Effect of whey protein isolate on intracellular glutathione and oxidant-induced cell death in human prostate epithelial cells

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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 whether 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 with 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 with 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.

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Keywords: Whey protein; Glutathione; Prostate; Oxidant; Antioxidant

1. 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., 2001). 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

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Abbreviations: BSA, bovine serum albumin; BSO, buthionine sulfoximine; DPBS, Dulbecco's phosphate buffered saline; GSH, reduced glutathione; GSSG, glutathione disulfide; MTT, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide; NAC, N-acetylcysteine; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBHP, t-butyl hydroperoxide; WPI, whey protein isolate.

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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 by-product 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., 2001). 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).

2. Materials and methods

2.1. Materials

Ultrafiltered whey protein isolate (WPI) and sodium caseinate (casein) were provided by New Zealand Milk Products (Santa Rosa, CA, USA). The glutathione assay kit was obtained from Calbiochem (San Diego, CA, USA). 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, USA). 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT) and buthionine sulfoximine (BSO) were obtained from Fisher Scientific. Tissue culture supplies were obtained from Invitrogen Corporation (Carlsbad, CA, USA).

2.2. 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% CO_2 and 90% relative humidity. After reaching 90% confluency, the cells were trypsinized and seeded into appropriate tissue culture vessels for each experiment.

2.3. 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 µ filter and stored at 4 °C. All calculations for concentration of hydrolyzed WPI and casein were based on 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 (BSA)] were also identified. A molecular weight marker was used for approximation of protein mass.

2.4. 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 (0–2000 µg/ml). Cells were incubated for 48 h, followed by addition of MTT dye (600 µM final concentration) to each well. After an additional 4 h of incubation, the growth medium was removed and the formazan crystals, formed by oxidation of the MTT dye, were dissolved with 100 µl of 0.04 M HCl in isopropanol. Absorbance was measured at 620 nm and cell viability was expressed as % control.

2.5. 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 (500 µg/ml) and hydrolyzed casein (500 µg/ml) for 48 h unless otherwise specified. Cells were also treated with BSO (500 µM), an inhibitor of GSH synthesis, and NAC (500 µM), 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 (DPBS). 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 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 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.

2.6. 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.

2.7. 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 ($n = 3$).

3. Results

3.1. Hydrolysis of WPI and effect on RWPE-1 cell viability

β-Lactoglobulin (18 kDa), α-lactalbumin (14 kDa) and BSA (69 kDa) were the principal proteins identified in the WPI (Plate 1). Treatment with proteolytic enzymes resulted in complete digestion of the major protein bands (Plate 1, lanes A and B). 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 with controls receiving no hydrolyzed protein ($P < 0.05$) (Fig. 1). 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 with 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.

3.2. Concentration and time dependent elevation of GSH by WPI

Treatment with hydrolyzed WPI for 48 h at concentrations of 250 and 500 µg/ml significantly elevated GSH by 60 and 64%, respectively ($P < 0.05$) (Fig. 2 A). A time-course study revealed that intracellular GSH
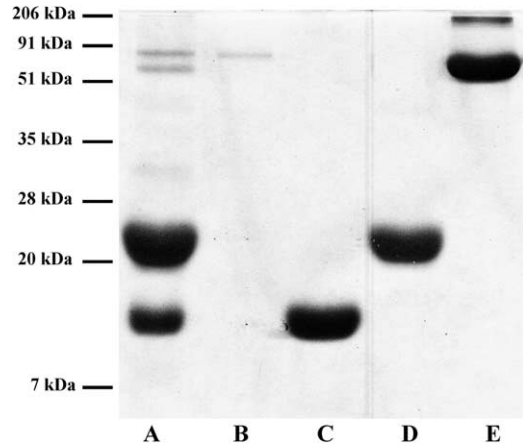

Plate 1. SDS–PAGE of WPI and identification of major proteins. (A) undigested WPI; (B) hydrolyzed WPI; (C) α-lactalbumin; (D) β-lactoglobulin; (E) bovine serum albumin (BSA).
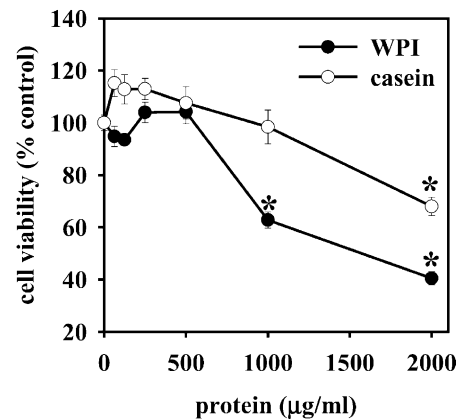

Fig. 1. Effect of hydrolyzed WPI and casein on viability of RWPE-1 cells. Cells were treated with each hydrolyzed protein source (0–2000 µg/ml) for 48 h, followed by determination of cell viability using the MTT assay ($n = 8$). Statistical difference from the control is designated by *$P < 0.05$.

was significantly elevated by 92% at 12 h post treatment with WPI (500 µg/ml) and remained elevated throughout the 48-h period compared with a control receiving no protein ($P < 0.05$) (Fig. 2B).

3.3. GSH synthesis and cysteine availability

WPI treatment (500 µg/ml) of RWPE-1 cells increased GSH by 64% compared with controls receiving no hydrolyzed protein; however, casein (500 µg/ml) did not significantly elevate GSH ($P < 0.05$) (Fig. 3A). Treatment with NAC (500 µM) significantly elevated intracellular GSH by 88% compared with 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) ($P < 0.05$) (Fig. 3B). GSH concentrations of cells treated with NAC (500 µM) in combination with BSO were not significantly different from controls ($P < 0.05$).
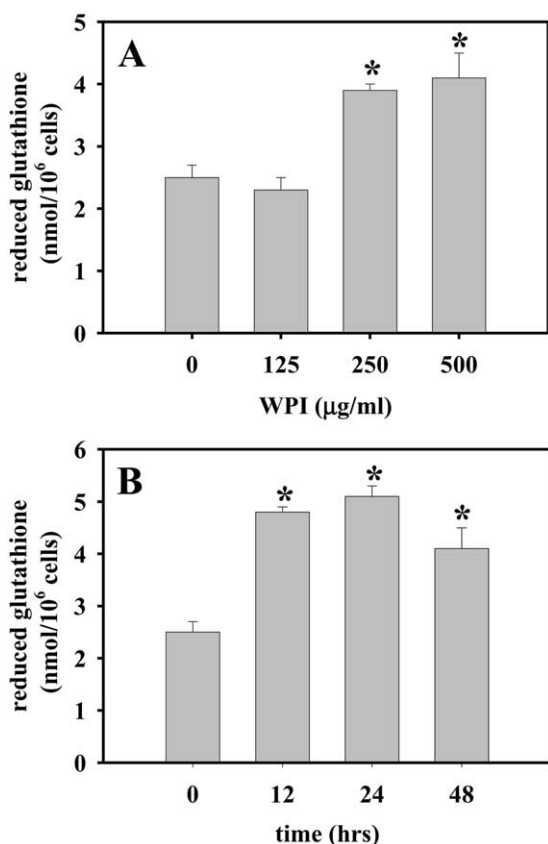
Fig. 2. Concentration and time dependent elevation of GSH by hydrolyzed WPI in RWPE-1 cells. (A) Cells were treated with hydrolyzed WPI (0–500 µg/ml) for 48 h. (B) Cells were treated with hydrolyzed WPI (500 µg/ml) for 0–48 h. Following treatments, GSH concentration was determined as described in Materials and methods ($n = 3$). Significant difference from the control is designated by *$P < 0.05$.
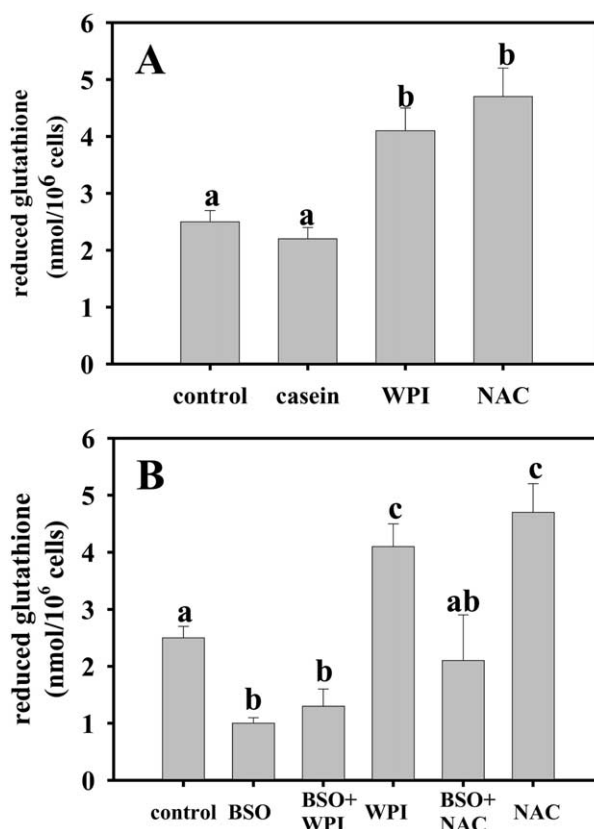

Fig. 3. Effect of cysteine availability and GSH synthesis on the elevation of GSH in RWPE-1 cells. (A) Cells were treated with hydrolyzed casein (500 µg/ml), hydrolyzed WPI (500 µg/ml), and NAC (500 µM) for 48 h. (B) Cells were treated with BSO (500 µM) alone and in combination with WPI (500 µg/ml) and NAC (500 µM) for 48 h. Following treatments, GSH content of the RWPE-1 cells was determined as described in Materials and methods ($n = 3$). Different superscripts designate statistically different groups ($P < 0.05$).

3.4. WPI and oxidant-induced cell death

The concentration of TBHP required to reduce RWPE-1 cell viability by 50% (IC_{50}) was 50 µM (Fig. 4A). At TBHP concentrations greater than 64 µM, cell viability was reduced by 98%. RWPE-1 cells treated with 64 µM TBHP had a 95% reduction in cell viability compared with 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 with controls (Fig. 4B). 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.

4. 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., 2001). 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%), BSA (1.4%), lactoferrin (0.06%) and other minor proteins (4%). The majority of whey proteins are cystine-rich, including β-lactoglobulin, α-lactalbumin and BSA, 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.
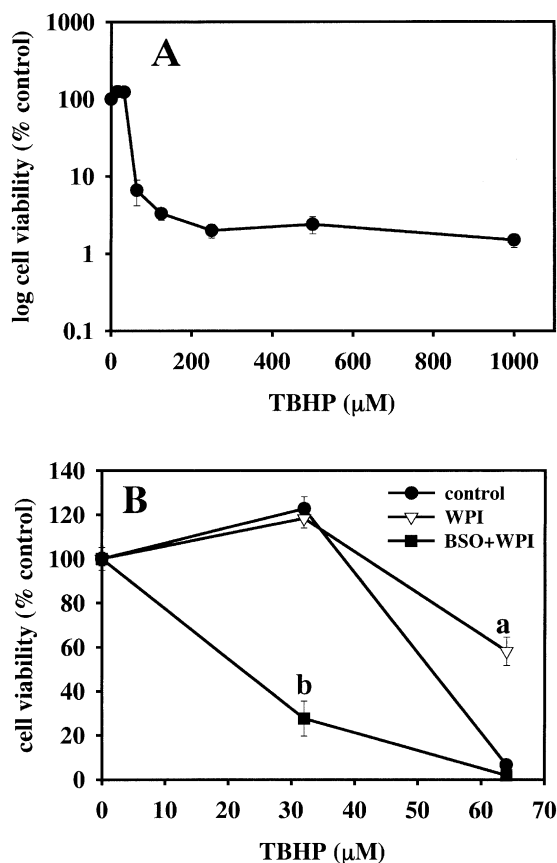
Fig. 4. Effect of TBHP treatment on oxidant-induced cell death in RWPE-1 cells. (A) Cells were treated with TBHP (0–500 µM) for 24 h. (B) Cells were pretreated with hydrolyzed WPI (500 µg/ml) alone and in combination with BSO (500 µM) for 24 h, followed by exposure to TBHP (0–64 µM) for an additional 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" ($P < 0.05$).

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 γ-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, α-casein, β-casein and κ-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 γ-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, 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 with 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.

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