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Increase in antioxidant activity by sheep/goat whey protein through nuclear factor-like 2 (Nrf2) is cell type dependent

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ABSTRACT

The aim of the present study was to investigate the molecular mechanisms through which sheep/goat whey protein exerts its antioxidant activity. Thus, it was examined whey protein's effects on the expression of transcription factor, nuclear factor-like 2 (Nrf2) and on the expression and activity of a number of antioxidant and phase II enzymes, superoxide dismutase (SOD), catalase (CAT), heme oxygenase 1 (HO-1), synthase glutamyl cysteine (GCS) and glutathione-s-transferase (GST), in muscle C2C12 and EA.hy926 endothelial cells. C2C12 and EA.hy926 cells were treated with sheep/goat whey protein (0.78 and 3.12 mg/ml) and incubated for 3, 6, 12, 18 and 24 h. Whey protein increased significantly the expression of Nrf2 only in EA.hy926 cells. Also, the expression of SOD, HO-1, CAT and the activity of SOD, CAT and GST were increased significantly in both cells types. The expression of GCS was increased significantly only in C2C12 cells. Sheep/goat whey protein was shown for the first time to exert its antioxidant activity through Nrf2-dependent mechanism in endothelial cells and Nrf2-independent mechanism in muscle cells. Thus, Nrf2 could be a target for food supplements containing whey protein in order to prevent oxidative stress damages and diseases related to endothelium.

1. Introduction

Oxidative stress is caused by the overproduction of reactive oxygen species (ROS) and electrophiles (Schieber and Chandel, 2014). ROS are produced in excessive amount by a wide variety of factors such as xenobiotics, drugs, heavy metals and ionized radiation (Klauning et al., 2010; Bhattacharyya et al., 2014). When ROS production exceeds cell detoxification ability, it can induce a chain of free radical reactions which may then destroy cellular biomolecules such as proteins, lipids, and DNA and eventually lead to diseases (Breimer, 1990; Meneghini, 1997). ROS have been implicated in more than 100 diseases such as atherosclerosis, cancer, diabetes and impaired reperfusion (Halliwell and Gutteridge, 1989). Therefore, cells must constantly control the levels of ROS and prevent their accumulation. Since most cells have been evolved in an oxygen environment, they have acquired complicated mechanisms to cope with ROS-induced toxicity.

One of the major cellular antioxidant responses is the induction of antioxidant enzymes by the cytosolic system Nrf2-Kelch-like ECH-associated protein 1 (Keap1), which is activated by a variety of natural and synthetic antioxidant agents (Nguyen et al., 2003). Under normal conditions, Keap1 anchors the transcription factor Nrf2 in the cytoplasm resulting in ubiquitination and proteasome

Abbreviations: AREs, antioxidant response elements; ATF4, activating transcription factor 4; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; DETAPAC, diethylenetriaminepentaactic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EH, epoxide hydrolases; EpREs, electrophiles response elements; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCS, synthase glutamyl cysteine; GR, reductase glutathione; GS, glutathione synthetase; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HO-1, heme oxygenase 1; HPAEC-PAD, high performance anion exchange with pulsed amperometric detection; IκBa, inhibitor of kappa B; KCl, potassium chloride; Keap1, kelch-like ECH-associated protein 1; KOH, potassium hydroxide; LC-MS/MS, liquid chromatography mass spectrometry/mass spectrometry; $MgCl_2$, magnesium chloride; NaCl, sodium chloride; NBT, nitroblue tetrazolium; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NrF2, nuclear factor-like 2; PCNA, proliferating cell nuclear antigen; PMF-1, polyamine-modulated factor 1; PVDF, polyvinylidene difluoride membranes; qNQO1, quinoneoxidoreductase; RIPA, radio-immunoprecipitation; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; sMaf, small musculoaponeurotic fibrosarcoma; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TR, thioredoxin reductase; Tris-HCl, tris-hydrochloride; UDPGTs, UDP-glucuronyltransferases; XO, xanthine oxidase.

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degradation, in order to maintain low levels of Nrf2. When cells are exposed to antioxidant agents or oxidative stress, a signal that can be phosphorylation and/or redox modification of the cysteine residue of Keap1 inhibits the enzymatic activity of the complex Keap1-CuI3-Rbx1 E3 ligase ubiquitin, resulting in reduced ubiquitination and degradation of Nrf2. As a result, the free Nrf2 translocates to the nucleus and in combination with other transcription factors [e.g. small musculoaponeurotic fibrosarcoma (sMaf), activating transcription factor 4 (ATF4), JunD, polyamine-modulated factor 1 (PMF-1)] activates the antioxidant response elements (AREs)/electrophiles response elements (EpREs) of many cytoprotective genes and of the same Nrf2 (Nguyen et al., 2003; Osburn and Kensler, 2008; Giudice and Montella, 2006; Kwak et al., 2003). The families of enzymes produced are classified into several categories: (a) xenobiotics metabolizing enzymes of phase II [e.g. glutathione-*S*-transferase (GSTs), UDP-glucuronyltransferases (UDPGTs), quinoneoxidoreductase (qNQO1), epoxide hydrolases (EH), aldehyde reductase aflatoxin B1 (AFAR), hemeoxygenase 1 (HO-1)], (b) antioxidant enzymes [e.g. synthase gamma-glutamylcysteine (g-GCS), superoxide dismutase (SOD), catalase (CAT), reductase glutathione (GR), thioredoxin reductase (TR)], (c) molecular chaperons/proteasome system, (d) DNA repair enzymes, and (e) anti-inflammatory proteins (e.g., HO-1) (Kwak et al., 2003; Hayes, and McLellan, 1999; Talalay et al., 2003; Li et al., 2002; Kelly et al., 2000).

In previous studies, we have shown that sheep/goat whey protein exerted antioxidant effects in muscle C2C12 cells (Kerasioti et al., 2014) and endothelial EA.hy926 cells (Kerasioti et al., 2016). Thus, the aim of the present study was to investigate the molecular mechanisms through which sheep/goat whey protein acts as antioxidant. Thus, it was examined the whey protein's effects on the transcription factor Nrf2 and on protein expression and activity of a number of enzymes (SOD-1, HO-1, CAT, GCS, GST) that are induced by this factor in C2C12 muscle cells and EA.hy926 endothelial cells.

2. Materials and methods

2.1. Sheep/goat whey protein

Sheep/goat whey protein was obtained from the Hellenic Protein S.A and its content was 80 g/100 g. In Table 1, the nutritional content, the profile fraction, the nucleotide content and the concentration of specific human-like oligosaccharides of sheep/goat whey protein, commercial bovine whey protein and human milk are presented. Liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) analysis was used to quantify nucleotides. High performance anion exchange with pulsed amperometric detection (HPAEC-PAD) on a gold electrode was used for the quantitative analyses of the mono- and oligosaccharides.

2.2. Cell culture conditions

C2C12 muscle cells and EA.hy926 endothelial cells were cultured in normal DMEM (Gibco, UK), containing 10% (v/v) Fetal Bovine Serum (FBS), 2 mM l-glutamine (Gibco, UK), 100 units/mL of penicillin, and 100 units/mL of streptomycin (Gibco, UK) in plastic disposable tissue culture flasks at 37 °C in 5% carbon dioxide.

2.3. Cell treatment with whey protein

The experimental design is shown in Fig. 1. C2C12 and EA.hy926 cells were seeded in culture flasks and incubated for 24 h. Then, the medium was removed and replaced with serum-free medium containing the whey protein at non-cytotoxic concentrations (0.78 mg/ml and 3.12 mg/ml) (Kerasioti et al., 2014, 2016) followed by incubation for 3, 6, 12, 18 and 24 h. Untreated cells were considered as controls.

After treatment, cells were lysed in radio-immunoprecipitation buffer [RIPA buffer; 50 mM Tris-hydrochloride (Tris-HCl), 150 mM sodium chloride (NaCl), 0.25% sodium dodecyl sulfate (SDS), 0.25% sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] containing protease inhibitors (Complete™ mini protease inhibitors, Roche, Switzerland) for the preparation of the whole cell lysate. Then, cell lysates were centrifuged at 16,250 g for 20 min at 4 °C. The supernatant was collected, and then the amount of protein was determined using the Bradford reagent (Sigma-Aldrich Ltd, Germany). For the preparation of the cytosolic and nuclear lysate, cells were lysed in cytosolic lysis buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-potassium hydroxide (HEPES-KOH) pH 7.9, 1.5 mM magnesium chloride ($MgCl_2$), 10 mM potassium chloride (KCl), 0.5 mM dithiothreitol (DTT), 0.5% Nonidet P-40] to which protease inhibitors were added (Complete™ mini protease inhibitors, Roche). Then, samples were incubated on ice for 20 min followed by centrifugation at 16.250g at 4 °C for 5 min. The supernatant was collected and the pellet was lysed in nuclear lysis buffer (10 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5% TritonX-100) to which protease inhibitors were added (Complete™ mini protease inhibitors, Roche). Then, samples were incubated on ice for 20 min followed by sonication for 30 s at periodic intervals of

Table 1
Nutritional content, profile fraction, nucleotide content and concentration of specific human-like oligosaccharides of sheep/goat whey protein (WP), commercial bovine whey protein (WP) and human milk.

Nutritional content (per 100 g)

	Sheep/goat	Bovine WP	Human milk
Energy	396 kcal/1678kj	394 kcal/1656 kj	65 kcal/272 kj
Proteins	80 g	80 g	1.1 g
Carbohydrates	10 g	6 g	7.4 g
Fats	4 g	7 g	3.4 g
Sodium	157 mg	165 mg	15 mg
Potassium	397 mg	550 mg	55 mg
Calcium	415 mg	445 mg	28 mg
Phosphorus	319 mg	315 mg	11 mg
Magnesium	73 mg	65 mg	0.7 mg

Profile fraction (per 100 g)

	Sheep/goat WP	**Bovine WP**	**Human milk**
Beta-lactoglobulin	47 g	35 g	–
Alpha-Lactalbumin	14 g	12 g	0.26 g
Glycomacropeptide	13 g	12 g	–
Lactoferrin		1 g	0.17 g
Lysosyme			0.05 g
Immunoglobulin A		8 g	0.1 g
Serum albumin	3 g	5 g	0.05 g

Nucleotide content (μmol/kg powder)

	5′ CMP	3′ CMP	5′ UMP	5′GMP	5′ IMP	5′ AMP	2′ UMP	3′ IMP	3′ GMP	3′ AMP	2′ GMP	2′AMP
Sheep/goat WP	117	18	642	144	12.3	99	75.2	0.6	13.5	11.7	8.2	9.2
Bovine WP	3.2	1.3	–	–	0.2	5.3	3.6	0.1	1.4	1.3	0.4	0.67
Human milk	9.2	–	2	–	0.1	2	0.1	–	1.5	0.9	0.5	0.4

Concentration of specific human-like oligosaccharides (mg/g)

	Lactose	3′-Sialyllactose	2′-Fucosyllactose
Sheep/goat WP	16	1.2	0.6
Bovine WP	3	0.3	0.2
Human milk	55	12	0.2