DAIRY PROTEINS AND LIPIDS IN THE CHEMOPREVENTION OF PROSTATE CANCER

DISSERTATION

A Dissertation

Presented in Partial Fulfillment of the Requirements for

the Degree Doctor of Philosophy in the

Graduate School of The Ohio State University

By

Kyle David Kent, M.S.

The Ohio State University
2004

Dissertation Committee:

Professor W. James Harper, Adviser
Professor Joshua Bomser
Professor Mark Failla
Professor Michael Mangino

Approved by

____________________
Adviser
Food Science and Nutrition
ABSTRACT

Prostate cancer is the second leading cause of cancer death in men, and diet is thought to play a role in the development of this disease. The “Westernized” diet, rich in red meat and dairy products, is associated with increased prostate cancer risk. Despite the negative perception of dairy products, milk contains several components, such as whey proteins and sphingomyelin (SM), which may play a role in prevention and/or treatment of prostate cancer. Whey proteins are a cysteine-rich protein source, and consumption of these proteins can elevate plasma concentrations of the antioxidant glutathione (GSH). Elevation of prostate GSH may reduce inflammation that is associated with cancer development. SM is a phospholipid that can induce apoptosis in cancer cells, and consumption of SM is associated with reduced incidence of colon cancer in mice. It is hypothesized that SM may also limit the proliferation of prostate cancer cells.

The present studies were designed to examine the role of whey proteins and SM in prostate cells to determine if there is a potential role for these dairy components in prostate cancer prevention and treatment. First, the effects of oxidative stress in non-cancerous prostate cells was examined. Next, we examined the elevation of GSH in non-cancerous prostate cells by hydrolyzed
whey proteins. Finally, the antiproliferative effects of SM in human prostate cancer cells were studied.

The results of the present study revealed that bovine pituitary extract, a supplement used in the growth medium of the non-cancerous prostate cells, provides significant protection against oxidant-induced cell damage and may represent a confounding variable when studying oxidative stress in cell lines requiring this supplement. Hydrolyzed whey proteins elevated GSH concentrations in non-cancerous prostate cells by 64% and this GSH elevation protected the cells against oxidant-induced cell death. Exogenous SM significantly reduced prostate cancer cell proliferation by 17% after 24 h incubation, and this reduction was, in part, due to apoptosis. Taken together, results from these studies suggest a potential role for whey proteins and SM in reducing the risk for development of prostate cancer.
Dedicated to my loving Jackie and my beautiful Lily
ACKNOWLEDGMENTS

I thank my adviser Dr. W. James Harper for providing the freedom to explore my own ideas and make my own mistakes.

I am grateful to Dr. Joshua Bomser for providing facilities and guidance throughout my research.

Finally I would like to thank my family for all of the love and support they have given me throughout my college career.
VITA

December 10, 1976……….Born – Springfield, Ohio

1999…………………………B.S. Food Science, The Ohio State University

1999 – 2001…………………M.S. Food Science, The Ohio State University

2001 – present………………Graduate Teaching and Research Associate, The Ohio State University

PUBLICATIONS

Research Publication


FIELDS OF STUDY

Major Field: Food Science and Nutrition
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>v</td>
</tr>
<tr>
<td>Vita</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>viii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xi</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 1: Bovine pituitary extract provides remarkable protection against oxidative stress in human prostate epithelial cells</td>
<td>9</td>
</tr>
<tr>
<td>Abstract</td>
<td>9</td>
</tr>
<tr>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>13</td>
</tr>
<tr>
<td>Results</td>
<td>20</td>
</tr>
<tr>
<td>Discussion</td>
<td>36</td>
</tr>
<tr>
<td>Chapter 2: Effect of whey protein isolate on intracellular glutathione and oxidant-induced cell death in human prostate epithelial cells</td>
<td>40</td>
</tr>
<tr>
<td>Abstract</td>
<td>40</td>
</tr>
<tr>
<td>Introduction</td>
<td>42</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>44</td>
</tr>
<tr>
<td>Results</td>
<td>48</td>
</tr>
<tr>
<td>Discussion</td>
<td>55</td>
</tr>
<tr>
<td>Chapter 3: Antiproliferative effects of dietary sphingolipids in androgen independent prostate cancer cells</td>
<td>59</td>
</tr>
<tr>
<td>Abstract</td>
<td>59</td>
</tr>
<tr>
<td>Introduction</td>
<td>62</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>67</td>
</tr>
<tr>
<td>Results</td>
<td>73</td>
</tr>
<tr>
<td>Discussion</td>
<td>86</td>
</tr>
<tr>
<td>Conclusion</td>
<td>97</td>
</tr>
<tr>
<td>References</td>
<td>99</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Sphingolipid-induced annexin V-FITC binding by PC-3 cells</td>
<td>84</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Involvement of BPE and EGF in protection against H$_2$O$_2$-induced cell death</td>
</tr>
<tr>
<td>1.2</td>
<td>BPE supplementation and RWPE-1 cell membrane integrity</td>
</tr>
<tr>
<td>1.3</td>
<td>BPE supplementation and H$_2$O$_2$-induced intracellular protein oxidation and DNA damage</td>
</tr>
<tr>
<td>1.4</td>
<td>BPE antioxidant activity and catalase inhibition</td>
</tr>
<tr>
<td>1.5</td>
<td>BPE antioxidant activity and charcoal stripping</td>
</tr>
<tr>
<td>1.6</td>
<td>The effect of treatment with heat or with proteolytic enzymes on BPE antioxidant activity</td>
</tr>
<tr>
<td>1.7</td>
<td>BPE fraction with antioxidant activity</td>
</tr>
<tr>
<td>1.8</td>
<td>BPE supplementation and intracellular ROS generation</td>
</tr>
<tr>
<td>1.9</td>
<td>Tyrosine kinase inhibition on BPE antioxidant activity</td>
</tr>
<tr>
<td>1.10</td>
<td>Simulation of BPE antioxidant effect by purified mitogens</td>
</tr>
<tr>
<td>2.1</td>
<td>SDS-PAGE of WPI and identification of major proteins</td>
</tr>
<tr>
<td>2.2</td>
<td>Effect of hydrolyzed WPI and casein on viability of RWPE-1 cells</td>
</tr>
<tr>
<td>2.3</td>
<td>Concentration and time dependent elevation of GSH by hydrolyzed WPI in RWPE-1 cells</td>
</tr>
<tr>
<td>2.4</td>
<td>Effect of cysteine availability and GSH synthesis on the elevation of GSH in RWPE-1 cells</td>
</tr>
<tr>
<td>2.5</td>
<td>Effect of TBHP treatment on oxidant-induced cell death in RWPE-1 cells</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>3.1</td>
<td>Concentration and time dependent effects of SM, C18-ceramide, and C6-ceramide on PC-3 cell proliferation</td>
</tr>
<tr>
<td>3.2</td>
<td>Concentration and time dependent effects of SM, C18-ceramide, C6-ceramide, and PD98059 on ERK 1/2 and MEK activation</td>
</tr>
<tr>
<td>3.3</td>
<td>ERK 1/2 inhibition and antiproliferative effects of C6-ceramide, SM, and C18-ceramide</td>
</tr>
<tr>
<td>3.4</td>
<td>Flow cytometric analysis of PC-3 cells treated with SM, C18-ceramide, C6-ceramide, and PD98059</td>
</tr>
<tr>
<td>3.5</td>
<td>Concentration and time dependent effects of SM, C18-ceramide, C6-ceramide, and PD98059 on Rb and cdc2 activation</td>
</tr>
<tr>
<td>3.6</td>
<td>Sphingolipid-induced DNA fragmentation</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

AT, aminotriazole; bFGF, basic fibroblast growth factor; BPE, bovine pituitary extract; BPH, benign prostate hyperplasia; BSO, buthionine sulfoximine; CLA, conjugated linoleic acid; DCF, dichlorofluorescein; DCFDA, dichlorofluorescin diacetate; DCFH, dichlorofluorescin; DNP, dinitriphenylhydrazine; DPBS, Dulbecco’s phosphate buffered saline without calcium and magnesium; EGF, epidermal growth factor; ERK 1/2, extracellular signal-regulated kinase; GCS, glucosylceramide synthase; GH, growth hormone; GPI, glycosphatidylinositol; GSH, reduced glutathione; GSSG, glutathione disulfide; GST, glutathione-S-transferase; HDL, high density lipoprotein; FITC, fluorescein isothiocyanate; KSFM, keratinocyte serum-free medium; LDH, lactate dehydrogenase; LDL, low density lipoprotein; MAPK, mitogen-activated protein kinase; MEK, mitogen activated protein kinase/ERK; MFGM, milk fat globule membrane; MTT, thiazolyl blue tetrazolium bromide; MWCO, molecular weight cutoff; NAC, N-acetylcysteine; PC, phosphatidylcholine; PD98059, ERK/MEK inhibitor; PE, phosphatidylethanolamine; PEITC, phenethylisothiocyanate; PIN, prostate intraepithelial neoplasia; PS, phosphatidylserine; Rb, retinoblastoma; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SM, sphingomyelin; SMase,
sphingomyelinase; TBHP, t-butyl hydroperoxide; TEM, transmission electron microscopy; VLDL, very low density lipoprotein; WPI, whey protein isolate; WPC, whey protein concentrate
INTRODUCTION

Diseases of the prostate


Damage resulting from chronic inflammation can lead to the development of benign prostate hyperplasia (BPH) and/or prostate cancer (König et al. 2004, Gonzalgo and Isaacs 2003, Lucia and Torkko 2004, Platz and DeMarzo 2004). BPH is a non-cancerous condition in which chronic inflammation stimulates polyclonal proliferation of cells in the transitional region of the prostate, causing enlargement of the prostate (DeMarzo et al. 1999). Chronic inflammation in the peripheral region of the prostate can lead to the development of prostate intraepithelial neoplasia (PIN) lesions, which are considered precursors for prostate cancer development (Gonzalgo and Isaacs 2003, DeMarzo et al. 2003). These mutated epithelial cells exhibit a relatively normal morphology but have
genetic alterations that lead to increased monoclonal cell proliferation (Gonzalgo and Isaacs 2003, DeMarzo et al. 2003). If further genetic damage occurs, PIN lesions have the potential to develop into metastatic prostate cancer (Gonzalgo and Isaacs 2003, DeMarzo et al. 2003). Cells in PIN lesions express lower levels of GSTP1 mRNA, which codes for the detoxification enzyme glutathione S-transferase π (GSTπ) (DeMarzo et al. 1999, Nelson et al. 2001b, DeWeese et al. 2001). This enzyme plays an important role in protecting the cell against the damaging effects of reactive oxygen species (ROS) and DNA adduct-forming electrophilic compounds (DeMarzo et al. 1999, Nelson et al. 2001b, DeWeese et al. 2001). As the cell repairs genetic damage caused by the absence of GSTπ, point mutations may occur leading to inactivation of other critical gene products. For example, as PIN lesions progress toward prostate cancer, the cells express lower levels of p27, an inhibitor of cyclin-dependent kinase activity (DeMarzo et al. 1999). This inhibition is critical for slowing progression through the cell cycle (DeMarzo et al. 1999). Further genetic damage causes decreased expression of tumor suppressor genes and ultimately leads to increased cell proliferation and the development of metastatic prostate cancer (Gonzalgo and Isaacs 2003, Elo and Visakorpi 2001).

**Prostate cancer epidemiology**

Prostate cancer is the second leading cause of cancer death and the most commonly diagnosed cancer in men (DeMarzo et al. 1999, Lin and Lange 2000). This disease is inherited in a small proportion of men; however, 90% of cases are believed to have environmental causes (DeMarzo et al. 1999). Age is the most
significant risk factor, with 85% of men over age 65 having some form of prostate cancer, but oxidative stress generated by physiological and environmental factors also appears to play a significant role (Grönberg 2003, Nelson et al. 2001a).

The most significant physiological risk factor for prostate cancer appears to be androgen exposure (DeMarzo et al. 1999). Androgens, such as testosterone, play an important role in stimulating proliferation of prostate cells; however, androgens also may increase the oxidative burden placed upon cells (DeMarzo et al. 1999). Prostate epithelial cells are androgen dependent, and androgen ablation therapy induces apoptosis in >90% of epithelial cells (DeMarzo et al. 1999). This therapy significantly reduces the size of the normal prostate and initiates apoptosis in the majority of prostate cancer cells (DeMarzo et al. 1999).

Environmental risk factors for prostate cancer are mainly associated with the diet. Diets containing large amounts of fat and red meat appear to be closely associated with increased cancer risk (Mettlin 1997, Grönberg 2003). Fat may contribute to increased oxidative stress by presenting the prostate with increased amounts of lipid peroxides (DeWeese et al. 2001, Dreher and Junod 1996, Grover and Martin 2002). Fats may also indirectly elevate androgen levels in the blood, indirectly increasing the oxidative burden placed upon prostate cells (Lin and Lange 2000). A few studies suggest that fat may bind to orphan receptors, normally reserved for androgens, on epithelial cells, stimulating cell proliferation and oxidative stress (DeMarzo et al. 1999).
Consumption of red meat may contribute increased prostate cancer risk by increasing fat intake, but it may also increase the risk of prostate cancer by presenting the prostate with heterocyclic amines (Lawson and Kolar 2002, Grönberg 2003, Nelson et al. 2001a, Shirai et al. 2002). Human prostate epithelial cells can metabolize heterocyclic amines to products that can damage DNA and cause mutations (Lawson and Kolar 2002, Nelson et al. 2001a).

These dietary risk factors are commonly found in “Westernized” diets (Grönberg 2003, Grover and Martin 2002, Nelson et al. 2002). “Westernized” diets are often poor in \( \omega-3 \) fatty acids and rich in \( \omega-6 \) fatty acids, and diets rich in \( \omega-6 \) fatty acids are associated with increased prostate cancer risk (Gil 2002, Terry et al. 2003, Simopoulos 2002). Prostate cancer risk incidence is significantly higher in the United States and United Kingdom than in Asian countries, such as China and Japan, indicating a potential cultural or dietary component of prostate cancer risk (Nelson et al. 2002, Grover and Martin 2002). Men in China and Japan consume significantly less fat and red meat than men in the US and UK (Nelson et al. 2002, Grover and Martin 2002). Chinese and Japanese men that re-locate to the US or UK and consume the typical diets assume the same prostate cancer risk as men in those countries within 6 months (Nelson et al. 2002, Grover and Martin 2002).

**Prostate cancer prevention strategies**

Diet represents a significant risk factor for prostate cancer, but it also represents a means to reduce this risk. Consumption of large amounts of fruits and vegetables is associated with reduced prostate cancer risk (Kucuk 2002).
More specifically, diets rich in fruit and vegetable-derived carotenoids, such as lycopene, are associated with decreased risk for prostate cancer (Kucuk 2002, Heber and Lu 2002). Lycopene has generated a great deal of interest for both its antioxidant properties and antiproliferative properties (Kucuk 2002, Heber and Lu 2002). Isoflavones are another group of phytonutrients with reported health benefits. Genistein, one of the better known isoflavones derived from sources such as soy beans, demonstrates antioxidant and antiproliferative properties as well (Kucuk 2002). In general, a diet that is low in fat and high in fruits and vegetables is associated with reduced incidence of prostate cancer.

**Dairy foods and prostate cancer**

With the epidemiological link between fat consumption and the development of prostate cancer, milk and dairy products have come under increasing scrutiny. Whole milk contains 3-4% fat (Broderick 2003) and many dairy products contain a large amount of medium chain saturated fatty acids, trans-fatty acids, and ω-6 fatty acids that are associated with increased risk of atherosclerosis and development of cancer (Gil 2002, Terry et al. 2003, Molkentin 1999, Simopoulos 2002). However, total energy intake correlates more strongly to prostate cancer risk than dairy fat consumption (Kristal et al. 2002).

Dairy products contain many components with reported anticarcinogenic activity, such as conjugated linoleic acid (CLA) and butyric acid. CLA is proposed to exert its anticancer effects by a variety of mechanisms ranging from functioning as an antioxidant to reducing cell proliferation (Parodi 1998). Butyric
acid has been reported to inhibit cell proliferation and induce apoptosis (Parodi 1998, Molkentin 1999).

The focus of the present study was to examine the biological effects of two components of milk that have not been extensively studied for their biological effects in the prostate: whey proteins and sphingomyelin (SM). Whey proteins are defined as the proteins that remain soluble in milk serum after coagulation of caseins at pH 4.6 and 20°C (Morr and Ha 1993). These proteins are a relatively cysteine-rich protein source, and cysteine is rate-limiting for cellular synthesis of glutathione (GSH) (Sen 1997, Anderson 1998, Griffith 1999, Lu 1999). Thus, hydrolysis of whey proteins may provide the needed substrate for elevation of intracellular GSH, which plays an important role in regulation of cellular redox state and protecting cells against oxidative damage that may lead to the development of cancer (Sen, 1997; Anderson, 1998; Griffith, 1999; Lu, 1999). This thiol also protects against the development of cancer by directly conjugating DNA-adduct forming compounds (Sen, 1997; Anderson, 1998; Griffith, 1999; Lu, 1999). The objective of the whey protein portion of the present study was to determine if hydrolyzed whey proteins can elevate intracellular GSH concentrations in non-cancerous human prostate epithelial cells (designated RWPE-1) and protect these cells against oxidative stress.

RWPE-1 cells are a non-tumorigenic cell line derived from the peripheral zone of the prostate where most prostate cancer arises (Webber et al. 2001). The RWPE-1 cell line contain 40-50 chromosomes per cell and express normal levels of p53, retinoblastoma (Rb), and androgen receptor (Bello et al. 1997).
Little work has been done with these cells concerning oxidative stress, so the first chapter of the dissertation involves a study of oxidative stress in these cells and how supplements used in the proliferation of these cells also protect the cells against oxidative stress.

The second dairy component examined in the present study was SM, a phospholipid commonly found in mammalian cell membranes. Most dairy products contain a significant proportion of this phospholipid trapped in the milk fat globule membrane (MFGM). The MFGM contains 22% of the total lipids in whey protein concentrates and accounts for 40% of the total phospholipids in whey protein concentrates (WPCs) (Houlihan and Goddard 1991). Phosphatidylethanolamine (PE), phosphatidylcholine (PC), and SM are found in equal ratios in the MFGM and whole milk, composing 20-35% of total phospholipids (Boyd et al. 1999, Molkentin 1999, Pfeuffer and Schrezenmeir 2001, Parodi 1999).

SM forms a significant proportion of mammalian plasma membranes (Houlihan and Goddard 1991, Virtanen et al. 1998, Lange et al. 1989), and hydrolysis of SM to ceramide occurs during periods of stress, resulting in activation of signal transduction cascades via activation of ceramide-dependent kinases and phosphatases (Parodi 1999, Tepper et al. 2000). Accumulation of ceramide leads to a variety of global cellular effects, such as cell cycle arrest and apoptosis (Parodi 1999, Molkentin 1999, Kolesnick 2002). Dietary SM inhibits the progression of colon cancer in mice and inhibits the formation of preneoplastic hepatic lesions in rats (Lemmonier et al. 2003, Silins et al. 2003);
however, the role of dietary SM has not been previously explored for its effects on prostate cancer cell proliferation. Therefore, the effects of exogenous SM upon the proliferation of androgen independent human prostate cancer cells (designated PC-3) were examined. PC-3 cells were derived from a metastatic tumor from the vertebra of a 62 year old Caucasian man (Webber et al. 1997, Bosland et al. 2000). These cells have a modal number of 62-64 chromosomes and no Y chromosome (Webber et al. 1997, Bosland et al. 2000). PC-3 cells produce wild type Rb and mutated p53, as well as large amounts of growth factors and growth factor receptors, allowing for autonomous growth (Webber et al. 1997, Bosland et al. 2000). These cells are unresponsive to androgen ablation therapy because they express low levels of androgen receptor, making these cells more difficult to treat than other types of prostate cancer (Webber et al. 1997, Bosland et al. 2000). Therefore, dietary strategies that limit the proliferation of these cells are of great importance.
CHAPTER 1

BOVINE PITUITARY EXTRACT PROVIDES REMARKABLE PROTECTION AGAINST OXIDATIVE STRESS IN HUMAN PROSTATE EPITHELIAL CELLS

Published:

ABSTRACT

Bovine pituitary extract (BPE) is routinely used as a mitogenic supplement in serum-free growth medium. In addition to its mitogenic activity, BPE contains a variety of growth factors and hormones with reported antioxidant activity. The present study examines the antioxidant potential of BPE in non-tumorigenic human prostate epithelial cells (RWPE-1). Treatment of RWPE-1 cells with BPE (50 µg/ml) provided significant protection against H₂O₂-induced cell death, DNA fragmentation, protein oxidation, and membrane damage. Treatment with heat (71°C, 10 min) and proteolytic enzymes reduced the antioxidant activity of BPE, suggesting proteins present in BPE may be responsible for the antioxidant activity. Residual catalase activity present in BPE was responsible for a portion (30%) of the antioxidant activity. Interestingly, RWPE-1 cells treated with BPE and H₂O₂ rapidly accumulated intracellular reactive oxygen species (ROS) to a
greater extent than cells receiving only \( \text{H}_2\text{O}_2 \). Pre-treatment of RWPE-1 cells with tyrosine kinase inhibitors (genistein, tyrphostin 47, AG-1296) prior to the addition of \( \text{H}_2\text{O}_2 \) diminished BPE protection against \( \text{H}_2\text{O}_2 \)-induced cell death, while treatment with purified mitogens commonly found in BPE, growth hormone and basic fibroblast growth factor, did not protect against oxidative damage. Taken together, these data suggest that BPE contains proteins or protein-complexes with remarkable antioxidant activity. These yet unidentified compounds appear to confer protection against \( \text{H}_2\text{O}_2 \)-induced cell death via tyrosine kinase-dependent pathways that increase intracellular ROS generation. The antioxidant activity of BPE may represent a confounding variable when studying oxidative stress in cells maintained in BPE-supplemented serum-free medium.
INTRODUCTION

Growth medium must be supplemented with nutrients and mitogens to support cell proliferation in vitro. Animal serum has traditionally been used at concentrations as high as 10% (v/v) to supplement growth medium; however, this serum is inherently variable in composition and may provide a potential source of experimental error (Barnes and Sato 1980, Froud 1999). Serum-free media have been developed which contain defined levels of nutrients and are supplemented with mitogens to support cell proliferation (Karasek 1983, Barnes and Sato 1980). A common mitogenic factor used to supplement serum-free medium is bovine pituitary extract (BPE) (Karasek 1983, Barnes and Sato 1980). BPE has approximately 70 times the mitogenic activity of animal serum (Asami et al. 1984). Although the exact composition of BPE is unknown, a variety of mitogens including, growth hormone (GH), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF)-like proteins, and platelet-derived growth factor are present in this extract (Hadden et al. 1989, Swope et al. 1995, Wang et al. 1995).

In addition to stimulating cell proliferation, EGF and BPE-associated mitogens can protect against oxidative stress. EGF protects rat pituitary cells against reactive oxygen species (ROS)-induced apoptosis by limiting cell membrane disruption and preventing DNA fragmentation (Yasuda et al. 1999). GH increases proliferation of rat cardiomyocytes and protects against H$_2$O$_2$-induced apoptosis (Gu et al. 2001). Administration of bFGF protects neurons against ROS-induced cell damage immediately following ischemic attack in rats (Liu et al. 1999). However, the antioxidant activity of BPE has not been studied.
The present study examines the antioxidant activities of BPE and EGF using a non-tumorigenic human prostate epithelial cell line (designated RWPE-1) (Rhim et al. 1994) that requires these mitogenic supplements for proliferation. Results from this study suggest that BPE, but not EGF, provides remarkable protection against H$_2$O$_2$-induced damage in RWPE-1 cells.
MATERIALS AND METHODS

Materials

Hydrogen peroxide (9.8M), aminotriazole (AT), genistein, ethidium bromide, trypsin inhibitor, porcine trypsin, bovine chymotrypsin, porcine GH, and bovine bFGF were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Thiazolyl blue tetrazolium bromide (MTT) and PD153035 were obtained from Fisher Scientific. AG-1296 and tyrphostin 47 were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Amicon Ultra size fractionation centrifuge tubes were purchased from Millipore Corporation (Bedford, MA). The Oxyblot Protein Oxidation Detection Kit was purchased from Serologicals Corporation (Norcross, GA). The lactate dehydrogenase (LDH) assay kit was purchased from Takara Bio (Japan). Keratinocyte serum-free medium (KSFM) with BPE and EGF supplements and Dulbecco’s phosphate buffered saline without calcium and magnesium (DPBS) were obtained from Invitrogen Corporation (Carlsbad, CA).

Cell culture

Adherent human prostate epithelial cells (RWPE-1, ATCC #CRL-11609) were maintained in KSFM supplemented with BPE (50 µg/ml) and EGF (5 ng/ml) at 37°C, 5% CO₂, and 90% relative humidity. After reaching 90% confluency, cells were trypsinized and seeded into appropriate tissue culture vessels for each experiment.
Cell viability

The MTT assay was used to assess the effect of the H$_2$O$_2$ treatment on cell viability. RWPE-1 cells were seeded in 96-well tissue culture plates at an initial concentration of 3x10$^4$ cells/well and incubated for 24 h. Cells were treated with medium containing H$_2$O$_2$ (24 h), followed by addition of MTT (600 µM final conc.) to each well. After 4 h incubation with MTT, the growth medium was removed and 100 µl of 0.04M HCl in isopropanol was added to each well. Absorbance was measured at 620 nm, and cell viability was expressed as % control.

Transmission electron microscopy

Transmission electron microscopy was used to visualize H$_2$O$_2$-induced morphological changes in RWPE-1 cells. The cells were seeded in 35 mm dishes at a concentration of 10$^6$ cells/dish and incubated (48 h) to 90% confluency. Cells were treated with H$_2$O$_2$ (800 µM) for 12 h followed by fixation with glutaraldehyde, osmium textroxide, and ethanol according to the procedure of Burry and Lasher (1978). The fixed cells were visualized by transmission electron microscopy (7500x magnification).

Measurement of cell membrane barrier integrity

Lactate dehydrogenase cannot cross the cell membrane into the extracellular space unless membrane integrity has been compromised (Gissel and Clausen 2001). Extracellular lactate dehydrogenase activity is therefore an effective indicator of cell membrane integrity and can indicate necrotic cell death. Lactate dehydrogenase (LDH) activity was measured using the LDH Cytotoxicity
Detection Kit. RWPE-1 cells were seeded in 96-well tissue culture plates at an initial concentration of $3 \times 10^4$ cells/well and incubated for 24 h. Cells were treated with $H_2O_2$ (2 h) in the presence and absence of BPE (50 µg/ml). Following the incubation, growth medium (100 µl) was removed from each well and added to the corresponding lanes of another 96 well plate along with a chromogenic LDH reaction solution (100 µl). The reaction was allowed to proceed in the dark for 30 min. Absorbance was measured at 492 nm and LDH activity was expressed as % control.

**Measurement of cellular protein oxidation**

To determine the extent of $H_2O_2$-induced protein oxidation, Western analysis was performed using the Oxyblot Protein Oxidation Detection Kit. RWPE-1 cells were seeded in 35 mm dishes at a concentration of $10^6$ cells/dish and incubated (48 h) to 90% confluency. Cells were treated with $H_2O_2$ (6 h) and washed with DPBS. The cells were scraped into lysis buffer, collected in microcentrifuge tubes, and lysed by sonication. Cell lysates were centrifuged (3000 x $g$, 1 min), and the resulting supernatants were collected. A portion of each sample (20 µg protein) was added to an Eppendorf tube along with 12% SDS (5 µl) and dinitriphenylhydrazine (DNP) (10 µl) for 15 min to derivatize oxidized protein residues. The derivatized samples (20 µg protein per lane) were electrophoresed on an SDS-PAGE gel (10% polyacrylamide). The derivatized proteins were transferred to nitrocellulose and relative amounts of oxidized protein were determined by Western analysis using the primary antibody rabbit
anti-DNP and the secondary goat anti-rabbit IgG (horseradish peroxidase-conjugated).

**DNA fragmentation**

To determine the extent of H$_2$O$_2$-induced DNA damage, DNA fragmentation was measured according to the method of Eastman (1995). RWPE-1 cells were seeded in 35 mm dishes at an initial concentration of 10$^6$ cells/dish and incubated until 90% confluent. Cells were treated with H$_2$O$_2$ (6 h), washed with DPBS, and collected by scraping in microcentrifuge tubes. The cells were pelleted by centrifugation (1500 x g, 5 min), and the supernatant was removed. The pellet was re-suspended in 30 µl sample buffer with RNase and resolved on agarose gel (2%) for 12 h (60 V). Following electrophoresis, the gel was stained for 1 h with ethidium bromide (2 µg/ml). DNA fragmentation was visualized and photographed under ultraviolet light.

**Inhibition of BPE catalase**

Catalase was inhibited in BPE-supplemented medium using AT. AT (500 µM) was added directly to BPE-supplemented medium, followed by sterilization of the medium by passage through a 0.22 µ filter. The AT-treated medium was incubated 6 h at 37°C prior to the addition of H$_2$O$_2$ at the indicated concentrations and addition to the cells.

**Charcoal stripping of BPE**

To remove steroids and lipid soluble antioxidants, BPE was treated with an activated charcoal solution prepared according to the procedure of Biswas and Vonderhaar (1987). The charcoal solution (2 ml) was centrifuged (600 x g, 5
min), the charcoal pellet was re-suspended with BPE (1 ml), and the resulting mixture was incubated at 55°C (30 min). The BPE-charcoal mixture was centrifuged (1250 x g, 5 min) to remove excess charcoal, and the charcoal-stripped BPE supernatant was sterilized by passage through a 0.22 µ filter.

**Enzymatic hydrolysis of BPE**

BPE was subjected to hydrolysis by a proteolytic enzyme solution to determine the effect of BPE proteins on protection against H₂O₂-induced cell death. The proteolytic enzyme solution was prepared by adding 10 mg trypsin (6000 U/mg) and 10 mg chymotrypsin (50 U/mg) to 5 ml DPBS. An enzyme inhibitor solution (6%) was also prepared by adding 30 mg trypsin inhibitor to 5 ml DPBS. Both solutions were sterilized by passage through a 0.22 µ filter. To 15 ml of BPE-supplemented medium (50 µg/ml), 1 ml of enzyme solution was added followed by incubation for 2 h at 37°C. Following incubation, 1 ml of trypsin inhibitor solution (6%) was added to block trypsin and chymotrypsin activity prior to addition to the cells. The extent of hydrolysis of the BPE was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 15% polyacrylamide), and molecular weight markers were used for approximation of protein mass.

**Heat treatment of BPE**

BPE was subjected to heat treatment to determine the effect of BPE proteins on the protection against H₂O₂-induced cell death. BPE (1 ml) was added to a sterile microcentrifuge tube and heated in a hot water bath (71°C, 10
min). The heat-treated BPE was vortexed prior to addition to growth medium to re-suspend any precipitated proteins.

**Size fractionation of BPE**

To approximate the size of the BPE protective component, BPE was fractionated using Amicon filter tubes with 10 kDa, 30 kDa, and 100 kDa molecular weight cutoffs (MWCO). BPE was diluted to 50% of initial protein concentration with DPBS and added to Amicon filter tubes. The tubes were then centrifuged (3200 x g, 30 min, 4°C) and the filtrate was collected. The resulting fractions were sterilized by passage through a 0.22 µ filter prior to addition to the cells.

**Dichlorofluorescein fluorescence**

To measure the amount of intracellular ROS accumulation following H₂O₂ treatment, a microplate method using dichlorofluorescin diacetate (DCFDA) was adapted from the method of Wang and Joseph (1999). DCFDA is converted to dichlorofluorescin (DCFH) in the cell, and in the presence of ROS, it is further converted to dichlorofluorescein (DCF) which fluoresces (Wang and Joseph, 1999). RWPE-1 cells were seeded in 96-well tissue culture plates at an initial concentration of 3x10⁴ cells/well and incubated for 24 h. For experiments involving genistein, cells were incubated with genistein (24 h) prior to the addition of dichlorofluorescin diacetate (DCFDA). Cells were incubated for 30 min with DCFDA (20 µM final). Growth medium containing DCFDA was removed, and the cells were washed with DPBS (200 µl). Growth medium containing H₂O₂ (200 µM) and BPE (50 µg/ml) was added to the cells, and relative fluorescence was
measured using a fluorescence plate reader set with an excitation wavelength of 485 ± 10 nm and an emission wavelength of 528 ± 10 nm. Measurements were taken at 90 sec intervals for 4 h and the results were expressed in arbitrary fluorescence units.

**Statistical analysis**

Statistical analyses were performed using one-way analysis of variance with Tukey’s LSD post hoc comparisons at a level of confidence of 95% (P<0.05). Data are expressed as mean ± standard error of the mean (n=3).
RESULTS

Effect of BPE and EGF supplementation on H$_2$O$_2$-induced cell death

Supplementation of growth medium with BPE (50 µg/ml) alone or in combination with EGF (5 ng/ml) provided significant (p<0.05) protection against H$_2$O$_2$-induced cell death compared to unsupplemented controls (Figure 1.1). In contrast, viability of cells incubated in unsupplemented medium, was reduced by 55%, 88%, and 92% when treated with 200, 400, and 800 µM H$_2$O$_2$, respectively (P<0.05). Similarly, cells supplemented with EGF alone had a 49%, 89% and 90% reduction in viability when treated with 200, 400, and 800 µM H$_2$O$_2$, respectively (P<0.05).
Figure 1.1: Involvement of BPE and EGF in protection against H$_2$O$_2$-induced cell death. RWPE-1 cells were incubated in medium supplemented with BPE (50 µg/ml) and EGF (5 ng/ml), medium supplemented with only BPE, medium supplemented with only EGF, and unsupplemented medium and treated with H$_2$O$_2$ (24 h). Cell viability was determined by MTT assay. Significant difference from the control is designated by * (P<0.05).
Effect of BPE on H$_2$O$_2$-induced oxidative damage

Representative transmission electron micrographs (TEM, 7500x), illustrating the effect of BPE and EGF supplementation on H$_2$O$_2$-induced changes in RWPE-1 cell morphology, are given in Figure 1.2A. No changes in cellular morphology were observed in control cells incubated with (+BPE) or without BPE (-BPE). In contrast, gross changes in membrane and cytoplasmic integrity were observed upon treatment of cells with H$_2$O$_2$ alone (-BPE, +H$_2$O$_2$). These H$_2$O$_2$-induced changes in cellular morphology were prevented with the addition of BPE (50 µg/ml) to the growth medium (+BPE, +H$_2$O$_2$).

The membrane barrier integrity of RWPE-1 cells was assessed by measuring extracellular LDH following H$_2$O$_2$ treatment, as shown in Figure 1.2B. Treatment of unsupplemented cells with 200 and 400 µM H$_2$O$_2$ significantly increased LDH activity in the extracellular medium by 30% and 138%, respectively (P<0.05), compared to control cells receiving no H$_2$O$_2$. In contrast, no significant H$_2$O$_2$-induced increase in LDH activity was observed in BPE-supplemented cells.
Figure 1.2: BPE supplementation and RWPE-1 cell membrane integrity.  

A  
Representative TEM micrographs (7500x) were obtained of BPE-supplemented and unsupplemented cells incubated with and without H2O2 (800 µM, 12 h).  

B  
BPE-supplemented and unsupplemented cells were treated with H2O2 (24 h), followed by analysis of the growth medium for LDH activity. Significant difference from the control is designated by * (P<0.05).
To further characterize the antioxidant effect of BPE on \( \text{H}_2\text{O}_2 \)-induced oxidative damage, protein oxidation and DNA fragmentation of RWPE-1 cells were measured (Figures 1.3A and 1.3B). \( \text{H}_2\text{O}_2 \) (800 \( \mu \text{M}, 6 \text{ h} \)) increased RWPE-1 protein oxidation in unsupplemented cells (Figure 1.3A, Lane 3).

Supplementation with BPE, however, reduced \( \text{H}_2\text{O}_2 \)-induced protein oxidation (Lane 4) to levels comparable to those observed in unsupplemented (Lane 1) and BPE-supplemented (Lane 2) cells that were not treated with \( \text{H}_2\text{O}_2 \).

Treatment of BPE-supplemented and unsupplemented RWPE-1 cells with 0 and 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) did not result in DNA fragmentation (Figure 1.3B, Lanes 1-4). Treatment of unsupplemented RWPE-1 cells with 800 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) resulted in DNA smearing, indicative of necrosis (Lane 5). BPE supplementation reduced the DNA smearing (Lane 6) to levels observed in the unsupplemented control (Lane 1).
Figure 1.3: BPE supplementation and H₂O₂-induced intracellular protein oxidation and DNA damage. **A** BPE-supplemented (50 µg/ml) and unsupplemented cells were incubated with H₂O₂ (800 µM, 6 h) followed by determination of oxidized protein by Western analysis. **B** BPE-supplemented and unsupplemented cells were treated with H₂O₂ (6 h) followed by assessment of DNA fragmentation by agarose gel electrophoresis.
The contribution of residual BPE catalase activity to the antioxidant activity of BPE is presented in Figure 1.4. As previously observed, \( \text{H}_2\text{O}_2 \) significantly reduced RWPE-1 cell viability, and this effect was prevented by BPE supplementation. Pre-treatment of BPE with the catalase inhibitor AT (500 µM, 6 h) caused no significant reduction in cell viability when treated with 400 µM \( \text{H}_2\text{O}_2 \).

**Figure 1.4:** BPE antioxidant activity and catalase inhibition. RWPE-1 cells were incubated in medium supplemented with BPE (50 µg/ml), medium supplemented with BPE and AT (500 µM), and unsupplemented medium and treated with \( \text{H}_2\text{O}_2 \) (24 h). Cell viability was determined by MTT assay. Significant difference from the control is designated by * (P<0.05). Significant difference from cells supplemented with unstripped BPE is designated by † (P<0.05).
The contribution of lipid soluble compounds to the antioxidant activity of BPE was determined via charcoal stripping of the extract (Figure 1.5). Following H$_2$O$_2$ treatment, the viability of cells supplemented with charcoal-stripped BPE (cBPE) was reduced by 8% compared to controls (P<0.05). In contrast, cell viability was reduced by 89% in unsupplemented cells following H$_2$O$_2$ treatment (P<0.05).

**Figure 1.5:** BPE antioxidant activity and charcoal stripping. RWPE-1 cells were incubated in medium supplemented with BPE (50 µg/ml), medium supplemented with charcoal stripped BPE (cBPE, 50 µg/ml), and unsupplemented medium and treated with H$_2$O$_2$ (800 µM, 24 h). Cell viability was determined by MTT assay. Significant difference from the BPE-supplemented control is designated by * (P<0.05).
Characterization of antioxidant constituents of BPE

The effect of proteolytic enzyme and heat treatment of BPE on RWPE-1 cell viability is given in Figure 1.6. Treatment of BPE with proteolytic enzymes (trypsin and chymotrypsin) and heat (71°C, 10 min) significantly reduced BPE antioxidant activity, resulting in reduction in cell viability to the level of the unsupplemented control.
**Figure 1.6:** The effect of treatment with heat or with proteolytic enzymes on BPE antioxidant activity. RWPE-1 cells were incubated in medium supplemented with BPE (50 µg/ml), medium supplemented with heat-treated BPE (hBPE, 50 µg/ml), medium supplemented with enzymatically hydrolyzed BPE (eBPE, 50 µg/ml), and unsupplemented medium and treated with H₂O₂ (24 h). Cell viability was determined by MTT assay. Significant difference from the control is designated by * (P<0.05). Significant difference from cells supplemented with undenatured BPE is designated by † (P<0.05).
The antioxidant activity of molecular weight fractions of BPE against H₂O₂-induced cell death is given in Figure 1.7. Following fractionation of BPE with 10 kDa, 30 kDa, and 100 kDa molecular weight cut-off filters, cells were supplemented with each filtrate and then treated with H₂O₂ (800 µM). The antioxidant activity of BPE was not present in fractions less than 100 kDa.

**Figure 1.7:** BPE fraction with antioxidant activity. BPE was fractionated using filters with 10 kDa, 30 kDa, and 100 kDa MWCO. BPE-supplemented (50 µg/ml) and unsupplemented cells were treated with H₂O₂ (800 µM, 24 h). Cell viability was determined by MTT assay. Significant difference from the BPE-supplemented control is designated by * (P<0.05).
The effect of BPE supplementation on accumulation of intracellular ROS is given in Figure 1.8. The rate of intracellular ROS accumulation was significantly higher in BPE supplemented cells versus unsupplemented controls following exposure to H$_2$O$_2$ (200 µM). After 1 h, H$_2$O$_2$-induced intracellular levels of ROS in BPE supplemented cells were approximately 3 times higher than in unsupplemented cells. Pre-treatment with the tyrosine kinase inhibitor genistein (100 µM, 4 h) reduced ROS accumulation in BPE supplemented cells.

**Figure 1.8:** BPE supplementation and intracellular ROS generation. RWPE-1 cells were pre-incubated with or without genistein (100 µM, 4 h) in fully supplemented medium. Cells were then incubated in BPE-supplemented (50 µg/ml) and unsupplemented medium containing H$_2$O$_2$ (200 µM). DCF fluorescence was measured and relative fluorescence was plotted.
The effect of tyrosine kinase inhibitors on H$_2$O$_2$-induced cell death is given in Figure 1.9. Pre-treatment of RWPE-1 cells with the tyrosine kinase inhibitors, genistein (50 µM), tyrphostin 47 (200 µM), and AG-1296 (200 µM), prior to treatment with 800 µM H$_2$O$_2$ significantly reduced the viability of BPE supplemented cells by 45%, 35%, and 18%, respectively, compared to BPE-supplemented controls, receiving no inhibitor (P<0.05). Pre-treatment with PD153035, an inhibitor of the EGF receptor, did not significantly reduce the viability of BPE supplemented cells when treated with H$_2$O$_2$. 
Figure 1.9: Tyrosine kinase inhibition on BPE antioxidant activity. RWPE-1 cells were pre-treated 24 h with genistein (GEN, 50 µM) in fully supplemented medium and 4 h in fully supplemented medium with PD153035 (PD, 100 nM), tyrphostin 47 (TYR, 200 µM), and AG-1296 (AG, 200 µM). Cells were then treated with BPE-supplemented medium (50 µg/ml) containing H₂O₂ (800 µM, 24 h) and the inhibitory compounds. Cell viability was determined by MTT assay. Significant difference from the BPE-supplemented control is designated by * (P<0.05).
Effect of GH and bFGF supplementation on H$_2$O$_2$-induced cell death

The effect of purified GH and bFGF on H$_2$O$_2$-induced cell death is given in Figure 1.10. Supplementation of cells with GH (2 IU/ml) and bFGF (100 ng/ml) did not protect against H$_2$O$_2$-induced cell death (Figure 1.10). Following treatment with 800 µM H$_2$O$_2$, GH-supplemented cell viability was significantly reduced by 85%, and bFGF-supplemented cell viability was reduced by 86% compared to BPE-supplemented controls (P<0.05). In comparison, the viability of cells incubated in the absence of BPE was reduced by 87% compared to BPE supplemented controls (P<0.05).
Figure 1.10: Simulation of BPE antioxidant effect by purified mitogens. RWPE-1 cells were incubated in BPE-supplemented medium (50 µg/ml), GH-supplemented medium (2 IU/ml), bFGF-supplemented medium (100 ng/ml), and unsupplemented medium, followed by treatment with H$_2$O$_2$ (800 µM, 24 h). Cell viability was determined by MTT assay. Significant difference from the BPE-supplemented control is designated by * (P<0.05).
DISCUSSION

BPE protects RWPE-1 cells against H₂O₂-induced cell damage

RWPE-1 cells are an ideal in vitro model to study prostate carcinogenesis because they are derived from the peripheral zone of the prostate where most prostate cancer develops (Webber et al. 2001). These cells are maintained in KSFM supplemented with BPE and EGF. BPE is included in the growth medium to stimulate proliferation, but the results of the present study demonstrate that this extract also has considerable antioxidant activity. BPE protected RWPE-1 cells against H₂O₂-induced DNA oxidation, protein oxidation, and cell membrane rupture, which led to necrotic cell death in unsupplemented cells.

Charcoal stripping and molecular weight fractionation of BPE

Charcoal stripping of BPE was performed to remove lipid soluble components that might contribute to the antioxidant activity of this extract. This process is routinely utilized to remove lipid components from growth medium (Biswas and Vonderhaar 1987). Charcoal stripping did not significantly reduce BPE antioxidant activity, suggesting that lipid soluble components were not responsible for protection against H₂O₂-induced cell death. Low molecular weight, water soluble antioxidants were separated from BPE via molecular weight fractionation. Fractions of BPE less than 100 kDa did not protect against H₂O₂-induced cell death, suggesting that water soluble antioxidants were also not responsible for the antioxidant activity.
BPE proteins may provide antioxidant activity

Heat denaturation and proteolysis significantly reduced the antioxidant activity of BPE, suggesting that proteins present in this extract were responsible for this activity. Inhibition of catalase in BPE-supplemented medium did not reduce RWPE-1 cell viability at moderate H₂O₂ concentrations (400 µM); however, at high H₂O₂ concentrations (800 µM) a portion of BPE antioxidant activity was removed, leading to a 30% reduction in cell viability. This suggests that catalase contributes, in part, to protection against H₂O₂-induced cell death. It is unclear, however, if inhibition of intracellular or BPE-associated catalase activity is responsible for the observed decrease in cell viability. BPE mitogenic proteins, such as GH (22 kDa) and bFGF (~20 kDa), represent another potential source of antioxidant activity. Many of these mitogenic proteins are relatively small in size but often form large oligomeric complexes and large complexes with macromolecules, such as heparin (Kurobe et al. 1986, Camacho-Hubner et al. 1991, DiGabriele et al. 1998, Plotnikov et al. 1999, Arunkumar et al. 2002). Therefore, these small mitogenic proteins may exert antioxidant activity in BPE fractions greater than 100 kDa.

Role of intracellular ROS formation and tyrosine kinase activity

BPE antioxidant activity appears to involve intracellular ROS generation and tyrosine kinase activity, both of which are components of intracellular signaling pathways activated by mitogens in response to oxidative stress. For example, GH can initiate intracellular signaling via tyrosine kinase activation, increase ERK activity, induce cell proliferation, and protect against oxidative
stress by elevating levels of intracellular antioxidant enzymes (Gu et al. 2001, Evans et al. 2000, Brown-Borg et al. 2002, Ryu et al. 2000, Huang et al. 2003). bFGF protects vascular endothelial cells and neurons against oxidant-induced damage and apoptosis (Yang and de Bono 1997, Alavi et al. 2003, Mark et al. 1997), and tyrosine kinase signaling pathways involving Raf, MAPK, and ERK2 activation have been implicated in its antioxidant activity (Alavi et al. 2003, Swope et al. 1995). EGF causes accumulation of sub-lethal levels of intracellular ROS through Ras-dependent tyrosine kinase signaling pathways in A431 human epidermoid carcinoma cells and PC-12 neurons (Bae et al. 1997, Mills et al. 1998), and ROS generation may be a necessary event for phosphorylation of the EGF receptor, an important step in protecting cells against oxidative stress (Meves et al. 2001).

The tyrosine kinase inhibitor genistein reduced the accumulation of ROS in BPE-supplemented cells upon treatment with H$_2$O$_2$, suggesting a link between tyrosine kinase activity and ROS accumulation. Pre-treatment of BPE-supplemented cells with genistein, and the tyrosine kinase inhibitors tyrphostin 47 and AG-1296, reduced cell viability following H$_2$O$_2$ exposure. Taken together, these results suggest that BPE initiates ROS generation and tyrosine kinase activation in RWPE-1 cells, and that these events are critical for protection against H$_2$O$_2$-induced cell death.

The results of the present study suggest that proteins and/or protein complexes present in BPE can initiate intracellular signaling cascades that protect RWPE-1 cells against H$_2$O$_2$-induced cell damage. These intracellular...
signals require ROS generation and activation of tyrosine kinases. Activation of these pathways is consistent with mitogen exposure; however, the proteins responsible for this activity have not yet been identified. The discovery of the protective effect of BPE against H₂O₂-induced cell death may represent a confounding variable when using BPE-supplemented medium in combination with oxidative stress. The results also suggest that pituitary mitogens may play an important role in limiting ROS-induced cellular damage associated with chronic diseases, such as prostate cancer.
CHAPTER 2

EFFECT OF WHEY PROTEIN ISOLATE ON INTRACELLULAR GLUTATHIONE AND OXIDANT-INDUCED CELL DEATH IN HUMAN PROSTATE EPITHELIAL CELLS

Published:

ABSTRACT

Cysteine is the rate-limiting amino acid for synthesis of the ubiquitous antioxidant glutathione (GSH). Bovine whey proteins are rich in cystine, the disulfide form of the amino acid cysteine. The objective of this study was to determine if enzymatically hydrolyzed whey protein isolate (WPI) could increase intracellular GSH concentrations and protect against oxidant-induced cell death in a human prostate epithelial cell line (designated RWPE-1). Treatment of RWPE-1 cells with hydrolyzed WPI (500 µg/ml) significantly increased intracellular GSH by 64 %, compared to control cells receiving no hydrolyzed WPI (P<0.05). A similar increase in GSH was observed with N-acetylcysteine (500 µM), a cysteine-donating compound known to elevate intracellular GSH. In contrast, treatment with hydrolyzed sodium caseinate (500 µg/ml), a cystine-poor...
protein source, did not significantly elevate intracellular GSH. Hydrolyzed WPI (500 µg/ml) significantly protected RWPE-1 cells from oxidant-induced cell death, compared to controls receiving no WPI (P<0.05). The results of this study indicate that WPI can increase GSH synthesis and protect against oxidant-induced cell death in human prostate cells.
INTRODUCTION

Accumulation of intracellular reactive oxygen species (ROS) during extended periods of oxidative stress is associated with the development of many chronic diseases, including heart disease and cancer (Devaux et al. 2001, Nelson et al. 2001b). Glutathione (γ-glutamylcysteinylglycine, GSH) is the most abundant non-protein thiol in mammalian cells and functions as an antioxidant to limit oxidant-induced damage to lipids, proteins, and genetic material (Sen 1997, Anderson 1998, Griffith 1999, Lu 1999). Depletion of intracellular GSH can lead to the accumulation of intracellular ROS (Esteve et al. 1999, Teramoto et al. 1999). Maintaining a high intracellular concentration of GSH is therefore critical for cellular defense against oxidative stress.

GSH, in its reduced form, can donate its sulfhydryl proton to quench ROS. Once oxidized, GSH forms a disulfide linkage with a second molecule of oxidized GSH, yielding glutathione disulfide (GSSG). Maintaining a high intracellular GSH:GSSG ratio provides optimal protection against oxidant-induced cell damage. Two primary cellular mechanisms are involved in maintaining a high ratio of GSH:GSSG. The first involves reduction of GSSG to the reduced, active form of GSH by glutathione reductase, and the second involves synthesis of reduced GSH using γ-glutamylcysteine synthetase and glutathione synthase (Sen 1997, Anderson 1998, Griffith 1999, Lu 1999). When sufficient intracellular quantities of glutamate, cysteine, and glycine are present, GSH synthesis may occur; however, this process is limited by the availability of cysteine (Sen 1997, Anderson 1998, Griffith 1999, Lu 1999). Increasing cysteine availability within
tissues using the cysteine donors N-acetylcysteine (NAC) and oxothiazolidine carboxylate can elevate tissue GSH concentrations and protect against oxidant-induced cell death (Levy et al. 1998, Ho et al. 1999, Kennedy et al. 1999, Cereser et al. 2001, Kamencic et al. 2001).

Diets that are rich in cystine-containing proteins can also elevate tissue GSH (Bounous 2000, Taniguchi et al. 2000). Cystine is the disulfide form of cysteine and is reduced to 2 moles of cysteine for use in cellular GSH synthesis (Anderson 1998). Bovine whey proteins, a byproduct of cheese manufacture, are a cystine-rich protein source (Morr and Ha 1993). Consumption of cystine-rich whey proteins can increase plasma GSH concentrations in humans (Kennedy et al. 1995, Micke et al. 2001) and also reduce the incidence of mammary and colon tumors in rats (Baruchel and Viau 1996, Sekine et al. 1997, Bounous 2000, Tsuda et al. 2000, Hakkak et al. 2001).

Human prostate tissue is particularly susceptible to oxidative stress. GSH and GSH-dependent enzymes protect the prostate from oxidative stress and may therefore be important in preventing prostate cancer development (DeWeese et al. 2001, Nelson et al. 2001b). The present study tests the hypothesis that whey proteins can provide the cystine necessary to increase GSH synthesis and protect against oxidant-induced cell death in a human prostate epithelial cell line, designated RWPE-1. This cell line is non-tumorigenic and represents an ideal model for the study of oxidative stress associated with prostate cancer development (Webber et al. 2001).
MATERIALS AND METHODS

Materials

Ultrafiltered whey protein isolate (WPI) and sodium caseinate (casein) were provided by New Zealand Milk Products (Santa Rosa, CA). The glutathione assay kit was obtained from Calbiochem (San Diego, CA). Reduced glutathione, N-acetylcysteine, t-butyl hydroperoxide (TBHP), porcine peptidase, porcine trypsin, and bovine chymotrypsin were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Thiazolyl blue tetrazolium bromide (MTT) and buthionine sulfoximine (BSO) were obtained from Fisher Scientific. Tissue culture supplies were obtained from Invitrogen Corporation (Carlsbad, CA).

Cell culture

Adherent human prostate epithelial cells (RWPE-1, ATCC #CRL-11609) were maintained in keratinocyte serum free medium supplemented with bovine pituitary extract (50 µg/ml) and epithelial growth factor (5 ng/ml) at 37°C, 5% CO₂, and 90% relative humidity. After reaching 90% confluency, the cells were trypsinized and seeded into appropriate tissue culture vessels for each experiment.

Hydrolysis of WPI and casein

WPI and casein (6 mg/ml) were each hydrolyzed with porcine trypsin (23,100 U), bovine chymotrypsin (186 U), and porcine peptidase (0.26 U) for 60 min at 37°C and pH 8.0 (Wong and Cheung 2001). Proteolytic enzymes were inactivated with heat treatment (85°C, 10 min) and samples were passed through a 0.2 micron filter and stored at 4°C. All calculations for concentration of
hydrolyzed WPI and casein were based upon the original concentration of undigested protein. The extent of hydrolysis of the WPI was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 15% polyacrylamide). Standards of each of the principal proteins for the WPI (α-lactalbumin, β-lactoglobulin, and bovine serum albumin) were also identified. A molecular weight marker was used for approximation of protein mass.

**Measurement of RWPE-1 cell viability**

RWPE-1 cells were seeded in 96-well tissue culture plates at an initial concentration of $10^5$ cells/ml and incubated for 24 h. After incubation, growth medium was removed and replaced with medium containing hydrolyzed WPI (0-2000µg/ml). Cells were incubated for 48 h, followed by addition of MTT dye (600µM final concentration) to each well. After an additional 4 h incubation, the growth medium was removed and the formazan crystals, formed by oxidation of the MTT dye, were dissolved with 100µl of 0.04M HCl in isopropanol. Absorbance was measured at 620 nm and cell viability was expressed as % control.

**Measurement of intracellular GSH**

RWPE-1 cells were seeded at an initial concentration of $10^6$ cells per T75 flask and incubated 24 h. The cells were then treated with hydrolyzed WPI (500µg/ml) and hydrolyzed casein (500µg/ml) for 48 h unless otherwise specified. Cells were also treated with BSO (500µM), an inhibitor of GSH synthesis, and NAC (500µM), a cysteine-donating compound which stimulates intracellular GSH production, for 48 h. Concentration dependent GSH elevation was
determined by treating the cells with hydrolyzed WPI (0-500 µg/ml) for 48 h.

Time dependent GSH elevation by WPI (500 µg/ml) was measured at several points over a period of 48 h. After each treatment, medium was removed and the cells were washed with Dulbecco’s phosphate buffered saline. Cells were scraped from flasks in 1.2 ml of DPBS and placed into microcentrifuge tubes. A portion of cell sample (0.2 ml) was used to determine cell number by light microscopy using a hemacytometer. The remaining sample (1 ml) was centrifuged at 1000 x g and cellular pellets were resuspended with 0.5 ml of 5% metaphosphoric acid. Cellular pellets were homogenized with a Teflon pestle and centrifuged at 10,000 x g to precipitate insoluble material. The resulting supernatant was assayed for GSH according to manufacturer’s recommendation. The concentration of GSH for each sample was determined from a standard curve and expressed as nmoles of GSH per 10^6 cells.

**Oxidant-induced cell death**

Cells were seeded in 96-well plates at an initial concentration of 10^5 cells/ml and incubated for 24 h. The growth medium was removed and the plates were incubated 24 h with hydrolyzed WPI (500 µg/ml) alone or in combination with BSO (500 µM). The oxidant t-butyl hydroperoxide (TBHP) (0-500 µM) was added to each plate and the cells were incubated for an additional 24 h. Following treatments, cell viability was measured by the MTT assay.
Statistical analysis

Statistical analyses were performed using one-way analysis of variance with Fisher’s least significant difference post hoc comparisons at a level of confidence of 95% (P<0.05). Data are expressed as mean ± standard error of the mean (n=3).
RESULTS

Hydrolysis of WPI and effect on RWPE-1 cell viability

β-lactoglobulin (18 kDa), α-lactalbumin (14 kDa), and bovine serum albumin (69 kDa) were the principal proteins identified in the WPI (Figure 2.1). Treatment with proteolytic enzymes resulted in complete digestion of the major protein bands (lanes A and B).

Figure 2.1: SDS-PAGE of WPI and identification of major proteins. (A) undigested WPI (B) hydrolyzed WPI (C) α-lactalbumin (D) β-lactoglobulin (E) bovine serum albumin.
RWPE-1 cell viability was significantly reduced by treatment with hydrolyzed WPI (40% at 1000 µg/ml) and casein (30% at 2000 µg/ml) compared to controls receiving no hydrolyzed protein (P<0.05) (Figure 2.2). Hydrolyzed WPI and casein did not significantly reduce the viability of RWPE-1 cells at concentrations less than 500 µg/ml and 1000 µg/ml, respectively, compared to the control containing no hydrolyzed protein (P<0.05). For subsequent experiments, 500 µg/ml was the highest concentration tested for both hydrolyzed WPI and casein.

**Figure 2.2**: Effect of hydrolyzed WPI and casein on viability of RWPE-1 cells. Cells were treated with each hydrolyzed protein source (0-2000 µg/ml) for 48 h, followed by determination of cell viability using the MTT assay. Statistical difference from the control is designated by * (P<0.05).
Concentration and time dependent elevation of GSH by WPI

Treatment with hydrolyzed WPI for 48 h at concentrations of 250 \( \mu g/ml \) and 500 \( \mu g/ml \) significantly elevated GSH by 60% and 64%, respectively \((P<0.05)\) (Figure 2.3A). A time course study revealed that intracellular GSH was significantly elevated by 92% at 12 h post treatment with WPI (500 \( \mu g/ml \)) and remained elevated throughout the 48 h period compared to a control receiving no protein \((P<0.05)\) (Figure 2.3B).
Figure 2.3: Concentration and time dependent elevation of GSH by hydrolyzed WPI in RWPE-1 cells. (A) Cells were treated with hydrolyzed WPI (0-500 µg/ml) for 48 h. (B) Cells were treated with hydrolyzed WPI (500 µg/ml) for 0-48 h. Following treatments, GSH concentration was determined as described in the Materials and Methods (n=3). Significant difference from the control is designated by * (P<0.05).
GSH synthesis and cysteine availability

WPI treatment (500 µg/ml) of RWPE-1 cells increased GSH by 64% compared to controls receiving no hydrolyzed protein; however, casein (500 µg/ml) did not significantly elevate GSH (P<0.05) (Figure 2.4A). Treatment with NAC (500 µM) significantly elevated intracellular GSH by 88% compared to controls (P<0.05).

GSH was significantly depleted by 50% in cells treated with BSO (500 µM) alone and in combination with WPI (500 µg/ml) (P<0.05) (Figure 2.4B). GSH concentrations of cells treated with NAC (500 µM) in combination with BSO were not significantly different from controls (P<0.05).
Figure 2.4: Effect of cysteine availability and GSH synthesis on the elevation of GSH in RWPE-1 cells. (A) Cells were treated with hydrolyzed casein (500 µg/ml), hydrolyzed WPI (500 µg/ml), and NAC (500 µM) for 48 h. (B) Cells were treated with BSO (500 µM) alone and in combination with WPI (500 µg/ml) and NAC (500 µM) for 48 h. Following treatments, GSH content of the RWPE-1 cells was determined as described in the Materials and Methods (n=3). Different superscripts designate statistically different groups (P<0.05).


**WPI and oxidant-induced cell death**

RWPE-1 cells treated with 64 µM TBHP had a 95% reduction in cell viability compared to control cells receiving no TBHP while cells treated with WPI (500 µg/ml) in combination with TBHP (64 µM) had only a 40% reduction in viability, compared to controls (Figure 2.5). Pretreatment with WPI (500 µg/ml) and BSO (500 µM) prior to exposure to 32 and 64 µM TBHP reduced cell viability by 70% and 95%, respectively.

![Graph showing cell proliferation](image)

**Figure 2.5:** Effect of TBHP treatment on oxidant-induced cell death in RWPE-1 cells. (A) Cells were treated with TBHP (0-500 µM) for 24 h. (B) Cells were pretreated with hydrolyzed WPI (500 µg/ml) alone and in combination with BSO (500 µM) for 24 h, followed by exposure to TBHP (0-64 µM) for an additional 24 h. Cellular viability was determined by the MTT assay (n=24). Statistical increase in cell viability from the control at a given TBHP concentration is designated by “a” and statistical decrease from the control is designated by “b” (P<0.05).
DISCUSSION

The development of prostate cancer is associated with cell damage resulting from the accumulation of intracellular ROS (DeWeese et al. 2001, Fleshner and Kucuk 2001, Nelson et al. 2001b). Epidemiological and experimental studies suggest that antioxidants can protect prostate tissue against oxidant-induced cell damage (Fleshner and Kucuk 2001). The present study tested the hypothesis that WPI could elevate GSH in human prostate cells, thereby improving protection against oxidant-induced cell damage.

The whey protein isolate used in this study consisted of 90.5% protein, including β-lactoglobulin (44.6%), α-lactalbumin (15.5%), glycomacropeptide (GMP) (15.9%), immunoglobulins (4.4%), proteose peptone (4.2%), bovine serum albumin (1.4%), lactoferrin (0.06%), and other minor proteins (4%). The majority of whey proteins are cystine-rich, including β-lactoglobulin, α-lactalbumin, and bovine serum albumin, which contain 2 cystine, 4 cystine, and 17 cystine per molecule, respectively (Morr and Ha 1993). These whey proteins were enzymatically hydrolyzed to amino acids and peptide fragments using trypsin, chymotrypsin, and peptidase, all of which are found in the human digestive tract. Hydrolyzed WPI significantly elevated intracellular GSH in RWPE-1 cells, while unhydrolyzed WPI did not, suggesting that only small peptides and/or amino acids liberated during the hydrolysis of WPI were used for GSH synthesis.

The specific mechanism of cystine uptake in RWPE-1 cells is not known. Shanker et al. (2001) found that the uptake of cystine in neurons is regulated
mainly by the sodium dependent transport mechanism system $X_{AG}$ (80-90%) and to a lesser extent by the sodium independent transporter $\gamma$-glutamyltranspeptidase (10-20%). In hepatic cells, the sodium independent transporter system $X_C$ is used to transport cysteine into the cell as glutamate is transported out of the cell (Lu 1999). Similar amino acid transport mechanisms have been identified in human prostate epithelial cells although their involvement in WPI peptide transport has not been characterized (Franklin et al. 1990, Frierson et al. 1997, Hanigan et al. 1999, McBean and Flynn 2001, Shanker and Aschner 2001).

In contrast to whey, casein proteins are relatively poor in cystine. The principal casein proteins, $\alpha$-casein, $\beta$-casein, and $\kappa$-casein, contain 0 cystine, 0 cystine, and 2 cystine per molecule, respectively (Brunner 1977). In the present study, treatment of RWPE-1 cells with hydrolyzed casein did not significantly elevate intracellular GSH suggesting that the cystine content of the protein source is responsible, in part, for the observed increase in GSH within the prostate epithelium. Interestingly, casein contains a large amount of methionine, which can be converted to cysteine via transsulfuration (Brunner 1977, Lu 1999). However, the contribution of transsulfuration to cysteine availability is hypothesized to be minimal because no significant elevation of GSH was observed in RWPE-1 cells treated with casein.

Elevation of cellular and tissue GSH concentrations using cysteine-donating compounds, such as NAC and oxothiazolidine carboxylate, suggest that cysteine is the rate limiting amino acid for GSH synthesis (Sen 1997, Anderson 1998,
Griffith 1999, Lu 1999). In the present study, NAC and WPI increased GSH concentrations to a similar extent in RWPE-1 cells, suggesting that both compounds may elevate GSH by increasing cysteine availability.

GSH synthesis in RWPE-1 cells can be blocked by BSO, a selective and irreversible inhibitor of \( \gamma \)-glutamylcysteine synthetase that non-covalently binds to the active site of this enzyme (Sen 1997, Anderson 1998, Griffith 1999). The inhibition of WPI and NAC-induced GSH elevation by BSO, suggests that the observed increase in cellular GSH results from an increase in GSH synthesis rather than the cycling of GSSG to GSH because BSO does not inhibit glutathione reductase activity (Walther et al. 2000).

As a consequence of BSO-induced depletion of GSH, cells become more susceptible to oxidant-induced cell death (Gardiner and Reed 1995, Teramoto et al. 1999). Dringen et al. (1998) have shown that astroglial cells with elevated GSH can more rapidly detoxify TBHP than those with depleted GSH. GSH depletion is associated with increased lipid oxidation, increased protein oxidation, membrane blebbing, and mitochondrial dysfunction (Esteve et al. 1999, Chen et al. 2000, Pocernich et al. 2000, Pocernich et al. 2001). Pretreatment of RWPE-1 cells with BSO and hydrolyzed WPI depleted intracellular GSH and increased their susceptibility to TBHP-induced cell death. In contrast, treatment with hydrolyzed WPI prior to treatment with TBHP led to significant protection against
oxidant-induced cell death compared to cells receiving no hydrolyzed WPI treatment. Consumption of dietary whey proteins may provide a useful strategy to elevate intracellular GSH and protect the prostate against ROS-induced cell damage.
CHAPTER 3

ANTIPROLIFERATIVE EFFECTS OF DIETARY SPHINGOLIPIDS IN ANDROGEN INDEPENDENT PROSTATE CANCER CELLS

ABSTRACT

Dietary sphingomyelin (SM) reduces the progression of colon cancer in mice and prevents the development of preneoplastic hepatic lesions in rats. Hydrolysis of SM, and the resulting accumulation of ceramide, activates a variety of intracellular signaling pathways, ultimately leading to cell cycle arrest and apoptosis. Exogenous ceramide has previously been used to induce apoptosis in a variety of cell types; however, little research has been done on the effects of exogenous SM in cancer cells.

The hypothesis of the present study was that exogenous SM could reduce the proliferation of androgen independent prostate cancer cells (designated PC-3) by inducing cell cycle arrest and apoptosis. Cell proliferation was determined using the MTT assay while apoptosis was assessed by Annexin V-FITC staining, flow cytometry, and DNA fragmentation. Activation of the mitogen-activated protein kinase (MAPK) cascade and cell cycle regulatory proteins was measured by immunoblotting. Exogenous SM (200 µg/ml, 24 h) reduced PC-3 cell proliferation by 17% compared to the untreated control (P<0.05). SM-induced apoptosis was
responsible, in part, for the observed reduction in cell proliferation, and cell cycle arrest did not appear to be involved in this effect. Similarly, C18-ceramide reduced cell proliferation by inducing apoptosis but not cell cycle arrest. In contrast, C6-ceramide (63 µM, 24 h) was a much more potent inducer of apoptosis, reducing cell proliferation by 46% (P<0.05). This reduction in cell proliferation was associated with arrest of the cell cycle in the G0/G1 phase and apoptosis.

The reduction in cell proliferation by SM and C18-ceramide was not associated with inhibition of mitogen activated protein kinase/ERK (MEK) and extracellular signal-regulated kinase (ERK 1/2) activity. However, the apoptotic effects of C6-ceramide were associated with inhibition of MEK and ERK 1/2 and activation of retinoblastoma (Rb). Inhibition of ERK 1/2 prior to treatment with C6-ceramide significantly reduced proliferation by 17% compared to cells treated with C6-ceramide alone (P<0.05), suggesting that ERK 1/2 inhibition may play an important role in mediating C6-ceramide-induced cytotoxicity.

The results of the present study suggest that dietary sphingolipids can induce apoptosis of PC-3 cells. The reduction in proliferation by exogenous SM and C18-ceramide was relatively small; however, as studies have shown in other cancer cell types, these dietary sphingolipids may be useful in the treatment of prostate cancer when used in conjunction with chemotherapy. Conversely, synthetic C6-ceramide was a more potent inducer of apoptosis than SM or C18-ceramide; however, the results of studies using this compound are difficult to compare with natural sphingolipids because the synthetic ceramides have
different membrane permeability than natural SM and long chain ceramide. This short chain ceramide does not mimic the effects of SM and long chain ceramide, suggesting that short chain ceramide may not accurately represent the effects of natural, long chain ceramide accumulation.
INTRODUCTION

Phospholipids and the mammalian plasma membrane

Mammalian plasma membranes have an asymmetric distribution of phospholipids with phosphatidylserine (PS) and phosphatidylethanolamine (PE) found exclusively in the inner leaflet of the membrane and phosphatidylcholine (PC) and SM found in the outer leaflet of the membrane (Virtanen et al. 1998, Bevers et al. 1999, van Engeland et al. 1998, Parodi 1999). In human erythrocyte plasma membranes, SM represents 25% of the total phospholipids and 42% of the outer leaflet phospholipids (as opposed to 9% of the inner leaflet) (Virtanen et al. 1998). The plasma membranes of human fibroblasts contain 90% of total cellular SM (Lange et al. 1989).

Cells maintain specific phospholipids in the inner and outer leaflets of the membrane by using ATP-dependent lipid transport mechanisms (Virtanen et al. 1998, Bevers et al. 1999). Aminophospholipid translocase rapidly moves PS and PE from the outer to inner leaflets, while floppase moves SM and PC to the outer leaflets (Bevers et al. 1999, van Engeland et al. 1998). Aminophospholipid translocase functions more rapidly than floppase, resulting in PS and PE localizing exclusively in the inner leaflet while SM and PC are distributed between the inner and outer leaflets (Bevers et al. 1999, van Engeland et al. 1998). This orientation may be altered by lipid scramblase during apoptosis, resulting in rapid translocation of inner leaflet phospholipids, such as PS, to the outer leaflet (Bevers et al. 1999). Drug resistance in cancer cells is thought to result from altered patterns of lipid translocation in the membrane. Multi-drug
resistant cells often express an additional lipid transporter, such as MDR1, which facilitates movement of lipid soluble chemotherapeutic drugs and SM from the inner to outer leaflet of the plasma membrane (Bevers et al. 1999).

**Membrane properties of SM**

Membrane SM has a high affinity for cholesterol, forming lipid rafts and increasing membrane fluidity while improving water impermeability (Ramstedt and Slotte 2002, Zager 2000, Spector and Yorek 1985, Zager 2000). SM/cholesterol lipid rafts also contain many glycophasphatidylinositol (GPI)-linked proteins, and these proteins can activate protein tyrosine kinases and signal transduction pathways within the cell (Hoessli et al. 2000). Hydrolysis of the phosphocholine headgroup of SM by sphingomyelinase (SMase) yields ceramide, causing oligomerization of these GPI-linked proteins and initiation of signal transduction cascades via activation of ceramide-dependent kinases and phosphatases (Parodi 1999, Tepper et al. 2000). In non-apoptotic cells, ceramide is normally found in low concentrations associated with SM/cholesterol lipid rafts; however, accumulation in the membrane can increase plasma membrane rigidity and permeability (van Blitterswijk et al. 2003, Mimeault 2002). Accumulation of plasma membrane ceramide has been linked to initiation of cell cycle arrest and apoptosis (Parodi 1999, Molkentin 1999, Kolesnick 2002).

There are three main forms of SMases: acid SMase, neutral SMase, and secretory SMase. Acid SMase is a soluble glycoprotein with optimal activity at pH 5 (Goñi and Alonso 2002, Levade et al. 1999). It is generally found in lysosomes; however, acid SMase can translocate to the plasma membrane outer
leaflet (Goñi and Alonso 2002, Levade et al. 1999). In the plasma membrane, acid SMase is functional at neutral pH and rapidly generates ceramide (Kolesnick 2002). In lysosomes, it is involved in hydrolysis of SM derived from endocytosis of low density lipoproteins (LDL) (Kolesnick 2002).

Neutral SMase is a plasma membrane-associated, magnesium dependent SMase with optimal activity at pH 7 (Goñi and Alonso 2002, Levade et al. 1999). This SMase is inhibited by high intracellular glutathione concentrations and is activated during periods of stress which alter the cellular redox state (Goñi and Alonso 2002, Levade et al. 1999, Andrieu-Abadie et al. 2001, Kolesnick 2002).

Secretory SMase is zinc dependent and is targeted to the Golgi instead of the lysosome (Goñi and Alonso 2002). This SMase is thought to play a role in atherogenesis by hydrolyzing LDL-associated SM and facilitating aggregation of oxidized LDL (Goñi and Alonso 2002).

**Modulation of ceramide metabolism in the treatment of cancer**

Ceramide generation occurs by two main mechanisms: SMase activation and de novo synthesis. Ceramide synthesis occurs in the endoplasmic reticulum, and newly generated ceramide is transferred to the Golgi for SM and glucosylceramide synthesis (Slimane and Hoekstra 2002, Ségui et al. 2002). Thus, glucosylceramide synthase (GCS) and SM synthase are used to prevent accumulation of de novo synthesized ceramide (Kolesnick 2002, Tepper et al. 2000, Itoh et al. 2003). In contrast, plasma membrane-derived ceramide is hydrolyzed by ceramidase to sphingosine which is ultimately converted to sphingosine-1-phosphate, an anti-apoptotic compound (Tepper et al. 2000).
Inhibition of GCS, SM synthase, and ceramidase is important in cancer treatment because many forms of cancer overexpress these enzymes, lowering their basal ceramide levels and increasing their resistance to chemotherapeutic drugs (Kester and Kolesnick 2003, Reynolds et al. 2004, Senchekov et al. 2001). For example, colon cancer cells contain only half the ceramide of their non-cancerous counterparts (Selzner et al. 2001), and multi-drug resistant leukemia cells have increased GCS and SM synthase activity, protecting them against drug-induced ceramide accumulation (Itoh et al. 2003).

Drugs that target actively proliferating cancer cells are not effective against androgen independent prostate cancer (PC-3 cells) because these cells have a low proliferation rate in vivo (Tang and Porter 1997). Therefore, strategies that increase ceramide accumulation and apoptosis in these cells may play an important role in their treatment. However, PC-3 cells are resistant to chemotherapy-induced ceramide accumulation (Wang et al. 1999). These cells overexpress ceramidase, and utilize GCS to prevent chemotherapy-induced ceramide generation (Seelan et al. 2000, Wang et al. 2003).

Dietary SM may play a role in the prevention and treatment of cancer. Dietary SM reduced the occurrence preneoplastic lesions in rat liver (Silins et al. 2003) and colon tumor formation in mice (Lemmonier et al. 2003). One of the major challenges in treating cancer appears to be overcoming the ability of the cells to detoxify ceramide. By providing excess substrate (SM) for ceramide generation, the capacity for cancerous cells to detoxify SM may be overwhelmed causing the cells to undergo cell cycle arrest and apoptosis. The present study
examined the effects of dietary sphingolipids on human prostate cancer cells (designated PC-3). These cells are androgen unresponsive and have demonstrated drug resistance (Tang and Porter 1997, Wang et al. 1999). While synthetic ceramide has been used with these cells previously (Wang et al. 1999), dietary SM has not been examined. Initial observations revealed that exogenous SM significantly reduced PC-3 cell proliferation. The objective of the study was to determine if the reduction in PC-3 cell proliferation was a result of apoptosis or cell cycle arrest and to determine the effects of sphingolipids on MAPK signaling in this cell line.
MATERIALS AND METHODS

Reagents

SM, C6-ceramide, PC, PD98059, thiazolyl blue tetrazolium bromide (MTT), ribonuclease A, and propidium iodide were obtained from Sigma-Aldrich Co. (St. Louis, MO). C18-ceramide was obtained from Fisher Scientific (Fairlawn, NJ). The Annexin V-FITC apoptosis detection kit and Suicide-Track DNA ladder isolation kit were purchased from Oncogene Research Products (San Diego, CA). The ECL Plus Western Blotting Detection Reagents kit was purchased from Amersham Biosciences (Piscataway, NJ). Phospho-p44/42 MAPK [Thr202/Tyr204] (p-ERK 1/2), p44/42 MAPK (total ERK 1/2), phospho-MEK1/2 [Ser217/221] (p-MEK), MEK1/2 (total MEK), phospho-Rb [Ser807/811] (p-Rb), Rb (4H1) monoclonal (total Rb), phospho-cdc2 [Tyr15] (p-cdc2), cdc2 (total cdc2), anti-rabbit horseradish peroxidase (HRP)-linked IgG, and anti-mouse HRP-linked IgG were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Media and tissue culture supplies were obtained from Invitrogen Corporation (Carlsbad, CA).

Cell culture

Human prostate cancer cells (PC-3, ATCC #CRL-1435) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C, 5% CO₂, and 90% relative humidity. Upon reaching 90% confluency, cells were trypsinized and seeded into the appropriate tissue culture vessels for each experiment.
**Lipid preparation**

C6-ceramide was solubilized in DMSO and added to growth medium at a solvent concentration at 0.5%. SM was solubilized in methanol and added to growth medium at a solvent concentration of 0.25%. C18-ceramide and PC were solubilized in a chloroform:methanol (1:1) solution and added to growth medium at a solvent concentration of 0.25%. Growth medium containing the lipids was sonicated for 30 min at 37°C followed by passage of the medium through a 0.2 µm sterile filter.

**Cell proliferation**

To determine the effects of SM, C6-ceramide, C18-ceramide, and PC on PC-3 cell proliferation, cells were seeded in 96-well tissue culture plates at an initial concentration of 2.5 x 10^4 cells/well. Following an overnight incubation, cells were treated with SM, C18-ceramide, C6-ceramide, and PC (150 µl/well) at the indicated concentrations and time intervals. MTT (600 µM final conc.) was added to each well (4 h), growth medium was aspirated, and 100 µl of 0.04 M HCl in isopropanol was added to each well. Absorbance was measured at 620 nm, and cell proliferation was expressed as a percentage of the untreated control.

For experiments involving PD98059, cells were seeded at an initial concentration of 1.5 x 10^4 cells/well. PC-3 cells were pre-treated with or without PD98059 (100 µM, 4 h) followed by treatment with or without C6-ceramide (63 µM, 24 h), SM (200 µg/ml, 24 h), and C18-ceramide (200 µg/ml, 24 h). Cell proliferation was determined by MTT assay.
Cell harvesting and immunoblotting

PC-3 cells were seeded in 60 mm dishes at an initial concentration of $10^6$ cells/dish. Following the indicated treatments (6 ml/dish) with SM, C18-ceramide, C6-ceramide, and PD98059, the cells were harvested and protein concentrations for each sample were determined using the bicinchoninic acid assay. Protein samples (20 µg) were subjected to electrophoresis on a 10% polyacrylamide gel (100V, 1.5 h) and transferred to nitrocellulose (100V, 2 h). Each nitrocellulose blot was probed using the specified primary antibody and incubated with the secondary anti-rabbit or anti-mouse HRP-conjugated IgG. The chemiluminescent (ECL Plus Western Blotting Detection Reagents) was applied to the nitrocellulose for 5 min followed by film exposure (15 s to 5 min depending on signal strength).

Flow cytometry

PC-3 cells were seeded in 60 mm dishes at an initial concentration of $10^6$ cells/dish. Following the indicated treatments (6 ml/dish) with SM, C18-ceramide, C6-ceramide, and PD98059 for 24 h, the cells were collected and analyzed according to the procedure of Darzynkiewicz et al. (1992). In brief, cells were trypsinized from the dishes and washed using phosphate buffered saline (PBS) containing 0.1% bovine serum albumin. The cells were fixed in 70% ethanol and stored at $4^\circ$C until analysis. For analysis, cells were washed using PBS, spun down at 1250 x $g$, and resuspended in 1 ml of propidium iodide staining solution (3.8 mM sodium citrate and 50 µg/ml propidium iodide in PBS) and 50 µl of ribonuclease A (10 mg/ml). The resulting cell suspension was
incubated for 30 min at room temperature in the dark and then counted immediately by flow cytometry.

**Annexin V-FITC binding**

PC-3 cells were seeded in 60 mm dishes at an initial concentration of $10^6$ cells/dish and treated (6 ml/dish) with SM (200 µg/ml), C18-ceramide (200 µg/ml), PD98059 (100 µM), and C6-ceramide (63 µM) for 24 h. Cells were also pre-treated with PD98059 (100 µM, 4 h) followed by treatment with C6-ceramide (63 µM, 24 h). Following the indicated treatments, cells were collected and analyzed according to the directions provided by the Annexin V-FITC apoptosis detection kit (Oncogene Research Products). In brief, cells were trypsinized from the dishes and washed using PBS. Annexin V-FITC (0.5 µg/ml final conc.) was added to each sample followed by 15 min incubation at room temperature in the dark. Medium containing Annexin V-FITC was removed and cells were resuspended in a propidium iodide solution (0.6 µg/ml propidium iodide final conc.). Each sample was stored on ice in the dark until analyzed by fluorescence microscopy. For each sample, a digital micrograph was taken (200x magnification) under bright field conditions, as well as under green and blue fluorescent filters. Cells expressing only green fluorescence under the blue filter were considered early apoptotic. Cells expressing green/red or red fluorescence were considered late apoptotic. The percentage of apoptotic cells was determined by dividing the total number of early and late apoptotic cells by the total number of cells as counted under bright field. Significant difference from the untreated control is designated by * (P<0.05).
DNA fragmentation

PC-3 cells were seeded in 60 mm dishes at an initial concentration of $10^6$ cells/dish. Following the indicated treatments (6 ml/dish) with SM, C18-ceramide, PC, and C6-ceramide for 48 h, the cells were collected and analyzed according to the directions provided by the Suicide Track DNA fragmentation kit (Oncogene Research Products). In brief, floating cells were collected by centrifugation (1250 x $g$) after the indicated treatments. Adherent cells were lysed in situ using the provided lysis/endonuclease inhibitor buffer (55 µl), and the lysate was used to resuspend the pelleted cells. The lysate was incubated with RNAse (37ºC, 1 h) followed by incubation at 50ºC (24 h) to aid in the isolation of DNA from the lysate. DNA was precipitated with acetic acid (3M) and isopropanol followed by centrifugation at 10,000 x $g$. Samples were progressively washed with 70% ethanol and 100% ethanol and allowed to air dry at room temperature. Samples were re-solubilized in high salt buffer, and a portion of each sample was loaded onto a 1.5% agarose gel. The DNA was subjected to electrophoresis at 60V for 4 h followed by staining with ethidium bromide. DNA was visualized by UV irradiation, and digital micrographs of the gel were recorded.
Data analysis

Statistical significance was determined using one-way analysis of variance with Tukey’s post hoc comparisons (n=3). Differences were considered significant at P<0.05.
RESULTS

Sphingolipids reduce PC-3 cell proliferation

The concentration dependent effects of SM, C18-ceramide, PC, and C6-ceramide on PC-3 cell proliferation (24 h) are given in Figure 3.1. Proliferation of PC-3 cells was significantly decreased by 14%, and 17% following treatment with 100 and 200 µg/ml SM, respectively (P<0.05), compared to untreated controls (Figure 3.1A). C18-ceramide reduced cell proliferation by 15-17% at all concentrations tested while PC did not affect proliferation. Cell proliferation was significantly decreased by 21%, 35%, and 46% following treatment with 16, 31, and 63 µM C6-ceramide, respectively (P<0.05), compared to untreated controls (Figure 3.1B).

PC-3 cells treated SM and C18-ceramide exhibited only modest reductions in proliferation over time (Figure 3.1C). SM (200 µg/ml) reduced PC-3 cell proliferation by 17%, 13%, and 18% at 24, 48, and 72 h post-treatment, respectively (P<0.05), compared to untreated controls. C18-ceramide (200 µg/ml) reduced proliferation by 17%, 19%, and 22% respectively (P<0.05), compared to untreated controls. PC (200 µg/ml) did not reduce cell proliferation. C6-ceramide (63 µM) reduced proliferation by 46%, 68%, and 72% at 24, 48, and 72 h post-treatment, respectively (P<0.05), compared to untreated controls (Figure 3.1D).
Figure 3.1: Concentration and time dependent effects of SM, C18-ceramide, and C6-ceramide on PC-3 cell proliferation. Cell proliferation was measured using the MTT assay. (A) PC-3 cells were treated for increasing time intervals with PC (200 µg/ml, closed circle), SM (200 µg/ml, open circle), and C18-ceramide (200 µg/ml, closed triangle). (B) Cells were treated 24 h with increasing concentrations of PC (closed circle), SM (open circle), and C18-ceramide (closed triangle). (C) PC-3 cells were treated for increasing time intervals with C6-ceramide (63 µM). (D) Cells were treated 24 h with increasing concentrations of C6-ceramide. Significant difference from the untreated control is designated by * (P<0.05).
Inhibition of MEK and ERK 1/2 by C6-ceramide but not SM and C18-ceramide

The concentration and time dependent effects of the sphingolipids on ERK 1/2 and MEK activation are given in Figure 3.2. Treatment with SM did not reduce ERK 1/2 or MEK activation at any concentration tested (Figure 3.2A). Conversely, SM increased ERK 1/2 activation at 6 and 12 h post-treatment. C18-ceramide did not reduce ERK 1/2 or MEK activation at any concentration tested, and instead increased ERK 1/2 activation through 12 h post-treatment (Figure 3.2B).

ERK 1/2 and MEK activation decreased in a concentration and time dependent manner following treatment with C6-ceramide (Figure 3.2C). The decrease in ERK 1/2 activation was greatest at 12 h post-treatment, with a small rebound in ERK 1/2 activation at 24 h post-treatment. The decrease in MEK activation was maximal at 6 h post-treatment, with a small recovery in ERK 1/2 activation at 12 h and 24 h post-treatment. Treatment with the MEK inhibitor PD98059 reduced activation of ERK 1/2 and MEK in a concentration and time dependent manner (Figure 3.2D). The reduction in ERK 1/2 activation was greatest at 6 h post-treatment, with no recovery of ERK 1/2 activation. Inhibition of MEK activation was maximal within 2 h post-treatment.
Figure 3.2: Concentration and time dependent effects of SM, C18-ceramide, C6-ceramide, and PD98059 on ERK 1/2 and MEK activation. PC-3 cells were treated with (A) SM, (B) C18-ceramide, (C) C6-ceramide, and (D) PD98059 followed by determination of activated and total ERK 1/2 and MEK by Western analysis.
ERK 1/2 inhibition enhances the antiproliferative effects of sphingolipids

Pre-treatment with PD98059 (100 µM, 4h) followed by incubation with control growth medium (24 h) significantly reduced cell proliferation by 8% (P<0.05) (Figure 3.3). Treatment with C6-ceramide (63 µM, 24 h), SM (200 µg/ml, 24 h), and C18-ceramide (200 µg/ml, 24 h) alone reduced cell proliferation by 41%, 15%, and 14%, respectively, compared to the untreated control (P<0.05). Pre-treatment with PD98059 (100 µM, 4 h) followed by treatment with C6-ceramide, SM, and C18-ceramide reduced cell proliferation by 58%, 20%, and 25%, respectively. The 17% change in proliferation found with ERK 1/2 inhibition and C6-ceramide treatment was significantly different from cells treated with C6-ceramide alone; however, the changes in proliferation found with ERK 1/2 inhibition and SM and C18-ceramide treatments were not significantly different from treatment with SM and C18-ceramide alone (P<0.05).
Figure 3.3: ERK 1/2 inhibition and antiproliferative effects of C6-ceramide, SM, and C18-ceramide. Cells were pre-treated with (+) or without (-) PD98059 (100 µM) for 4 h followed by incubation with (+) or without (-) C6-ceramide (63 µM, 24 h), SM (200 µg/ml, 24 h), and C18-ceramide (200 µg/ml, 24 h). Statistical difference from the untreated control is designated by * (P<0.05). Statistical difference between cells treated with PD98059 and sphingolipids versus sphingolipids alone is designated by ** (P<0.05).
**Exogenous sphingolipids and PC-3 cell cycle**

Treatment with SM significantly increased the population of apoptotic cells by 11% compared to the untreated control (P<0.05) without affecting the proportions of cells in the G₁, S, and G₂ phases (Figure 3.4B). C18-ceramide (Figure 3.4C) increased apoptosis by 9%; however, this was not statistically different from the untreated control (P<0.05). C18-ceramide did not alter the ratios of cells in the G₁, S, and G₂ phases compared to the control.

Treatment with C6-ceramide alone (Figure 3.4D) significantly increased the population of apoptotic cells by 39% compared to the untreated control (P<0.05). C6-ceramide also increased the population of cells in the G₂ phase of the cell cycle by 44% while reducing the populations of G₁ and S phase cells by 29% and 15%, respectively, compared to the untreated control (P<0.05). Cells pre-treated with PD98059 followed by treatment with C6-ceramide (Figure 3.4E) followed a similar pattern, increasing apoptosis by 34%, while increasing the G₂ population by 41% and reducing the populations of G₁ and S phase cells by 26% and 15%, respectively. No statistical differences were observed in cell cycle or apoptosis data for cells treated with C6-ceramide alone and cells treated with a combination of PD98059 and C6-ceramide (P<0.05). Treatment with PD98059 alone (Figure 3.4F) increased apoptosis by 9% and significantly increased the population of cells in the G₂-phase by 9% relative to the untreated control (P<0.05). PD98059 had no significant effects on the G₁ or S populations.
Figure 3.4: Flow cytometric analysis of PC-3 cells treated with SM, C18-ceramide, C6-ceramide, and PD98059. (A) Cells were left untreated or treated with (B) SM (200 µg/ml, 24 h), (C) C18-ceramide (200 µg/ml, 24 h), (D) C6-ceramide (63 µM, 24 h), (E) PD98059 (100 µM, 4 h) followed by C6-ceramide (63 µM, 24 h), and (F) PD98059 (100 µM, 24 h). Significant difference from the untreated control is designated by * (P<0.05).
Sphingolipid-induced activation of cell cycle regulatory proteins

The concentration and time dependent effects of C6-ceramide, SM, and C18-ceramide on Rb and cdc2 activation are given in Figure 3.5. Treatment with SM and C18-ceramide did not affect activation of Rb or cdc2 at any concentration tested (Figures 3.5A and 3.5B). Rb activation (dephosphorylation) increased in a concentration and time dependent manner following treatment with C6-ceramide (Figure 3.5C). The increase in Rb activation was greatest at 6 h post-treatment. No change in cdc2 activation was observed following treatment with C6-ceramide.

Treatment with PD98059 did not affect activation of Rb at any concentration tested (Figure 3.5D). Activation of cdc2 (dephosphorylation) increased in a concentration and time-dependent manner.
Figure 3.5: Concentration and time dependent effects of SM, C18-ceramide, C6-ceramide, and PD98059 on Rb and cdc2 activation. PC-3 cells were treated with (A) SM, (B) C18-ceramide, (C) C6-ceramide, and (D) PD98059 followed by determination of activated and total Rb and cdc2 by Western analysis.
**Sphingolipid-induced apoptosis**

SM (200 µg/ml) significantly increased the amount of apoptotic cells by 3% compared to the untreated control (P<0.05) (Table 3.1). C18-ceramide (200 µg/ml) significantly increased the amount of apoptotic cells by 3.6% compared to the control (P<0.05). The increase in total apoptosis induced by SM and C18-ceramide was represented by an increase in the population of early apoptotic cells. C6-ceramide significantly increased the population of apoptotic cells by 55.7% compared to the untreated control, and represented a significant increase in the population of late apoptotic cells (P<0.05). Cells pre-treated with PD98059 followed by treatment with C6-ceramide similarly increased the population of apoptotic cells by 46.1% compared to the untreated control (P<0.05). This increase in apoptosis was not significantly different from cells treated with C6-ceramide alone and was composed entirely of late apoptotic cells. Treatment with PD98059 alone did not significantly increase apoptosis relative to the untreated control.
Table 3.1: Sphingolipid-induced apoptosis determined by annexin V-FITC staining. Statistical difference from the untreated control is designated by * (P<0.05).

<table>
<thead>
<tr>
<th></th>
<th>total apoptosis</th>
<th>early apoptosis</th>
<th>late apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>3.6 ± 0.3%</td>
<td>1.5 ± 0.3%</td>
<td>2.1 ± 0.3%</td>
</tr>
<tr>
<td>SM</td>
<td>6.6 ± 0.8% *</td>
<td>3.6 ± 0.8% *</td>
<td>3.0 ± 0.7%</td>
</tr>
<tr>
<td>C18-ceramide</td>
<td>7.2 ± 0.6% *</td>
<td>4.4 ± 0.6% *</td>
<td>2.7 ± 0.3%</td>
</tr>
<tr>
<td>C6-ceramide</td>
<td>59.3 ± 0.4% *</td>
<td>3.5 ± 1.0%</td>
<td>55.8 ± 0.7% *</td>
</tr>
<tr>
<td>PD98059</td>
<td>10.5 ± 1.5%</td>
<td>2.6 ± 0.9%</td>
<td>7.9 ± 0.7%</td>
</tr>
<tr>
<td>C6-ceramide+PD98059</td>
<td>49.7 ± 18.3% *</td>
<td>3.3 ± 0.7%</td>
<td>46.4 ± 17.6% *</td>
</tr>
</tbody>
</table>

Sphingolipid-induced DNA fragmentation

C6-ceramide (63 µM) induced DNA fragmentation with a laddering pattern (Figure 3.6). SM (200 µg/ml) induced DNA fragmentation with a smearing pattern. No clear DNA fragmentation was observed when cells were treated with SM (100 µg/ml) or PC (200 µg/ml).
Figure 3.6: Sphingolipid-induced DNA fragmentation. PC-3 cells were treated with C6-ceramide (63 µM), SM (100 and 200 µg/ml), and PC (200 µg/ml) for 48 h followed by determination of DNA fragmentation by agarose gel electrophoresis.
DISCUSSION

Dietary SM absorption and transport

Humans consume 115-140 g of sphingolipids per year from sources such as meat and dairy products, and rats fed diets containing 1% SM have shown no toxic effects (Merrill et al. 1997, Vesper et al. 1999, Ahn and Schroeder 2002a). Human digestion and absorption of SM is not well understood; however, it is known that the alkaline SMase has been found in bile and the pancreas secretes neutral SMase that may hydrolyze dietary SM (Vesper et al. 1999). SM is believed to be hydrolyzed to ceramide and sphingosine throughout the intestinal tract via the action of SMase, glucoceramidase, and ceramidase (Merrill et al. 1997, Vesper et al. 1999, Parodi 1999). Rats fed radiolabeled SM converted it to ceramide during digestion, and it was taken up as such by the intestines with small amounts of the radiolabel appearing in the liver within 30-60 min post-treatment (Schmelz et al. 1994). Approximately 25% of dietary SM is excreted (10% as SM, 80-90% as ceramide, 3-6% as sphingosine) (Vesper et al. 1999). The other 75% is absorbed by intestinal cells, re-synthesized into SM, and passed into the circulatory system via chylomicrons (Parodi 1999, Vesper et al. 1999).

Circulating plasma sphingolipids are found in the greatest amounts in LDL followed by very low density lipoproteins (VLDL) and high density lipoproteins (HDL) (Vesper et al. 1999). LDL and HDL contain mainly SM while VLDL contains mainly ceramide (Vesper et al. 1999). HDL contains approximately equal proportions of PC and SM, and LDL contains significantly more SM than
PC (Schiller et al. 2001). The chain length of SM increases with increasing lipoprotein density (Myher and Kuksis 1981).

**Cellular absorption of sphingolipids**

Cells can take up exogenous phospholipids, including SM, without affecting cell viability, and up to 20-30% of these phospholipids remain in the plasma membrane (Spector and Yorek 1985). This absorption has also been demonstrated by *Giardia lamblia*, a primitive eukaryotic cell, which efficiently absorbs exogenous SM, PC, and ceramide (Stevens et al. 1997).

Exogenous SM and ceramide can be endocytosed via tubulovesicular compartments in the plasma membrane (Slimane and Hoekstra 2002). These sphingolipids can also be taken up by lipoprotein receptor-mediated endocytosis, where they are directed to lysosomes for degradation (Jansen et al. 2001, Levade et al. 1999). In the present study, 5.7% of exogenous SM was taken up by PC-3 cells within 24 h (data not shown). Interestingly, Spence et al. (1983) showed that more SM (20 to 50 times more) is hydrolyzed by human fibroblasts, suggesting that some cells may take up long chain ceramide when treated with exogenous SM.

Ceramide generated from mammalian SM typically contains acyl chains composed of 16 to 24 carbons (Ramstedt and Slotte 2002). Exogenous long chain ceramide accumulates in the outer leaflet of the plasma membrane and is unable to penetrate the plasma membrane, contributing to its limited signaling effects and lower cytotoxicity than synthetic short chain ceramide (Shabbits and Mayer 2003, van Blitterswijk et al. 2003, Silins et al. 2003, Mimeault 2002). Short
chain ceramide, such as C6-ceramide, can penetrate the plasma membrane and accumulate in the Golgi where it is converted to long chain ceramide via ceramide synthase (Ogretmen et al. 2002). Thus, exogenous short chain ceramide can significantly increase endogenous long chain ceramide concentrations.

**Cytotoxicity of exogenous sphingolipids**

Exogenous SM can exert antiproliferative effects upon cells and has been shown to deplete ATP and initiate phospholipase A2-induced damage in human renal proximal tubule cells (Zager 2000). The effects of exogenous SM and C18-ceramide on PC-3 cell proliferation have not been previously been studied, and these sphingolipids have been the subject of only limited study in other cell lines. Thus, the aim of the present study was to examine the effects of exogenous SM and long chain ceramide on PC-3 cell proliferation. In PC-3 cells, SM and C18-ceramide reduced cell proliferation by 17% after 24 h incubation. Annexin V-FITC staining revealed significant increases in apoptosis for cells treated with C18-ceramide (3.6% increase) and SM (3% increase). Flow cytometric analysis of SM-treated cells showed a significant 11% increase in apoptosis. A statistically similar increase (9%) in apoptosis was found upon treatment with C18-ceramide; however, this did not achieve statistical difference from the untreated control. SM-treated cells exhibited DNA fragmentation; however, no clear laddering pattern was observed that would clearly indicate apoptotic cell death. Taken together, the results suggest that apoptosis may, in part, explain the observed reductions in cell proliferation by SM and C18-ceramide.
The effects of short chain ceramide on induction of cell cycle arrest and apoptosis have been documented in a variety of cell lines (Ahn and Schroeder 2002b, Jayadev et al. 1995, Wang et al. 1999). C6-ceramide has previously been shown to induce apoptosis in PC-3 cells (Wang et al. 1999), so it was included as a positive control in the present study. However, C6-ceramide causes ceramide accumulation by a different mechanism than exogenous SM, suggesting a limitation of studies comparing the antiproliferative effects of natural and synthetic sphingolipids (Mimeault 2002). C6-ceramide exhibited a higher degree of cytotoxicity in PC-3 cells than SM and C18-ceramide, reducing cell proliferation by 46% after 24 h. Annexin V-FITC staining and flow cytometry revealed 52% and 39% increases in apoptosis, respectively, compared to the untreated control. Additionally, the characteristic laddering of fragmented DNA was observed in cells treated with C6-ceramide. These results suggest that C6-ceramide-induced apoptosis was responsible for the observed reduction in proliferation.

The importance of SM in cancer treatment has been established in a variety of studies. In multidrug resistant cancers, the MDR1 transporter causes movement of inner leaflet SM to the outer leaflet, and this is thought to contribute to drug resistance (Bevers et al. 1999). Therefore, increasing the inner leaflet concentration of SM may play an important role in inducing apoptosis of PC-3 cells. Increasing SM concentration also increases the substrate for SMase, and this may generate sufficient concentrations of ceramide to overwhelm the capacity of these cells to metabolize ceramide.
Another important role for exogenous SM may be as a delivery vehicle for chemotherapeutic drugs. Vincristine delivered in liposomes formed from SM and cholesterol overcomes the drug resistance of melanoma and improves the effectiveness of the drug in non-resistant melanoma (Leonetti et al. 2004). Exogenous SM administered to mice with human colonic tumor xenografts had no effect on tumor growth; however, the combination of SM and 5-fluorouracil induced significantly more apoptosis than 5-fluorouracil alone (Modrak et al. 2002). Thus, SM-derived liposomes not only deliver the drug to the cells more efficiently but also provide substrate for ceramide generation.

**Sphingolipid-induced effects on MAPK signaling**

The MAPK cascade is involved in pro-apoptotic and anti-apoptotic signaling (Makin and Dive 2001). Within the MAPK cascade are the anti-apoptotic ERK 1/2 pathway and the pro-apoptotic p38 and JNK pathways (Makin and Dive 2001). The ERK 1/2 pathway is of particular interest because sequential activation of Ras/Raf/MEK/ERK 1/2 causes upregulation of anti-apoptotic genes (Vermeulen et al. 2003).

In most cases, ceramide accumulation results in activation of JNK and inhibition of ERK 1/2 activity, reducing cell proliferation and increasing susceptibility to apoptosis (Ruvolo 2001, Kolesnick 2002, Coroneos et al. 1996, Bourbon et al. 2001, Lee et al. 1996, Franklin and McCubrey 2000). Phosphatase inhibitors have been used successfully to prevent ceramide-induced ERK 1/2 inhibition, suggesting that ceramide-activated protein
phosphatases may play a role in this inhibition (Lee et al. 1996, Kitatani et al. 2001).

However, several chemotherapeutic drugs that cause endogenous ceramide generation also activate JNK and ERK 1/2 (MacKeigan et al. 2000, Suyama et al. 2004). Inhibition of ERK 1/2 activation using compounds such as PD98059 increases drug-induced apoptosis in a variety of cell lines, including PC-3 cells (MacKeigan et al. 2000, Zelivianski et al. 2003, Xiao et al. 2004). Taken together, the results of these studies suggest that ERK 1/2 inhibition is an important event in initiating apoptosis of cancer cells.

The role of exogenous sphingolipids in the modulation of MAPK signaling in PC-3 cells has not been previously studied. In the present study, C6-ceramide inhibited ERK 1/2 and MEK activity within 6 h post-treatment while inducing apoptosis. In contrast, PD98059 more rapidly inhibited ERK 1/2 and MEK activity while modestly reducing cell proliferation by 8% after 24 h. These results suggest that ERK 1/2 inhibition alone is not sufficient to cause the reductions in proliferation found upon treatment with C6-ceramide. In fact, the PI3K/Akt pathway has been shown to play a larger role in regulating proliferation of PC-3 cells (Gao et al. 2003).

In contrast to C6-ceramide, SM and C18-ceramide reduced cell proliferation without inhibiting ERK 1/2 activity. Instead, these natural sphingolipids increased its activation. Hydrolysis of plasma membrane SM activates tyrosine kinases and ERK 1/2 in rat endothelial cells (Czarny et al.
In leukemia cells and melanoma cells, ceramide accumulation results in ERK 1/2 activation and growth arrest (Ragg et al. 1998, Han et al. 2002).

Interestingly, inhibition of ERK 1/2 prior to the addition of exogenous sphingolipids led to further reductions in PC-3 cell proliferation. In the case of C6-ceramide, this reduction was greater than that provided by C6-ceramide and PD98059 alone. This suggested a synergistic effect between ERK 1/2 inhibition and ceramide-induced cell death. SM and C18-ceramide, on the other hand, exhibited more of an additive effect. PD98059 reduced proliferation by 8%, which approximately matched the change in cell proliferation found when cells were pre-treated with this compound prior to treatment with the SM and C18-ceramide.

Taken together, these results suggest that SM and C18-ceramide induce apoptosis by a different mechanism than C6-ceramide. Despite structural similarities to C6-ceramide, SM and C18-ceramide may have vastly different effects due to their different cellular localizations.

**Effects of SM/ceramide on cell cycle**

The mammalian cell cycle can be divided into three distinct phases: the G₀/G₁ phase in which the cells have just divided and are quiescent or awaiting initiation of DNA synthesis, the S phase in which DNA synthesis is occurring in preparation for mitosis, and the G₂/M in which the cell has already doubled its DNA content and is preparing to divide into daughter cells (Slingerland and Tannock 1998). Between each of these phases of the cell cycle are located
checkpoints designed to prevent cells from dividing uncontrollably and to prevent damaged cells from dividing (Slingerland and Tannock 1998). These checkpoints can arrest the cell cycle in any of the three major phases of the cell cycle, resulting in reduced cell proliferation and/or apoptosis. Cell cycle checkpoints are governed by regulatory proteins, such as Rb and cdc2, which are activated by alteration of phosphorylation state (Slingerland and Tannock 1998).


G2/M arrest of the cell cycle is usually associated with inhibition of cdc2 activity (Smits and Medema 2001). This protein is synthesized during S phase of the cell cycle and complexes with cyclin B (Smits and Medema 2001). In the phosphorylated form, the kinase activity of cdc2 is inhibited, preventing changes in microtubule and filament structure necessary for progression through mitosis.
Cdc2 activates itself following translation by activating Cdc25, a phosphatase that is necessary for activating the cdc2/cyclin B complex (Smits and Medema 2001). During G2/M arrest, cdc2 is maintained in its inactive (phosphorylated) form (Smits and Medema 2001).

In the present study, C6-ceramide induced a large amount of apoptosis as determined by flow cytometry. These results also showed a significant increase in cells in the G2 phase, suggesting G2/M arrest of the cell cycle. C6-ceramide has previously been shown to weakly inhibit cdc2 activity (Lee et al. 2000); however, no inhibition of cdc2 activation was observed in the PC-3 cells to correspond with G2/M arrest. Rb activation was observed with C6-ceramide treatment, but it was unclear if this corresponded to G0/G1 arrest. Most of the G1 cells were undergoing apoptosis after 24 h treatment with C6-ceramide. Thus, the increase in G2 phase cells may result from the lack of G1 population when the peak ratios are calculated. Blockage of the cell cycle at G1 and the subsequent induction of apoptosis may also explain the decrease in S phase cells. Therefore, the cells in the G2 peak may represent cells resistant to C6-ceramide treatment or cells that were already in the S or G2 phases of the cell cycle before treatment began. This may explain why no change in cdc2 phosphorylation was observed.

Treatment with PD98059 prior to treatment with C6-ceramide led to similar effects on the cell cycle as C6-ceramide alone. Treatment with PD98059 alone led to a slight increase in S phase cells and increased cdc2 activation without activating Rb. SM and C18-ceramide increased the amount of apoptosis in PC-3
cells without affecting cell cycle. No changes in Rb or cdc2 activation were observed with these treatments. The lack of evidence for cell cycle arrest by SM and C18-ceramide lends support for the hypothesis that these sphingolipids exert their antiproliferative effects by inducing apoptosis in PC-3 cells.

Conclusion

Reducing cell proliferation by modulating SM and ceramide content represents one strategy for preventing or slowing the proliferation of prostate cancer. SM is a common dietary component that is efficiently absorbed as ceramide and sphingosine during digestion, re-synthesized into SM, and circulated via lipoproteins. Thus, it is feasible that dietary SM can be delivered to cells throughout the body. The results of the present study suggest that SM reduces proliferation of androgen independent prostate cancer cells by a mechanism, in part, involving apoptosis. Exogenous C18-ceramide had a similar effect, suggesting that SM may exert its antiproliferative effects by increasing cellular ceramide concentrations. SM used in conjunction with chemotherapy improves the effectiveness of the treatment, so increasing dietary SM in combination with chemotherapy may be an effective strategy for the treatment of prostate cancer.

The synthetic ceramide, C6-ceramide, dramatically reduced PC-3 cell proliferation by inducing apoptosis. C6-ceramide has vastly different cellular effects than SM and long chain ceramide in PC-3 cells. These differences are
presumably due to the marked difference in membrane permeability between these sphingolipids. C6-ceramide has vastly different effects of MAPK signaling and cell cycle regulatory proteins, and inhibition of ERK 1/2 appears to play an important role in modulating C6-ceramide toxicity.
CONCLUSION

Several studies have associated dairy products with cancer development; however, dairy products may contain several components that could prevent the development of this disease. The present study examined how dairy proteins and lipids could play a role in limiting the progression of prostate cancer. Dairy proteins can protect against oxidative damage that could lead to the development of prostate cancer while dairy sphingolipids may exert antiproliferative effects in prostate cancer cells.

The cystine-rich whey proteins represent a way to prevent the development of prostate cancer. The relatively high cystine content may elevate intracellular GSH concentrations. Oxidative stress is an important contributor to the development of prostate cancer, so elevating GSH concentrations with whey proteins may be one strategy by which to limit oxidative stress in prostate tissue and prevent the development of this disease. Further studies should be performed using an animal model to confirm that dietary whey proteins can, in fact, elevate prostate GSH.

Dairy sphingolipids, on the other hand, may reduce the proliferation of cells that have been transformed to prostate cancer cells. In the present study, dietary SM induced apoptosis in prostate cancer cells, significantly reducing cell populations within 24 h. Sphingolipids may have strong anticarcinogenic
properties alone; however, the combination of sphingolipids with chemotherapy may represent a means to enhance the antiproliferative effects of the chemotherapeutic drugs. As with the whey proteins, in vivo testing of dietary sphingolipids should be performed to see if the in vitro antiproliferative effects can be replicated in vivo. If the in vivo animal studies show similar effects as the in vitro studies, then testing can proceed to human clinical trials.
REFERENCES


106


