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Whey protein boosts the antioxidant profile of rats by enhancing the activities of crucial antioxidant enzymes in a tissue-specific manner

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ABSTRACT

Whey protein, a by-product of cheese industry, is harmful for the environment (i.e., surface and subterranean waters, soil) and, therefore, for humans due to its high polluting burden. Concomitantly, it has been reported that it is a mixture with potent antioxidant action since it is rich in cysteine residues, which are necessary for glutathione synthesis *in vivo*. On this basis, this study intended to examine the role of whey protein on the intensification of tissue antioxidant arsenal. To this end, a dose of sheep/goat whey protein equal to 1 g/kg of body weight/day dissolved in drinking water was administered to rats for 28 consecutive days. According to our findings, whey protein improved the antioxidant profile of liver, small intestine, lung and muscle whereas it did not affect the redox state of kidney. Our results were based on the alterations found in the protein expression of glutamate cysteine ligase, catalase and superoxide dismutase-1 measured in all tissues and the activity of glutathione S-transferase evaluated in muscle. Although tissue-specific, it is obvious that the action of whey protein is biologically beneficial and could serve as a biofunctional constituent for foods able to improve redox profile when administered against redox-related diseases.

1. Introduction

Whey protein is, along with caseins, one of the basic constituents of milk. It is a by-product of cheese manufacture in dairy industry with high polluting burden. Indeed, it has been calculated that its polluting potential is equal to a biochemical oxygen demand (BOD) approximately 175-fold higher than the sewage system of modern cities (Smithers, 2008). Therefore, it causes serious environmental problems when discarded without control. Except for its detrimental role, however, whey protein is considered a compound with high biological value (Kerasioti et al., 2018). It constitutes a milk component with known health benefits due to its antioxidant, antiinflammatory, antiviral and antitumor properties, either individually or as part of biofunctional foods (Smithers, 2008; Kerasioti et al., 2012). Its basic components are α-lactalbumin, albumin, β-galactoglobulin, immunoglobulin and antioxidant enzymes (i.e., glutathione peroxidase, GPx), whereas it plays its important biological role by its cysteine residues that are essential antioxidant moieties (Marshall, 2004). Due to the aforementioned actions, whey protein is routinely used by athletes of resistance and endurance sports (Smithers, 2008).

Previous studies of our group have shown that whey protein acts as a crucial antioxidant *in vitro* by improving the redox status of endothelial cells (Kerasioti et al., 2016a). In addition, it has been proposed that it exerts its antioxidant activity through modulation of the nuclear factor erythroid 2-related factor 2 (Nrf2), but in a cell type-dependent manner (Kerasioti et al., 2016b). Furthermore, the levels of various biomarkers of oxidative damage [i.e., protein carbonyls, thiobarbituric acid reactive substances, (TBARS)] have been improved both *in vitro* and *in vivo* indicating the wide beneficial role of this putatively polluting compound (Kerasioti et al., 2016a, 2018). However, its action on the protein levels of enzymes, such as catalase (CAT), superoxide dismutase (SOD) and glutamate cysteine ligase (GCL) in experimental animal tissues has not been yet investigated.

Therefore, in the present study we focused on the study of SOD, CAT

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Abbreviations

BOD	biochemical oxygen demand
CAT	catalase
CDNB	1-chloro-2,4-dinitrobenzene
DTT	dithiothreitol
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
γ-GC	gamma-glutamylcysteine
GPx	glutathione peroxidase
GR	glutathione reductase
GCL	glutamate cysteine ligase
GSH	glutathione
GSSG	oxidized glutathione
GST	glutathione-S-transferase
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
Nrf2	nuclear factor erythroid 2-related factor 2
$O_2^{\cdot-}$	superoxide radical
PBS	phosphate buffered saline
Prx	peroxiredoxins
PVDF	polyvinylidene difluoride membrane
ROOH	organic hydroperoxides
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances

and GPx, the three first-line-of-defense antioxidant enzymes, as well as of GCL, glutathione-S-transferase (GST) and peroxiredoxins (Prx) after administration of whey protein in rats for 28 days. SOD is the main enzymatic mechanism for the defense against superoxide radicals ($O_2^{\cdot-}$) and is the first line of defense against oxidative stress. SOD catalyzes the conversion reaction of $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2). CAT is present in every cell type, and especially in peroxisomes, cellular structures that use oxygen to detoxify toxic substances and produce H_2O_2. It catalyzes the conversion of H_2O_2 into water (H_2O) and molecular oxygen, thus completing the detoxification process initiated by SOD (Chelikani, 2004). GPx catalyzes the reduction of H_2O_2 or organic hydroperoxides (ROOH) into H_2O and alcohols, respectively, using GSH or in some cases thioredoxin or glutaredoxin as the electron donor (Kurutas, 2016). Additionally, GCL is the first enzyme that is involved in the biosynthetic pathway of GSH production, involving the ATP-dependent condensation of cysteine and glutamate to form the dipeptide gamma-glutamylcysteine (γ-GC) (Franklin et al., 2009). Prxs are a family of peroxidases that reduce hydroperoxides into H_2O protecting cellular molecules from oxidative damage and especially lipid peroxidation (Rhee, 2016). The three aforementioned enzymes are indicative biomarkers of tissue antioxidant profile. The potential alterations induced by whey protein administration will offer new insight to its way of action in the biochemical and molecular level and, therefore, contribute to a commercialized utilization of this by-product in favour of human and environmental health. We have hypothesized that, as is the case *in vitro* (Kerasioti et al., 2014), whey protein will act as an antioxidant *in vivo*, in a tissue dependent manner though. This study is part of a series of studies that have been conducted by our group and approach holistically (i.e., *in vitro*, in cell lines and *in vivo*) the role of sheep/goat whey protein on animal and human redox status.

2. Materials and methods

2.1. Sheep/goat whey protein

Sheep/goat whey protein was obtained from the Hellenic Protein S.A (Athens, Greece) and its content was 80 g in 100 g. It consists of β-lactoglobulin (47 g/100 g), α-lactalbumin (14 g/100 g), glycomacropeptide (13 g/100 g) and serum albumin (3 g/100 g). According to its nutritional composition, it comprises proteins (80 g/100 g), carbohydrates (10 g/100 g), fats (4 g/100 g), sodium (157 mg/100 g), potassium (397 mg/100 g), calcium (415 mg/100 g), phosphorus (319 mg/100 g) and magnesium (73 mg/100 g).

2.2. Experimental animals

Twelve six-month old male Wistar rats divided in two groups (6 rats/group) [control group (weighing 470 ± 6.3 g); whey group (weighing 465 ± 14.3 g)] were used for this experiment, which was performed in the Veterinary Medicine School of Aristotle University of Thessaloniki in accordance to the Helsinki Declaration and National standards (Permission code EL54BIO10). The experimental protocol was approved by the National Veterinary Administration authorities on July 13th, 2018 (Licence No.: 135973/851). The animals were housed in cages individually under controlled temperature (20–22 °C) and humidity (50–70%) and a 12-h light/dark cycle. Furthermore, they were acclimated for one week in the animal facility before the experiment took place. All animals were treated in accordance with the guiding principles of the European Community Council Directive (2010/63/EU) for the care and use of laboratory animals.

2.3. Study design

The animals were randomly divided into two groups (i.e., 6 rats per group) as follows: the control group that was fed with the standard commercial diet containing corn, soybean meal, barley, bran, milk paste and molasses (purchased from Viozois S. A. Ioannina, Greece) and the experimental group, which was fed with the standard commercial diet plus sheep/goat whey protein in a dose equal to 1 g/kg of body weight/day dissolved in drinking water. According to the literature, the maximum dose of administered whey protein in experiments that examine its effects on oxidative stress is 150 mg/rat (Hamad et al., 2011). However, whey protein has been administered at a dose of 1 g/kg of body weight/day without been toxic in experiments that examine its effects on blood pressure of hypertensive rats (Costa et al., 2005). Thus, we have selected to administer the maximum dose (i.e., 1 g/kg of body weight/day), which corresponds to 470 mg/rat in order to observe a maximum response, given that this dose does not induce toxicity. The exact daily volume of drinking water for each rat was determined and standardized by preliminary experiments in order to be sure that the rats consume the desired concentration of the control and experimental feed. The duration of the experiment was 28 days. Previous studies have shown that this is an appropriate time period in order to observe the effects of whey protein on a wide range of parameters, such as its antioxidant and hepatoprotective action (Hamad et al., 2011; Gad et al., 2011). Furthermore, a previous study of our group has also observed the beneficial antioxidant properties of whey protein after a 28-day administration regimen (Kerasioti et al., 2018). The general health of the animals was observed daily. At the end of the treatment period, all animals were anaesthetized with isoflurane (IsoFlo®, Abbot). Then, the liver, quadriceps muscle, lung, small intestine and kidney were excised, snapped-frozen in liquid nitrogen and stored at −80 °C until analyses.

2.4. Tissue preparation

The tissue samples were thawed and prepared as follows: 100 mg of tissue were homogenized with 500 μl of phosphate buffered saline [PBS (0.01 M, pH = 7.4)] and a cocktail of protease inhibitor tablet

(Complete™ mini protease inhibitors, Roche, Basel, Switzerland) was added. The homogenate was vigorously vortexed and a brief sonication treatment on ice was applied. The homogenate was then centrifuged (10,000 × *g*, 15 min, 4 °C) and the supernatant (i.e., the tissue lysate) was collected and stored at −80 °C until analyses. The activities of GST, GPx and Prx were measured in liver and quadriceps muscle, whereas the protein expression levels of GCL, SOD-1 and CAT were evaluated in all harvested tissues.

2.5. Protocol for the determination of GST activity

The determination of GST activity was based on the spectrophotometric method of (Habig and Jakoby, 1981) as previously described (Veskoukis et al., 2016). More specifically, 920 μl of phosphate buffer (100 mM, pH = 7.4) were mixed with 50 μl of GSH (1 mM) and 20 μl of 1-chloro-2,4-dinitrobenzene (CDNB, Sigma-Aldrich) and the samples were incubated for 5 min at 30 °C. Then, the liver and quadriceps muscle samples were added (the protocol requires > 10 μg of total protein in the tested sample) and the change in absorbance was monitored at 340 nm for 5 min using a spectrophotometer (HITACHI, U-1900 UV/VIS). The conjugation of the thiol group of glutathione to CDNB is accompanied by an increase in the absorbance. The samples containing the tissue lysate alone were used as blanks. GST activity was normalized on the basis of the total protein concentration of each sample.

2.6. Protocol for the determination of GPx activity

The determination of GPx activity was based on the measurement of nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) oxidation according to the spectrophotometric method of (Flohé and Günzler, 1984) as previously described (Veskoukis et al., 2016). More specifically, oxidized glutathione (GSSG) produced upon reduction of an organic hydroperoxide by GPx is instantly and continuously reduced by an excess of glutathione reductase (GR) activity providing a constant level of GSH. The concomitant oxidation of NADPH to $NADP^+$ is monitored spectrophotometrically at 340 nm. Fifty hundred microliters of phosphate buffer (100 mM, pH = 7) was mixed with 100 μl of GSH (10 mM), 100 μl of GR (2.4 U/ml) and 100 μl of liver or quadriceps muscle lysate (diluted 1:10) and the samples were incubated for 10 min at room temperature (RT). Then, 100 μl of NADPH (1.5 mM) was added and a second incubation for 3 min at RT was followed. The mixture was transferred in a cuvette where 100 μl of pre-warmed t-butyl hydroperoxide (t-BOOH) (12 mM) was added and the decrease in absorption at 340 nm was monitored for 5 min. GPx activity in the tissue lysates was normalized on the basis of the total protein concentration of each sample.

2.7. Protocol for the determination of Prx activity

The determination of Prx activity was based on the spectrophotometric method of (Nelson and Parsonage, 2011) as previously described (Veskoukis et al., 2018). More specifically, 20 μl of liver lysate or 10 μl of quadriceps muscle lysate (diluted 1:2) were mixed with 0.167 mM dithiothreitol (DTT) and 0.5 mM t-BOOH. The samples were incubated in the dark at RT for 30 min. Then, 50 μl of each sample was mixed with 950 μl of FOX reagent (25 mM ammonium ferrous sulfate diluted in 2.5 M sulphuric acid, 100 mM sorbitol and 125 μM xylenol orange). Then, the samples were incubated in the dark at RT for 30 min and the absorbance was monitored at 560 nm before and after incubation. Prx activity in the tissue lysates was normalized on the basis of the total protein concentration of each sample.

2.8. Determination of protein expression levels of GCL, SOD-1 and CAT with western blot analysis

In order to determine the protein expression levels of GCL, SOD-1 and CAT, 40 μg of protein measured using the Bradford assay were used. Tissue lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an 8% polyacrylamide gel. The proteins were then transferred onto a polyvinylidene difluoride membrane (PVDF). The membranes were blocked overnight with 5% non-fat milk in a buffer (13 mM of Tris, 150 mM of NaCl, pH = 7.5) containing 0.2% Tween-20. Then, they were probed with goat anti-human/mouse SOD-1 (1:1600; Santa Cruz Biotechnology Inc., TX, USA) or rabbit anti-human/mouse GCL (1:1600; Santa Cruz Biotechnology) or goat anti-human/mouse CAT (1:1400; R&D systems) primary antibodies for 1 h at RT. The membranes were then incubated with a polyclonal horseradish peroxidase conjugated goat anti-rabbit (1:5000) or anti-goat (1:3000) secondary antibodies for 30 min at RT. The membranes were re-probed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10000) as an internal control. The optical density of the protein bands was measured by the Alpha View quantification software (Alpha Innotech, San Jose, CA, USA).

2.9. Statistical analysis

The data were analyzed with one-way ANOVA followed by Dunnett's test using the statistical package for social sciences (SPSS, Inc., Chicago, Ill, version 21.0). The normality of distribution for all dependent variables was tested through the Kolmogorov-Smirnov test and the homogeneity of variance was assessed by the Levene's test. All results are expressed as mean ± SD (i.e., standard deviation). The level of the statistical significance was set at $P < 0.05$.

3. Results

3.1. General animal data

The general animal data, namely the mean body weight, the mean food intake, the mean water intake and the mean organ weight for both animal control and whey groups are depicted in Table 1. No statistically significant differences were observed between the two groups concerning the body weight, the food and water intake and the organ weight.

3.2. GST, GPx and prx activities

Regarding GST activity in quadriceps muscle, a significant main effect of treatment was found, whereas no effect was observed in liver. Specifically, GST activity was significantly increased in quadriceps muscle by 22% ($P = 0.030$) in the whey group compared to the control

Table 1
The general animal data.

Animal group	Mean body weight (g)	Mean food intake (g/day)	Mean water Intake (ml/day)	Mean organ weight (mg)
Control	470 ± 6.3	19.2 ± 2.3	47 ± 3.2	1010 ± 30 (liver) 850 ± 22 (muscle)
Whey	465 ± 14.3	19 ± 3.2 plus 1 g of whey/kg b.w/day	46.4 ± 2.7	1091 ± 37 (liver) 867 ± 27 (muscle)

group, whereas, in liver its activity was not affected ($P > 0.05$). Regarding GPx and Prx activities, no effect was found both in liver and quadriceps muscle ($P > 0.05$), thus their activities remained unaffected following the administration of whey protein (Tables 2 and 3). Furthermore, all dependent variables follow the normality of distribution ($P > 0.05$ for all dependent variables according to the Kolmogorov-Smirnov test), whereas for all dependent variables the homogeneity of variance was equal ($P > 0.05$ for all dependent variables according to the Levene's test).

3.3. GCL, CAT and SOD-1 expression levels

Our results indicated an important tissue specificity of the action of whey protein (Fig. 1). A significant main effect of treatment was observed in liver CAT, in all enzyme expression levels in small intestine, lung and quadriceps muscle ($P < 0.05$), whereas no effect was found in kidney ($P > 0.05$). In particular, in liver, CAT expression levels were the only ones significantly affected by whey administration [i.e., they were reduced by 64% ($P = 0.040$) compared to the control group]. In small intestine, GCL levels were significantly increased by 46% ($P = 0.045$), while CAT and SOD-1 levels were reduced by 41% ($P = 0.015$) and 79% ($P = 0.004$) respectively in the whey group compared to the control group. In lung, GCL protein levels were increased by 93% ($P = 0.049$) while CAT and SOD-1 levels were reduced by 42.5% ($P = 0.012$) and 58% ($P = 0.047$) respectively in the rats that were administered with whey protein. In quadriceps muscle, GCL and SOD-1 expression levels were increased by 100% ($P = 0.002$) and 68% ($P = 0.015$) respectively, whilst CAT expression levels were decreased by 30% ($P = 0.035$) in the whey group compared to the control group. Finally, in kidney, no significant differences were observed to any of the tested enzyme levels following whey protein administration. Furthermore, all dependent variables follow the normality of distribution ($P > 0.05$ for all dependent variables according to the Kolmogorov-Smirnov test), whereas for all dependent variables the homogeneity of variance was equal ($P > 0.05$ for all dependent variables according to the Levene's test).

4. Discussion

The present study examined the influence of sheep/goat whey protein on the redox status of the most vital tissues of rats after 28 days of administration. Given that this by-product of cheese industry is rich in cysteines that constitute the precursor residues for GSH synthesis *in vivo* and that it is a potent *in vitro* antioxidant, the main objective of this study was to examine its redox-altering action in rat tissues. According to our results, whey protein exerted beneficial antioxidant action on the tested tissues but the profile of each tissue is differentiated. Specifically, the GCL protein levels were increased in small intestine, lung and muscle. Regarding all the aforementioned tissues, CAT levels were decreased as was the case for SOD-1 with the exception of muscle. It appears that there is no need for the antioxidant mechanism to be further reinforced since GSH synthesis is enhanced, a finding that has been previously observed (Kerasioti et al., 2018). Although seemingly muscle is differentiated from this profile, its antioxidant arsenal is also strengthened since SOD-1 protein levels and GST activity are also increased. The levels of GCL in liver remained unaffected, however SOD-1 levels were decreased. It seems that, on the basis of previous relative results of our research team (Kerasioti et al., 2018), there is no need for their activation since whey acts protectively through different mechanisms compared to small intestine, lung and muscle that are not related to glutathione synthesis. In particular, whey protein was found to improve tissue redox status of rats in general by decreasing lipid peroxidation and protein oxidation and by enhancing GSH concentration levels (Kerasioti et al., 2018). We report that the enzymes whose expression in the protein level was evaluated herein act complementarily and the effects of whey on them are appraised in combination with our previous results (Kerasioti et al., 2018). These findings denote the promising activity of sheep/goat whey protein, a compound/putative pollutant, as a constituent of functional foods.

The potential use of a compound as a component of functional foods acquires the precondition of its examination in *in vivo* experimental modalities. However, a holistic approach investigating the *in vitro* antioxidant action of this specific compound (i.e., *in vitro* assays and in cell lines) is imposed. This experimental pathway has been previously meticulously analyzed by our group and, thus, it has been revealed that the measurement of a battery of redox biomarkers is able to help researchers reach the desirable conclusions (Veskoukis et al., 2012, 2019; Priftis et al., 2015). Under this frame, the examined sample of the sheep/goat whey protein was firstly studied for its putative *in vitro* antioxidant role. Indeed, it has been reported that whey protein improves the antioxidant status of muscle cells by increasing GSH levels and by decreasing reactive oxygen species and TBARS concentration (Kerasioti et al., 2014). A similar beneficial action was observed when endothelial cells were incubated with the same functional mixture. Interestingly, the examined redox biomarkers of biomolecule oxidation (i.e., GSSG, TBARS and protein carbonyls) were decreased whereas the GSH levels were enhanced implying a protective role of whey protein (Kerasioti et al., 2016a). This investigation was also conducted in the protein expression level and it was observed that the expression levels of several antioxidant enzymes were also enhanced and that at the molecular level whey protein acts through the Nrf2 pathway (Kerasioti et al., 2016b). Whey protein acts as a potent antioxidant in endothelial cells even when it is an encapsulating agent for an extract derived from olive oil mill wastewaters, which are by-products of olive oil generation process and serious polluting agents (Kreatsouli et al., 2019). Furthermore, whey protein increases catalase, SOD and GPx activities in C2C12 myoblasts, whereas the cells were able to develop even in the presence of H_2O_2 as an oxidizing agent (Xu et al., 2011). Whey protein also acted beneficially *in vitro* against oxidative damage induced by H_2O_2 in rat pheochromocytoma cells (Zhang et al., 2012).

Apart from its *in vitro* antioxidant role, however, several studies have shown that whey protein is also a strong antioxidant *in vivo*.

Table 2
The effects of sheep/goat whey protein on GST (A), GPx (B) and Prx (C) activities on rat liver. The results of the descriptive statistics parameters are presented.

Descriptive statistics parameters	Redox biomarkers in liver					
	GST (U/mg protein)		GPx (U/g protein)		Prx (U/mg protein)	
	Control	Whey	Control	Whey	Control	Whey
Mean	40.5	40.4	107.9	115.5	0.14	0.15
SD	7.2	11.2	12.9	14.2	0.01	0.02
Minimum	39.9	40.4	93.2	99.1	0.13	0.13
Maximum	57.6	69.9	124.1	132.1	0.14	0.18
Median	43.7	44.0	107.6	113.2	0.14	0.14

GST: glutathione S-transferase; **GPx**: glutathione peroxidase; **Prx**: peroxiredoxin.

Table 3
The effects of sheep/goat whey protein on GST (A), GPx (B) and Prx (C) activities on rat muscle. The results of the descriptive statistics parameters are presented.

Descriptive statistics parameters	Redox biomarkers in muscle					
	GST (U/mg protein)		GPx (U/g protein)		Prx (U/mg protein)	
	Control	Whey	Control	Whey	Control	Whey
Mean	4.5	5.5[a]	32.6	41.5	0.71	0.68
SD	0.8	0.6	6.6	19.2	0.18	0.14
Minimum	3.6	4.9	25.9	22.9	0.49	0.51
Maximum	5.4	6.5	41.8	66.7	0.93	0.83
Median	4.5	5.4	30.8	35.5	0.74	0.68

[a] Statistically significant compared to the control. **GST**: glutathione S-transferase; **GPx**: glutathione peroxidase; **Prx**: peroxiredoxin.

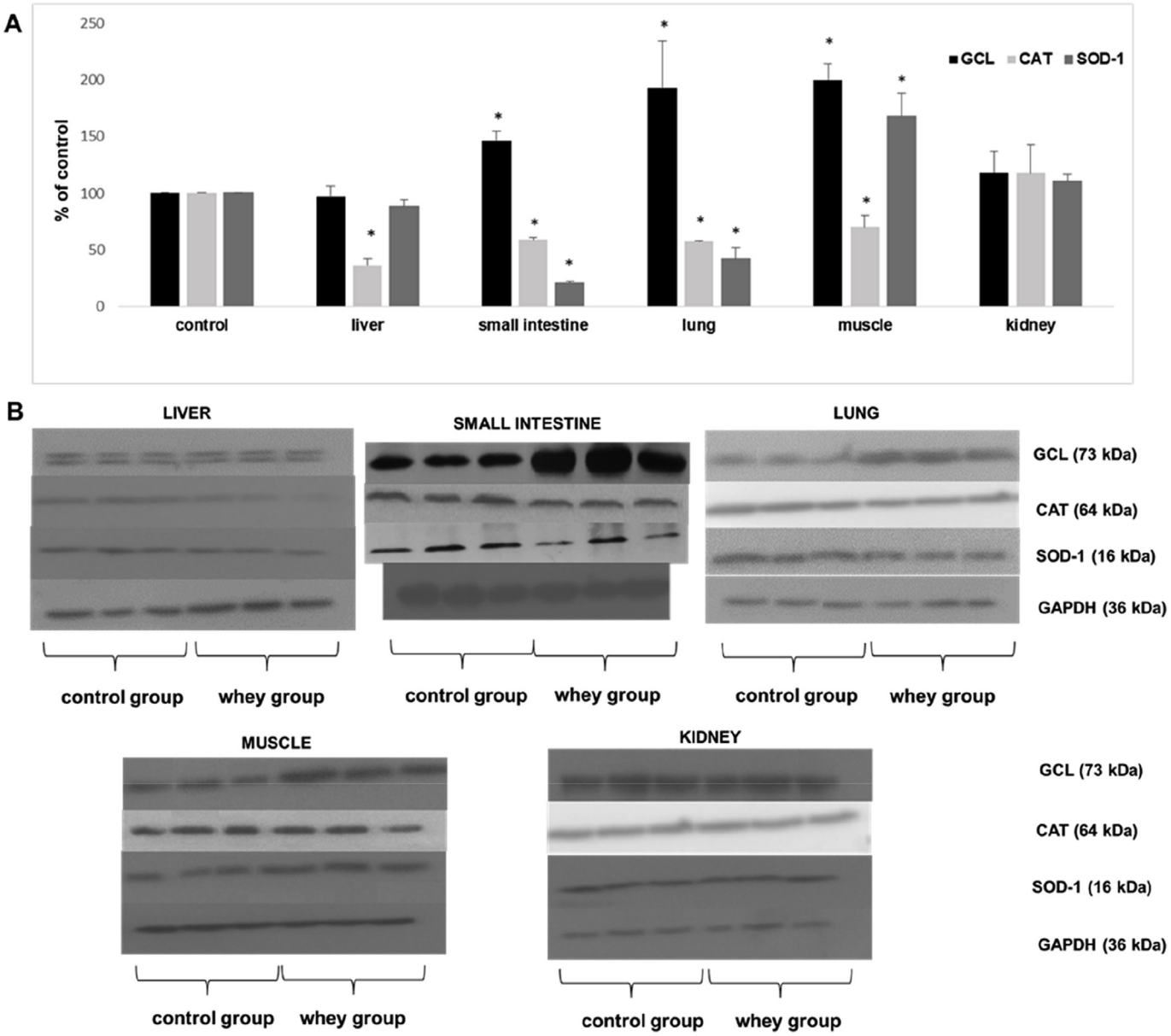

Fig. 1. The effects of sheep/goat whey protein on the expression of GCL, CAT and SOD-1 in the tested rat tissues. (A): The quantification of the results in comparison to the control. (B): The protein bands of the three aforementioned enzymes and GAPDH obtained from three animals of each group using western blot analysis. The first three bands indicate the results in the control animals and the next three in the experimental group. *: Statistically significant compared to the control. The comparisons presented refer to the protein expression of each enzyme between the control and the whey group of each tissue. GCL: glutamate cysteine ligase; CAT: catalase; SOD-1: superoxide dismutase-1; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Indeed, it has been observed that when whey protein is used as a dietary supplement in rats it improves tissue redox status by decreasing protein carbonyl and TBARS concentrations and by enhancing catalase activity and GSH levels (Kerasioti et al., 2018). Of note, whey protein reinforces the antioxidant profile of a wide range of tissues apart from the ones examined in the present investigation. Specifically, it enhances the GSH levels and the total antioxidant capacity in pancreas, whereas it decreases protein oxidation measured through protein carbonyl concentration in brain and spleen, whereas it also acts protectively against lipid peroxidation in brain, pancreas and spleen (Kerasioti et al., 2018). Although the molecular mechanism of the *in vivo* action of sheep/goat whey protein is largely unknown, a relevant study has proposed that it protects against mammalian target of rapamycin (mTOR) overactivation in rats with a mode of action that mimics fasting (Kerasioti et al., 2019). Interestingly, it has been demonstrated that whey protein reduces the concentrations of numerous amino acids in plasma and it partially prevents the p70-S6K1 (Thr389) expression, thus leading to the putative inhibition of the adverse, disease-related effects induced by mTOR overactivation as amino acids are important regulators of mTOR complex 1 (mTORC1). This beneficial action of whey protein could be aptly parallelized with the mode of action of intermittent fasting. Interestingly, during fasting, plasma amino acids, as well as insulin levels are decreased. The disturbance of the balance between food intake and energy needs increases the adenosine monophosphate/adenosine triphosphate (AMP/ATP) ratio, promoting the mTORC1 inhibition and autophagy activation. Due to the above effects, fasting is applied for the attenuation or the symptom alleviation of type 2 diabetes mellitus, cardiovascular diseases, neurodegenerative disorders, aging and abnormal weight loss. Therefore, our finding implies that sheep/goat whey protein could potentially be used for building eating habits with a similar pattern of action with fasting in order to help patients that suffer from disorders that mTORC1 overactivation is implicated in. Moreover, whey protein acts also beneficially in exercise context as it has been found that it improves several physiological parameters, such as body composition in rats after resistance training (Avila et al., 2018). Additionally, it appears that whey protein as part of biofunctional foods leads to a normal glycemic response (Manthou et al., 2014) and it is an antioxidant and anti-inflammatory agent in cyclists (Kerasioti et al., 2012, 2013) implying its positive role as part of food constituents. In the same page, it has been reported that whey protein decreases food intake and body fat in rats (Zhou et al., 2011) and it protects rat brain against aging-induced oxidative stress and neurodegeneration (Garg et al., 2018). Furthermore, whey protein, due to its very important biological properties, has been found to mitigate the oxidative stress related damages induced by toxic agents. Indeed, it ameliorates the potassium dichromate induced hepatic injury (Bashandy et al., 2018), the paracetamol induced oxidative stress (Athira et al., 2013), whereas it induces hepatoprotective effects in liver against CCl_4 (Gad et al., 2011).

Based on the above, whey protein is an essential antioxidant and antimutagenic agent both *in vitro* and *in vivo*. However, studies in the literature stress also its pro-oxidant role when administered in excessively high concentrations, as is the case for the increased protein intake under specific circumstances. In particular, it has been previously pointed out that diet with excess in proteins causes adverse effects on organs that play key role in metabolism and especially liver (Díaz-Rúa et al., 2017). One of the most notable disadvantages for excess protein intake through diet is the enhanced generation of urea and ammonia that cause overload in the hepatic function (Jean et al., 2001; Diez-Fernandez et al., 2017). Except the aforementioned abnormal outcome on liver it has been proposed that protein overload induces increased reactive oxygen species generation and, finally, oxidative stress (Bee et al., 2018). Being on the same page, whey protein impairs the physiological function of rat liver via inducing oxidative stress (i.e., increased lipid peroxidation) when administered in high concentration (Żebrowska-Gamdzyk et al., 2018). Subsequently, it appears that the administered dosage, the composition of whey protein and the tissue studied are decisive factors regarding its beneficial or harmful biological role.

It becomes evident that whey protein is a strong *in vitro* and *in vivo* antioxidant. Furthermore, it can be used as a constituent of biofunctional foods to exert its beneficial role in blood and tissues of experimental animals and humans. In particular, whey protein seems to be a high promising nutritional intervention against the prevention of oxidative stress-related pathologies or the alleviation of specific symptoms on human health. This study is part of a series of experiments indicating that sheep/goat whey protein, a compound with high pollutant burden when discarded in the environment, is a promising food constituent that acts protectively against oxidative stress. The results of the present study and previous relevant studies of our group show that whey protein acts as a biofunctional component improving the antioxidant status of rats and productive animals (Kerasioti et al., 2013, 2018; 2019). Based on these findings, the next step of our research is to investigate the molecular mechanisms for this highly beneficial role of whey protein *in vivo*, thus revealing the reasons why it is considered a very important supplement in animal and human nutrition.

5. Conclusions

The results of the present study indicate that sheep/goat whey protein exerts beneficial antioxidant action in a tissue specific manner by altering the protein expression of a number of antioxidant enzymes. Sheep/goat whey protein can act as a biofunctional component by improving the antioxidant status of rats. Thus, whey protein, a by-product of cheese manufacturing with a high pollutant load is a promising food constituent that acts protectively against oxidative stress.

CRediT authorship contribution statement

Aristidis S. Veskoukis: Writing - original draft. **Efthalia Kerasioti:** Methodology, Investigation, Formal analysis. **Zoi Skaperda:** Investigation. **Porfirios Apostolos Papapostolou:** Investigation. **Charitini Nepka:** Validation, Visualization. **Demetrios A. Spandidos:** Writing - review & editing. **Eftihia Asprodini:** Writing - review & editing. **Ioannis Taitzoglou:** Methodology. **Demetrios Kouretas:** Conceptualization, Methodology, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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