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Prostate cancer risk is significantly lower in Asian countries compared to the US, which has prompted interest in the chemo-preventative action of dietary components such as soy and green tea commonly found in Asian diets, such as soy and green tea. Studies have suggested that soy isoflavones and green tea catechins exert anti-carcinogenic effects; however, the effects of dietary soy and green tea on prostate cancer development *in vivo* are understudied. We proposed that soy and green tea, containing a mixture of bioactive components, would be more potent chemo-preventative agents than individual supplements.

Using an *in vitro* system, we observed that soy extract induced significantly more apoptosis and Bax expression than the individual soy isoflavones genistein or daidzein. Similarly, green tea induced more apoptosis than epigallocatechin gallate (EGCG), by decreasing inhibitors of apoptosis proteins, XIAP, cIAP-1 and cIAP-2. Notably, soy extract and green tea did not induce cell cycle arrest or apoptosis in non-cancerous benign prostate hyperplasia (BPH-1) cells, suggesting that the effects of whole foods were tumor-cell specific.

Chronic inflammation and nuclear factor-kappa B (NFκB) have been implicated in prostate cancer development, suggesting that factors that inhibit NFκB may serve as effective chemo-preventative agents. We therefore examined the effects of dietary soy, green tea, and the combination of soy and green tea *in vivo* on inflammation, NFκB activation and cancer development using a hormone-induced prostate cancer model. The combination of both soy and green tea decreased prostate inflammatory infiltration and cytokine (TNFα, IL-6 and IL-1β) levels, increased Bax expression, and

decreased prostate hyperplasia. Interestingly, these effects were not apparent in soy alone or tea alone treated animals. The combination of soy and green tea also suppressed NFκB p50 activity via induction of IκBα. Together these results suggest that the combination of soy and green tea may inhibit hormone-induced pro-inflammatory NFκB signals that contribute to prostate cancer development.

These studies suggest that food products that bear a combination of active compounds may be more efficacious as chemo-preventative agents than individual compounds, and combination of different dietary compounds may offer additional beneficial effects. This “whole food” based approach is significant for the development of dietary recommendations for prostate cancer prevention.

Dietary Soy and Green Tea in the Prevention of Prostate Cancer

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Anna Hsu, Author

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Dietary Soy and Green Tea in the Prevention of Prostate Cancer

Chapter 1 Introduction

1.1 General Introduction

Prostate cancer is the most common type of cancer found in American men, accounting for 10% of male cancer-related deaths (1). Risk factors of prostate cancer include age, family history, ethnicity, and hormonal status, but there is compelling evidence that suggest that environmental factors, such as diet and lifestyle, play more important roles in prostate carcinogenesis than genetic predispositions (2). Epidemiological studies have shown some discrepancy between the distribution of the incidence of latent prostatic lesions and the incidence of prostate cancer in men around the world. The incidence of latent prostatic lesions in men appears uniform across Asian and Western countries, but prostate cancer outcomes and mortality rates are considerably higher in Western countries (3). Moreover, prostate cancer rates have been increasing gradually in major industrialized cities in Asia. For example, between 1978 and 1997, prostate cancer incidence has risen to more than doubled in Singapore (4). It has been postulated that a greater extent of westernization and adaptation of Western diet could be contributing factors to the greater prostate cancer risk in these Asian cities. The goal of this literature review is to examine the interactions among diet, inflammation and sex hormones in prostate cancer development.

1.2 Chronic Inflammation, Nuclear Factor kappa B (NFκB) and Prostate Cancer

The connection between chronic inflammation and cancer was first noted by Virchow in 1863. He hypothesized that a combination of some classes of irritants, tissue injuries and associated inflammation enhanced cell proliferation and became the origin of cancer. Chronic inflammation has been estimated to contribute to as much as one third of all cancers (5). Chronic inflammation may act as both initiators and/or promoters in the carcinogenesis process. Cells that mediate inflammatory responses are major contributors of *in vivo* production of reactive oxygen species (ROS).

Polymorphonuclear leukocytes (PMNs, which include neutrophils, eosinophils and basophils) and mononuclear phagocytes (monocytes and macrophages) produce superoxide, nitric oxide, hydrogen peroxide, and hypochloric acid in attempts to defend against pathogens. High concentrations and the persistent presence of these ROS are mutagenic and can lead to tissue damage. Furthermore, damaged tissues generate proliferation signals for cell growth and repair. Both increased ROS and cell proliferation contribute to the initiation and promotion of carcinogenesis, and in combination, they provide an optimal microenvironment for tumorigenesis.

Inflammation is implicated as a major risk factor for prostate cancer. Population studies have found an increased relative risk of prostate cancer in men with prior histories of prostatitis (inflammation of the prostate) (6). Benign prostatic hyperplasia (BPH), a condition that often precedes and co-exists with prostate cancer, is an inflammatory disease. Specifically, almost all human BPH specimens showed inflammatory infiltration and high expression of pro-inflammatory cytokines, including interleukin-17 (IL-17) that promotes stromal growth and chronic inflammation (7). Several inflammatory pathways are promising targets for intervention including the cyclooxygenase-2 (COX-2) and Nuclear Factor kappa B (NFκB) pathways. Inhibition of inflammatory processes through non-steroidal anti-inflammatory drugs (NSAIDs) that block COX-2, effectively decreased prostate tumor growth (8, 9). Interestingly, COX-2 is over expressed in human prostate adenocarcinomas compared with normal prostate tissues (10-12). The NFκB pathway has gained much interest in recent years because of its influence on many cellular responses that contribute to carcinogenesis, including regulation of cell cycle, cell proliferation, apoptosis and inflammation. The deregulation of COX-2 and the NFκB pathway increased cell proliferation by up-regulating anti-apoptotic protein, BcL2 (13, 14), promoting angiogenesis (15), and inhibiting immune surveillance via production of immunosuppressive interleukins, such as IL-10 (16). Thus, inflammation plays a major role in cancer etiology and may provide selective growth advantages for cancer cells. Targeting aberrant NFκB activation in cancer cells may restore deregulated cellular functions by inducing apoptosis and decreasing uncontrolled cell proliferation and chronic inflammation; thus strategies to mitigate NFκB may have important preventative or therapeutic values against cancer.

Constitutive activation of NFκB is responsible for numerous diseases (17). Potential toxicity associated with NFκB blockage leads to liver apoptosis (18), but the first link implicating NFκB as a possible target for chemoprevention was based on the recognition that c-Rel, which encodes an NFκB subunit, is the cellular homolog of the v-Rel oncogene,. Upon stimulation, IκB kinase (IKK) is activated. Activated IKK phosphorylates IκB that is bound to NFκB and targets IκB for poly-ubiquitination and degradation. Free NFκB homodimers and heterodimers then translocate into the nucleus and transactivate hundreds of genes involved in inflammatory responses and cell proliferation (19, 20). NFκB activity is negatively self-regulated by transcriptional up-regulation of its inhibitor, IκBα. Initially, it appeared IκBα solely prevented nuclear localization of activated NFκB, but recent studies suggest that IκBα also directly interacts with DNA-bound NFκB. IκBα dissembles NFκB from DNA by changing conformation of p65 subunits, and channels NFκB to the cytoplasm via its nuclear export signal (NES) (21). The IκBs are controlled by upstream kinases, the IKKs. IκBs are regulated through phosphorylation by IKKs, especially IKKβ. Recent data suggest that IKKα activity is essential for prostate cancer metastasis. Activation of IKKα increases NFκB activity while represses Maspin, a metastasis suppressor (22). NFκB activation also up-regulates essential factors that facilitated endothelial adhesion in PC3 cells (23). The complexity of NFκB pathway suggests that total blockage of NFκB in healthy cells is not ideal, but the multiple levels of regulation in the NFκB pathway also provide many possible targets for chemoprevention in cancer cells.

Many *in vitro* studies have shown that targeting NFκB was effective in inducing cell death in prostate cancer cells. Blocking NFκB activity in PC3 cells by transfection of mutant IκBα suppresses angiogenesis and cell invasion (24). Strategies utilizing IκBα-super repressors or inhibitors of NFκB activity to sensitize prostate epithelial cells to chemotherapy have shown promising results (25, 26). Even though there are limited *in vivo* studies investigating the impact of NFκB in prostate cancer, these studies suggest that NFκB is a crucial mediator of tumorigenesis in several cancer models. Deletion of IKKβ is associated with an increase in epithelial apoptosis during tumor promotion and a decrease in tumor incidence in a colitis-associated cancer mouse model (27). TNFα-induced NFκB activation does not affect tumor initiation, but

is essential for the promotion of hepatitis-induced hepatocellular carcinoma in mice (28). Over-expression of NFκB promotes tumor growth due to its effects on inflammation, apoptosis, and cell proliferation. Among the array of genes regulated by NFκB, cyclin D1 influences cell growth and differentiation (21, 29). NFκB also amplifies the expression of other inflammatory signals, including COX-2 and pro-inflammatory cytokines, such as IL-6, by acting as a transcriptional activator (30). NFκB mediates constitutive activation of IL-6 and this has been implicated as a possible mechanism in prostate cancer progression (31). Furthermore, NFκB intimately interacts with the homeostasis of steroid hormones. For example, androgen receptor (AR) expression antagonizes NFκB activity. Prostate cancer cell lines without endogenous androgen receptor expression, such as malignant PC3 cells, have constitutive NFκB activity, whereas cell lines that express AR (LnCap cells) have low basal NFκB activity (32). Upon the loss of AR expression and androgen dependency, deregulation of NFκB becomes a major promoting factor for transformation of cancer cells to malignancy and poor prognosis.

Overall, both pharmacological and genetic strategies have provided strong support for the rationale of using agents that target inflammatory pathways as a cancer chemoprevention strategy. Importantly, many factors found in the diet also can target these pathways and possibly exert anti-inflammatory and cancer preventive properties.

1.3 Diet and prostate cancer

Human migration studies emphasize the importance of diet and lifestyle on prostate cancer development. For instance, first-generation Asian migrants (high soy and tea intake population) have a lower incidence of prostate and mammary cancers than subsequent generations of Asian Americans (33). Prostate cancer rates in Chinese (24 per 100,000), Japanese (29.6 per 100,000) and Filipino (56.8 per 100,000) men born in China, Japan and the Philippines were about half of those born in the US (44.4, 42.2, and 111.3 per 100,000, respectively) (2). These observations have prompted studies to investigate and compare dietary components in the Asian diet, such as soy and green tea that may have cancer preventative properties such as soy

and green tea. The differences in consumption of these two dietary components between the Asian and US populations are very large. A typical soy-rich Japanese diet consists of 25 to 100 mg soy isoflavones/day (1 serving of traditional fermented soy food contains about 25 mg soy isoflavones), while the typical American diet contains about 1 to 3 mg soy isoflavones/day. The green tea consumption in Asian countries averages ~360 to 480 mL per day; whereas only 8% of the American population regularly consumes ~180 mL of green tea per day (34). The differences in dietary intake of soy and green tea are hypothesized to contribute to the observed differences in prostate cancer outcomes between the two populations.

Various mechanisms for the anti-carcinogenic properties of soy or green tea have been proposed, but we favor the likelihood that inflammation, especially as dependent on the NFκB pathway, to be a target of inhibition by these agents. However, it is unclear whether either dietary soy or green tea has significant impacts on chronic inflammation and NFκB *in vivo*. Regular intakes of nonsteroidal anti-inflammatory drugs (NSAIDs) appear to reduce prostate cancer growth by decreasing inflammation in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model (9). The studies in this dissertation examined dietary soy and green tea as a dietary chemo-preventative strategy by attenuating chronic inflammation and inhibiting prostate carcinogenesis. If increasing the consumption of soy or green tea may prevent prostate cancer development, these dietary recommendations would provide a more cost-effective and a safer chemo-preventative strategy than using pharmacologic agents. The delineation of the molecular events by which these nutrients interfere with prostate cancer will reveal novel understandings of the factors involved in prostate carcinogenesis and key molecular targets for prostate cancer prevention.

1.3.1 Soy and Prostate Cancer

Soy foods have been the subject of considerable investigation since the 1960's, largely due to the potential health effects of soy isoflavones. Soybeans are the only food to contain nutritionally relevant amounts of isoflavones. There are currently over 500 papers published on soy isoflavones in 2008, compared with only 12 papers in 1985 based on a Medline search. In 1999, the United States Department of

Agriculture (USDA) established an online database of the isoflavone contents of foods (http://www.nal.usda.gov/fnic).

Soy isoflavones are classified as phytoestrogens, and have been suggested to modulate endogenous hormone homeostasis because their phenolic ring structures resemble estradiol and can bind to estrogen receptors, acting as either an estrogen agonist or antagonist (56, 57). The primary isoflavones in soybeans are genistein (4'5, 7-trihydroxyisoflavone), daidzein (4', 7-dihydroxyisoflavone) and glycitein (4', 7-dihydroxy-6-methoxyisoflavone), and their respective β-glycosides, genistin, daidzin, and glycitin (**Figure 1.1**). Isoflavones are phytoalexins, substances formed by the host plant tissue in response to environmental stimuli and possess properties that enhance the survival of the soybean. Therefore, isoflavone concentrations vary depending on environmental conditions; normally isoflavone levels increase with increasing environmental stresses. Nevertheless, soybeans contain ~1.2-3.3 mg of isoflavones/g dry weight. Soy isoflavones exist predominantly as glycosides in soybeans, but fermentation and processing significantly alter the aglycone contents of soy products. The contents of soy isoflavones in commonly available soy products are summarized in **Table 1.1**.

Soybean products have been widely used as dietary supplements and food ingredients. The details of soybean processing are reviewed in Phytoestrogens and Health (35). Briefly, soybeans are cleaned, heated, cracked and dehulled. The soybean cotyledons are then flaked to produce full-fat flakes. These flakes are then extracted with hexane to remove oil. The defatted soy flakes are the starting material for commercial soy protein production. As shown in **Figure 1.2**, defatted soy flakes are processed into three major commercial soy protein products: soy flour, soy protein concentrate and soy protein isolate. By-products of manufacturing soy protein concentrate and isolate are soy soluble (molasses) and soy whey, which are then used for isolation of soy isoflavones.

Epidemiological studies suggest that consumption of soy foods are associated with lower risk of prostate cancer (36). The plasma and serum phytoestrogen concentrations in Japanese men are at least ten-fold higher than their Caucasian counterparts in the UK (37, 38). Such observations led to hypotheses that soy or

components in soy exerts anti-cancer effects, and most have attributed these effects to soy isoflavones, especially the isoflavone genistein (39). The protective properties of soy against prostate cancer are not only apparent for a low-intake population. Even among Japanese men with relatively high soy intake compared to Caucasians, higher consumption of soy food and soy isoflavones was associated with a decrease in localized prostate cancer (40), possibly through induction of apoptosis and cell cycle arrest in prostate cancer cells. The anti-carcinogenic effects of genistein are summarized in several reviews (41, 42). Both *in vitro* and *in vivo* studies showed protective effects of soy isoflavones against cancer development. Soy isoflavones reduce serum androgen and estrogen levels in Japanese men (43). Soy isoflavones inhibit several steroidogenic enzymes, such as 5α-reductase (44), aromatase (37), 17β-hydroxysteroid oxidoreductase (45), and cytochrome P450 that is responsible for estrogen hydroxylation(46). Furthermore, dietary genistein decreases androgen receptor mRNA and protein expression in rat prostates (47), and in men (48), thus limiting the contribution of androgen receptor (AR) deregulation in prostate carcinogenesis. Daily consumption of soy grits decreases PSA levels and the free/total PSA ratio in prostate cancer patients (49), and total and free testosterone concentrations are also inversely correlated with soy intake in Japanese men (43). Soy isoflavones also have non-hormone properties and act through hormone-independent pathways. Genistein is a known inhibitor of protein tyrosine kinase (50) and topoisomerase II (51). *In vitro,* genistein inhibits angiogenesis by decreasing endothelial cell proliferation (52, 53), up-regulating p21, a major cell cycle inhibitory protein and down-regulating cyclin B (54), and elicits a reversible dose-dependent inhibition on human gastric cancer cells via G2/M arrest (55). Soy constituents, especially isoflavones, appear to target different stages of the carcinogenesis pathway.

Of all the anti-carcinogenic mechanisms, the anti-inflammatory effects of soy constituents are relatively understudied. Nevertheless, previous studies have indicated that soy isoflavones exert inhibitory effects on NFκB. Genistein regulates immune function in mice by modulating humoral and cell-mediated immunity (56). *In vitro*, , genistein inhibits constitutive NFκB/Ap-1 activities in breast cancer cells (57) and inhibits NFκB DNA binding and induces apoptosis by inactivating Akt kinase in prostate cancer PC3 cells. Such inhibitory effects are shown to be cell-specific

because non-tumorigenic prostate epithelial cells, CRL2221, are not affected by genistein (58, 59). This ability to target cancerous cells is highly desirable for chemo-preventative agents. Soy isoflavones may also target molecular pathways that regulate cell proliferation in the prostate. Soy isoflavone concentrate that contains various soy isoflavones was shown to modulate cell cycle by inducing G2/M arrest and increasing p21 expression in PC3 cells (60). Dietary genistein inhibits expression of EGF and ErbB2/Neu receptors in rat dorsolateral prostate in a dose-dependent manner *in vivo* (61).

Notiably, many of these studies used beyond physiological levels of genistein, higher than attainable from diet. There is also emerging evidence demonstrating that acute administration of high levels of soy isoflavones may not be beneficial and may have toxicities (62-64). In contrast, long term treatments at low doses, such as found with dietary intake, may be safer and still exert similar beneficial effects. In addition, combinations of isoflavones appear to be more effective in inhibiting prostate cancer cell growth than individual soy isoflavones. Concentrations of soy isoflavonoids detected in prostatic fluids were sufficient in inhibiting prostatic epithelial cell growth *in vitro* (65). More importantly, whether chemo-preventative agents are metabolized and local physiological concentrations can be achieved for significant effects determines the effectiveness of many potential chemo-preventative agents. Soy isoflavonoids were shown to accumulate in the prostate gland (66). Also, long-term consumption of soy improves the ability of Caucasian men to produce active soy isoflavone metabolite such as equol and increases bioavailability of soy isoflavones because of changes in gut microflora (66, 67). Therefore, increased dietary consumption of soy could be a promising and implementable strategy for prostate cancer prevention.

1.3.2 Green Tea and Prostate Cancer

Green tea (*Camellia sinensis*) is one of the most widely consumed beverages, especially in Asia. Green tea is different from black or Oolong tea by methods of processing (**Figure 1.3**). Green tea is prepared by pan-frying or steaming fresh leaves to heat inactivate oxidative enzymes followed by drying, whereas Oolong and black tea are prepared by additional steps of fermentation.

Epidemiological studies suggest protective properties of green tea against the development of stomach (68), lung (69), pancreatic and colorectal (70), and breast cancer (71). The bioactive components of green tea are the catechins that include epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC), and theaflavins. Catechins are especially concentrated in green tea, accounting for 30-40% of its dry weight (72). During fermentation, most of the catechins are converted to oligomeric theaflavins and polymeric thearubigins in black tea, resulting in only 3-10% catechins, 2-6% theaflavins and >20% thearubigins in black tea (73). However, most studies ascribe the beneficial effects of green tea to the most abundant catechin in green tea, EGCG. Other than its well evidenced anti-cancer effects, EGCG demonstrated beneficial effects against Parkinson's (74) and Alzheimer's disease (75), stroke (76), obesity (77), and diabetes (78, 79). These beneficial effects of green tea polyphenols are summarized in several reviews (80, 81).

EGCG exerts anti-carcinogenic effects at multiple stages of the carcinogenesis pathway. Noticeably, EGCG is a strong antioxidant and effective inhibitor for carcinogen activation. EGCG induces phase-II detoxifying enzymes, such as glutathione peroxidase and QR, cell cycle arrest, apoptosis, and attenuates inflammation and tumor promotion (82). EGCG suppresses growth of human gastric cancer xenografts in mice through the inhibition of tumor invasion and metastasis by regulating expression of matrix metalloproteinases (MMP) and inhibiting angiogenesis by reducing vascular endothelial growth factor (VEGF) production (82, 83). The role of EGCG in prostate cancer prevention and treatment are summarized by Stuart et.al (84).

Among the protective effects of EGCG, its ability to limit tumor promotion is well established (39). EGCG exerts pro-apoptotic effects through inhibition of NFκB *in vitro* by inducing kinase (NIK) and subsequent activation of the IκB-kinase (IKK)/NIK signaling complex, as shown in human lung cancer cells (85). In JB6 mouse epidermal cells, EGCG decreased phosphorylated-IκBα, hence blocking the degradation of NFκB inhibitor proteins (86). Decreasing activation of NFκB in cancer cells subsequently increased the ratio of the pro-apoptotic protein Bax to anti-apoptotic protein BcL-2 (13). Similar to soy isoflavones, the pro-apoptotic effects of

EGCG appeared to be tumor-specific because while EGCG significantly decreased NFκB activation in epidermoid carcinoma (A431) cells, normal human epidermal keratinocytes (NHEK) were unaffected (87). EGCG suppressed abnormal over-production of pro-inflammatory mediators in mouse macrophage cells (RAW2647) (88) and *in vivo*, EGCG supplementation decreased downstream markers in the NFκB pathway, such as oncogene cyclin D, COX-2 and prostaglandin E_2 production and reduced NMBA-induced esophageal tumors (29). One possible mechanism by which EGCG inhibits NFκB may be the attenuation of p-ERK activation and subsequent mitigation of NFκB and COX-2 as has been shown in mouse skin cells and human mammary epithelial cells, but not *in vivo* (89).

In vitro studies have proposed several other mechanisms of action against prostate cancer by EGCG. It was reported that EGCG inhibited cell proliferation, inhibited the activity of the epidermal growth factor receptor (EGFR) and caused cell cycle arrest in prostate cancer cells. EGCG also inhibited production of MMPs in androgen-independent DU145 cells, thus prevented angiogenesis and metastasis (90). EGCG attenuated NFκB at different levels along the pathway *in vitro*. However, there are limited studies investigating the effects of green tea or green tea polyphenols on inflammation and prostate carcinogenesis *in vivo*. One study showed that oral infusion of green tea polyphenols at human physiologically achievable concentrations (0.1%, about 6 cups of green tea per day) inhibited prostate cancer development and distant-site metastasis in the TRAMP model (91). They also showed that GTP infusion decreased IGF-1/IGFBP-3 ratio, a predictor in prostate cancer progression (91-94). Follow up studies showed that a green tea polyphenol mixture and EGCG lowered levels of IGF-1 in the dorsolateral prostate of TRAMP mice and significantly reduced the activation of PI3K/AKT and ERK1/2 pathways, and protein levels of VEGF, MMP-2 and -9 (95, 96). These findings suggested that a green tea polyphenol mixture inhibited growth signaling, progression and invasion of malignant tumors. Furthermore, one study using male athymic nude mice inoculated with human prostate cancer cells lines, PC3 and LnCap 104-R, showed that EGCG supplementation (1 mg/mouse/day, i.p., 14 days) significantly attenuated growth of both androgen dependent and independent tumors (96, 97).

Nevertheless, the effects of green tea on prostate hormone homeostasis, inflammation and NFκB *in vivo* are still unclear. The Noble rat model incorporates elements of hormone, inflammation and prostate cancer, which has enabled the investigation of the efficacy of dietary agents such as green tea as potential anti-inflammatory and chemoprevention agents *in vivo*.

1.3.3 "Whole Food" Approaches for Cancer Prevention

More studies in recent years have started to take a "holistic" instead of a "reductionist" approach in searching for effective disease preventive agents. Consuming a food instead of a supplement containing high levels of a specific bioactive component from that food may be more effective in preventing disease. This approach has become a more appealing and economic consideration for disease prevention. Moreover, high levels of a specific bioactive agent may also elicit toxicity as well as eliminating possible cooperative interactions among different constituents of the food. Different components of a whole food may work synergistically together, or form specific food matrix effects that provide greater benefits, which are omitted from using only a single component of whole food. Previous studies have demonstrated that dietary lycopene, one of the bioactive components of tomatoes, is effective in reducing prostate cancer. Interestingly, a diet supplemented with whole tomato product (dried tomato powder) was more effective in reducing prostate cancer mortality than dietary supplementation with equivalent levels of lycopene alone (98). These results indicated that other compounds in tomatoes, such as folate, vitamin C, β-carotene, or phenolic compounds may enhance the beneficial effects of lycopene. These studies further supported the whole-food based approach to prostate cancer prevention. Similarly, both soy and green tea contain mixtures of bioactive components, thus a whole food approach may be more effective than using individual soy isoflavones or tea catechins for chemoprevention. Soybeans contain numerous biologically active constituents including phytate (99, 100), phytosterols (101), oligossacharides, α-linolenic acid (102), vitamin E (103) and soy protein/peptides (104). In addition to catechins, green tea contains vitamin C (6 mg/100 mL green tea, 10 g tea/430 mL water, 90ºC, 1 min), caffeine (0.02 g/100 mL green tea), and other nutrients (105). In addition, treatment dosages higher than physiological concentrations may not always be beneficial. There is emerging evidence that suggests acute administration of high

levels of soy isoflavones had no protective effects against breast cancer in post menopausal women (62-64). High levels of soy isoflavones may be detrimental to breast cancer development. High levels of daidzein had a slight stimulatory effect on MCF-7 breast cancer tumor growth in ovariectomized athymic mice. Therefore, the timing of soy isoflavone supplementation may be important for breast cancer patients (106). Similar to the toxicity induced by high levels of soy isoflavones, EGCG at high concentrations may also elicit adverse effects. High levels of EGCG were shown to produce hydrogen peroxide, and its pro-oxidant activity inhibited gap junctional intercellular communications in rat liver cells, which was linked to carcinogenesis (107, 108).

On the contrary, low dosages of a combination of bioactive compounds may eliminate the possible toxicity associated with high levels of one compound and induce additional/synergistic effects among them. This again follows the rationale that whole foods containing multiple antioxidants and nutrients will target and sequester various points of the carcinogenesis pathway. For example, soy contains several compounds besides isoflavones that have been suggested to have anti-carcinogenic effects, such as saponins and protease inhibitors (109-111). These compounds may target different mechanisms that become deregulated during the development of cancer, such that some nutrients act as anti-oxidants while others target cell proliferation or angiogenesis. Studies showed that combinations of five isoflavones were more efficacious than genistein or daidzein treatments alone (65). Furthermore, combination of different foods can provide novel chemo-preventative strategies. Zhou et al. showed that combination of soy phytochemical concentrates and green tea reduced angiogenesis, IGF-1 and estrogen-dependent breast (112) and prostate tumorigenicity in mice (113).

1.4 Steroid Hormones and Prostate Cancer

Targeting steroid hormone metabolism has been the primary strategy in combating prostate cancer for many years. Recent studies noted that changes in estrogen/testosterone balance in older men might contribute to prostate pathology.

1.4.1 Testosterone and dihydrotestosterone

Testosterone and dihydrotestosterone (DHT) are the two major androgens responsible for growth and differentiation of prostate tissue. 5-α-reductase converts testosterone to DHT within the prostate. Both testosterone and DHT binds and activates the androgen receptor, but DHT has a greater affinity for the receptor, thus exerts more potent androgenic effects. Activation of androgen receptors (AR) results in enhanced transcription of genes involved in cellular proliferation such as epidermal growth factors (EGFs) and insulin growth factor-I (IGF-I) (114). Traditional prostate cancer therapy targets such properties of androgens, but total androgen ablation therapies have not succeeded in eradicating the disease because prostate cancer generally progresses to a hormone refractory state, limiting the efficacy of this approach. AR expression is maintained throughout prostate cancer progression including the majority of hormone refractory prostate cancers. Most identified AR mutations from hormone refractory prostate cancer are capable of transcriptional activity, suggesting that AR function is not the major cause of androgen ablation failure, but rather, dysregulation of AR signal transduction cascades, alteration in the expressions of AR co-regulators, and mutations in AR that allows transcriptional activation by ligands other than testosterone and DHT (114). Treatment strategies that target levels of AR may be a promising approach in prostate cancer therapy.

1.4. 2 Estrogens

Estrogens also play a role in prostate carcinogenesis and facilitates the transformation of prostate cells to higher malignant states (115, 116). Estrogen is proposed to be tumorigenic because it can act as both an initiator and/or a promoter for cancer development. Estrogens affect the rate of cell proliferation through receptor-mediated processes and enables genetic errors that lead to malignant phenotypes. Certain metabolites of endogenous estrogens, such as estradiol-3, 4-quinones, have been shown to form depurinated DNA adducts. The apurinic sites produced in the DNA by error-prone basic excision repair can lead to critical mutations that initiate cancers. The strongest carcinogenic estrogen metabolite is 4-hydroxyestradiol (4-OHE_2), followed by 2-OH E_2 and E_2 (117). 4-OHE_2 serves as a co-oxidant and strongly stimulates production of pro-inflammatory products such as prostaglandins (118). Furthermore, prostate regions susceptible to carcinoma induction were found to have less protein expressions of enzymes that normally

detoxify 4-catechol estrogens including catechol-*o*-methyltransferase (C*O*MT), glutathione (GSH) and quinone reductase (QR) (119). Nevertheless, targeting only testosterone or estrogen may neglect an important aspect of the inter-conversion between androgens and estrogens by aromatase and overlook the complexity of the regulation among these hormones. The disruption of the balance between estrogen and testosterone seems to be more applicable to prostate carcinogenesis than the deregulation of one hormone.

Androgens and estrogens both contribute to normal prostate cell growth and differentiation, but an imbalance in the hormone homeostasis may disrupt redox balance in the prostate. Estrogen facilitates the loss of androgen dependency and transformation to more aggressive-growing cells in androgen-dependent prostate cancer LnCap cells. During such transformation, the redox balance in the estrogen/testosterone metabolism shifts towards estradiol production and activation of testosterone and DHT, leading to cellular proliferative pressures and unregulated prostatic growth (120). Prostate tissues are able to produce estrogens. Human benign prostatic hyperplasia specimens have enhanced expression of enzymes involved in conversion of circulating dehydroepiandrosterone (DHEA) to E_2. Furthermore, LnCap, an androgen-dependent prostate cancer cell line, can synthesize active E_2; thus, local biosynthesis of estrogen may contribute to development and progression of prostate pathology such as BPH-1 and prostate cancer (121). Observational studies also suggest that higher circulating estradiol contributes to higher prostate cancer risk in African American men compared to Caucasian men in the US (122). In the Noble rat model (discussed in detail in the next section), chronic combined administration of testosterone and 17-β-estradiol, in the absence of a xenobiotic carcinogen, results in nearly 100 % incidence of prostate tumors after 50 weeks of treatment, with pre-cancerous lesions seen as early as 16 weeks (115, 123-125). The prostate lesions are localized to the dorsolateral lobes of the rat prostate, which is morphologically similar to the peripheral zone in the human prostate where cancer usually develops. Interestingly, the incidences of prostate cancer in rats receiving only testosterone or estradiol treatments are markedly lower than the combine treatment group, suggesting that elevation of both testosterone and estrogen are required for prostate carcinogenesis.

1.5 The Noble Rat Model

It was proposed that the imbalance of estrogen and testosterone in elderly men contributes to prostate cancer. Androgen ablation therapy (AAT) has been in use for decades for combating prostate cancer, but despite the varied means in which it is achieved, AAT has not been completely effective and ablated patients eventually relapse. Relapsing is associated with disease progression into androgen-independent state, for which no known treatment is yet known. Estrogen action in prostate has gained increasing attention over the years. Estrogen acts both as an endocrine indirectly acting on the balance of androgen productions via the pituitary systematically and in a paracrine fashion, acting directly on prostate tissues locally. To further complicate the story, the presence of two estrogen receptor subtypes, α and β, appear to have significantly different roles in the prostate, with ERα mediating the adverse and ERβ mediating the beneficial effects of estrogen (126). The complexity of hormonal signaling and balance in the prostate has rendered it very difficult to pin-point a specific target for intervention.

One major challenge of prostate cancer research is finding a representative animal model system that closely reflects aspects and stages of the human disease. Key anatomical differences between the rodent and human prostates have led to concerns about whether rodent models are suitable for studying human prostate cancer. There is currently no model that captures all features of human prostate cancer initiation and progression. Moreover; most existing model systems utilize toxic carcinogens or transgenic strategies to induce prostate cancer. Both methods induce aggressive disease progression that does not recapitulate the characteristics of prostate cancer in human, which develops slowly over several decades (127). Furthermore, levels of carcinogen exposures in human are rarely equivalent to what the animals receive in these models. Rodents do not spontaneously develop prostate cancer, and the Noble rat model is one of the few model systems that induce prostate cancer without relying on genetic modification or SV40T antigens. In our studies, we used an established rodent model of prostate carcinogenesis that administered elevated hormone treatments to Noble rats to induce an inflammatory response and tumors in the prostate (see **Figure 1.4)**(124, 128). The Noble rat model was

developed by Dr. Robert L. Noble in 1987 (129). He noticed that elevated levels of estradiol plus testosterone in Noble rats induced inflammation in the prostate, which eventually progressed to prostate tumors. Chronologically, Noble rats exposed to elevated levels of testosterone and estradiol show NFκB activation and inflammation in the prostate after 4 weeks, precancerous lesions at 16 weeks, and prostate tumors beyond 50 weeks of hormone treatments. By using the Noble rat model, we can study the molecular interactions among diet, sex hormonal microenvironments of the prostate, inflammation and cancer development.

Preliminary data showed that after 8 weeks of hormone treatments, rats showed inflammatory infiltration in the dorsolateral prostate characterized by the invasion of polymorphonuclear leukocytes (PMN; mainly neutrophils) and mononuclear cells (monocytes and macrophages) into the prostate stroma (**Figure 1.5**). The appearance of inflammation and PIN-like lesions preceded the development of cancer in the same anatomical locations in the prostate (130). Studies suggest that hormone-induced inflammatory responses increase oxidative and nitrosative stress in the prostates of Noble rats (131) and the inflammation is found in close association with PIN-like lesions (132). It is postulated that estrogen metabolites form DNA adducts that would eventually lead to carcinogenesis. Cavalieri et al. showed that when treated with 4-catechol estrogen metabolites, which could be oxidized to form reactive catechol estrogen quinones and further generated DNA depurinating adducts, there were fewer glutathione (GSH) conjugates formed by catechol-*O*-methyltransferase (C*O*MT) in the dorsolateral lobes than the ventral lobes in Noble rats. This suggested that dorsolateral lobes, which are more susceptible to carcinoma induction, have less protection by catechol metabolizing enzymes, such as C*O*MT and GSH (119). Hormone induced inflammation may act as an initiator in early stages of prostate carcinogenesis by increasing oxidative imbalance in the prostate, act as promoters that encourage regenerative proliferation of damaged cells and provide growth advantages for cancerous cells. Elevated estradiol and testosterone result in deregulation of signaling pathways and chronic immune responses (6). However, the exact mechanisms by which hormones induce inflammation have not yet been elucidated.

Based on cDNA microarray and gene profiling studies, genes involved in cellular proliferation and apoptosis are affected by hormone treatments in this model, but whether the changes are directly induced by estrogen or testosterone remains inconclusive (133, 134). Nevertheless, long term hormone treatments for 12 months up-regulate genes that facilitate malignancy and metastasis, such as matrix metalloproteinase-7 (MMP-7) and testosterone-repressed prostatic message-2 (TRPM-2) proteins (133, 135). The adenocarcinomas observed in the Noble rat model are microscopic, invasive tumors that mainly originate near the peri-urethral ducts of the dorsolateral prostate and the ventral prostates are not involved in tumor development (123). The prostate lesions are localized to the dorsolateral lobes of the rat prostate, which morphologically mimics the peripheral zone in the human prostate where cancer usually develops. Interestingly, the incidences of prostate cancer in rats receiving only testosterone or estradiol treatments are markedly lower than the combine treatment group.

Several studies have utilized the Noble rat model to investigate the effects of dietary compounds on prostate cancer incidence and latency such as dietary fat (136) and retinoids (137). Christov et al. concluded from their studies that hormone induced prostate intraepithelial neoplasia (PIN) in 80-100% of Noble rats after 36 weeks of testosterone and estradiol treatments, and the PIN lesions in Noble rats were useful intermediate endpoints in assessing efficacy of chemo-preventative agents (137). Yatkin et.al. found that dietary soy (7.61% extracted and toasted soy protein and 4.25% soy oil) decreased inflammatory foci in the prostate and marginally decreased development of obstructive voiding in Noble rats (137, 138). This is the only previous study focused on the anti-inflammatory effects of phytochemicals in the prostate using this model.

To administer the hormone treatments, noble rats at sexual maturity (11 to 12 weeks old) were implanted with two 3 cm silicone tubes containing ~14 mg of testosterone and one 2 cm tube containing ~14.8 mg of estradiol (123). Tubes were packed with hormones via vacuum suctioning, and ends of the tubes sealed with silicone type medical adhesive. Tubes were inserted subcutaneously between the shoulder blades and were replaced every 8 weeks**.** In our study design, male Noble rats were randomly assigned to groups according to hormone treatments and dietary

treatments. There were four dietary treatment groups- control, soy, green tea, and soy+tea combination group, see **Figure 1.6**. According to the "whole-food" approach, whole food containing multiple components would be more effective than individual bioactive component in attenuating carcinogenesis. Therefore, our study used soy and green tea instead of soy isoflavones and tea polyphenols as the dietary treatments. We evaluated inflammation and NFκB activation in the prostate at the early time points of the study, and assessed hyperplasia, and pre-cancerous lesions such as prostate intraepithelial neoplasia (PIN) in the prostate at later time points.

The Noble rat model has inherent problems like many other prostate cancer animal models, such that the prostate physiology in rats is different than human. Rat prostates have six distinctive lobes, whereas human prostate are characterized by zones and contains no lobes (139). Furthermore, Noble rats do not spontaneously develop prostate cancer. The gut microflora also plays an important role in metabolism and uptake of phytochemicals. In particular, soy isoflavones depend heavily on gut microflora for metabolizing to their bioactive compounds. Soy glycosides in soybeans, namely daidzin, genistin and glycitin, are hydrolyzed by mammalian enzymes and the gut microflora to form active aglycones, daidzein, genistein and glycitein, respectively. There are large differences in inter-individual metabolism due to different microflora in humans, and this may have major health implications (140-142). Our model assumes that Noble rats are genetically homogenous and quantities of gut microflora are similar among individual rats. Furthermore, studies have shown that after β-glucuronidase hydrolysis, soy isoflavone aglycones are absorbed and bioavailable in rats' prostates (143, 144).

In summary, the Noble rat model avoids the usage of carcinogens or transgenic strategies to induce prostate tumors. The slow progression and distinctive stages of disease progression observed in this model mimics the human condition. Overall, the Noble rat model is suitable for understanding the intricate relationships among chronic inflammation, hormonal microenvironment and prostate carcinogenesis. This model allows us to investigate novel mechanisms *in vivo* by which sex hormones initiate and promote prostate cancer, and the efficacies of cancer preventative agents in attenuating these processes.

1.6 Dissertation Specific Aims and Hypothesis

Prostate cancer is the second leading cancer-related cause of death in American men. Population studies indicate that prostate cancer rate is lower in Asian countries, especially China, than Western countries. However, the difference in prostate cancer rate between Asian and Western countries cannot be fully accounted for by genetic differences. Moreover, migration studies indicate that when Asian immigrants move to the US, their prostate cancer rates increase considerably. Such observations suggest that modifiable factors such as diet and lifestyle are important determinants of prostate cancer progression. Dietary components in Asian cuisines, such as soy and green tea, hence are prospective chemo-preventative agents in prostate cancer research. Soy isoflavones and green tea polyphenols have been proposed to exert anti-carcinogenic effects through several mechanisms including induction of apoptosis, cell cycle arrest and reducing inflammation in cancer cells. Notably, inflammation has been implicated as an important contributor in prostate cancer.

Previous studies have shown that chronic inflammation and uncontrolled cell proliferation in the prostate are linked to tumor initiation and neoplastic conversion (6, 7, 10). More specifically, NFκB is suggested as the mediator between chronic inflammation and carcinogenesis (27, 28). NFκB is a transcription factor that initiates and amplifies inflammatory cascades and cell proliferation. Prolonged activation of NFκB causes sustained oxidative stress and uncontrolled cell proliferation, and dysregulation of NFκB pathway has been implicated in various diseases (17, 28), including prostate cancer (22, 24). The cause of chronic inflammation and NFκB activation in the prostate is not well understood, but some investigators have postulated that inflammation is caused by exogenous toxins or by endogenous hormone imbalance. Notably, aging is usually associated with decrease in testosterone and increase in estrogen levels in men, and the balance between estrogen and testosterone are shown to influence immune responses and inflammation in the prostate (26). Estrogen receptors found in prostates are implicated in tumorigenesis and pro-inflammatory conditions, whereas estrogen receptor decoys (phytoestrogens and ICI 182,780) are effective in decreasing downstream pro-inflammatory cytokine productions (145). Therefore, dietary regimens that target hormone-induced inflammation and cell proliferation may be

effective chemoprevention against prostate cancer. Whether soy or green tea protects the prostate from hormone-induced chronic inflammation and carcinogenesis by modulating NFκB pathway *in vivo* is currently unclear. This gap in knowledge will limit our understanding of prostate cancer pathology and development of efficacious preventative strategies.

The long-range goal of this research project is to gain a better understanding of the molecular and cellular mechanisms involved in the initiation and progression of prostate cancer, and to develop effective dietary preventative strategies to reduce the incidence of this disease. The objective of these studies is to validate the mechanisms that link hormonal (estrogens/testosterone) stimulation of chronic inflammation and uncontrolled prostate cell proliferation, and to evaluate the effectiveness of dietary strategies that target these processes. Our central hypothesis states that prostate carcinogenesis will be attenuated by inhibiting hormone-induced prolonged activation of NFκB. Our three specific aims are:

Aim 1. To evaluate and compare the effects of whole soy and green tea versus individual soy isoflavones and green tea polyphenols in inducing apoptosis, cell cycle arrest, and inhibiting* NFκB *activation in prostate cancer epithelial cells in vitro.

Our working hypothesis is that soy extract or green tea with combined active components will provide more potent anti-carcinogenic effects on prostate cancer cells than individual bioactive components of soy or green tea, namely soy isoflavones (genistein and daidzein) and green tea polyphenols (EGCG). These effects will be apparent in both the early stage, androgen-dependent prostate cancer LnCap cells and late stage, androgen-independent prostate cancer PC-3 cells.

Aim 2. To evaluate the effects of dietary soy and green tea on sex hormone-induced prostate carcinogenesis in Noble rats.

Our working hypothesis is that elevated hormone levels will lead to chronic inflammation and prostate cancer growth, and dietary modifications, specifically soy and green tea, will decrease inflammation and prostate tumor outcome.

Aim 3. To identify the molecular mechanism(s) by which soy and green tea may inhibit hormone-induced inflammation in prostate tissues of Noble rats.

Our working hypothesis is that dietary soy or green tea treatments will inhibit NFκB activation, sequester the inflammatory cascade, induce apoptosis, and modulate hormone homeostasis, resulting in attenuation of prostate carcinogenesis.

Table 1.1 Soy isoflavone contents in soy products.

	Soy isoflavone content
Traditional soy foods	25 mg/ serving
Soymilk	0.3 mg/mL
Tofu	0.75- 0.9 mg/g
Soybeans (dry weight)	~1.2- 3.3 mg/g
Cooked soybeans	~ 0.88 mg/g

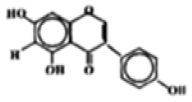
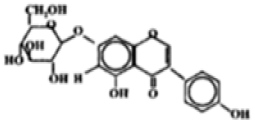
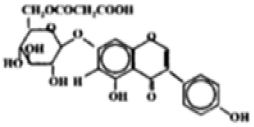
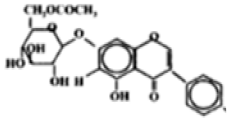
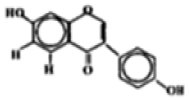
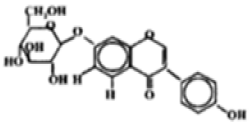
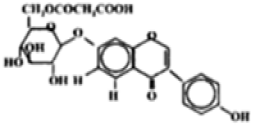
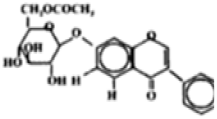
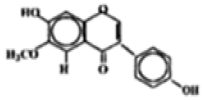
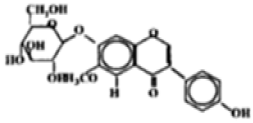
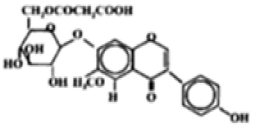
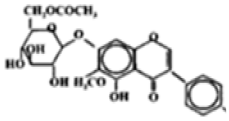
AGLYCONES	GLYCOSIDES	MALONYL-GLYCOSIDES	ACETYL-GLYCOSIDES
 genistein	 genistin	 6″-*O*-malonylgenistin	 6″-*O*-acetylgenistin
 daidzein	 daidzin	 6″-*O*-malonyldaidzin	 6″-*O*-acetyldaidzin
 glycitein	 glycitin	 6″-*O*-malonylglycitin	 6″-*O*-acetylglycitin

Figure 1.1 Chemical structures of 12 isoflavone isomers found in soy.

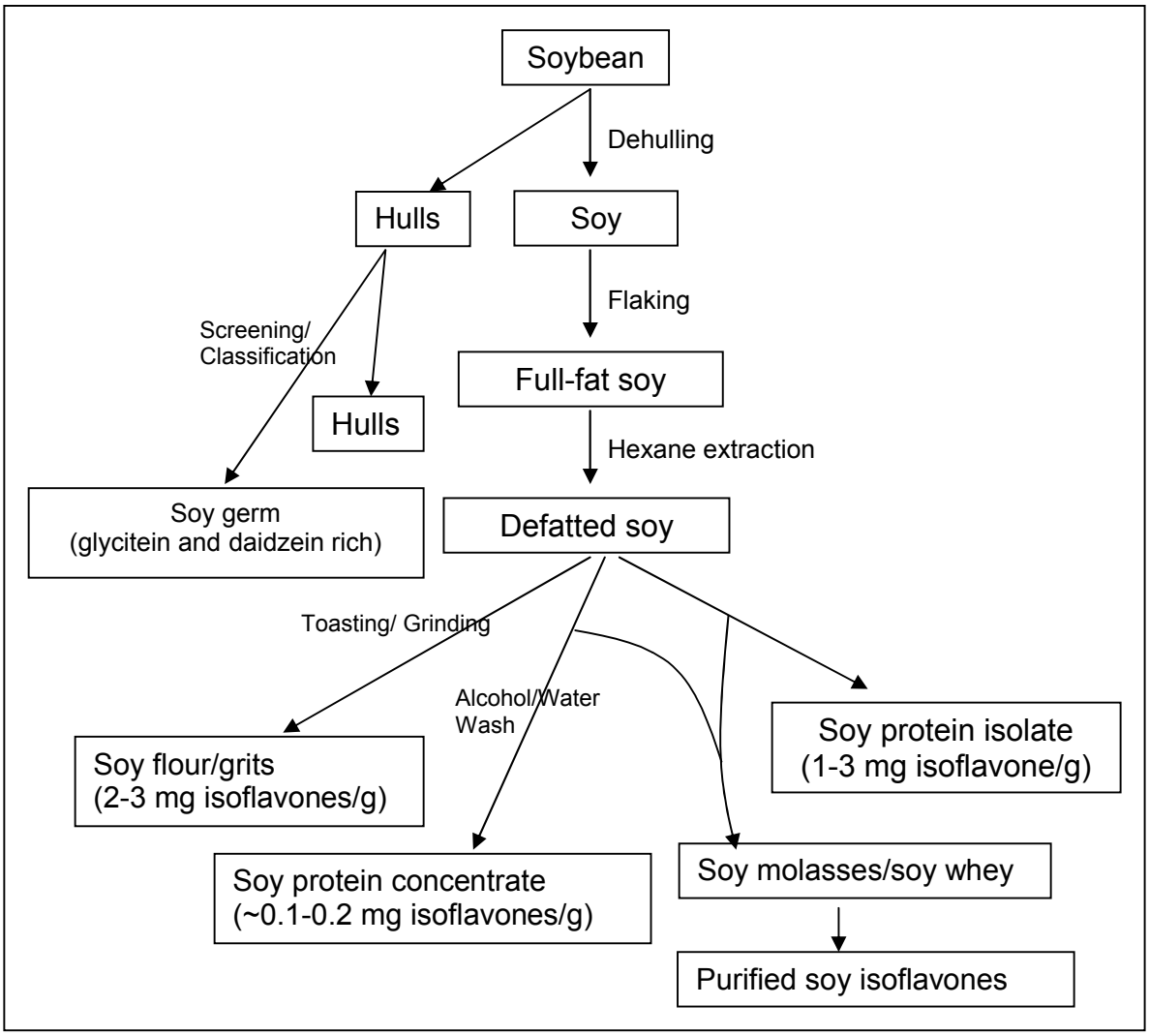


Figure 1.2 Stages of soybean processing

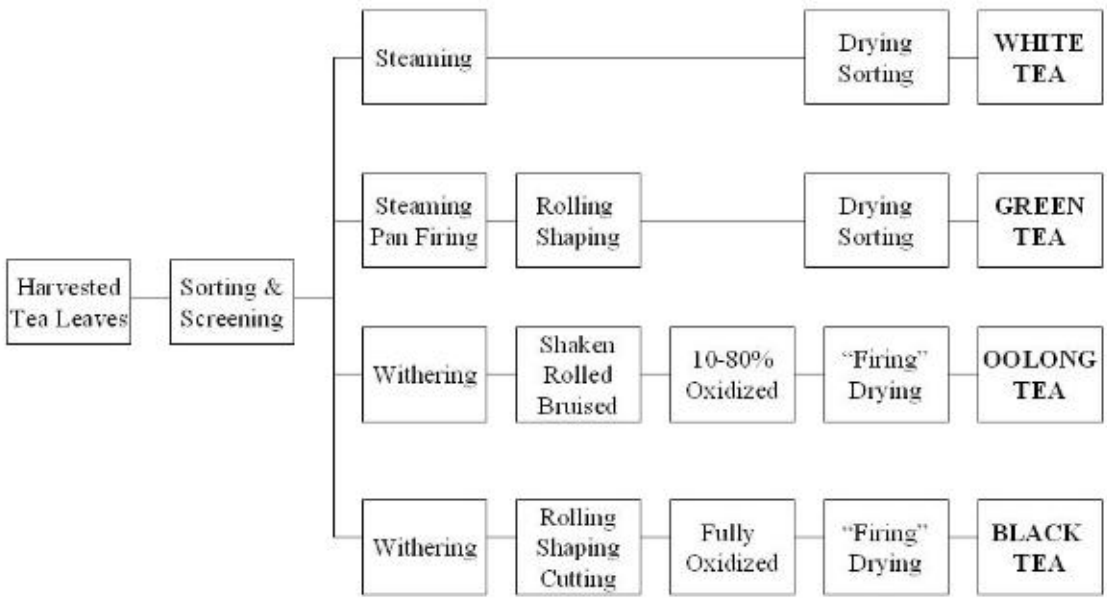


Figure 1.3 Processing of tea.

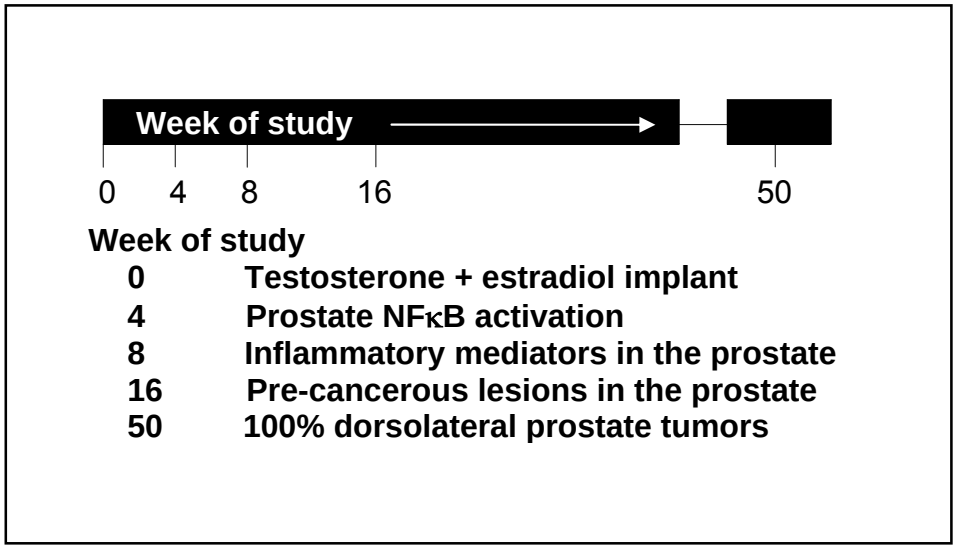


Figure 1.4 Chronology of Noble rat model. Chronological events in the Noble rat model from prostate inflammation to carcinogenesis, as established by other investigators.

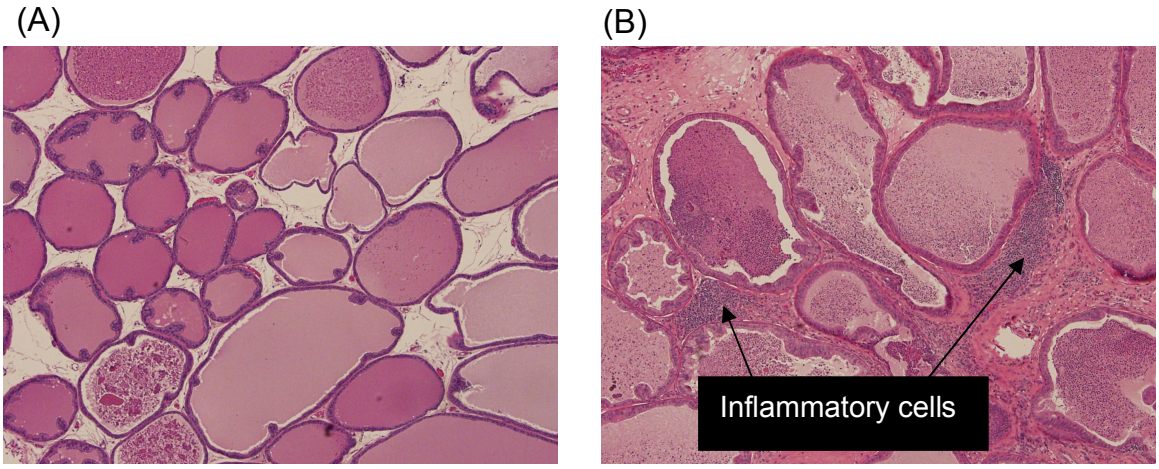


Figure 1.5 Hormone-induced inflammations in the prostate. Presence of inflammatory cells (PMNs) in the dorsolateral prostate in rats trested with empty implants (A), or implants containing both testosterone and estradiol (B) (both H&E staining; 10×).

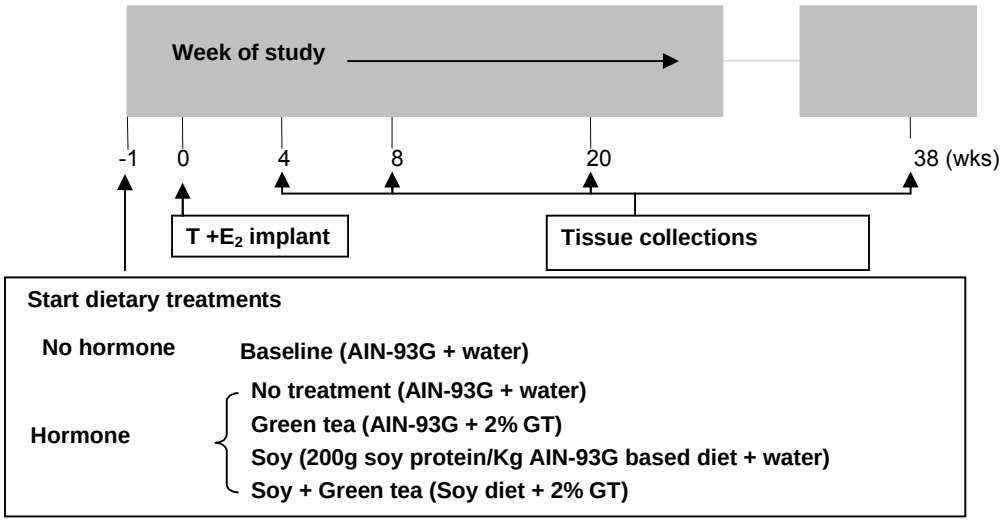


Figure 1.6 Noble rat dietary treatment plan. Rats were randomly assigned to five groups: baseline without hormone, no treatment (AIN-93G diet), green tea, soy, and soy+tea groups. Testosterone and estradiol were implanted subcutaneously into hormone-treated rats at sexual maturity (11 weeks of age; time point 0) in the no treatment, green tea, soy and soy+tea groups. Rats were sacrificed and tissue samples collected after 4, 8, 20 and 38 weeks of hormone treatments.

Chapter 2 Differential effects of whole soy extract and soy isoflavones on apoptosis in prostate cancer cells

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Provisional acceptance; *Experimental Biology and Medicine*, 2009

2.1 Abstract

Previous studies have suggested that soy isoflavones exert anti-carcinogenic effects against prostate cancer. We propose that soy extracts, containing a mixture of soy isoflavones and other bioactive components are a more potent chemo-preventative agent than individual soy isoflavones. We compared the apoptotic effects of whole soy extracts and individual soy isoflavones, genistein and daidzein, on prostate cancer cells. The soy extract contained 50% w/w of total isoflavones with approximately 1:5.5:3.5 ratios of genistin, daidzin, and glycitin, respectively. BPH1, LnCap and PC3 cells were treated with varying concentrations of soy extract, genistein or daidzein and analyzed for cell cycle alterations and induction of apoptosis. At equal concentration (25 μM), soy extract induced significantly higher percentage of cells undergoing apoptosis than did genistein or daidzein ($p<0.001$). No significant changes in cell cycle arrest or apoptosis were observed in non-cancerous benign prostate hyperplasia (BPH-1) cells treated with soy extract, suggesting that the effects of soy extract may be tumor-cell specific. On the contrary, both genistein and daidzein induced apoptosis in BPH-1 cells suggesting individual isoflavones may have cytotoxicity in non-cancerous cells. Soy extracts also increased Bax expression in PC3 cells, but no significant changes in NFκB activation were detected suggesting that the induction of apoptosis was independent of the NFκB pathway. Food products that bear a combination of active compounds may be more efficacious and safer as chemo-preventative agents than individual compounds. This “whole food” based approach is significant for the development of public health recommendations for prostate cancer prevention.

KEYWORDS: Soy isoflavones, prostate cancer, apoptosis, nuclear factor kappa B

2.2 Introduction

Prostate cancer is the most common type of cancer found in American men (1), however prostate cancer rates are considerably lower in Asian countries. Migration studies have shown that prostate cancer rate increases considerably when Asian migrants moved to the United States. Asian migrants (high soy and tea intake population) have lower incidence of prostate and mammary cancers than subsequent generations of Asian Americans (33). Incidence of prostate cancer in Chinese, Japanese and Filipino men born in China, Japan and the Philippines, respectively, were about half of those born in the US (2). These observations suggest that the etiology of prostate cancer is highly influenced by environmental lifestyle factors such as diet. Dietary factors found in the Asian diet, such as increased soy, may contribute to the decrease prostate cancer risk. A typical soy-rich Japanese diet consists of 25 to 100 mg soy isoflavones/day (1 serving of traditional fermented soy food contains about 25 mg of soy isoflavones), while the typical American diet contains about 1 to 3 mg soy isoflavones/day (34, 35). The circulating soy isoflavone concentrations in Japanese men are at least ten-fold higher than Caucasian men (38, 146) and increased soy consumption has been associated with lower risk of prostate cancer (36, 49). Even when soy consumption is moderately high, a dose-dependent inverse relationship with soy intake and localized prostate cancer incidence has been observed in Japanese populations (40). Although soy consumption appears to contribute to decrease risk in prostate cancer, the precise mechanisms are still unclear.

Both *in vitro* and *in vivo* studies have theorized possible protective mechanisms of soy isoflavones, such as genistein, against cancer development (39, 41, 42). Soy isoflavones, which are classified as phytoestrogens, act as both estrogen agonists and antagonists by differentially binding to estrogen receptor α or β (45, 46) and/or altering enzymes involved in hormone metabolism (37, 45, 46). Furthermore, soy isoflavone concentrates also induce G2/M arrest and p21 expressions in androgen-independent PC3 prostate cancer cells (60) and may act through hormone-independent pathways that target cell cycle or apoptotic mechanisms. For instance, genistein is a known inhibitor of protein tyrosine kinase (50), topoisomerase II (51),

and upregulates p21 in various cancer cells (147-149). Genistein also inhibits NFκB, a transcription factor that regulates inflammatory & cellular proliferation pathways, and subsequently induces apoptosis in breast cancer cells (57) and prostate cancer cells (58, 59).

Nevertheless, there are still questions to whether using high levels of soy isoflavones in human is feasible and safe. Previous *in vitro* studies have often utilized supra-physiological levels (≥ 50 μM) of genistein in their treatment regimes to elicit effects, which may not be safe *in vivo* (55, 150). However, acute administration of high levels of soy isoflavones may not be beneficial (62-64). In contrast, longer term treatment of these bioactive compounds at low doses, such as found in whole foods may be a safer approach. Furthermore, the different components of a whole food may be able to work synergistically together to provide additional benefits.

The present study was undertaken to compare the effectiveness of a soy extract with individual soy isoflavones in inhibiting prostate cancer cell growth via regulation of apoptosis, cell cycle and NFκB activation pathways. These findings from this study will help establish the foundation for using soy as chemo-preventative dietary strategy for prostate cancer.

2.3 Materials and Methods

Cell Culture: Benign prostate hyperplasia (BPH-1) cells were generously donated by Dr. Simon Hayward (Vanderbilt University Medical Center, Nashville, TN), and malignant androgen-independent prostate cancer epithelial cells (PC3) and early-stage androgen-dependent prostate cancer cells (LnCap) were obtained from America Type Culture Collection (Manassas, VA). All cells were grown and maintained in RPMI 1640 with glutamine (Mediatech Inc., Manassas, VA) supplemented with 1% penicillin-streptomycin (Mediatech Inc., Manassas, VA) and 5 % fetal bovine serum (Hyclone, Logan, UT) for BPH-1 and 10% for PC3 and LnCap cells at 5% CO_2 atmosphere at 37ºC.

Treatments: Crude soy extract (AdvantaSoy™ Clear 900, Cargill, Minneapolis, MN), genistein (Sigma-Aldrich, Saint Louis, MO) and daidzein (Sigma-Aldrich) powders were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Control treatment

consisted of DMSO at a final concentration matching the dose of treated cells and less than 0.1% of final volume. The soy extract contains 50% w/w of total isoflavone content with approximate 1:5.5:3.5 ratios of genistin, daidzin, and glycitin respectively. Based on manufacturer's analyses, soy extract also contained 5% protein, 3% ash, and 10-20% saponins. Soy extract concentrations were calculated by using the proportional sum of molecular weights of soy isoflavone aglycone and glycosides in the soy extract. The treatment concentrations were calculated as the total soy isoflavone contents using the combined molecular weight. The majority of the soy isoflavones in the soy extract were confirmed to be in their conjugated, glycosylated, forms (97%) with only about 3% as free aglycones by HPLC. Previous studies have suggested that genistein exhibited its anti-carcinogenic effects in prostate cancer cells. However, the dosages used in previous *in vitro* studies were higher than physiologically attainable levels(39). Also, genistein exhibited biphasic effects on cancer cell growth *in vitro*, stimulating cell growth at low concentrations (less than 10 μM) and inhibiting proliferation at high concentrations (more than 10 μM) (41). To establish the effective dose range for soy isoflavones in our model system, we performed dose response experiments where cells were treated with increasing doses (from 25 μM to 100 μM) of individual soy isoflavones (genistein or daidzein) or soy extract for 24, 48, and 72 hours.

Multicaspase Assay: After 48-hour treatment with soy extract, genistein or daidzein, adherent and floating cells were harvested and stained with sulforhodamine- valyl-alanyl- aspartyl- fluoromethyl- ketone (SR-VAD-FMK), a fluorochrome-conjugated caspase inhibitor, and 7-amino-actinomycin D (7-AAD) according to manufacture's instructions (Guava Technologies, Hayward, CA). Cell populations were quantified using Guava personal flow cytometer (Guava Technologies).

Cell Cycle: After 48-hour treatment with soy extract, genistein or daidzein, adherent and floating cells were trypsinized and fixed in ice cold 70% ethanol at 0.5 million cells per ml. After incubation at -20ºC for 12 hours, cell pellets were resuspended in cellular DNA staining solution containing 40 mg/ml propidium iodide (Sigma-Aldrich) and 100 μg/ml of RNase (Sigma-Aldrich). Cell cycle kinetics was analyzed using Guava personal flow cytometer. Histograms were constructed using MultiCycle V3.0 software.

Western blot analysis: Nuclear and cytosolic extracts from treated cells were collected using the Nuclear Extract Kit (ActiveMotif, Carlsbad, CA) according to manufacturer's instructions. Protein concentrations were determined by the Bradford assay (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA) (151). Nuclear protein levels of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2, and proteins in the NFκB pathway including p50, p65 (nuclear), IKKβ, IκBα, and p-IκBα (cytosolic) were qualitatively evaluated by Western blotting using the Invitrogen NuPAGE Western blotting system (NuPAGE Novex, Invitrogen, Carlsbad, CA). Cell cycle markers such as p53 (CalbioChem, San Diego, CA) and cMyc were also assessed. All primary antibodies specific against proteins of interest were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Ponceau S red staining and β-actin protein levels were used as protein loading controls. Secondary antibodies were conjugated with horseradish peroxidase (HRP) (Bio-Rad, Hercules, CA), and proteins were detected by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA) and imaged by Alpha Innotech photodocumentation system. Densitometry and quantifications were performed using NIH Image J software.

NFκB Enzyme-Linked ImmunoSorbent Assay (ELISA): NFκB DNA binding activity was assessed by using transcription factor p65 and p50 ELISA kits (ActiveMotif, Carlsbad, CA). Oligonucleotides containing the NFκB consensus site (5'-GGGACTTTCC-3') were immobilized on a 96-well plate. The active form of NFκB in the nuclear extracts were bound to the oligonucleotides on the plate and detected colorimetrically by spectrophotometry at absorbance of 450 nm.

Statistics: Experiments were performed independently for at least three times with triplicates for each biological sample. Data was analyzed using Graph Pad Prism V4.0. One-way ANOVA and Dunnett's Multiple Comparison test were used to evaluate the statistical difference; * indicated $p<0.05$, and ** indicated $p<0.01$.

2.4 Results

Soy extract induced cell cycle arrest and caspase activation.

We observed an induction in cell cycle arrest and apoptosis in prostate cancer cells 48 hours post treatment with both individual isoflavones and soy extracts. Greater than or equal to 100 μM genistein or daidzein was needed to induce caspase activation and cell cycle arrest in LnCap and PC3 cells (**Fig. 2.1**). In contrast, 25 μM of soy extract was able to induce caspase activation in LnCap and PC3 cells. In androgen-independent PC3 cells, treatment with equivalent doses of soy extract (25 μM) increased caspase activation and significantly increased cell population in SubG1, but not in cells treated with individual soy isoflavones (**Fig. 2.2**). Similarly, androgen-dependent LnCap cells had greater sensitivity to caspase induction with soy extracts. Interestingly, the pro-apoptotic effects of soy extracts were more prominent in PC3 cells than LnCap cells, as indicated by the differences in percentage of cells undergoing apoptosis. Despite the increase in caspase activity in LnCap exposed to soy extract, the percent cells in subG1 phase of cell cycle were unaffected. On the contrary, soy extracts increased caspase activity as well as the percent cells undergoing apoptosis in PC3 cells. There were no significant differences in necrotic cell populations among treatments for both the LnCap and PC3 cells (data not shown), suggesting that perhaps there is a delay in the LnCap cells in response to soy extracts in the transition from early apoptotic events to the DNA fragmentations that would be measured by the cell cycle analysis. No significant differences in cell cycle arrest or caspase activation were found in BPH-1 cells treated with soy extracts. However, in BPH-1 cells, 50 μM of genistein and daidzein induced caspase activation, suggesting cytotoxicity with individual isoflavones even at lower concentrations.

Soy affected BAX protein expression.

To investigate the subsequent events in the apoptotic pathways in prostate cancer cells, the protein expression of Bax, BcL2, cMyc and p53 in nuclear extracts of treated cells were measured by western blotting. Soy extract, genistein and daidzein increased Bax expression in PC3 cells (**Fig. 2.3B**), but not in LnCap cells (**Fig. 2.3A**).

There were no significant alterations in p53, BcL2 and cMyc expression among treatment groups (data not shown).

No effect of soy extract on NFκB activation pathways.

NFκB is a major mediator of inflammatory responses, cell proliferation and apoptosis. Expression of NFκB and its major regulatory proteins including IKKβ, p-IκBα, and IκBα were evaluated. Soy extract did not significantly change the translocation of p50 or p65. However, there was a trend for daidzein to increase the level of p50 translocation (p= 0.078, t-test) (**Fig. 2.4A**). There were no significant changes in NFκB binding activity with treatments (data not shown). There were no changes in NFκB inhibitory proteins in PC3 cells or LnCap cells associated with soy extract treatment (**Fig. 2.4B**), but daidzein induced higher levels of phosphorylated-IκBα (p-IκBα) targeted for degradation. The increased degradation of IκBα would account for the increase in p50 observed in the daidzein group.

As indicated previously, no significant changes in apoptosis and cell cycle were observed in BPH-1 cells treated with soy extract. Similarly, there were no significant changes in NFκB pathway with soy extract treatments observed in BPH-1 cells (data not shown). These results suggest that the anti-carcinogenic and pro-apoptotic effects of soy may be specific to tumor cells.

2.5 Discussion

Our study is one of the first to directly compare the effects of soy extract and individual soy isoflavones in inhibiting prostate cancer cell proliferation at physiologically attainable concentrations. Results from present study suggested that at equivalent physiological concentrations soy extract was more effective at inducing apoptosis in prostate cancer cells than individual soy isoflavones. Moreover, the anti-proliferative effects of soy extract were specific to prostate cancer cells, especially late stage prostate cancer PC3 cells. Both genistein and daidzein induced apoptosis in non-cancerous BPH-1 cells, whereas soy extracts had no effects. These data indicate that the combination of compounds found in soy extracts may be a more efficacious, safe and specific strategy for prostate cancer prevention.

Physiological levels of soy isoflavones are normally between 1 to 2 μM (65, 66), but higher concentrations (equal and greater than 25 μM) such as those observed in Japanese men can be achieved by regular consumption of soy (34). High levels of genistein and daidzein (≥100 μM) were required to induce anti-proliferative effects in prostate cancer cells (**Fig. 2.1)**. This finding agreed with previous studies, which demonstrated that relatively high doses of soy isoflavones (50-100 μM) were necessary to inhibit cell growth in breast and prostate cancer cells (57, 58, 150, 152). Furthermore, the soy extracts used in our study contained mostly conjugated soy isoflavones. The bioavailabilities of conjugated soy isoflavones are known to be lower than their aglycones, thus, the actual concentrations of active compounds available to treated cells were likely lower than 25 μM in our study (153). Despite this issue, our results showed that this limitation did not affect the potency of soy extract. Our observations agreed with previous studies that soy extract containing predominantly glycosides was used for inducing anti-carcinogenic effects in cell culture studies and that soy isoflavones glycosides were completely hydrolyzed and metabolized by prostate cancer cells (154).

One of the mechanisms by which prostate cancer cells retain growth advantages may be through constitutive activation of NFκB pathway (24). NFκB modulates cellular inflammatory responses, cell proliferation, cell cycle and apoptosis. Previous studies

by Davis et al. showed that 50 μM genistein inhibited NFκB nuclear translocation by reducing phosphorylation of IκBα (58). Our study showed that soy did not exert significant effects on NFκB. On the contrary, daidzein appeared to slightly increase p50 protein expressions by increasing degradation of IκBα in PC3 cells (**Fig. 2.4A**). Thus, physiological concentrations of soy isoflavones have markedly different responses in prostate cancer cells than supra-physiological ones. Physiological concentrations of soy may be more effective at the initiation and promotion stages of cancer when NFκB acts to promote chronic inflammation, rather than at later stages where NFκB is already constitutively activated and deregulated. Future *in vivo* chemoprevention studies that investigate the effects of soy on NFκB and inflammation in prostate carcinogenesis will have more clinical values and implications.

Soy extract induced caspase activation (**Fig. 2.2**) without changes in Bax expressions in LnCap cells. On the contrary, soy extract induced caspase activation and cell cycle arrest in PC3 cells through Bax (**Fig. 2.3C)** and independent of NFκB activation. Soy isoflavones also exerted differential effects in LnCap and PC3 cells. In LnCap cells, soy isoflavones had no effects on caspases or Bax, but in PC3 cells, genistein and daidzein both up-regulated Bax without affecting caspase activation. A possible explanation may be that soy isoflavones regulate Bax expression, but other components of soy extract can additionally target other downstream proteins, such as the anti-apoptotic caspases regulators, inhibitor of apoptosis (IAP) (155). IAPs have been shown to protect cells from Bax-mediated mitochondrial disruption and inhibit activated caspases (156, 157), and prostate cancer cells, especially PC3 and DU145 cells, over-expressed IAPs (158). The combination of up-regulated Bax and down-regulated IAPs could translate into caspase activation and apoptosis observed in soy extract treatments, but not individual soy isoflavones treatments. Furthermore, previous studies indicated that decreases in the expression of caspases 3 was correlated with increase prostate cancer Gleason grade (159), and caspase 3 may be a critical factor in genistein-mediated anti-tumor effects in breast cancer cells (160). Therefore, identification of specific caspases and regulators of caspases in future studies will further clarify the targets of chemoprevention by soy treatments.

Soy contains several compounds in addition to isoflavones that may have anti-carcinogenic effects, such as saponins and protease inhibitor (109-111). Although we did not measure the composition of these compounds in the soy extract, the Cargill manufacturer estimated the approximate content of saponins to be 10 to 20% in the soy extract. The possible synergy of these other phytochemicals and their effects are an important area for future mechanistic studies. Importantly, the combinations or matrix of bioactive compounds in whole food may have greater anti-cancer effects than individual bioactive components. For example, dietary supplementation with whole tomato product (tomato powder) was more effective than equal level of lycopene supplement alone in inhibiting prostate cancer growth *in vivo* (98). In soy research, a previous study has also shown that combined soy isoflavones were more efficacious than genistein or daidzein in inhibiting prostate epithelial cell growth *in vitro* (65). In addition, the matrix of combined soy isoflavonoids and other compounds may provide more stability for the soy isoflavonoids. Studies done by Setchell et al. indicated that the biologically active aglycones are very stable and released intact after processing or intestinal metabolism (161-163). It is possible that that the isoflavones show differential stability either individually or combined with each other, and is an important area of future research. Following the same rationale, whether soy isoflavones interact with other components of soy and soy combine with other foods to form additional or synergistic benefits against prostate cancer cell growth will be of interest for future investigations.

In summary, soy extract was more effective in inducing cell cycle arrest and apoptosis than soy isoflavones in both early-stage (LnCap) and late-stage (PC3) prostate cancer cells. Soy extract induced cell cycle arrest at SubG1, activated caspases and increased pro-apoptotic protein Bax expressions through NFκB- independent pathways. Importantly, the pro-apoptotic effects of soy extract appear to be tumor-cell specific. Whole food approach (soy extract) limited toxicity towards healthy cells while effectively inhibited prostate cancer cell proliferation, but future *in vivo* studies are needed to evaluate and compare the effects of soy and soy isoflavones on prostate cancer. Taking the whole-food approach may be more efficacious, safer and cost effective than individual compounds for the development of evidence-based public health recommendations and chemo-preventative strategy for prostate cancer.

Acknowledgements:

The authors thank Dr. William Helferich for the soy isoflavone HPLC measurements.

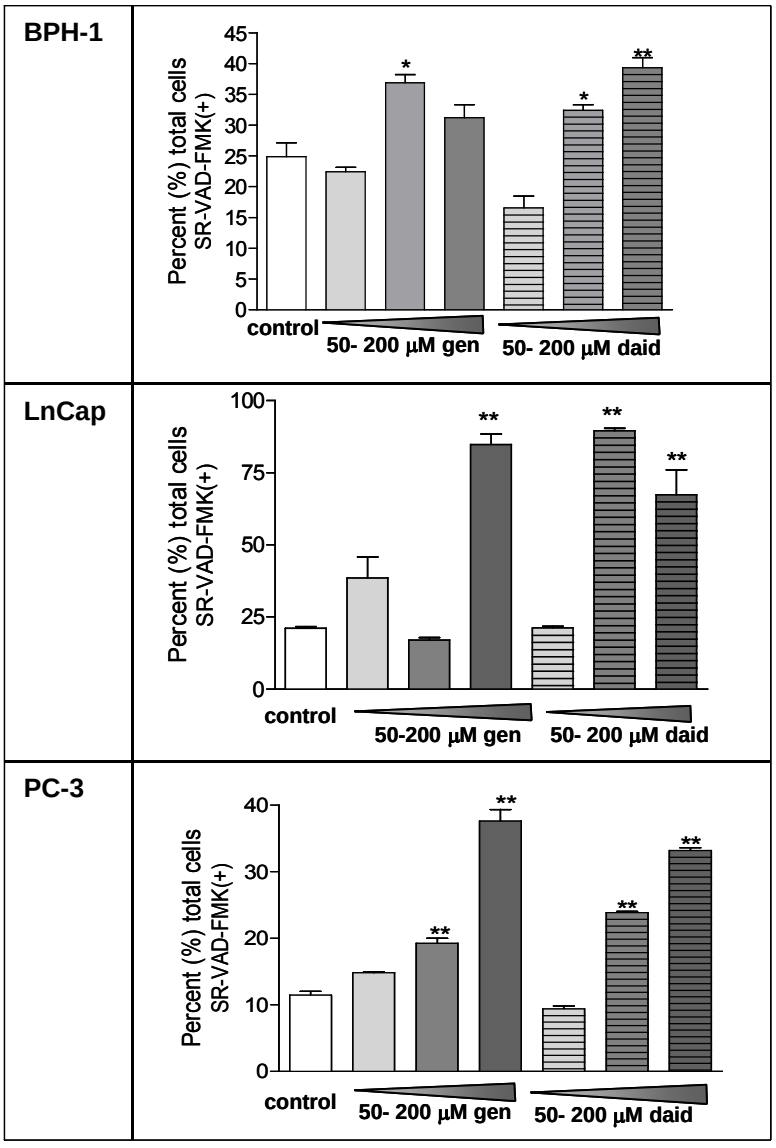

Figure 2.1 High doses of genistein induced cell cycle arrest and apoptosis in BPH-1, LnCap and PC3 cells. BPH-1, LnCap, and PC3 cells were treated with varying concentrations (25, 50, 100 and 200 μM) of genistein (gen) or daidzein (daid) for 48 hours, and labeled with SR-VAD-FMK stain (fluorochrome-conjugated caspase inhibitor) to assess multicaspase activity by flow cytometry. Greater than 100μM of genistein and daidzein induced caspase activation in LnCap and PC3 cells, but ≥50 μM of genistein and daidzein also activated caspase activation in BPH-1 cells. Control treatment was consisted of media with <0.1% DMSO. Values represent means ± SEM, n=3. * $p<0.05$, ** $p<0.01$ compared to control.

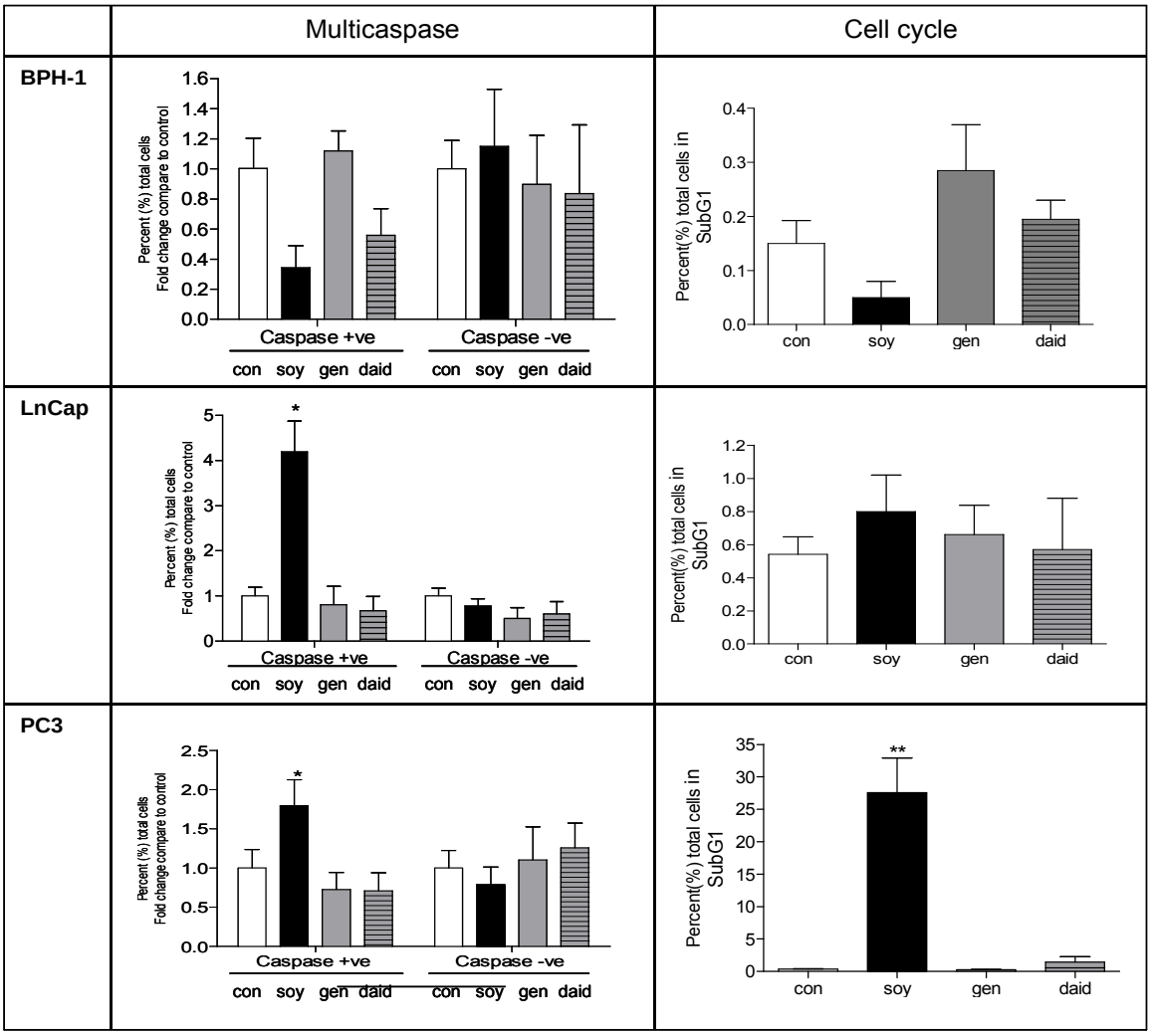

Figure 2.2 25 μM of soy extract induced apoptosis in PC3 and LnCap cells with no effect at equal concentrations of soy isoflavones. BPH-1, LnCap, and PC3 cells were treated with 25 μM of soy extract (soy), genistein (gen) or daidzein (daid) for 48 hours. Multicaspase activity and cell cycle analysis were measured by flow cytometry. Soy extract induced higher caspase activation, indicated by more SR-VAD-FMK positively stained cells (caspase +ve cells), than genistein or daidzein at equal concentration (25 μM) in both LnCap and PC3 cells. Soy extract also resulted in more cell population in SubG1 stage of the cell cycle in PC3 cells. No significant changes in caspase activity or cell cycle were observed in BPH-1 cells treated by soy extract or individual soy isoflavones. Control treatment was consisted of media with <0.1% DMSO. Values represent means ± SEM, n=3. * $p<0.05$, ** $p<0.01$ compared to control.

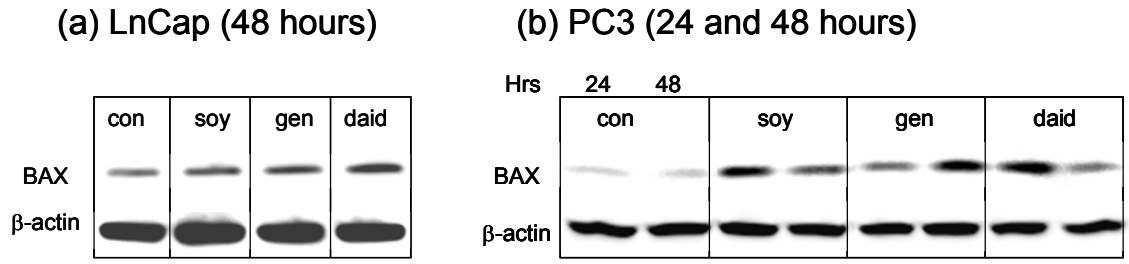

Figure 2.3 Soy and soy isoflavones increased Bax expression. Protein expression levels of Bax in nuclear extracts of treated cells were measured by western blotting. (a) LnCap and (b) PC3 cells were treated with 25 μM of soy extract (soy), genistein (gen), or daidzein (daid). Control treatment was consisted of media with <0.1% DMSO. Soy extract, genistein and daidzein increased Bax compared to control in PC3 cells, but not in LnCap cells. Representative blot of triplicate experiments are shown.

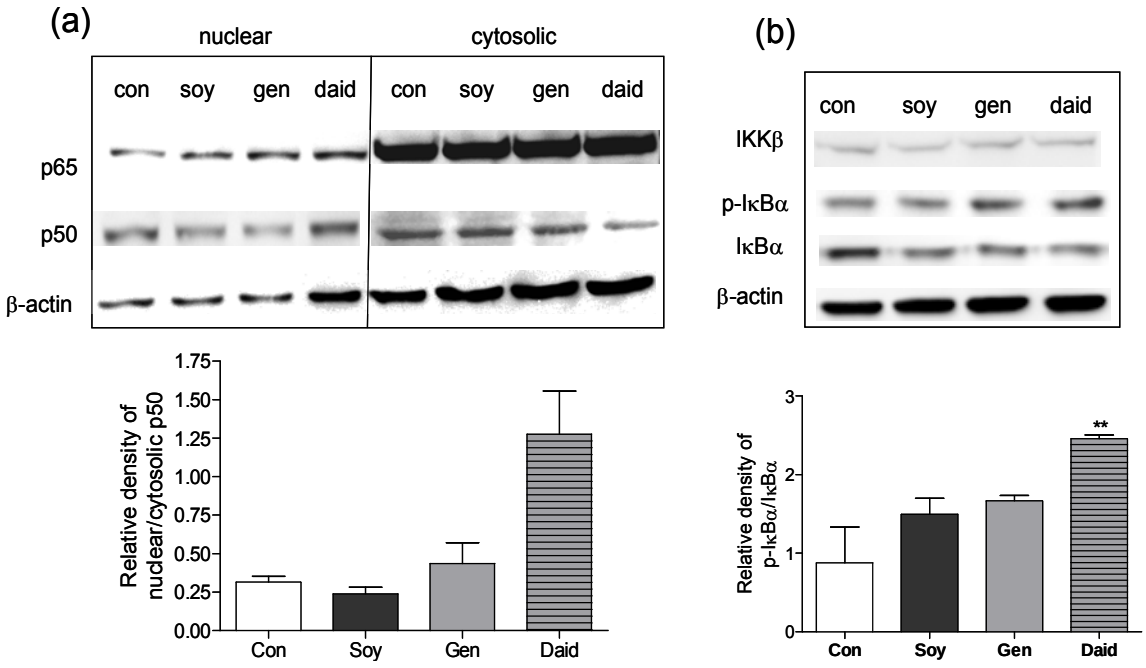

Figure 2.4 No effect of soy extract on NFκB activation, but an increase with daidzein treatment in PC3 cells after 48 hours. (a) Western blots of nuclear and cytosolic extracts from PC3 cells treated with DMSO (con), 25 μM of soy extract (soy), genistein (gen), or daidzein (daid) and raised against p50 and p65 antibodies. Relative density of nuclear/cytosolic p50 was obtained by dividing OD of nuclear p50 to cytosolic p50 after normalized to respective β-actin. (b) Cytosolic extract of treated PC3 cells was raised against IKKβ, p-IκBα, and IκBα. Relative density of p-IκBα/IκBα were obtained by dividing OD of p- IκBα to IκBα and normalized to β-actin. Daidzein increased nuclear p50 and cytosolic p-IκBα compared to control, but no significant changes were observed in cells treated with soy extract or genistein. Representative blot of triplicate experiments are shown. Densitometry values represent means ± SEM, n=3. **p<0.01 compared to control.

Chapter 3 Green tea is more effective than EGCG in inducing caspase dependent apoptosis in prostate carcinoma cells

Anna Hsu, Tammy M. Bray and Emily Ho

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3.1 Abstract

Green tea, particularly its catechins such as epigallocatechin gallate (EGCG), possesses anti-proliferative and anti-carcinogenic effects against prostate cancer. There has been increasing interest in possible synergistic effects of using whole-foods versus individual bioactive compounds. Taking this whole food approach, we hypothesized that green tea containing a mixture of catechins and other bioactive components, would be more potent and effective than individual green tea catechins as prostate cancer chemo-preventative agents. The objective of this study was to investigate and compare the efficacy of green tea with EGCG to inhibit cell proliferation and induce apoptosis in prostate cancer cells. Androgen dependent (LnCap) and androgen-independent (PC3) prostate cancer cells were treated with varying concentrations of green tea or EGCG, and analyzed for cell viability, caspase activation and cell cycle alterations. Both EGCG and green tea significantly decreased viability of LnCap and PC3 cells, but green tea induced caspase activation at lower concentrations than did EGCG. Green tea (50 μM) significantly decreased protein expression of pro-caspase 3, 8 and major inhibitors of apoptosis (XIAP, cIAP-1 and cIAP-2), but EGCG at equal concentrations did not induce similar effects in LnCap cells. In PC3 cells, green tea induced higher Bax expression than EGCG alone. No significant changes in apoptosis, cell cycle or caspases were observed in non-cancerous benign prostate hyperplasia (BPH-1) cells. These results suggested that other active compounds in green tea may provide additional beneficial effects and green tea may be more efficacious as chemo-preventative agents than individual compounds.

KEYWORDS: green tea, catechins, EGCG, prostate cancer, apoptosis, caspases, inhibitor of apoptosis proteins (IAP)

3.2 Introduction

Prostate cancer is the most common type of cancer found in American men (1); moreover, prostate cancer rates are considerably lower in Asian countries. Epidemiological studies have shown that the incidence of latent prostatic lesions in men appear to be uniform across Asian and Western countries, but prostate cancer outcome is considerably higher in Western countries (3). Migration studies indicate that prostate cancer rates increase when Asian men migrate move to the United States (2). The first generation Asian migrants with high soy and tea intake have lower incidences of prostate and mammary cancers compared with subsequent generations of Asian Americans (34). Together, these data suggest that the etiology of prostate cancer is highly influenced by environmental factors such as diet. Green tea (*Camellia sinensis*) is one of the most widely consumed beverages, especially in Asia. It has been postulated that higher green tea consumption may contribute to the decrease prostate cancer risk in the Asian male population.

The Asian population consumes as much as three fold-higher levels of green tea on a regular basis than does the US population. Green tea consumption in Asian countries averages ~360-480 mL/day (2-3 cups); whereas only 8% of the American population regularly consumes ~180 mL/day (33). Proposed bioactive components of green tea are polyphenolic compounds called catechins that include epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC), and theaflavins. Epidemiological studies demonstrate the protective properties of green tea against the development of stomach (68), lung (69), pancreatic and colorectal (70), and breast cancer (71). Most studies ascribe the beneficial effects of green tea to its most abundant catechin in green tea, EGCG. EGCG exerts anti-carcinogenic effects at multiple stages of carcinogenesis. Notably, EGCG is not only a potent antioxidant; it is also an effective inhibitor of carcinogen activation by inducing phase-II detoxifying enzymes, including glutathione peroxidase and quinone reductase (QR). At the post-initiation stage of carcinogenesis, EGCG may induce cell cycle arrest, apoptosis, and attenuate inflammation and tumor promotion (82). Although green tea consumption appears to contribute to decrease prostate cancer risk, most of the mechanistic studies have been focused on individual catechins, and

the effects of whole green tea are relatively understudied. For instance, previous studies have demonstrated that high levels of EGCG (≥100 μM) induced caspase-mediated apoptosis (164, 165), but the effects of whole green tea on prostate cancer cell proliferation and specific mechanisms are still unknown. In contrast to the usage of high levels of EGCG, longer term treatment of bioactive compounds at low doses, such as found in whole foods (green tea) may be a safer and more efficacious approach. Different components of a whole food may work additionally/synergistically together to target different molecular pathways in the carcinogenesis pathway.

This study compares the effectiveness of green tea with EGCG in inhibiting prostate cancer cell growth via regulation of apoptosis, cell cycle and caspase pathways. We hypothesize that green tea will be more effective than EGCG in all of these processes. The findings from this study will help establish the foundation for using green tea as a chemo-preventative dietary strategy for prostate cancer.

3.3 Materials and Methods

Cell Culture: Benign prostate hyperplasia (BPH-1) cells were generously donated by Dr. Simon Hayward (Vanderbilt University Medical Center, Nashville, TN), and malignant androgen-independent prostate cancer epithelial cells (PC3) and early-stage androgen-dependent prostate cancer cells (LnCap) were obtained from America Type Culture Collection (Manassas, VA). All cells were grown and maintained in RPMI 1640 with glutamine (Mediatech Inc., Manassas, VA) supplemented with 1% penicillin-streptomycin (Mediatech Inc., Manassas, VA) and 5 % fetal bovine serum (Hyclone, Logan, UT) for BPH-1 and 10% for PC3 and LnCap cells at 5% CO_2 atmosphere at 37ºC.

Treatments: EGCG (Sigma Aldrich, Saint Louis, MO) was dissolved in 0.05% citric acid (Sigma Aldrich). Control treatments included a no treatment group which consisted of media only, and a citrate treated group with final concentration matching the highest concentrations of treated cells. The green tea treatment concentrations were normalized based on the tea EGCG content. Based on previous HPLC measurements, 2% green tea contains about 550μg/ml EGCG. Two percent green

tea was shown previously to be the most effective dosage in a colon cancer cell model (166).To establish the effective dose range for catechins in our model system, we performed dose response experiments. Cells were treated with increasing doses (from 25 μM to 100 μM) of EGCG or green tea at different time points (3, 6, 24, 48 hours). Forty-eight hour treatments showed most significant changes; thus was used as study time. Two percent organic Chunmee green tea (Harney & Sons, Salisbury, CT) was made from brewing green tea leaves for 2 minutes in boiling water followed by filtration with coffee filter paper. A final concentration of 0.05% citric acid (Sigma-Aldrich, Saint Louis, MO) was added into freshly brewed and cooled green tea (167).

Multicaspase Assay: After 48-hour treatment with green tea or EGCG, adherent and floating cells were harvested and stained with sulforhodamine- valyl- alanyl- aspartyl-fluoromethyl- ketone (SR-VAD-FMK), a fluorochrome-conjugated caspase inhibitor, and 7-amino-actinomycin D (7-AAD) according to manufacture's instructions (Guava Technologies, Hayward, CA). Cell populations were quantified using Guava personal flow cytometer (Guava Technologies).

Cell Cycle: After 48-hour treatment with green tea or EGCG, adherent and floating cells were trypsinized and fixed in ice cold 70% ethanol at 0.5 million cells per ml. After incubation at -20ºC for 12 hours, cell pellets were resuspended in cellular DNA staining solution containing 40 mg/ml propidium iodide (Sigma-Aldrich) and 100 μg/ml of RNase (Sigma-Aldrich). Cell cycle kinetics was analyzed using the Guava personal flow cytometer. Histograms were constructed using MultiCycle V3.0 software.

Western blot analysis: Nuclear and cytosolic extracts from treated cells were collected using the Nuclear Extract Kit (ActiveMotif, Carlsbad, CA) according to the manufacturer's instructions. Whole cell lysates were isolated by lysing cells with immunoprecipitation (IP) buffer (25mM HEPES, pH 7.4; 150mM NaCl; 1mM EDTA; 0.5% Triton X-100) followed by flash freeze/thaw treatment and centrifugation. Protein concentrations were determined by the Bradford assay (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA) (151). Primary antibodies specific against Bax, caspases 8, cIAP-2 were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Caspase 3, and PARP antibodies were purchased from BD Pharmingen (San Jose, CA); XIAP and cIAP-1 antibodies were purchased from R&D Systems

(Minneapolis, MN); cleaved caspase 6 and 7 antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA), and BcL-2 antibody was from Upstate (Billerica, MA). Bax, Bcl-2 (nuclear); pro-caspase 3, 8, cIAP-2, XIAP, cIAP-1 (whole cell lysates) were qualitatively evaluated by the Invitrogen NuPAGE Western blotting system (NuPAGE Novex, Invitrogen, Carlsbad, CA). Ponceau S red staining and β-actin protein levels were used as protein loading controls. Secondary antibodies were conjugated with horseradish peroxidase (HRP) (Santa Cruz), and proteins were detected by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA) and imaged by Alpha Innotech photodocumentation system. Densitometry and quantifications were performed using NIH Image J software.

Statistics: Experiments were performed independently at least three times in triplicate for each biological sample. Data was analyzed using Graph Pad Prism V4.0. One-way ANOVA and Dunnett's Multiple Comparison test were used to evaluate the statistical difference. * indicated $p<0.05$, and ** indicated $p<0.01$.

3.4 Results

Green tea and EGCG exerted cancer cell-specific cytotoxicity

Both green tea and EGCG at the highest concentration (100 μM) significantly decreased cell viability in both androgen dependent LnCap and androgen-independent PC3 cells (**Fig. 3.1**). Green tea was more efficacious than EGCG in inhibiting cell proliferation in PC3 cells because a decrease in cell viability was observed at lower concentrations (50 μM). In both LnCap and PC3 cells, both the highest green tea and EGCG concentrations induced caspase activation, but green tea induced caspase activation at lower concentrations (≥ 25 μM). At least 100μM of EGCG was needed to induce caspase activation, but at a concentration of 25 μM green tea increased both caspase activation and cells undergoing apoptosis in SubG1 stage of cell cycle (**Table 3.1**). Together, these data suggest that green tea at an equal concentration of EGCG was more effective than EGCG alone in inhibiting cell proliferation and inducing apoptosis in prostate cancer cells. Green tea and EGCG did not significantly decrease cell viability or induce caspase activation in benign hyperplasia (BPH-1) cells, but EGCG appeared to induce G2/M arrest at the highest concentration, suggesting that very high concentrations of EGCG may induce cytotoxic effects in non-cancerous prostate cells.

Differential effects of green tea on caspase pathways in LnCap and PC3 cells

Further examination of specific caspases and regulators of caspases indicated that 25μM of green tea induced the cleavage of caspase-3 and -8 in LnCap cells (**Fig. 3.2, left panel**). Cleaved caspase-6 and -7 were below detection levels in the prostate cancer cells (data not shown). Green tea also demonstrated differential effects on the caspase pathways between the LnCap and PC3 cells. In LnCap cells, 25 μM of green tea effectively stimulated cleavage of pro-caspase-3 and -8, as indicated by decreased pro-caspase protein expressions, but no significant changes were observed in PC3 cells. Furthermore, there was a greater degree of PARP cleavage with green tea treatment than with EGCG in LnCap cells; similar effects were not observed in PC3 cells.

Differential effects of green tea on Bax in LnCap and PC3 cells

To investigate the subsequent events in the pro-apoptotic pathways, the protein expression of Bax and BcL2 in extracts of treated cells were measured by Western blotting. There were no significant differences in BcL2 among groups (data not shown). Green tea increased Bax expression in PC3 cells, but not in LnCap cells (**Fig. 3.3**). In LnCap cells, 25 μM of EGCG increased Bax expression compared to media-treated cells, but it appeared that citrate also induced a basal level of Bax expression. In contrast, green tea effectively increased Bax expression compared to EGCG in PC3 cells in a dose-dependent manner.

The differential response in caspase and Bax expressions between LnCap and PC3 cells suggested that green tea treatments may decrease cell proliferation by activating different apoptotic pathways. While there were no significant differences in the expression of apoptosis inhibitors with EGCG treatments, 50 μM green tea potently decreased XIAP, cIAP-1 and cIAP-2, which are major inhibitors of caspases in LnCap cells (**Fig. 3.4**). These findings explain the increase in cleavage of pro-caspases and caspase activation in LnCap cells treated with green tea. On the contrary, green tea increased higher Bax expression than EGCG in PC3 cells, but had no significant effects on caspase expressions.

3.5 Discussion

The anti-carcinogenic effects of supra-physiological levels of EGCG and green tea polyphenol concentrates are well-established in different cancer models, including prostate cancer cells (95, 168-170), but the efficacy of green tea has been largely untested. Green tea, with other bioactive constituents beside EGCG, may target the entire antioxidant/anti-inflammatory/anti-proliferation network in cancer cells; therefore, provide additional beneficial effects than individual catechins. Furthermore, taking the whole food approach better reflects the dietary patterns of the Asian population that consumes green tea on daily basis. To date there have been few studies that have directly compared the efficacy of green tea, as a whole food and EGCG, its putative bioactive component, in inhibiting prostate cancer cell proliferation and apoptosis. Results from this study suggested that green tea was more effective at inducing apoptosis in prostate cancer cells than EGCG alone, although both significantly decreased cell viability and increased apoptosis at high concentrations. Importantly, green tea did not induce apoptosis in non-cancerous cells, suggesting that its effects were tumor-cell specific, which is a highly desirable property for chemo-preventative agents. Green tea appeared to exert pro-apoptotic effects through different pathways depending on the stage of cancer. Green tea induced caspase activation through inhibition of inhibitors of apoptosis proteins in early-stage androgen dependent LnCap cells, while increasing Bax protein expression in late-stage androgen-independent PC3 cells. Moreover, the anti-proliferative effects of green tea were achieved at lower concentrations than EGCG alone, especially in late stage prostate cancer PC3 cells. These findings indicate that green tea may be an efficacious, safe and specific strategy for prostate cancer prevention.

EGCG and GT treatments elicited different expression profiles for caspase and inhibitor of apoptosis proteins in LnCap and PC3 cells (**Fig. 3.2**). In LnCap cells, green tea induced caspase activation possible through the inhibition of major IAPs without changing Bax protein expression. In contrast, in PC3 cells green tea induced caspase activation and cell cycle arrest via up-regulation of Bax (**Fig. 3.3)** and independent of the IAP pathways. Inhibitors of apoptosis proteins inhibit activation of caspases and protect cells from Bax-mediated mitochondrial disruption (156, 157). Previous studies also indicated that EGCG effectively inhibited X chromosome linked

inhibitor of apoptosis protein (XIAP) in pancreatic cancer cells (165). Therefore, targeting IAPs may be a effective strategy in overcoming apoptosis resistance and increasing sensitivity of prostate cancer cells to chemo-preventative agents (171, 172). Androgen-independent prostate cancer cells, especially PC3 and DU145 cells, have been shown to over-express IAPs (158). Green tea and EGCG did not elicit inhibitory effects on caspases or IAPs in PC3 cells even at the highest concentrations, suggesting that these molecular targets may be too extensively deregulated in PC3 cells, and the treatment concentrations used in the this study was insufficient to exert significant effects. Nevertheless, a slight decrease in cIAP-1 was observed with the ≥50 μM of green tea treatment.

The increase in apoptosis observed with green tea treatments may be mediated by other pathways, such as the Bax/BcL pathway. PARP cleavage has been used as an indicator for cells undergoing apoptosis. In this study, green tea increased activation of caspases and caused subsequent PARP cleavage in LnCap cells more than EGCG did alone. We also compared the apoptotic response between green tea and a green tea extract that was composed of ≥90% green tea polyphenols, predominantly EGCG (unpublished data). Green tea was more effective in inducing apoptosis in prostate cancer cells than the polyphenol-rich green tea extracts. Combined with the findings in this study, the pro-apoptotic effects of green tea may not be attributable solely to EGCG or polyphenols. Therefore, identification of specific apoptotic pathways and regulators in future studies will clarify the targets of chemoprevention by green tea treatments.

In summary, green tea was more effective in inducing apoptosis and decreasing cell viability than EGCG in both early-stage (LnCap) and late-stage (PC3) prostate cancer cells. Green tea induced cell cycle arrest at SubG1, caspase activation and apoptosis in prostate cancer cells through different apoptotic pathways based on the stage of cancer. Green tea up-regulated pro-apoptotic protein Bax protein expressions in PC3 cells while it induced caspase activation through IAP-dependent pathways in LnCap cells. The pro-apoptotic effects of green tea and EGCG at physiologically more relevant concentrations appear to be tumor-cell specific. The whole food approach (green tea) limited toxicity towards healthy cells while effectively inhibited prostate cancer cell proliferation, but future *in vivo* studies are needed to evaluate and

compare the effects of green tea and catechins on prostate cancer. Taking the whole-food approach may be more efficacious, cost effective and safer than individual compounds for the development of evidence-based public health recommendations and chemo-preventative strategy for prostate cancer.

Table 3.1 EGCG and green tea altered cell cycle distribution in BPH-1, LnCap and PC3 cells. BPH-1, LnCap, and PC3 cells were treated with 25, 50, and 100 μM of EGCG or green tea (GT) for 48 hours. Cell cycle kinetics was measured by flow cytometry and analyzed by MultiCycle software. Green tea induced apoptosis (G1 arrest) at lower concentrations than EGCG alone in both LnCap and PC3 cells. Control treatments include media (ntx) and citrate (matched to 100 μM GT).

		% cells in phase			
Cell Line	Treatment	G1	S	G2/M	SubG1
BPH-1	Ntx	61.88 ± 2.48	32.05 ± 1.95	7.29 ± 2.47	4.37 ± 0.40
	Citrate	59.29 ± 3.24	33.06 ± 2.10	7.64 ± 1.98	2.41 ±1.24
	25 (μM EGCG)	61.83 ± 1.11	25.16 ± 0.82	13.01 ± 0.30	1.96 ±1.33
	50	72.26 ± 1.56	24.27 ± 2.78	3.48 ± 1.48	2.72 ±0.67
	100	35.58 ± 3.75**	41.67 ± 4.19	22.75 ± 1.14**	3.72 ±0.66
	25 (μM GT)	57.02 ± 3.98	35.07 ± 3.01	6.42 ± 2.86	6.45 ± 0.63
	50	72.29 ± 2.10	24.73 ± 4.66	4.48 ± 3.63	3.72 ±0.76
	100	39.28 ± 3.73**	28.39 ± 3.62	29.73 ± 1.95**	4.44 ±0.65
LnCap	Ntx	59.69 ± 1.29	25.74 ± 2.18	14.63 ± 1.56	0.15 ±0.04
	Citrate	63.24 ± 0.70	23.84 ± 1.74	12.92 ± 1.21	0.07± 0.03
	25 (μM EGCG)	60.91 ± 1.53	27.12 ± 2.50	11.98 ± 1.47	0.11 ±0.04
	50	62.28 ± 0.25	25.65 ± 1.33	12.25 ± 1.30	0.42 ±0.07
	100	60.33 ± 2.50	23.16 ± 4.59	16.52 ± 3.18	5.37 ±2.61
	25 (μM GT)	62.11 ± 0.56	25.38 ± 1.24	12.51 ± 1.25	0.36 ±0.11
	50	64.18 ± 1.05	21.40 ± 1.20	14.41 ± 1.24	3.03 ±0.70*
	100	55.40 ± 5.20	30.00 ± 8.84	24.79 ± 7.87	10.27 ± 2.21**
PC3	Ntx	44.06 ± 1.65	31.33 ± 2.02	24.62 ± 1.65	0.47 ± 0.34
	Citrate	47.46 ± 2.30	28.73 ± 3.25	23.75 ± 1.13	0.09 ± 0.04
	25 (μM EGCG)	43.98 ± 1.27	29.47 ± 0.79	26.47 ± 0.55	1.04 ± 0.50
	50	23.94 ± 1.94**	45.82 ± 4.32	29.25 ± 4.51	5.02 ± 1.45
	100	34.20 ± 3.63*	35.78 ± 2.26	33.08 ± 1.72	8.33 ± 2.09*
	25 (μM GT)	38.71 ± 2.68	35.10 ± 5.33	26.20 ± 5.25	7.47 ± 1.89*
	50	29.28 ± 2.83*	32.24 ± 5.32	36.28 ± 3.96*	10.40 ± 2.68**
	100	44.56 ± 1.91	28.70 ± 1.31	26.75 ± 0.61	9.33 ± 1.21*

Values represent means ± SEM, n=6. * $p<0.05$, ** $p<0.01$ compared to citrate.

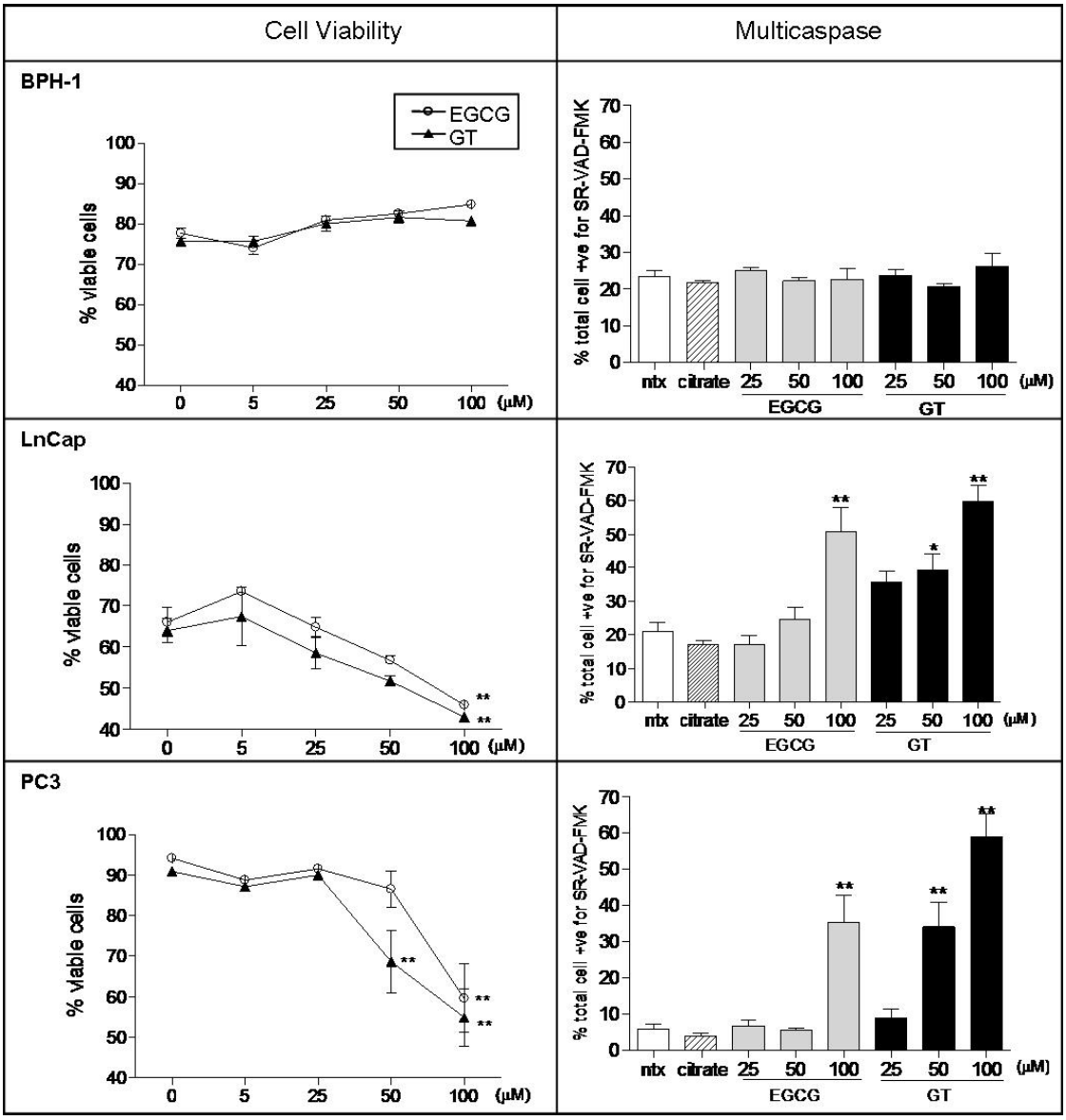

Figure 3.1 Green tea and EGCG induced apoptosis and inhibited cell proliferation in LnCap and PC3, but not BPH-1 cells. BPH-1, LnCap, and PC3 cells were treated with 25, 50, and 100 μM of EGCG or green tea (GT) for 48 hours. Cell viability, multicaspase activity and cell cycle kinetics were measured by flow cytometry. Green tea induced higher caspase activation, indicated by more SR-VAD-FMK positively stained cells (caspase +ve cells) at lower concentrations than EGCG alone in both LnCap and PC3 cells. No significant changes were observed in BPH-1 cells. Control treatments include media (ntx) and citrate (matched to 100 μM GT). Values represent means ± SEM, n=3. * $p<0.05$, ** $p<0.01$ compared to citrate.

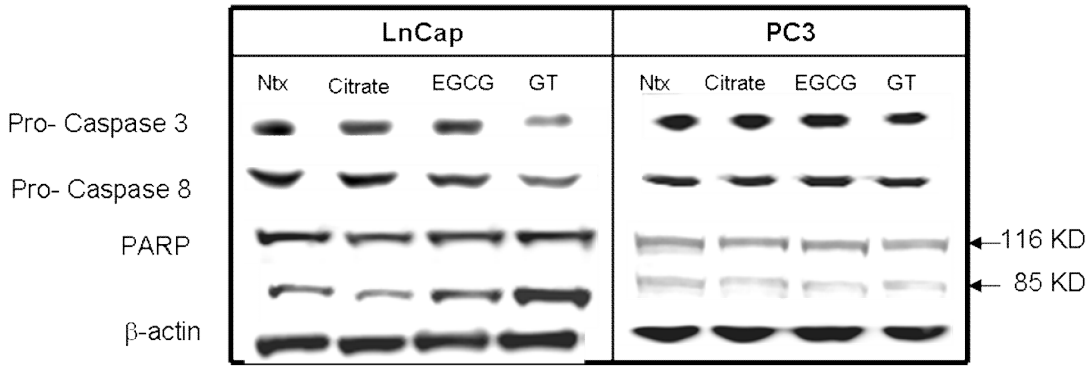

Figure 3.2 Green tea increased caspase activation and PARP-cleavage in LnCap, but not PC3 cells. LnCap and PC3 cells were treated with 25 μM of EGCG or green tea (GT) for 48 hours, and then whole cell lysates and nuclear extracts were isolated. Protein expressions of caspases and PARP were evaluated by Western blotting. GT was more effective in decreasing pro-caspase 3, 8, and increasing PARP cleavage than EGCG in LnCap cells, but similar effects were not observed in PC3 cells. Control treatments included media (ntx) and citrate (matched 25 μM GT). Representative blots of triplicates are shown.

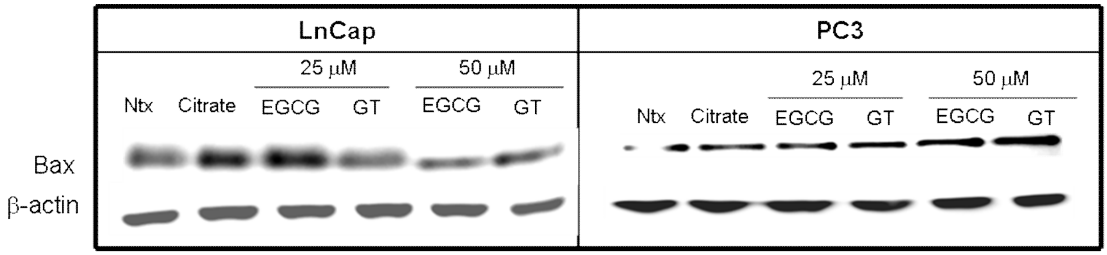

Figure 3.3 Green tea and EGCG elicited differential Bax protein expressions in LnCap and PC3 cells. LnCap and PC3 cells were treated with 25 and 50 μM of EGCG or green tea (GT) for 48 hours, and nuclear extracts were isolated. Protein expression levels of Bax were measured by Western blotting. Green tea up-regulated Bax in PC3 cells, but not in LnCap cells. Control treatments include media (ntx) and citrate (matched to 50 μM GT). Representative blots of triplicates are shown.

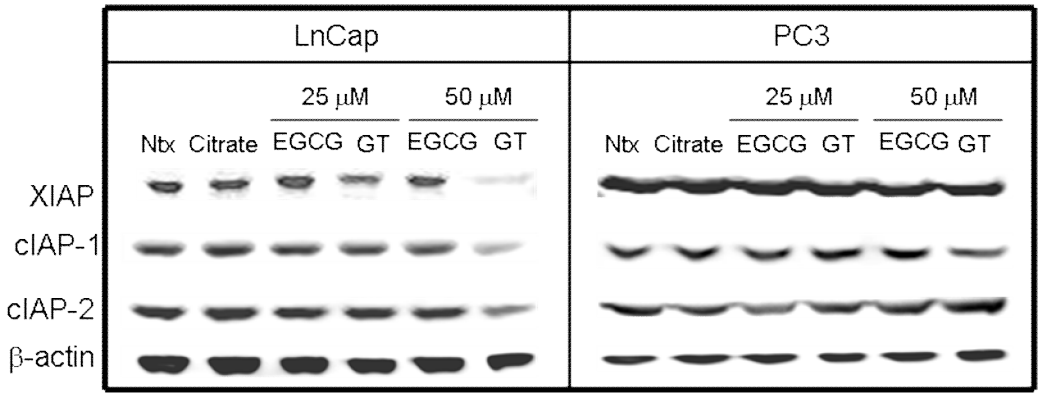

Figure 3.4 Green tea decreased IAPs in LnCap and PC3 cells. LnCap and PC3 cells were treated with 25 and 50 μM of EGCG or green tea (GT) for 48 hours, and whole cell lysates were isolated. Protein expressions of inhibitor of apoptosis proteins (IAPs) were evaluated by Western blotting. GT was more effective in decreasing XIAP, cIAP-1 and cIAP-2 than EGCG at equal concentrations in LnCap cells. Green tea also decreased cIAP-1 in PC3 cells, but not other IAPs. Control treatments include media (ntx) and citrate (matched to 50 μM GT). Representative blots of triplicates are shown.

Chapter 4 Dietary soy and tea mitigate chronic inflammation and prostate cancer via NFκB pathway in the Noble rat model

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4.1 Abstract

Chronic inflammation and nuclear factor-kappa B (NFκB) have been implicated in prostate cancer development; thus, dietary factors that inhibit NFκB may serve as effective chemo-preventative agents. Prostate cancer risk is significantly lower in Asian countries compared to the US, which has prompted interest in the potential chemo-preventative action of Asian dietary components such as soy and green tea. This study examined the effects of dietary soy and green tea on NFκB activation and inflammation *in vivo* using a hormone-induced rat model for prostate cancer. Male Noble rats implanted with estradiol and testosterone subcutaneously were divided into 4 dietary groups: control, soy, green tea, or soy+ green tea. NFκB activation and inflammatory cytokines were measured post implantation. The combination of soy and green tea suppressed NFκB p50 binding activity and protein levels via induction of IκBα. Soy and green tea also decreased prostate inflammatory infiltration, increased Bax/BcL2 ratio, and decreased protein expression of TNFα, IL-6 and IL1-β compared to control. Soy and green tea attenuated prostate malignancy by decreasing prostate hyperplasia. These effects were not apparent in groups treated with soy or green tea alone. The ongoing i*n vivo* studies thus far suggest that combination of foods, such as soy and green tea, may inhibit hormone-induced pro-inflammatory NFκB signals that contribute to prostate cancer development.

KEYWORDS: Soy, green tea, inflammation, Noble rat, prostate cancer, nuclear factor kappa B

4.2 Introduction

Prostate cancer is the second leading cancer-related cause of mortality in American men (1). Epidemiological studies have shown that the incidence of latent prostatic lesions in men appear to be uniform across Asian and Western countries, but prostate cancer outcome is considerably higher in Western countries (3). Migration studies indicate that prostate cancer rates increase considerably when Asian migrants move to the United States (2), and the first generation Asian migrants with high soy and tea intake have lower incidences of prostate and mammary cancers (34). Together, these findings suggest that the etiology of prostate cancer is highly influenced by environmental factors such as diet. A typical soy-rich Japanese diet consists of 25-100 mg soy isoflavones/d whereas the typical American diet contains ~1-3 mg soy isoflavones/d (34, 173). In addition, green tea consumption in Asian countries averages 360- 480 mL/d only whereas 8% of Americans regularly consume ~180 mL/d (33).

Soy and green tea consumption has been associated with a lower risk of prostate and several other cancers (68-71). Soybeans and green tea contain bioactive components that have anti-carcinogenic properties, with soy isoflavones and tea catechins as the two major constituents, respectively. The anti-cancer effects of soy isoflavones and green tea catechins may target different stages of the carcinogenesis pathway by inhibiting proliferation (60, 80), angiogenesis and metastasis (53, 83), activating phase II enzymes or modulating immune functions (34, 56, 170). Nonetheless, a gap in knowledge exists as to whether dietary whole soy or green tea modulates chronic inflammation that would otherwise contribute to prostate carcinogenesis *in vivo* (27). Nuclear factor-kappa B (NFκB), a transcription factor that regulates immune responses and cell proliferation, is constitutively active in prostate cancer, and mediates chronic inflammation and carcinogenesis in colitis and hepatitis mouse models (27, 28). Molecular strategies that target NFκB have been shown to suppress prostate cancer progression (22). Soy isoflavones and green tea catechins have been shown to modulate NFκB *in vitro* (59, 85, 174). However, the *in vivo* effects of dietary soy and green tea on hormonal microenvironments, NFκB activation, and inflammation in the prostate have not been investigated.

This study sought to examine the effects of dietary soy and green tea on prostate carcinogenesis using the hormone-induced prostate cancer Noble rat model. The outcome of the proposed investigations will enhance our understanding of the significance of NFκB regulation and chronic inflammation in prostate carcinogenesis. We hypothesized that soy or green tea would attenuate prostate cancer development in Noble rats by inhibiting NFκB activation and mitigating inflammatory responses implicated in the development of prostate cancer. The study design also examined the potential for synergism, by investigating the effects of combined soy and green tea treatments on prostate cancer development.

4.3 Materials and Methods

Animal care and sacrifice: Five to six- week-old male Noble rats were housed in pairs, provided laboratory rodent diet (Harlan Teklad, Kent, WA) devoid of soy isoflavones and tea catechins, and acclimated to the temperature and humidity controlled environment with a 12 h dark: light cycle until 10-wk of age. At 10 wk of age (one week prior to hormone implantation), rats were fed *ad libitum* with the treatment diets. There were five different treatment groups including one sham group and four hormone-treated groups (**Fig. 4.1**). The baseline sham without hormone group was fed AIN-93G diet and deionized water. The hormone treated groups included no treatment (AIN-93G+ water), green tea (AIN-93G+ 2% green tea), soy (200 g soy protein/kg AIN-93G based diet+ water), and soy+tea combination (200 g soy protein/kg AIN-93G based diet+ 2% green tea). Diets were formulated commercially (Research Diets Inc., New Brunswick, NJ). Chunmee green tea (Harney & Sons, Salisbury, CT) was brewed for 2 minutes in boiling water followed by filtration. A final concentration of 0.05% citric acid (Sigma-Aldrich, Saint Louis, MO) was added into freshly brewed and cooled green tea (167). Green tea was replaced every other day. Green tea and soy treatment concentrations were determined based on previous studies (167, 175). After 4, 8, 20 and 38 w of hormone treatment, blood was collected via cardiac puncture while rats were anaesthetized under 2.5% isoflurane in 100% O_2. Plasma and serum were obtained by centrifugation from tubes containing EDTA. Spleen and liver were also excised from rats, flash frozen with

liquid nitrogen and stored at -80ºC. Prostates were divided for tissue extracts, fixation in paraformaldehyde for histology and flash frozen for storage in -80ºC.

Hormone treatments: Noble rats exposed to elevated levels of testosterone plus estradiol have NFκB activation and inflammation in the prostate after 4 weeks, precancerous lesions at 16 weeks, and prostate tumors by 50 weeks (124, 128). At sexual maturity (11 wk of age), rats in the hormone treated groups were implanted subcutaneously with two 3 cm silicone tubes (0.62 ID × 0.125 OD × 0.32 wall, VWR, West Chester, PA) containing ~14 mg of testosterone and one 2 cm tube containing ~14.8 mg of estradiol (Sigma-Aldrich) (123). Tubes were prepared via vacuum suctioning, and tube endings were sealed with silicone type medical adhesive (Dow Corning, Midland, MI). Tubes were soaked in 1× Phosphate Buffer Saline (PBS) at 37ºC for at least 12 hours and wiped with 70% ethanol prior to implantation. Rats were anaesthetized with 2.5% isoflurane in 100% O_2 prior to subcutaneous insertion of the tubes between the shoulder blades, and tubes were replaced every 8 weeks.

EGCG concentration: Concentrations of EGCG in green tea and plasma soy isoflavones were measured by HPLC to validate the levels of these compounds added to the diets. High Performance Liquid Chromatography (HPLC) were used to measure EGCG and caffeine contents in the green tea via a Shimadzu VP series instrument equipped with a 25 cm × 4.6 mm, 5 μm particle size, reverse-phase column (Supelcosil™ LC-18) following established methods (176). The mobile phase was composed of methanol (buffer A) and water (buffer B). Chloroacetic acid was added to both solvents to reach a final concentration of 0.3% (w/v) and the pH was adjusted to 4.5 using 1 N NaOH. The gradient program used for separation of the tea components was as follows: 10% buffer A at 0 min, increased linearly to 40% buffer A at 50 min and returned to 10% buffer A at 60 min. The flow rate was set at 1 ml/min with detection at 273 nm. The EGCG and caffeine fractions were identified by comparing retention times to standards, 0.05% EGCG (Calbiochem, Darmstadt, Germany) and 0.05% caffeine (Alfa Aesar, Ward Hill, MA) in 0.5% citric acid.

Plasma soy isoflavone concentrations: Plasma isoflavones (genistein, diadzein, and equol) were measured by using HPLC Coularray as described previously (177). In brief, serum was buffered with ammonium acetate (pH 4.6), mixed with internal

standard (4-hydroxybenzophenone; 110 μmol/L), and subjected to enzymatic hydrolysis (overnight, 37ºC) using 200 U β-glucuronidase and 15 U sulfatase prepared ammonium acetate (pH 4.6). After incubation, proteins were precipitated by using acetonitrile (1 mL), the sample was delipidated using hexane (3 mL), and the isoflavones were extracted 3 times using 3 mL methyl tert-butyl ether. Extracts were combined and dried under nitrogen gas, reconstituted in mobile phase A, and injected on the HPLC system. The sample was separated by binary gradient at 1 ml/min on a C18 Luna, 250× 4.6 mm, 5 μm (Phenomenex, Torrance, CA) by using 25 mmol potassium phosphate buffer/L (pH 2.7) as mobile phase A and methanol:acetonitrile:mobile phase A (50:30:20) as mobile phase B. The gradient was delivered as follows: 50% B to 65% B from 0 to 20 min, a linear gradient to 75% B from 20 to 30 min, and a linear gradient to 100% from 30 to 35 min. Initial conditions were restored over 2 min, and the system was equilibrated at 50% B for 12 min before subsequent injection. Analytes were detected by using potential settings of 325, 450, 575, and 700 mV and were quantified on their dominant channel. Plasma isoflavone concentrations were calculated by using area ratios for standards and the internal standard, and the lower limit of quantification was ≈20 nmol/L for each analyte.

Plasma estradiol concentrations: Plasma estradiol and testosterone concentrations were measured by radioimmunoassay (RIA) (Diagnostic Systems Laboratories, Webster, TX). Plasma (200 μL) was mixed with estradiol antiserum, followed by addition of radioactive [I-125]-labeled estradiol. The amount of antibody bound [I-125]-estradiol was inversely proportional to the concentrations of estradiol in the samples. Sample counts per minute (CPMs) were taken by Cobra II Auto-Gamma Counter (Packard Instruments, Meriden, CT), and plasma estradiol levels were extrapolated from a standard curve. Testosterone levels were measured under similar procedures.

NFκB DNA binding activity and cytokine levels: *NFκB DNA binding activity*- p65 and p50 DNA binding activities were measured by Transcription Factor Assay Kit (ActiveMotif, Carlsbad, CA). Nuclear extract were obtained from prostate tissue using Nuclear Extraction kit (ActiveMotif). Oligonucleotides containing the NFκB consensus site (5'-GGGACTTTCC-3') were immobilized on a 96-well plate. The active forms of

NFκB in the nuclear extracts were bound to the oligonucleotides on the plate, and DNA binding activities were measured colorimetrically at 450 nm. *Tumor Necrosis Factor-alpha (TNFα), Interleukin-1 beta(IL-1β) and Interleukin-6 (IL-6) ELISA*- protein levels of TNFα, IL-1β and IL-6 were measured by Rat TNF-alpha ELISA and Rat IL-1-beta Tissue Culture ELISA Ready-SET-Go kits (eBiosciences, San Diego, CA) respectively.

Western blot analysis: Protein levels of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2, inhibitors of apoptosis proteins (cIAP-1 and cIAP-2), and proteins in the NFκB pathway including p50, p65 (nuclear), IKKα, IKKβ, IκBα, and p-IκBα (cytosolic) in treated cells were qualitatively evaluated by the Invitrogen NuPAGE Western blotting system (NuPAGE Novex, Invitrogen, Carlsbad, CA). Nuclear and cytosolic extracts were obtained as described above. All primary antibodies specific against proteins of interest were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), except IKKα and IKKβ were purchased from Cell Signaling Technology Inc. (Danvers, MA). Ponceau S red staining and β-actin protein levels were used as protein loading controls. Secondary antibodies were conjugated with horseradish peroxidase (HRP) (Bio-Rad, Hercules, CA), and proteins were detected by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA) and imaged by Alpha Innotech photodocumentation system (Alpha Innotech Corp., San Leandro, CA). Densitometry and quantifications were performed using NIH Image J software.

Prostate histology and scoring: Freshly excised prostate dorsolateral lobes were fixed in 10% paraformaldehyde in PBS over night. One-step sections (3.5 μm) were prepared onto slides and stained with Harris hematoxylin, followed by 1% eosin. Slides were then dehydrated in 95% and 100% ethanol, cleared in xylene and mounted with Permount. The sections were examined by light microscopy in a blinded fashion for morphology and prostate lesions by a board certified veterinary pathologist (CL) from the Veterinary Diagnostic Laboratory at Oregon State University. Prostate acini infiltrated by inflammatory cells, prostate stromal volume (10× magnification) and epithelium thickness (40× magnification) were scored using a histomorphometric software (OsteoMeasure system, OsteoMetrics, Inc., Atlanta, GA).

Statistics: Measurements were performed independently for three times with multiple biological samples (n=4 to 6). Data were analyzed using GraphPad Prism V4.0. One-way ANOVA and Tukey-Krammer Multiple Comparison test were used to evaluate the statistical differences. Values not sharing the same superscript letter differ $p<0.05$.

4.4 Results

Estradiol plus testosterone increased prostate mass and circulating estradiol:

The average food consumption was approximately 15 g of diet/rat/day, and the green tea group consumed an average of 45 ml green tea/ day, which contained 60 mmol of EGCG. There were no significant differences in food intake among groups, but hormone treatments decreased body mass (**Table 4.1**), as reported previously (115, 125, 132). Similar to prior work in this model (116, 123), prolonged estradiol and testosterone treatments increased prostate mass and circulating estradiol level without affecting circulating testosterone level. All the hormone-treated groups had no significant changes in prostate size, body weight or plasma hormone levels among dietary treatment groups.

Catechin and isoflavone levels:

Animals in the tea groups ingested ~1.33 mmol/ml EGCG throughout the study, similar to a prior report (166). Plasma genistein, daidzein, equol and combined concentrations of all three isoflavones increased with both soy and soy+tea treatment compared to rats fed AIN-93G diets (**Table 4.2**). The plasma soy isoflavone concentrations in soy+tea group were significantly higher than the soy alone group, suggesting that consumption of tea might have augmented soy isoflavone uptake or reduced elimination.

Dietary soy and green tea reduced prostate malignancy:

Progression to prostate malignancy in the Noble rat model is characterized by morphological changes including thickening of the stroma and epithelial cells, both preceding neoplasia in the prostate (123, 129). Hormone treatment significantly increased prostate epithelial thickness and stromal volume (**Table 4.3**). Green tea alone and in combination with soy, decreased stromal volume, but only the soy+tea group significantly reduced epithelial thickness. Importantly, the incidence of hyperplasia was significantly reduced to baseline levels with combined soy+tea treatment, providing direct evidence for its efficacy in reducing progression to prostate cancer. No apparent decrease in the incidence of hyperplasia was observed in soy or green tea treated groups alone.

Inflammatory markers:

The Noble rat model is characterized by inflammatory response in the prostate at early time points (6, 129, 131, 132). As early as 4 w post-implantation, hormone treatments increased the infiltration of inflammatory cells including macrophages, B- and T-cells into the prostate, which appeared to be decreased by combined soy+tea treatment (data not shown). At 20 w, the soy + tea group had quantitatively fewer prostate acini that were infiltrated by inflammatory cells compared with other hormone-treated groups (**Fig. 4.2A**), including acini surrounded by inflammatory cells and acini with inflammatory cells infiltrated into the epithelium and lumen. In contrast, soy or green tea alone did not show a decrease in infiltrating inflammatory cells compared to hormone-implanted animals fed the AIN-93G diet (no treatment group).

To profile chronic inflammatory responses, TNFα, IL-6 and IL-1β were assessed (**Fig. 4.2B-D**). Hormone implantation had no effect on TNFα and IL-6, but significantly increased the concentration of IL-1β compared to no hormone baseline group. Dietary treatment of soy+tea decreased protein levels of TNFα, IL-6 and IL-1β significantly. At 20 weeks, the transcript levels of IL-1β and IL-6 were increased following hormone treatment, but soy+tea treatment decreased levels of IL-1β and IL-6 mRNA expression in the prostate (data not shown). Notably, the transcript levels of TNFα at 20 weeks were very low, supporting the view that activation of TNFα was likely an early, transient inflammatory response.

Dietary soy and tea decreased inflammation via NFκB pathway

At 8 weeks post implantation, there were no significant changes in p65 DNA binding activity among treatment groups, but hormone treatments increased p50 DNA binding activity (**Fig. 4.3**). Green tea, but not soy, significantly decreased the hormone-induced p50 binding. However, the treatment of soy+tea more effectively decreased NFκB p50 DNA binding activity, suggesting the combination of dietary agents might exert additional inhibitory effects on NFκB DNA binding activity.

Thus, NFκB p65 and p50 protein levels were measured at 8 weeks post hormone implantation in prostate nuclear extracts. Hormone treatments increased nuclear p50

protein levels, which was inhibited by tea, and more so by soy+tea treatment, whereas soy alone had no effect (**Fig. 4.4A**). No significant changes in nuclear p65 were detected among groups. NFκB is primarily regulated by a cascade of regulatory proteins (19). Protein expressions of several major regulator proteins in the NFκB pathway including IKKα, IKKβ, IκBα and p-IκBα were measured. The levels of IKKα and IKKβ were below detection, but there was a trend for decrease in p-IκBα in the soy+tea group (**Fig. 4.4B**), suggesting a decrease in degradation of NFκB inhibitory proteins. Interestingly, hormone treatments did not significantly affect NFκB regulatory proteins, implying that there may be other points of deregulation or mechanisms by which hormone increased NFκB protein and DNA binding ability. Furthermore, inhibition of NFκB might provide higher apoptotic potentials in the soy+tea treated prostates, such that the Bax/BcL2 ratio was increased.

Dietary soy and tea affected apoptotic pathways:

Although no significant differences in prostate mass occurred among all the hormone-treated groups, there was evidence of greater apoptotic signaling in the animals receiving soy+tea in combination. The expression of pro-apoptotic Bax protein and anti-apoptotic BcL-2 protein were evaluated via Western blotting, and the ratio of Bax/BcL-2 was determined as a marker for apoptosis. Soy or green tea treatment alone did not significantly change the Bax/BcL-2 ratio, but the treatments in combination tended ($p=0.052$) to increase Bax and decrease BcL2 levels (**Fig. 4.5a**). Inhibitors of apoptosis proteins (IAPs) inhibit caspase activities, positively modulate the NFκB pathways and are over-expressed in prostate cancer cells (155). There was a non-significant trend of increasing protein expressions of cIAP-1 ($p=0.13$) with hormone treatments that was decreased with soy+tea treatment (**Fig. 4.5b**). These findings suggested that there was an increase in pro-apoptotic pathways with combined soy and green tea consumption.

4.5 Discussion

Prostate cancer is the most common type of cancer found in American men (178). Low prostate cancer incidence in Asian countries has prompted interest in dietary components in Asian diets, such as soy and tea, as cancer chemoprevention agents. When studying the combinational effects of dietary soy and green tea on hormone-induced chronic inflammation and prostate cancer, we found marked differences in responses between single food and combined dietary strategies. We demonstrated that dietary soy and green tea in combination decreased prostate inflammation and pre-cancerous lesions via attenuation of NFκB and downstream apoptotic pathways. Soy or green tea alone did not exert similar inhibitory actions, suggesting that the interactions between soy and green tea provided additional benefits against prostate cancer. Thus, the concurrent ingestion of soy and green tea may have synergistic activities that mitigate the risk for developing prostate cancer, particularly in the US where the consumption of these foods are limited.

There are many concerns about the *in vivo* bioavailability of soy phytochemicals, but previous studies have shown that soy isoflavonoids accumulate in the prostate gland (66). The prostate produces active soy isoflavone metabolites and the bioavailabilities of soy isoflavones improve with long-term consumption of soy (66, 67). Our results were consistent with previous observations that soy isoflavones were present at detectable levels in the plasma of soy treated rats (179). The soy+tea group had higher soy isoflavone concentrations than the soy group (**Table 4.1**). It is possible that green tea enhanced the absorption of soy, making soy isoflavones more bioavailable in the prostate. Plasma EGCG was not measured in the present study, but it is possible that soy constituents affected bioavailability of green tea catechins. One previous study reported that co-treatment of genistein increased uptake of EGCG in human colon cancer cells and mice (180). Our study did not explore such mechanisms or examine the effects of other soy constituents on bioavailability of green tea catechins. Green tea may improve absorption of soy constituents by increasing activities of lactase phlorizin hydrolase (LPH) and β-glucosidase that hydrolyze isoflavone glucosides to form aglycones and/or activities of glucoronidases that metabolize isoflavones for digestion and absorption. Current knowledge of the

effects of green tea on β-glucosidase, LPH and glucuronidases responsible for soy metabolism is very limited. Previous studies have indicated that green tea increased hepatic UDP-glucuronosyl transferase activity in rats (181, 182), which might contribute to the glucoronidation of soy metabolites for more efficient transport to target tissues (173). Green tea may also selectively enhance growth of gut bacteria that are crucial for soy isoflavone metabolism and absorption. Studies have showed that green tea had selective bactericidal properties (183), but no studies have investigated the effects of green tea on intestinal flora that are responsible for soy metabolism. Future work on these topics will be essential for understanding the interactions between soy and green tea *in vivo*.

Previous findings have suggested that blocking inflammation is an effective strategy to prevent prostate cancer progression (8, 9). Inhibition of the COX-2 pathway by non-steroidal anti-inflammatory drugs (NSAIDs) effectively decreased prostate tumor growth (8, 9), but long-term NSAIDs usage also elicited adverse gastrointestinal and vascular effects (184, 185). Prostate regions susceptible to carcinoma induction also have lower expressions of anti-oxidative enzymes, including catechol-*O*-methyltransferase (C*O*MT), glutathione (GSH) and quinone reductase (QR) (119). When prostate cells transform to more aggressive cancerous cells, the redox balance in estrogen/testosterone metabolism shifts towards production of estradiol and activation of testosterone and DHT, leading to proliferative pressure on cells and unregulated prostatic growth (120). In addition, carcinogenic estrogen metabolites such as 4- hydroxyestradiol (4-OHE_2) can serve as a co-oxidants and strongly stimulate production of pro-inflammatory prostaglandins (117, 118). The NFκB pathway influences many cellular responses that attribute to carcinogenesis, such as regulation of cell cycle, apoptosis and inflammation, and also intimately interacts with hormonal homeostasis. With the loss of redox balance and androgen dependency, deregulation of NFκB becomes a major promoting factor for transformation to malignancy and poor prognosis (32). Targeting NFκB may have important prevention or therapeutic values against prostate inflammation and cancer.

Results from the present study suggested that hormone treatments increased NFκB p50 DNA binding activities and protein expressions without affecting p65 (**Fig. 4.3-4**). NFκB p50 has lower affinity for the IκBα regulatory element compared to p65 (20);

therefore a selective increase in p50 subunits may lack adequate feedback mechanisms and contribute to a chronic inflammatory response. Dietary soy+tea appeared to mitigate NFκB at several levels. The combined soy+tea treatment significantly decreased protein levels of p-IκBα, NFκB p50 protein expressions and DNA binding activity. Thus, the combined treatment of soy+tea may inhibit NFκB activation via decreasing phosphorylation and subsequent degradation of its inhibitory unit, IκBα. Further examination of possible candidate upstream kinases that control IκB phosphorylation are an important area of future interest. Interestingly, soy or green tea alone did not exhibit similar protective effects as the combination treatment. Under the present conditions, neither soy nor green tea alone significantly affected inflammatory infiltration and inflammatory cytokine levels in the prostate. However, green tea decreased p50 DNA binding activity, and soy showed a trend for restoring levels of p-IκBα to that of the baseline group. Green tea also reduced hormone-induced prostate stroma enlargement, but neither soy nor green tea alone changed prostate hyperplasia outcome. Thus, soy or green tea alone might target different molecular endpoints further upstream in the NFκB pathway. Previous studies have shown that EGCG inhibits NFκB inducing kinase (NIK) and subsequent IκB-kinase (IKK)/NIK signaling complex in human lung cancer cells (85), and genistein inhibits proteasome activity responsible for degradation of IκBα (186). It is possible that dietary levels of soy or green tea alone were not sufficient, but given in combination, they targeted different pathways, and the anti-inflammatory and anti-cancer effects were amplified.

The current study shows a lack of chemo-preventative effects with dietary soy or green tea alone, which is in contrast to prior studies that showed high concentrations of soy isoflavone or green tea extracts induced protective effects in other models (95, 187-189). However, similar to our study, Cohen et al. utilized lower concentrations of soy protein isolate (≤20% by weight) in the diet and showed a lack of preventative effects against hormone refractory prostate tumor growth in rats (190). Previous studies utilizing green tea polyphenol extracts containing high concentrations of catechins showed efficacy in inhibiting inflammation and prostate tumorigenicity (95, 170, 191), but the concentrations they used do not reflect normal human consumption. Studies looking specifically at the combined effects of soy and green

tea are limited. One report indicated that genistein increased bioavailability of EGCG, but also increased intestinal tumorigenesis in $APC^{min/+}$ mice (180). Utilizing high levels of individual bioactive agents, rather than lower levels of agents in combination, may induce adverse effects or ignore cooperative interactions among several components (62, 64). Zhou et al. reported that combination of soy phytochemical concentrate and brewed green tea synergistically inhibited prostate tumorigenicity and metastasis possibly through modulating serum testosterone and DHT in a mouse prostate cancer model (113, 192). Nevertheless, they also found that green tea alone was not sufficient in inducing beneficial effects. Moreover, this study utilized a xenograph model to introduce prostate tumors, which limits the ability to examine chemoprevention at early stages of carcinogenesis. In contrast to these previous studies, the Noble rat model permitted investigation of preventative effects of dietary compounds at the early initiation and promotion stages of prostate cancer, and allowed the examination of chronic inflammation and NFκB activation. Further studies are needed to pinpoint the precise mechanism leading to the synergy between soy and green tea.

In summary, dietary soy and green tea treatments worked together, but not alone, in inhibiting inflammatory cytokine production and inducing apoptosis, possibly through NFκB-dependent pathways. Mitigation of NFκB resulted in attenuation of inflammation in the prostates and inhibited prostate carcinogenesis. Combination of soy and green tea may have provided additional beneficial effects by targeting multiple points along the NFκB pathway, and/or by targeting other mechanisms, such as improving bioavailability of active compounds in the prostate. Overall, we conclude that dietary modifications incorporating soy and green tea, which together target inflammatory and apoptotic pathways have preventative and therapeutic values against prostate cancer development. The precise mechanism warrant further study.

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Table 4.1 Animal characteristics. Hormone treatments led to enlarged prostates, decreased body weights, and elevated plasma estradiol levels after 20 weeks of hormone treatments. No significant difference in food intake or plasma testosterone levels was observed.

	Food Intake (g/rat/day)	Prostate Mass[1]	Body Mass (g)	Plasma Estradiol (log pg/mL)	Plasma Testosterone (ng/mL)
Baseline	15.27±0.46	0.71±0.03[a]	398.3±23.9[a]	ND[a]	0.63±0.13
Hormone treated					
No treatment	14.94±0.50	1.29±0.07[b]	305.3±6.8[b]	1.44±0.11[b]	0.54±0.13
Tea	14.33±0.45	1.26±0.04[b]	291.2±6.3[b]	1.46±0.11[b]	0.97±0.35
Soy	14.07±0.39	1.29±0.08[b]	288.5±2.9[b]	1.67±0.23[b]	1.10±0.29
Soy+Tea	15.50±0.39	1.32±0.11[b]	315.3±7.5[b]	1.99±0.26[b]	0.92±0.40

Value represent means ±SEM, n=4 for Baseline and n= 6 for all other groups.
[1] Values in the prostate mass column were normalized to body weight and multiplied by 100
ND, not detectable
Values not sharing the same superscript letter differ $p<0.05$.

Table 4.2 Plasma soy isoflavone concentrations. Plasma soy isoflavone concentrations in rats were measured by HPLC. Rats in the soy and soy+tea groups had higher plasma concentrations of genistein, daidzein and equol than rats fed the control AIN-93G diet.

	Genistein	Daidzein	Equol	Total (genistein +daidzein+ equol)
	μmol/L			
AIN-93G	ND[a]	0.21±0.03[a]	ND[a]	0.21 ±0.03[a]
Soy	0.41±0.09[b]	0.24±0.02[b]	0.75±0.05[b]	1.19 ± 0.06[b]
Soy + Tea	077±0.13[c]	1.23±0.11[c]	1.62 ±0.20[c]	3.40 ± 0.37[c]

Value represent means ±SEM, n= 6.
ND, not detectable
Values not sharing the same superscript letter differ $p<0.05$.

Table 4.3 Prostate malignancy markers and hyperplasia incidence at 38 weeks post implantation. Epithelium thickness, stromal volume and hyperplasia incidence at 38 weeks post implantation. Tea and soy+tea group decreased stromal volume, but only soy+tea group decreased all three markers of prostate malignancy.

	Epithelium layer thickness (μm)	**Stromal volume** Percent (%) total volume	**Hyperplasia incidence** Percent (%)
Baseline	16.80 ± 1.43[b]	14.10 ± 0.68[b]	16.7
Hormone treated			
No treatment	23.64 ± 0.59[a]	31.61 ± 3.74[a]	50
Tea	22.84 ± 0.82[a]	30.50 ± 2.92[b]	66.7
Soy	20.93 ± 0.87[a]	23.91 ± 3.06[a]	50
Soy+Tea	18.69 ± 1.73[b]	22.85 ± 2.65[b]	16.7

Values represented means ±SEM, n=4 for Baseline and n= 6 for all other groups.
Values not sharing the same superscript letter differ $p<0.05$.
Hyperplasia incidence represents percent animals in a treatment group with prostate hyperplasia.

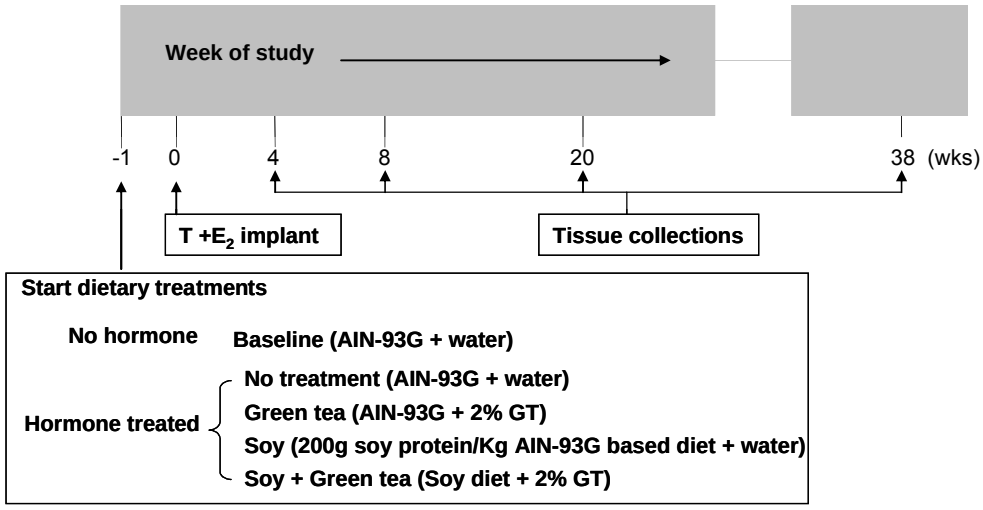

Figure 4.1 Dietary treatment plan. Rats were randomly assigned to five dietary treatment groups: baseline without hormone, no treatment (AIN-93G diet), green tea, soy, and soy+tea groups. Testosterone and estradiol were implanted subcutaneously between shoulder blades of hormone-treated rats at sexual maturity (11 weeks of age; time point 0) in the no treatment, green tea, soy and soy+tea groups. Rats were sacrificed and tissue samples collected after 4, 8, 20 and 38 weeks of hormone treatments.

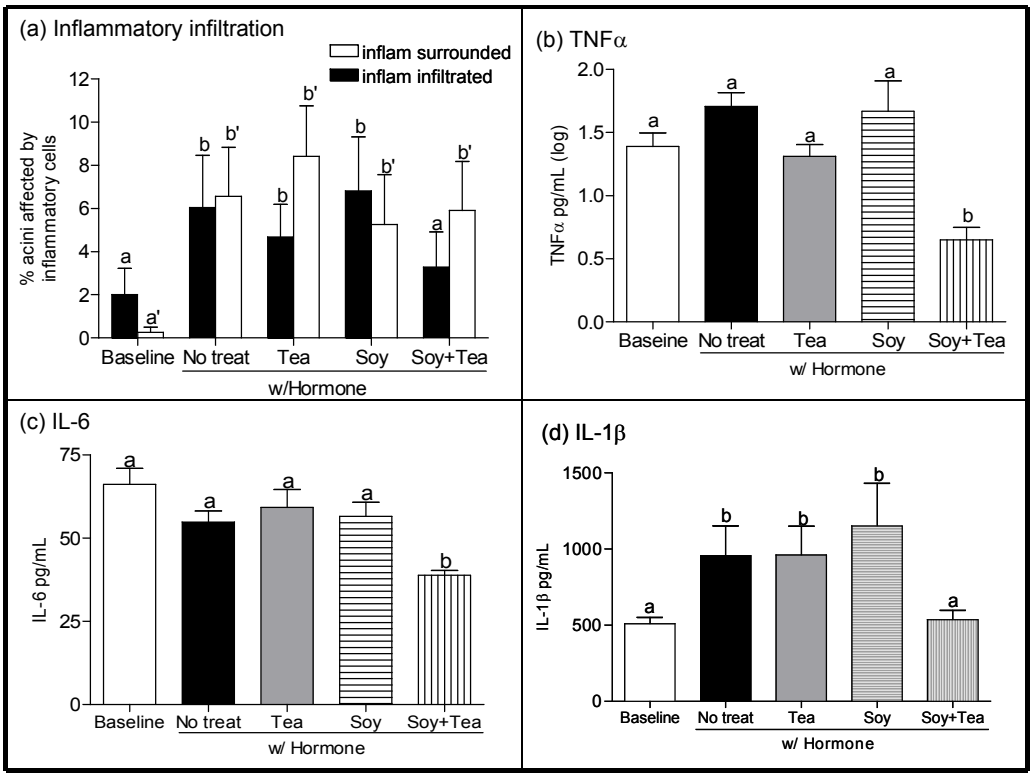

Figure 4.2 Dietary soy and green tea decreased inflammatory infiltrates, TNFα, IL-6 and IL-1β levels in the prostate of hormone-implanted rats. (a) Scores of inflammatory infiltration in rat prostate at 20 weeks post implantation. At 20 weeks post implantation, soy and tea combination treatments decreased inflammatory infiltration into the rat prostate acini more than other treatments. Protein levels of TNFα (b), IL-6 (c) and IL-1β (d) in prostate cytosolic portions were measured by enzyme immunosorbent assay (ELISA). Hormone treatments increased inflammatory cytokines at 4 weeks post implantation, but soy and tea treatment significantly decreased levels of TNFα, IL-6 and IL-1β. Values represented means ±SEM, n= 4 for baseline, and n= 6 for all other groups. Values not sharing the same superscript letter differ $p<0.05$.

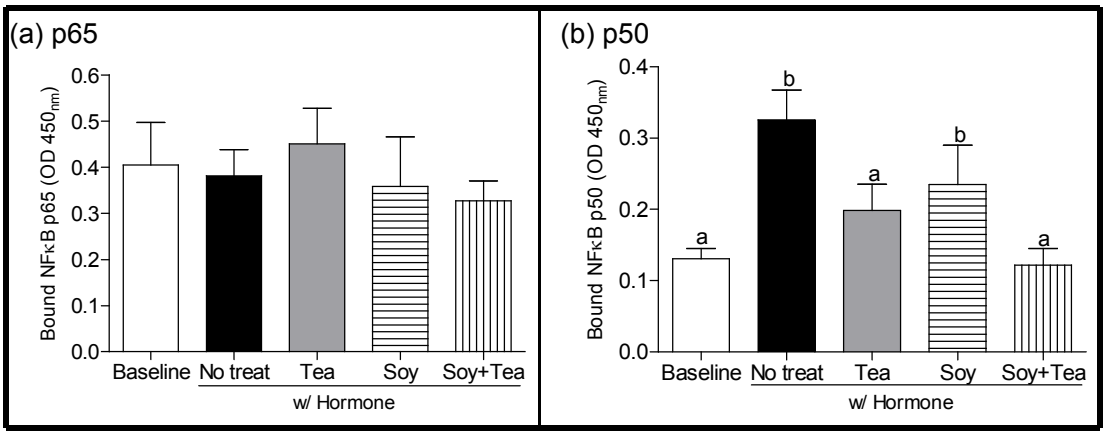

Figure 4.3 Dietary tea and soy+tea decreased NFκB p50 binding activity in the prostate. NFκB DNA binding abilities were measured by enzyme immunosorbent assay (ELISA). Dietary treatment of Soy+Tea significantly decreased NFκB activity in the prostates at 8 weeks post implantation. There were no differences in p65 binding activities (a) among all groups. Hormone implants elevated p50 binding activities (b), but soy and tea significantly decreased p50 activities. Values represent means ±SEM, n= 4 for baseline, and n= 6 for all other groups. Values not sharing the same superscript letter differ $p<0.05$.

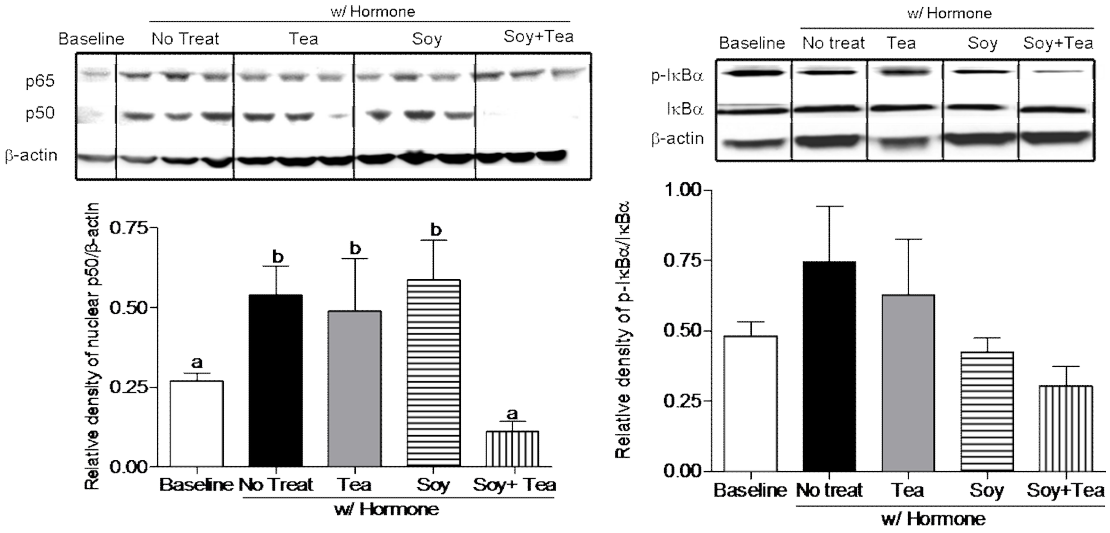

Figure 4.4 Dietary soy and green tea decreased nuclear NFκB p50 translocation in the prostate. Western blots of NFκB in the prostates at 8 weeks post-implantation. Hormone treatments increased nuclear p50 protein levels, and a trend for increased cytosolic p- IκBα in the prostates. Soy+Tea group decreased protein levels of p50, possibly due to decrease p-IκBα. Representative blots are shown. Densitometry values represent means ±SEM, n= 4 for baseline, and n= 6 for all other groups. Values not sharing the same superscript letter differ $p<0.05$.

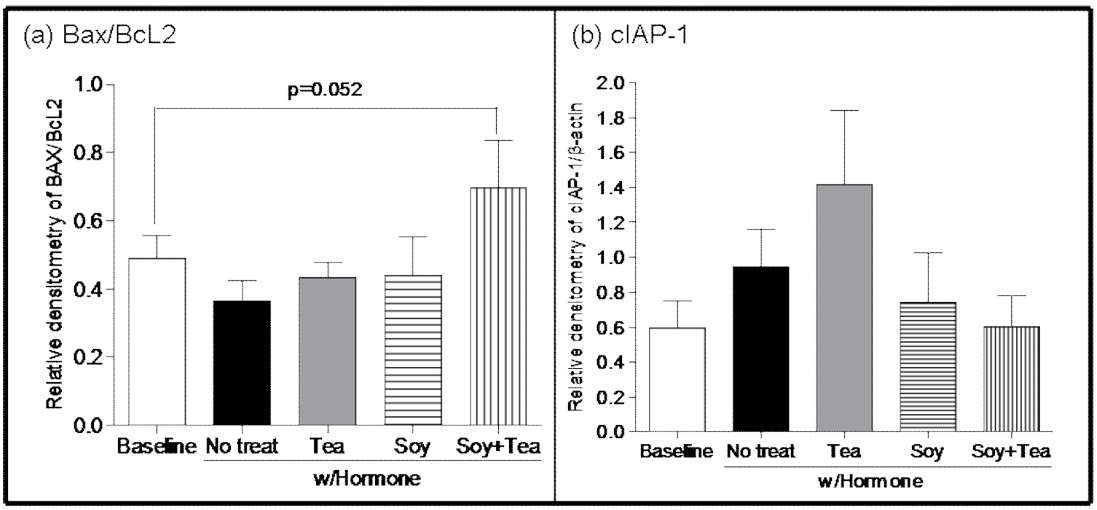

Figure 4.5 Dietary soy and green tea induced higher BAX/BcL2 ratio and decreased cIAP-1. Protein expression levels of Bax, BcL2 and cIAP-1 of prostate tissues were measured by western blotting. Soy+Tea group increased pro-apoptotic protein Bax and decreased anti-apoptotic protein Bcl2 protein expressions at 4 weeks post implantation. Soy+Tea group also showed a non-significant trend of decreasing protein expressions of inhibitor of apoptosis, cIAP-1 at 8 weeks post implantation. The densitometry values represented means ±SEM, n= 4 for baseline, and n= 6 for all other groups. p=0.052 compared to no treatment group.

Chapter 5 General Conclusions

Diet and nutrition contributes to prevention of most chronic diseases and are major focus of prostate cancer research. In the US, prostate cancer is the most prevalent non-cutaneous cancer in men and one of the leading causes of cancer-related deaths. In contrast, men from Asian countries have a 10 to 15-fold lower rate of prostate cancer than US men. Migration studies further emphasize the impact of diet and lifestyle. For instance, Asian migrants (high soy and tea intake population) has lower incidence of prostate and mammary cancers than subsequent generations of Asian Americans (33). Incidences of prostate cancer in Chinese (24 per 100,000), Japanese (29.6 per 100,000) and Filipino (56.8 per 100,000) men born in China, Japan and the Philippines were about half of those born in the US (44.4, 42.2, and 111.3 per 100,000, respectively) (2). Moreover, there is a gradual increase of prostate cancer rate in major industrialized cities in China, reflecting that greater extent of westernization and adaptation of western diet is associated with increase prostate cancer risk (4). These observations have prompted studies in recent years to investigate and compare different dietary components in the Asian diet that may have cancer preventative properties such as soy and green tea.

Most studies have taken a reductionist approach by focusing in specific individual bioactive compounds, as a “silver bullet”. Even though many bioactive compounds at pharmaceutical levels have anti-cancer effects, studies using physiological levels of nutrients have not been promising. For example, high concentrations of soy isoflavone concentrates and green tea polyphenol extracts induced protective effects against prostate cancer (187), but dietary levels of soy protein isolate (≤20% by weight) (190) and green tea (113) alone failed to demonstrate protective effects against prostate tumor growth in rats and mice, respectively. Also, even though selenium and vitamin E showed great promise as chemo-preventative agents against prostate cancer, in clinical trials there was a lack of significant protective effects of selenium and vitamin E alone against prostate cancer in the Selenium and Vitamin E Cancer Prevention Trail (SELECT) (193). Utilizing high levels of individual bioactive agents may also induce adverse effects and/or ignore the cooperative interactions among several dietary compounds (62, 64). For example, the Alpha-Tocopherol Beta Carotene (ATBC) trial suggested that high levels of β-carotene had no beneficial

effects against cancer development and may in fact increase lung cancer incidence in smokers (194). Furthermore, it has been suggested that acute administration of high levels of soy isoflavones may stimulate breast cancer cell growth in post-menopausal women (62-64). On the contrary, more recent studies have indicated that “whole food” or combination of dietary compounds may offer additional beneficial effects. For instance, consuming green tea instead of individual constituents of green tea may offer more beneficial effects (192). Dietary supplementation with whole tomato product (tomato powder) was more effective than equal level of lycopene supplement alone in inhibiting prostate cancer growth *in vivo* (98). These findings further advocated for the whole-food based approach to cancer prevention. Nevertheless, the effects and efficacy of whole soy and green tea in prevention of prostate cancer have remained largely understudied. Therefore, the focus of this dissertation was to compare the effectiveness of whole soy and green tea with individual bioactive compounds using *in vitro* systems, and to further investigate the effects of dietary soy and green tea on chronic inflammation and prostate carcinogenesis *in vivo*.

Most mechanistic studies have attributed the anti-cancer properties of soy and green tea to their most prominent constituents, namely soy isoflavones and green tea catechins. The protective properties of soy against prostate cancer are not only apparent for a low-intake population. Even among Japanese men with relatively high soy intake compared to Caucasians, higher consumption of soy food and soy isoflavones was associated with a decrease in localized prostate cancer (40). The anti-carcinogenic effects of soy isoflavones, especially genistein, are extensive (41, 42). Both *in vitro* and *in vivo* studies have theorized possible protective mechanisms of soy isoflavones against cancer development (39, 41, 42). Soy isoflavones, which are classified as phytoestrogens, act as both estrogen agonists and antagonists by differentially binding to estrogen receptor α or β (45, 46) and/or altering enzymes involved in hormone metabolism (37, 45, 46). Furthermore, soy isoflavone concentrates induce cell cycle arrest and up-regulate tumor suppressor genes in androgen-independent PC3 prostate cancer cells (60). Soy may also act through hormone-independent pathways that target cell cycle or apoptotic mechanisms. For instance, genistein is a known inhibitor of protein tyrosine kinase (50), topoisomerase II (51), and upregulates p21 in various cancer cells (147-149). Genistein also induces

apoptosis in a NFκB-dependent fashion in breast cancer cells (57) and prostate cancer cells (58, 59).

EGCG has been shown to be a potent chemo-preventative agent and exerts anti-carcinogenic effects at multiple stages of the carcinogenesis. Noticeably, EGCG is a strong antioxidant and effective inhibitor for carcinogen activation. EGCG induces phase-II detoxifying enzymes, such as glutathione peroxidase and QR, cell cycle arrest, apoptosis, and attenuates inflammation and tumor promotion (82). EGCG suppresses growth of human gastric cancer xenografts in mice through inhibiting tumor invasion and metastasis by regulating expressions of matrix metalloproteinases (MMPs) and angiogenesis by reducing vascular endothelial growth factor (VEGF) production (82, 83). The role of EGCG in prostate cancer prevention and treatment are summarized by Stuart et.al (84). Although soy and green tea consumption appears to contribute to decrease prostate cancer risk, the precise mechanisms have been focused on individual soy isoflavones and green tea catechins, and the mechanistic effects of whole soy and green tea are understudied.

The *in vitro* studies presented in this dissertation indicated that a soy crude extract and green tea were more effective in inhibiting prostate cancer cell proliferation than individual soy isoflavones (genistein) and catechins (EGCG). Furthermore, we explored some possible mechanisms by which these dietary compounds may inhibit proliferation. Our investigation with soy demonstrated that crude soy extract induced apoptosis possibly through Bax-dependent pathways. The ability of any cancer therapeutic agent to target cancer cells is highly desirable. Importantly, the addition of soy extract to non-cancer benign prostate hyperplasia (BPH) cells had no effects on apoptosis, suggesting that the pro-apoptotic effects of whole soy extracts were tumor-cell specific. In contrast, individual soy isoflavones, specifically genistein and daidzein induced cytotoxicity in BPH cells, reinforcing that high level of individual compounds may not be safe for healthy cells. These data suggest that a whole food approach with soy may be a more selective and a safer strategy for prostate cancer prevention. Similarly, our investigation with green tea and EGCG indicated that even though both green tea and EGCG effectively inhibited prostate cancer cell proliferation and apoptosis at high concentrations, green tea was more effective at lower, more physiologically relevant concentrations. Furthermore, green tea appeared to induce pro-

apoptotic effects through caspase-dependent pathways by down-regulating inhibitor of apoptosis proteins, which were shown to be over-expressed in prostate cancer cells (158). Similar to soy extract, green tea did not exert cytotoxicity towards BPH-1 cells at concentrations that induced apoptosis in malignant prostate cancer cells. This again suggested that green tea exerted tumor-specific effects and was not cytotoxic to non-cancerous cells. Whole foods may exert additional beneficial effects by targeting multiple points of carcinogenesis pathway. Our findings reinforce the whole food approach as an effective cancer prevention strategy and may help identify possible mechanisms by which soy or green tea exerts anti-cancer effects in the prostate.

Besides diet and nutrition, chronic inflammation has been implicated as another major contributor of increase prostate cancer risk. There is growing evidence that suggests that the imbalance in estrogen and testosterone homeostasis leads to chronic inflammation, and prostate cancer development, especially through NFκB(17). NFκB is a transcription factor responsible for modulating cell proliferation, apoptosis, immune and inflammatory responses in the body. When the NFκB pathway is disrupted, cells lose their abilities to control inflammation and cellular proliferation. Also, prolonged inflammation triggered by constitutive activation of NFκB generates many harmful oxidants that further damage the tissues, and the combination of these events can result in cancer. Previous studies have indicated a correlation between intakes of non-steroidal anti-inflammatory drugs (NSAIDs) and a decrease prostate cancer incidence (8, 9). The complexity of hormonal signaling and balance in the prostate has rendered it very difficult to pin-point a specific target for intervention, and finding a suitable model that encompasses all these elements is challenging. In our studies, we chose to use the Noble rat model to better understand the timing of inflammatory response and identify possible inflammatory targets, such as NFκB, during sex-hormone-induced prostate carcinogenesis *in vivo*. This model would allow us to characterize the effects of dietary modifications on inflammation and tumor development in the prostate. Noble rats were administered with combined testosterone and estradiol treatments to induce inflammation and tumors in the prostate. The Noble rats develop inflammation in the prostate prior to tumor development, and tumors induced originate in the dorsolateral lobe of the prostate, which is considered to be the most homologous to the peripheral zone of the human

prostate. The Noble rat model was a suitable model for studying the molecular interactions among diet, sex hormonal microenvironments, inflammation and cancer of the prostate *in vivo*.

Using the Noble rat model, we demonstrated that dietary soy and green tea in combination decreased prostate inflammation and pre-cancerous lesions via attenuation of NFκB and downstream apoptotic pathways *in vivo*. The combination of soy and green tea effectively increased pro-apoptotic signals such as Bax, decreased pro-inflammatory cytokines and inflammatory infiltration into the prostate and prostate hyperplasia incidence. The anti-inflammatory effects of soy and green tea were mediated through inhibition of NFκB p50 DNA binding activity and protein expressions. The targets of inhibition appeared to be upstream regulator proteins, including IκBα and cIAP-1. Soy or green tea alone did not demonstrate similar effects, suggesting that the interactions between soy and green tea provided additional benefits against prostate cancer. This study suggests that interactions among several foods may potentiate the activities of any single supplement. Furthermore, the most effective strategy for the prevention of prostate cancer may involve enhancing the entire antioxidant/ anti-inflammation/ anti-proliferative network. Whole foods are more efficacious than single nutrient supplements because they contain an array of nutrients and phytochemicals that can target a wide range of biological functions and cellular pathways that are deregulated during cancer development. As we have discussed in chapter 4, the beneficial effects of combining soy and green tea were likely results of the attenuation at multiple points of the carcinogenesis pathway, including NFκB. One of our unpublished *in vitro* data indicated that combining genistein and EGCG were not more effective in inducing apoptosis than genistein or EGCG alone in prostate cancer cells, suggesting that the combinational effects could possibly be due to other compounds or food matrix formed between soy and green tea. Furthermore, little is known about the metabolism of these phytochemicals and how they influence each other *in vivo*. One major finding in our *in vivo* study indicated that soy and green tea combination increased plasma soy isoflavone concentration in the rats. It is possible that the nutrient bioavailability and metabolism interactions between soy and green tea are important, and future mechanistic studies are needed to understand the synergism between soy and green tea in prostate cancer prevention.

In conclusion, this dissertation supported using soy and green tea as chemo-preventative strategies against prostate inflammation and carcinogenesis. Our findings emphasized that food products that bear a combination of active compounds may be more efficacious and safer as chemo-preventative agents than individual compounds, and combination of different dietary foods might offer additional beneficial effects. This “whole food” based approach was significant for the development of public health recommendations for prostate cancer prevention. This dissertation also identified apoptosis and NFκB as key pathways of regulation by soy and green tea in attenuation of prostate carcinogenesis. Overall, our findings suggested that increasing both soy and green tea consumption had the potential to help reduce prostate cancer incidence and health care cost in the US. It had also provided more understanding of the relationship between chronic inflammation and prostate cancer, and provided the basis for examining specific molecular pathways that contribute to prostate cancer in future studies.

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