13. Ozawa, H. Steroid Hormones, their receptors and neuroendocrine system. J Nippon Med Sch, *72:* 316-325, 2005.
14. Adams, T. E., Epa, V. C., Garrett, T. P., and Ward, C. W. Structure and function of the type 1 insulin-like growth factor receptor. Cell Mol Life Sci, *57:* 1050-1093, 2000.
15. Langcake P, P. R. The production of resveratrol by Vitis vinifera and other members of the vitaceae as a response to infection or injury. Physiol Plant Pathol, *9:* 77-86, 1976.

16. Soleas, G. J., Diamandis, E. P., and Goldberg, D. M. Resveratrol: a molecule whose time has come? And gone? Clin Biochem, *30:* 91-113, 1997.
17. Celotti, E., Ferrarini, R., Zironi, R., and Conte, L. S. Resveratrol content of some wines obtained from dried Valpolicella grapes: Recioto and Amarone. J Chromatogr A, *730:* 47-52, 1996.
18. Sato, M., Suzuki, Y., Okuda, T., and Yokotsuka, K. Contents of resveratrol, piceid, and their isomers in commercially available wines made from grapes cultivated in Japan. Biosci Biotechnol Biochem, *61:* 1800-1805, 1997.
19. Sanders, T. H., McMichael, R. W., Jr., and Hendrix, K. W. Occurrence of resveratrol in edible peanuts. J Agric Food Chem, *48:* 1243-1246, 2000.
20. Wenzel, E. and Somoza, V. Metabolism and bioavailability of trans-resveratrol. Mol Nutr Food Res, *49:* 472-481, 2005.
21. Vitaglione, P., Sforza, S., Galaverna, G., Ghidini, C., Caporaso, N., Vescovi, P. P., Fogliano, V., and Marchelli, R. Bioavailability of trans-resveratrol from red wine in humans. Mol Nutr Food Res, *49:* 495-504, 2005.
22. Goldberg, D. M., Yan, J., and Soleas, G. J. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin Biochem, *36:* 79-87, 2003.
23. Yu, C., Shin, Y. G., Chow, A., Li, Y., Kosmeder, J. W., Lee, Y. S., Hirschelman, W. H., Pezzuto, J. M., Mehta, R. G., and van Breemen, R. B. Human, rat, and mouse metabolism of resveratrol. Pharm Res, *19:* 1907-1914, 2002.
24. Marier, J. F., Vachon, P., Gritsas, A., Zhang, J., Moreau, J. P., and Ducharme, M. P. Metabolism and disposition of resveratrol in rats: extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model. J Pharmacol Exp Ther, *302:* 369-373, 2002.
25. Delmas, D., Jannin, B., and Latruffe, N. Resveratrol: preventing properties against vascular alterations and ageing. Mol Nutr Food Res, *49:* 377-395, 2005.
26. Hao, H. D. and He, L. R. Mechanisms of cardiovascular protection by resveratrol. J Med Food, *7:* 290-298, 2004.
27. Pervaiz, S. Chemotherapeutic potential of the chemopreventive phytoalexin resveratrol. Drug Resist Updat, *7:* 333-344, 2004.
28. Poulsen, H. E., Prieme, H., and Loft, S. Role of oxidative DNA damage in cancer initiation and promotion. Eur J Cancer Prev, *7:* 9-16, 1998.
29. Goldstein, B. D. and Witz, G. Free radicals and carcinogenesis. Free Radic Res Commun, *11:* 3-10, 1990.
30. Epe, B. Role of endogenous oxidative DNA damage in carcinogenesis: what can we learn from repair-deficient mice? Biol Chem, *383:* 467-475, 2002.
31. Ignatowicz, E. and Baer-Dubowska, W. Resveratrol, a natural chemopreventive agent against degenerative diseases. Pol J Pharmacol, *53:* 557-569, 2001.
32. Bhat, K. P. and Pezzuto, J. M. Cancer chemopreventive activity of resveratrol. Ann N Y Acad Sci, *957:* 210-229, 2002.
33. Lee, S. K., Mbwambo, Z. H., Chung, H., Luyengi, L., Gamez, E. J., Mehta, R. G., Kinghorn, A. D., and Pezzuto, J. M. Evaluation of the antioxidant

potential of natural products. Comb Chem High Throughput Screen, *1:* 35-46, 1998.
34. Stojanovic, S., Sprinz, H., and Brede, O. Efficiency and mechanism of the antioxidant action of trans-resveratrol and its analogues in the radical liposome oxidation. Arch Biochem Biophys, *391:* 79-89, 2001.
35. Jang, M. and Pezzuto, J. M. Effects of resveratrol on 12-O-tetradecanoylphorbol-13-acetate-induced oxidative events and gene expression in mouse skin. Cancer Lett, *134:* 81-89, 1998.
36. Cadenas, S. and Barja, G. Resveratrol, melatonin, vitamin E, and PBN protect against renal oxidative DNA damage induced by the kidney carcinogen KBrO3. Free Radic Biol Med, *26:* 1531-1537, 1999.
37. Breinholt, V. M., Molck, A. M., Svendsen, G. W., Daneshvar, B., Vinggaard, A. M., Poulsen, M., and Dragsted, L. O. Effects of dietary antioxidants and 2-amino-3-methylimidazo[4,5-f]- quinoline (IQ) on preneoplastic lesions and on oxidative damage, hormonal status, and detoxification capacity in the rat. Food Chem Toxicol, *41:* 1315-1323, 2003.
38. Cao, Z. and Li, Y. Potent induction of cellular antioxidants and phase 2 enzymes by resveratrol in cardiomyocytes: protection against oxidative and electrophilic injury. Eur J Pharmacol, *489:* 39-48, 2004.
39. Ciolino, H. P. and Yeh, G. C. Inhibition of aryl hydrocarbon-induced cytochrome P-450 1A1 enzyme activity and CYP1A1 expression by resveratrol. Mol Pharmacol, *56:* 760-767, 1999.
40. Gerhauser, C., Klimo, K., Heiss, E., Neumann, I., Gamal-Eldeen, A., Knauft, J., Liu, G. Y., Sitthimonchai, S., and Frank, N. Mechanism-based in vitro screening of potential cancer chemopreventive agents. Mutat Res, *523-524:* 163-172, 2003.
41. Gonzalez, F. J. and Gelboin, H. V. Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. Drug Metab Rev, *26:* 165-183, 1994.
42. Murray, G. I. The role of cytochrome P450 in tumour development and progression and its potential in therapy. J Pathol, *192:* 419-426, 2000.
43. Surh, Y. Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. Mutat Res, *428:* 305-327, 1999.
44. Afaq, F., Adhami, V. M., and Ahmad, N. Prevention of short-term ultraviolet B radiation-mediated damages by resveratrol in SKH-1 hairless mice. Toxicol Appl Pharmacol, *186:* 28-37, 2003.
45. Li, Z. G., Hong, T., Shimada, Y., Komoto, I., Kawabe, A., Ding, Y., Kaganoi, J., Hashimoto, Y., and Imamura, M. Suppression of N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol. Carcinogenesis, *23:* 1531-1536, 2002.
46. Subbaramaiah, K., Chung, W. J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J. M., and Dannenberg, A. J. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J Biol Chem, *273:* 21875-21882, 1998.
47. Martin, A. R., Villegas, I., La Casa, C., and de la Lastra, C. A. Resveratrol, a polyphenol found in grapes, suppresses oxidative damage and stimulates

apoptosis during early colonic inflammation in rats. Biochem Pharmacol, *67:* 1399-1410, 2004.
48. Kundu, J. K., Chun, K. S., Kim, S. O., and Surh, Y. J. Resveratrol inhibits phorbol ester-induced cyclooxygenase-2 expression in mouse skin: MAPKs and AP-1 as potential molecular targets. Biofactors, *21:* 33-39, 2004.
49. Nielsen, M., Ruch, R. J., and Vang, O. Resveratrol reverses tumor-promoter-induced inhibition of gap-junctional intercellular communication. Biochem Biophys Res Commun, *275:* 804-809, 2000.
50. Francis, G. E. and Domine, M. Growth and differentiation control. Cancer Chemother Biol Response Modif, *13:* 230-285, 1992.
51. Green, D. R., Bissonnette, R. P., and Cotter, T. G. Apoptosis and cancer. Important Adv Oncol 37-52, 1994.
52. Kundu, J. K. and Surh, Y. J. Molecular basis of chemoprevention by resveratrol: NF-kappaB and AP-1 as potential targets. Mutat Res, *555:* 65-80, 2004.
53. Gusman, J., Malonne, H., and Atassi, G. A reappraisal of the potential chemopreventive and chemotherapeutic properties of resveratrol. Carcinogenesis, *22:* 1111-1117, 2001.
54. Huang, C., Ma, W. Y., Goranson, A., and Dong, Z. Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. Carcinogenesis, *20:* 237-242, 1999.
55. Kuo, P. L., Chiang, L. C., and Lin, C. C. Resveratrol- induced apoptosis is mediated by p53-dependent pathway in Hep G2 cells. Life Sci, *72:* 23-34, 2002.
56. Zhou, H. B., Yan, Y., Sun, Y. N., and Zhu, J. R. Resveratrol induces apoptosis in human esophageal carcinoma cells. World J Gastroenterol, *9:* 408-411, 2003.
57. Surh, Y. J., Hurh, Y. J., Kang, J. Y., Lee, E., Kong, G., and Lee, S. J. Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. Cancer Lett, *140:* 1-10, 1999.
58. Aggarwal, B. B., Bhardwaj, A., Aggarwal, R. S., Seeram, N. P., Shishodia, S., and Takada, Y. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res, *24:* 2783-2840, 2004.
59. Warwick, G. P. Effect of the cell cycle on carcinogenesis. Fed Proc, *30:* 1760-1765, 1971.
60. Collins, I. and Garrett, M. D. Targeting the cell division cycle in cancer: CDK and cell cycle checkpoint kinase inhibitors. Curr Opin Pharmacol, *5:* 366-373, 2005.
61. Kastan, M. B. and Bartek, J. Cell-cycle checkpoints and cancer. Nature, *432:* 316-323, 2004.
62. Hartwell, L. H. and Kastan, M. B. Cell cycle control and cancer. Science, *266:* 1821-1828, 1994.
63. Sherr, C. J. The Pezcoller lecture: cancer cell cycles revisited. Cancer Res, *60:* 3689-3695, 2000.
64. Fontecave, M., Lepoivre, M., Elleingand, E., Gerez, C., and Guittet, O. Resveratrol, a remarkable inhibitor of ribonucleotide reductase. FEBS Lett, *421:* 277-279, 1998.

65. Ahmad, N., Adhami, V. M., Afaq, F., Feyes, D. K., and Mukhtar, H. Resveratrol causes WAF-1/p21-mediated G(1)-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. Clin Cancer Res, *7:* 1466-1473, 2001.
66. Banerjee, S., Bueso-Ramos, C., and Aggarwal, B. B. Suppression of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-kappaB, cyclooxygenase 2, and matrix metalloprotease 9. Cancer Res, *62:* 4945-4954, 2002.
67. Ragione, F. D., Cucciolla, V., Borriello, A., Pietra, V. D., Racioppi, L., Soldati, G., Manna, C., Galletti, P., and Zappia, V. Resveratrol arrests the cell division cycle at S/G2 phase transition. Biochem Biophys Res Commun, *250:* 53-58, 1998.
68. Liang, Y. C., Tsai, S. H., Chen, L., Lin-Shiau, S. Y., and Lin, J. K. Resveratrol-induced G2 arrest through the inhibition of CDK7 and p34CDC2 kinases in colon carcinoma HT29 cells. Biochem Pharmacol, *65:* 1053-1060, 2003.
69. Carbo, N., Costelli, P., Baccino, F. M., Lopez-Soriano, F. J., and Argiles, J. M. Resveratrol, a natural product present in wine, decreases tumour growth in a rat tumour model. Biochem Biophys Res Commun, *254:* 739-743, 1999.
70. Joe, A. K., Liu, H., Suzui, M., Vural, M. E., Xiao, D., and Weinstein, I. B. Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin Cancer Res, *8:* 893-903, 2002.
71. Cutolo, M., Sulli, A., Seriolo, B., Accardo, S., and Masi, A. T. Estrogens, the immune response and autoimmunity. Clin Exp Rheumatol, *13:* 217-226, 1995.
72. Bowers, J. L., Tyulmenkov, V. V., Jernigan, S. C., and Klinge, C. M. Resveratrol acts as a mixed agonist/antagonist for estrogen receptors alpha and beta. Endocrinology, *141:* 3657-3667, 2000.
73. Klinge, C. M., Risinger, K. E., Watts, M. B., Beck, V., Eder, R., and Jungbauer, A. Estrogenic activity in white and red wine extracts. J Agric Food Chem, *51:* 1850-1857, 2003.
74. Lu, R. and Serrero, G. Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. J Cell Physiol, *179:* 297-304, 1999.
75. Fujimoto, N., Nomura, M., and Matsumoto, T. Current and future status of prostate cancer chemoprevention. Expert Rev Anticancer Ther, *6:* 59-71, 2006.
76. Klein, E. A. Can prostate cancer be prevented? Nat Clin Pract Urol, *2:* 24-31, 2005.
77. Iwasaki, M. and Tsugane, S. [Risk factors and current chemoprevention studies in prostate cancer]. Nippon Rinsho, *63:* 321-326, 2005.
78. Pandini, G., Mineo, R., Frasca, F., Roberts, C. T., Jr., Marcelli, M., Vigneri, R., and Belfiore, A. Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. Cancer Res, *65:* 1849-1857, 2005.
79. Wolk, A. Diet, lifestyle and risk of prostate cancer. Acta Oncol, *44:* 277-281, 2005.

80. Harkonen, P. L. and Makela, S. I. Role of estrogens in development of prostate cancer. J Steroid Biochem Mol Biol, *92:* 297-305, 2004.
81. Takahashi, Y., Hursting, S. D., Perkins, S. N., Wang, T. C., and Wang, T. T. Genistein affects androgen-responsive genes through both androgen- and estrogen-induced signaling pathways. Mol Carcinog, *45:* 18-25, 2006.
82. Sunny, L. A low fat diet rich in fruits and vegetables may reduce the risk of developing prostate cancer. Asian Pac J Cancer Prev, *6:* 490-496, 2005.
83. Schulman, C. C. and Zlotta, A. R. Prevention of prostate cancer. Recent Results Cancer Res, *163:* 199-211; discussion 264-196, 2003.
84. Sgambato, A., Ardito, R., Faraglia, B., Boninsegna, A., Wolf, F. I., and Cittadini, A. Resveratrol, a natural phenolic compound, inhibits cell proliferation and prevents oxidative DNA damage. Mutat Res, *496:* 171-180, 2001.
85. Hsieh, T. C. and Wu, J. M. Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. Exp Cell Res, *249:* 109-115, 1999.
86. Cardile, V., Scifo, C., Russo, A., Falsaperla, M., Morgia, G., Motta, M., Renis, M., Imbriani, E., and Silvestre, G. Involvement of HSP70 in resveratrol-induced apoptosis of human prostate cancer. Anticancer Res, *23:* 4921-4926, 2003.
87. Morris, G. Z., Williams, R. L., Elliott, M. S., and Beebe, S. J. Resveratrol induces apoptosis in LNCaP cells and requires hydroxyl groups to decrease viability in LNCaP and DU 145 cells. Prostate, *52:* 319-329, 2002.
88. Kuwajerwala, N., Cifuentes, E., Gautam, S., Menon, M., Barrack, E. R., and Reddy, G. P. Resveratrol induces prostate cancer cell entry into s phase and inhibits DNA synthesis. Cancer Res, *62:* 2488-2492, 2002.
89. Lin, H. Y., Shih, A., Davis, F. B., Tang, H. Y., Martino, L. J., Bennett, J. A., and Davis, P. J. Resveratrol induced serine phosphorylation of p53 causes apoptosis in a mutant p53 prostate cancer cell line. J Urol, *168:* 748-755, 2002.
90. Denmeade, S. R., Lin, X. S., and Isaacs, J. T. Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. Prostate, *28:* 251-265, 1996.
91. Brawer, M. K. Prostate cancer: where we have been, where we are, where we are going. World J Surg, *24:* 1200-1204, 2000.
92. Mitchell, S. H., Zhu, W., and Young, C. Y. Resveratrol inhibits the expression and function of the androgen receptor in LNCaP prostate cancer cells. Cancer Res, *59:* 5892-5895, 1999.
93. Narayanan, N. K., Narayanan, B. A., and Nixon, D. W. Resveratrol-induced cell growth inhibition and apoptosis is associated with modulation of phosphoglycerate mutase B in human prostate cancer cells: two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis and mass spectrometry evaluation. Cancer Detect Prev, *28:* 443-452, 2004.
94. Shih, A., Zhang, S., Cao, H. J., Boswell, S., Wu, Y. H., Tang, H. Y., Lennartz, M. R., Davis, F. B., Davis, P. J., and Lin, H. Y. Inhibitory effect of epidermal growth factor on resveratrol-induced apoptosis in prostate cancer cells is mediated by protein kinase C-alpha. Mol Cancer Ther, *3:* 1355-1364, 2004.

95. Greenlee, R. T., Hill-Harmon, M. B., Murray, T., and Thun, M. Cancer statistics, 2001. CA Cancer J Clin, *51:* 15-36, 2001.
96. Willett, W. C. Diet and health: what should we eat? Science, *264:* 532-537, 1994.
97. Kris-Etherton, P. M., Hecker, K. D., Bonanome, A., Coval, S. M., Binkoski, A. E., Hilpert, K. F., Griel, A. E., and Etherton, T. D. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. Am J Med, *113 Suppl 9B:* 71S-88S, 2002.
98. Hain, R., Bieseler, B., Kindl, H., Schroder, G., and Stocker, R. Expression of a stilbene synthase gene in Nicotiana tabacum results in synthesis of the phytoalexin resveratrol. Plant Mol Biol, *15:* 325-335, 1990.
99. Bhat, K. P. L., Kosmeder, J. W., 2nd, and Pezzuto, J. M. Biological effects of resveratrol. Antioxid Redox Signal, *3:* 1041-1064, 2001.
100. Ho, S. M. Estrogens and anti-estrogens: key mediators of prostate carcinogenesis and new therapeutic candidates. J Cell Biochem, *91:* 491-503, 2004.
101. Miller, N. J. and Rice-Evans, C. A. Antioxidant activity of resveratrol in red wine. Clin Chem, *41:* 1789, 1995.
102. Kim, Y. A., Rhee, S. H., Park, K. Y., and Choi, Y. H. Antiproliferative effect of resveratrol in human prostate carcinoma cells. J Med Food, *6:* 273-280, 2003.
103. Narayanan, B. A., Narayanan, N. K., Re, G. G., and Nixon, D. W. Differential expression of genes induced by resveratrol in LNCaP cells: P53-mediated molecular targets. Int J Cancer, *104:* 204-212, 2003.
104. Wolf, D. A., Herzinger, T., Hermeking, H., Blaschke, D., and Horz, W. Transcriptional and posttranscriptional regulation of human androgen receptor expression by androgen. Mol Endocrinol, *7:* 924-936, 1993.
105. Nelson, P. S., Clegg, N., Arnold, H., Ferguson, C., Bonham, M., White, J., Hood, L., and Lin, B. The program of androgen-responsive genes in neoplastic prostate epithelium. Proc Natl Acad Sci U S A, *99:* 11890-11895, 2002.
106. Huang, H. and Tindall, D. J. The role of the androgen receptor in prostate cancer. Crit Rev Eukaryot Gene Expr, *12:* 193-207, 2002.
107. Parnes, H. L., Thompson, I. M., and Ford, L. G. Prevention of hormone-related cancers: prostate cancer. J Clin Oncol, *23:* 368-377, 2005.
108. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst, *82:* 1107-1112, 1990.
109. Takahashi, Y., Lavigne, J. A., Hursting, S. D., Chandramouli, G. V., Perkins, S. N., Barrett, J. C., and Wang, T. T. Using DNA microarray analyses to elucidate the effects of genistein in androgen-responsive prostate cancer cells: identification of novel targets. Mol Carcinog, *41:* 108-119, 2004.
110. Schuurmans, A. L., Bolt, J., Veldscholte, J., and Mulder, E. Regulation of growth of LNCaP human prostate tumor cells by growth factors and steroid hormones. J Steroid Biochem Mol Biol, *40:* 193-197, 1991.

111. Oesterling, J. E. Prostate-specific antigen and diagnosing early malignancies of the prostate. J Cell Biochem Suppl, *16H:* 31-43, 1992.
112. Risbridger, G. P., Bianco, J. J., Ellem, S. J., and McPherson, S. J. Oestrogens and prostate cancer. Endocr Relat Cancer, *10:* 187-191, 2003.
113. Gehm, B. D., McAndrews, J. M., Chien, P. Y., and Jameson, J. L. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc Natl Acad Sci U S A, *94:* 14138-14143, 1997.
114. Matsumura, A., Ghosh, A., Pope, G. S., and Darbre, P. D. Comparative study of oestrogenic properties of eight phytoestrogens in MCF7 human breast cancer cells. J Steroid Biochem Mol Biol, *94:* 431-443, 2005.
115. Friedrichsen, D. M., Hawley, S., Shu, J., Humphrey, M., Sabacan, L., Iwasaki, L., Etzioni, R., Ostrander, E. A., and Stanford, J. L. IGF-I and IGFBP-3 polymorphisms and risk of prostate cancer. Prostate, *65:* 44-51, 2005.
116. Yu, H. and Berkel, H. Insulin-like growth factors and cancer. J La State Med Soc, *151:* 218-223, 1999.
117. Papatsoris, A. G., Karamouzis, M. V., and Papavassiliou, A. G. Novel insights into the implication of the IGF-1 network in prostate cancer. Trends Mol Med, *11:* 52-55, 2005.
118. Kehinde, E. O., Akanji, A. O., Mojiminiyi, O. A., Bashir, A. A., Daar, A. S., and Varghese, R. Putative role of serum insulin-like growth factor-1 (IGF-1) and IGF binding protein-3 (IGFBP-3) levels in the development of prostate cancer in Arab men. Prostate Cancer Prostatic Dis, *8:* 84-90, 2005.
119. Kimura, G., Kasuya, J., Giannini, S., Honda, Y., Mohan, S., Kawachi, M., Akimoto, M., and Fujita-Yamaguchi, Y. Insulin-like growth factor (IGF) system components in human prostatic cancer cell-lines: LNCaP, DU145, and PC-3 cells. Int J Urol, *3:* 39-46, 1996.
120. Gupta, C. The role of estrogen receptor, androgen receptor and growth factors in diethylstilbestrol-induced programming of prostate differentiation. Urol Res, *28:* 223-229, 2000.
121. Orio, F., Jr., Terouanne, B., Georget, V., Lumbroso, S., Avances, C., Siatka, C., and Sultan, C. Potential action of IGF-1 and EGF on androgen receptor nuclear transfer and transactivation in normal and cancer human prostate cell lines. Mol Cell Endocrinol, *198:* 105-114, 2002.
122. Umayahara, Y., Kawamori, R., Watada, H., Imano, E., Iwama, N., Morishima, T., Yamasaki, Y., Kajimoto, Y., and Kamada, T. Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. J Biol Chem, *269:* 16433-16442, 1994.
123. Lee, A. V., Weng, C. N., Jackson, J. G., and Yee, D. Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. J Endocrinol, *152:* 39-47, 1997.
124. Thissen, J. P., Ketelslegers, J. M., and Underwood, L. E. Nutritional regulation of the insulin-like growth factors. Endocr Rev, *15:* 80-101, 1994.
125. Serrero, G. and Lu, R. Effect of resveratrol on the expression of autocrine growth modulators in human breast cancer cells. Antioxid Redox Signal, *3:* 969-979, 2001.

126. Iwamura, M., Sluss, P. M., Casamento, J. B., and Cockett, A. T. Insulin-like growth factor I: action and receptor characterization in human prostate cancer cell lines. Prostate, *22:* 243-252, 1993.
127. Mueller, S. O., Simon, S., Chae, K., Metzler, M., and Korach, K. S. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ERalpha) and ERbeta in human cells. Toxicol Sci, *80:* 14-25, 2004.
128. Baserga, R., Peruzzi, F., and Reiss, K. The IGF-1 receptor in cancer biology. Int J Cancer, *107:* 873-877, 2003.
129. Gingrich, J. R., Barrios, R. J., Kattan, M. W., Nahm, H. S., Finegold, M. J., and Greenberg, N. M. Androgen-independent prostate cancer progression in the TRAMP model. Cancer Res, *57:* 4687-4691, 1997.