RESEARCH ARTICLE

Peptides from water buffalo cheese whey induced senescence cell death *via* ceramide secretion in human colon adenocarcinoma cell line

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Scope: Milk proteins are a source of bioactive peptides. Recent studies have indicated that protein-derived peptides released in buffalo cheese acid whey exert a cytomodulatory effect in human epithelial colon cancer (CaCo2) cells. The aim of the present study was to explain the molecular mechanism involved in the response of CaCo2 cells to oxidative stress in the presence of peptide fractions of buffalo cheese whey, purified and characterized by mass spectrometry.

Methods and results: We demonstrated that treatment of CaCo2 treated with H_2O_2 (H-CaCo2) cells with a partially purified peptide sub-fraction (f3) from buffalo cheese acid whey induced a reduction of mitochondrial superoxide anion with subsequent decrease in heat shock protein 70 and 90 expression. Moreover, we observed a 5-fold decrease in cyclin A expression and cell cycle arrest in G1/G0 phases. These responses were associated with increased activity of alkaline phosphatase and beta-galactosidase, markers of differentiation and senescence respectively.

Conclusions: The structural characterization of the active peptide fraction and the elucidation of the effects induced by its treatment on H-CaCo2 cells *in vitro* demonstrated an activity of this peptide sub-fraction in the modulation of cell cycle, thus suggesting potential application for the development of nutraceuticals as well as health-promoting functional foods.

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1 Introduction

The intestinal fully differentiated tissues are characterized by an arresting of cells in the quiescent state with an enterocyte turn over within 48–72 h [1]. Because the intestine sits at the interface between the organism and its luminal environment, it represents a critical defense barrier against luminal toxic agents [2]. Thus, in addition to being exposed to luminal nutrients, the intestinal mucosa is constantly injured by reactive oxygen species endogenously and exogenously generated [3]. Imbalance between the production of reactive oxygen species and antioxidant defenses, plays an important role in the initiation of the oxidative stress [4] and the imposition of a severe oxidant stress typically results in cytotoxicity. The oxidative stress can induce phase transition in the regular cell cycle from a quiescent state to a proliferative, apoptotic, or necrotic one. When concentrations of oxidants are very high, the cellular response may activate different pathways and cell death can occur either by necrosis or apoptosis [5]. Additionally,

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Abbreviations: **ALP,** alkaline phosphatase; **BWW,** buffalo milk waste whey; **CaCo2,** human epithelial colon cancer; **f3,** purified peptide subfraction; **H-CaCo2,** human epithelial colon cancer treated with H_2O_2; **HE,** Hydroethydine; **Hsp,** heat shock protein; **MEM,** Minimum Essential Medium; **PI,** propidium iodide

unless the concentrations of oxidants are cytotoxic, necrotic cell death is not necessarily an obligatory endpoint of oxidative stress [6]. Adenocarcinoma cells are remarkably resistant to injury by radiation, as well as immunological, and chemotherapeutic agents [7]. As a consequence, these tumor cells are very difficult to treat and proliferate rapidly, even under conditions that may be adverse for normal cells. This is partially due to the endogenous overexpression of heat shock protein (Hsp) family. Hsps are believed to bind and protect critical cellular proteins, preventing their denaturation by adverse factors or conditions. Although Hsp expression can be induced by a variety of stressful stimuli, certain neoplasms, including human intestinal T84, HT-29, and human epithelial colon cancer (CaCo2) cell lines, express constitutively high levels of Hsps even under non-stress conditions [7]. The anti-apoptotic action of Hsp is due to their stimulation of Akt activity [8]. Moreover, cancer cells are characterized by an increase in their intracellular concentration of biomolecules such as ceramides [9]. The intracellular levels of these sphingolipids are elevated in tumor cells after irradiation or therapy with anticancer drugs, such as doxorubicin [10], implicating endogenous ceramides as important mediators of cancer therapy [11]. It has also been shown that exogenous ceramides induce cell death in a variety of human cancer cell types [12]. Interestingly, cancer cells seem to be more susceptible to the toxic effect of exogenous ceramides than normal cells [13–15], rendering exogenous ceramides promising novel drugs for chemotherapy. The sphingolipid ceramides can be formed by sphingomyelin breakdown or through *de novo* synthesis. They are intimately involved in normal and cancerous cells death, growth, differentiation, and senescence. This latter is a physiological process that leads to irreversible growth arrest, accompanied by characteristic phenotype changes (such as induction of senescence-associated β-galactosidase activity). Besides to ''replicative senescence'' resulting from shortening of telomeres at the ends of the chromosomes [16], an ''accelerated senescence'' pathway not involving telomeres shortening, but other factors as DNA damage have been described [17]. Growth arrest in both replicative and accelerated senescence induces cell cycle arrest. Food constituents which are known to prevent the development of colorectal cancer have been shown to enhance apoptosis following DNA damage and this may reflect an important mechanism of cancer prevention [18]. These constituents include butyrate [19, 20], flavonoids [21], and glucosinate breakdown products from brassicas [22]. Several bioactive peptides derived from milk proteins are potential modulators of various regulatory processes in the body and thus may exert beneficial physiological effects such as antioxidant activity [23]. The antioxidant activity of milk protein hydrolysates has been interestingly described [24–27], and also individual peptide released after hydrolysis from α_s-casein have been shown to possess free radical scavenging activities inhibiting enzymatic and nonenzymatic lipid peroxidation [28]. Moreover, a number of recent studies have shown that milk-derived peptides exert cytomodulatory activity playing a role in the regulation of cell growth, differentiation, and apoptosis [29–31]. A previous study on the CaCo2 cell line suggested that the complex peptide fraction of the buffalo milk waste whey (BWW), resulting from production of buffalo Mozzarella cheese had antioxidant and cytomodulatory properties [32]. In the present investigation, BWW peptide subfractions were characterized by complementary MS techniques. We studied the effects of BWW sub-fractions on: (i) mitochondrial oxidative stress and expression of Hsp 70 and Hsp 90; (ii) cell cycle and differentiation; and (iii) production of ceramides and senescence. These effects were investigated on CaCo2 cell line in which oxidative damage was induced with hydrogen-peroxide (human epithelial colon cancer treated with H_2O_2, H-CaCo2), to simulate the response of intestinal cells which are constantly exposed to luminal oxidants during food digestion.

2 Methods and materials

2.1 Materials

BWW deriving from Mozzarella di Bufala Campana Protected Denomination of Origin cheese was provided by a dairy local farm of the Salerno area, Italy. To prevent undesired peptide hydrolysis, immediately after collection, samples were added with a protease inhibitor cocktail, aliquoted (50 mL), refrigerated, and stored at −20°C until used. All chemicals and reagents were of analytical or higher grade from Sigma (St. Louis, MO, USA). HPLC-grade ACN and methanol (MetOH) were purchased from Carlo Erba (Milano, Italy). DMEM, PBS (composed of 0.1 M PBS containing NaCl 0.138 M, KCl 0.0027 M, pH 7.4), Minimum Essential Medium (MEM), nonessential amino acids, streptomycin, penicillin, L-glutamine, fetal bovine serum, and Nonidet P40 were obtained from Gibco-BRL (Grand Island, NY). Tissue culture plasticware was furnished from Becton Dickinson (Lincoln Park, NJ, USA). Hydrogen peroxide (H_2O_2) 1 M solution was prepared immediately before use. Hydroethydine (HE) was purchased from Invitrogen Srl (Milan, Italy).

2.2 Extraction of peptides from BWW

BWW was collected at the proper stage of the technological process of Buffalo Mozzarella cheese manufacturing. The peptide mixture was extracted from BWW as described previously [32]. The peptide extracts were dried using a Savant concentrator (Speed Vac, Milan, Italy) and stored at −20°C either for structural analysis or further purification by RP-HPLC or biological assays.

2.3 Large-scale fractionation by RP-HPLC of peptide extract from BWW

To assay inhibition properties of simplified peptide sets, BWW peptide extract was fractionated using an HP 1100 modular HPLC apparatus (Agilent, Palo Alto CA, USA). The sample was loaded onto a 218TP54, 5 μm RP column C_{18}, 250 mm × 4.6 mm column (Vydac, Hesperia, CA). Solvent A was water containing 0.1% v/v TFA and solvent B was ACN containing 0.1% v/v TFA. A linear gradient from 5 to 70% solvent B was applied over 60 min with a flow rate of 1 mL/min, after 5 min at isocratic elution (5% solvent B). UV detection was carried out at 220 and 280 nm using a multi-wave length detector. For each run, 100 μL of a 23.5 mg/mL solution of peptide extracts from BWW were injected; eluted peptide fractions were manually collected at 10 min time intervals, obtaining on the whole five different peptide subfractions, labelled in chronological order f1–f5. Due to limited sensitivity of CaCo-2 cell growth inhibition assay, subfractions collected at the same retention times from three HPLC runs were joined together and used for biological assays.

2.4 Extraction of sphingolipids from growth media of preconfluent cells with and without purified peptide subfraction treatment

Spingolipids were extracted form cell culture media of preconfluent cell by adding an equal volume of a mixture of chloroform/methanol 80:20. After vortexing and centrifuging for 5 min at 4000 rpm (Minifuge centrifuge, Heraeus, Osterode, Germany), the upper phase was transferred to a new vial for MALDI-TOF-MS analysis.

2.5 MALDI-TOF MS analysis

MALDI-TOF MS experiments were carried out loading peptide or lipid mixtures (1 μL from a solution 0.02 μg/μL in H_2O/0.1% v/v TFA) on the stainless steel target together with 1 μL of matrix 2,5-dihydroxybenzoic acid (10 mg in 1 mL MetOH/0.1% v/v TFA) for lipid analysis and α-cyano-4-hydroxycinnamic acid (10 mg in 1 mL 50% v/v ACN/0.1% v/v TFA) for peptides. Spectra were acquired on a PerSeptive Biosystems (Framingham, MA, USA) Voyager DE-PRO mass spectrometer, equipped with a N_2 laser (337 nm, 3 ns pulse width) operating either in linear or in reflector positive ion mode, using the Delay Extraction technology. Accelerating voltage was 20 kV and typically 250 laser pulses were acquired for each mass spectrum. In the analysis of sphingolipids, laser power was maintained at the lowest possible values in order to prevent in source fragmentation. The external mass calibration was performed with low mass peptide standards (PerSeptive Biosystem). To check repeatability, spectra were acquired in triplicate at least. Post-Source Decay fragmentation spectra were acquired after isolation of the appropriate peptide precursor ions using timed ion selection. Fragment ions were refocused onto the final detector by stepping the voltage applied to the reflector and the individual segments were finally stitched together using software Data Explorer developed by Applied Biosystems.

2.6 *nano*-ESI MS and *nano*-ESI MS/MS analysis

Before *nano*-ESI MS analysis, each peptide subfractions was purified from residual salts by loading onto $ZipTip_{C18}$ Reversed Phase pre-packed micro-columns (Millipore, Bedford, MA, USA), previously equilibrated with 0.1% TFA, washing with 0.1% TFA, and eluting by aqueous 50% v/v ACN containing 0.1% v/v TFA. Flow direct injection *nano*-ESI MS and *nano*-ESI MS/MS experiments of peptide subfractions, manually collected from BWW RP-HPLC, were carried out using an hybrid quadruple-orthogonal acceleration time of flight Q-star Pulsar (PESciex, Toronto, Ontario, Canada) equipped with a *nanospray* source (Protana, Odense, Denmark), operating in positive ion mode. The samples were diluted, doubling the volume, in a solution 2:1 v/v H_2O+5% acetic acid/AcN and introduced in the source trough borosilicate needles, gold coated (Protana). Needle voltage was 800 V and orifice voltage was set at 40 V to minimize fragmentation; air at the pressure of 10 psi was used as ''curtain gas''. In *nano*-ESI MS/MS experiments, N_2 was used as collision gas; Q0 and Q2 quadrupole voltages were 58.0 and 9.9 V, respectively.

2.7 Cell culture

CaCo2 cells (American Type Culture Collection, Rockville, MD) were grown at 37°C in h-glucose MEM containing: 1% (by vol) MEM nonessential amino acids and supplemented with 10% (by vol) decomplemented fetal bovine serum (Flow, McLean, VA), 100 U/mL penicillin, 100 μg/mL streptomycin, 1% L-glutamine, and 1% sodium pyruvate. After incubation for 4 h, the cells were washed with 1% PBS to remove unattached dead cells and treated for 30 min with 50 μM H_2O_2 (H-CaCo2). The preconfluent H-CaCo2 (21 passage) were incubated with 0.08 mg/mL of BWW and RP-HPLC purified peptide sub-fractions (f1, f2, f3, f4, f5) for 24 h at 37°C. All experiments were performed on triplicate cultures. After 24 h, the cell number was determined with a haemocytometric counter and cell proliferation was determinated through CyQuant cell proliferation assay Kit (Invitrogen) with dye fluorescence measurement at 480 nm excitation maximum and 520 nm emission maximum. Cell proliferation was expressed in percentage of proliferation compared with the control. All data are the mean ± SD of three experiments.

2.8 Flow cytometry analysis

H-CaCo2 cells were seeded in 12 multi-well plates at the density of 25×10^5 cells/plate. After 24 h of incubation with 0.08 mg/mL of f3 sub-fraction, the cells were washed in PBS, pelleted in centrifuged, and directly stained in a propidium iodide (PI) solution (50 mg PI in 0.1% sodium citrate, 0.1% NP40, pH 7.4) for 30 min at 4°C in the dark for cell cycle analysis. The experiments were performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The PI fluorescence was collected as FL2 (linear scale) by the ModFIT software (Becton Dickinson). For the evaluation of intracellular DNA content, at least 20 000 events for each point were analyzed in at least three different experiments giving a SD lower than 5%. H-CaCo2 cells were seeded in 12 multi-well plates at the density of 25×10^5 cells/plate and then incubated with 0.08 mg/mL of f3 subfraction for mitochondrial superoxide anion analysis. The superoxide anion levels were measured by HE staining. The cells were incubated for 1 h with 20 μg/mL HE stock solution (2.5 mg/mL). After this, they were scraped, washed twice with PBS, and the pellet was added to 1 mL PBS. Dye accumulation was analyzed by FACScan flow cytometer (FACScan, Becton Dickinson) by the CellQuest software. For each sample, 2×10^4 events were acquired. Analysis was carried out by triplicate determination in at least three separate experiments.

2.9 Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was used as a marker of colonic differentiation degree. The H-CaCo2 cells were washed and lysed with 0.25% sodium deoxycholate essentially as described by Herz *et al.* [33]. ALP activity was determined using the Sigma Diagnostics ALP reagent (No. 245). Total cellular protein content of samples was determined by the Bradford microassay using the Coomassie Protein Assay Reagent Kit (Pierce). ALP activity was calculated as units of activity for milligram of protein.

2.10 Western blot assay

The effect of f3 subfraction on expression of Hsp 70, Hsp 90, Akt (also named protein kinase B) and Cyclin A was determined by Western blot. H-CaCo2 treated and untreated (control) were lysed using an ice cold lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton) supplemented with a mixture of protease inhibitors containing antipain, bestatin, chymostatin, leupeptin, pepstatin, phosphoramidon, pefabloc, EDTA, and aprotinin (Boehringer, Mannheim, Germany). Equivalent protein samples were resolved on 8–12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Germany). For immunodetection, membranes were incubated overnight with specific antibodies at the concentrations recommended by the Manufacturer. All antibodies were diluted in Tris-buffered saline/Tween 20–1% milk powder. This step was followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (anti-mouse-IgG 1:2000, anti-rabbit-IgG 1:6000; Biosource, Germany). Protein bands were detected by enhanced chemiluminescence (ECL-kit, Amersham, Germany). Total Akt expression was monitored by Western blot analysis with an antibody recognizing Akt independently from its phosphorylation state. For the measure of the activity, Akt was immunoprecipitated with a specific anti-Akt antibody and its activity was measured in an *in vitro* kinase assay using GSK-3a as substrate. Immunoprecipitated Akt kinase was used to phosphorylate glycogen synthase kinase-3a (GSK-3a). GSK-3a phosphorylation was measured by Western blot analysis with a specific anti-phospho-GSK-3a/b antibody. Reactions were carried out according to the instructions of the Akt kinase assay kit from New England Biolabs.

2.11 β-Galactosidase assay

Senescence was assessed by β-galactosidase staining. Cells were washed with PBS and fixed with 2% formaldehyde and 0.2% glutaraldehyde at room temperature for 5 min. After two washes with PBS, the cells were incubated at 37°C for 4 h in a humidified chamber with freshly prepared senescence-associated β-galactosidase activity staining solution (1 mg/mL X-Gal in DMF, 40 mM citric acid, and phosphate buffer, pH 6.0, 5 mM potassium ferrocyanide, 150 mM sodium chloride, and 2 mM magnesium chloride). After 1 day, cells were washed twice with PBS at room temperature for 10 min, and staining was visualized and captured using an optical microscope (Carl Zeiss, USA).

3 Results

3.1 MS characterization and proliferation effect of BWW subfractions on H-CaCo2 cell lines

As evidenced by RP-HPLC analysis (Fig. 1A), BWW produced during ''Mozzarella di Bufala Campana Protected Denomination of Origin'' manufacturing is a very complex mixture of peptides which derived from hydrolysis of caseins by endogenous proteases (*e.g.* plasmin), proteolytic enzymes of rennet and endo-/exopeptidases from lactic acid bacteria. Peptide subfractions were obtained by RP-HPLC purification of BWW by collecting eluates at 10 min time intervals. Five peptide subfractions (f1, f2, f3, f4, and f5) were tested for 24 h in H-CaCo2 cell line to assay proliferation effects (Fig. 1B). BWW reduced H-CaCo2 proliferation of about 30% compared with the control sample represented by H-CaCo2 cells untreated with peptide extract. f1, f2, f4, and f5 had no statistically significant effect on

cellular proliferation compared with control cells, whereas only f3 showed a marked antiproliferative effect (67% growth reduction), with respect to the unfractionated BWW (p value >0.0001). To further investigate its composition and biological effects, f3 was characterized by MALDI-TOF MS. This analysis allowed us to identify the single peptide components and to hypothesize the presence of active sequences and of their precursors (Supporting Information Table S1). The peptides mainly derived from κ-cn, *g*-CMP (*glyco*-caseinmacropeptide), and β-cn, whereas only three peptides were from αs_1- and αs_2-cn. Peptides identity was confirmed by Post-Source Decay fragmentation mass spectra and *nano*-ESI/MS/MS. A limited number of peptides remained unidentified.

3.2 The antioxidant effect of f3 reduced Hsp 70 and 90 expression and Akt activity

The antioxidant effect of f3 on H-CaCo2 cell line was investigated through analysis of the intracellular redox status of H-CaCo2 cells treated with f3 by evaluating mitochondrial superoxide anion production (Supporting Information Fig. S2). HE was used to measure mitochondrial superoxide anion production of vital cells. The adduct mean fluorescence intensity decreased from 6.2% in the control to 2.5% in the cells treated with f3. This indicated a corresponding decrease in the superoxide anion production.

This finding led us to investigate if the decrease of superoxide anion production might influence the expression level of Hsp 70 and 90, which are significantly elevated in many cancers, and in the case of some tumor types this is linked with poor prognosis and a muted response to chemotherapy. In the specific case of CaCo2 adenocarcinoma cell line, it has been previously reported that high Hsp levels are expressed constitutively even under nonstress conditions [34]. This expression has been associated with enhanced survival to conditions such as oxidant- and thermal-induced stress. In Fig. 2, the expression levels of Hsp 70 and Hsp 90 are shown. Figure 2A shows the Western blot analysis of Hsp 70 and Hsp 90 in H-CaCo2 cells treated with 0.08 mg/mL of f3 for 24 h. Treatment with f3 decreased

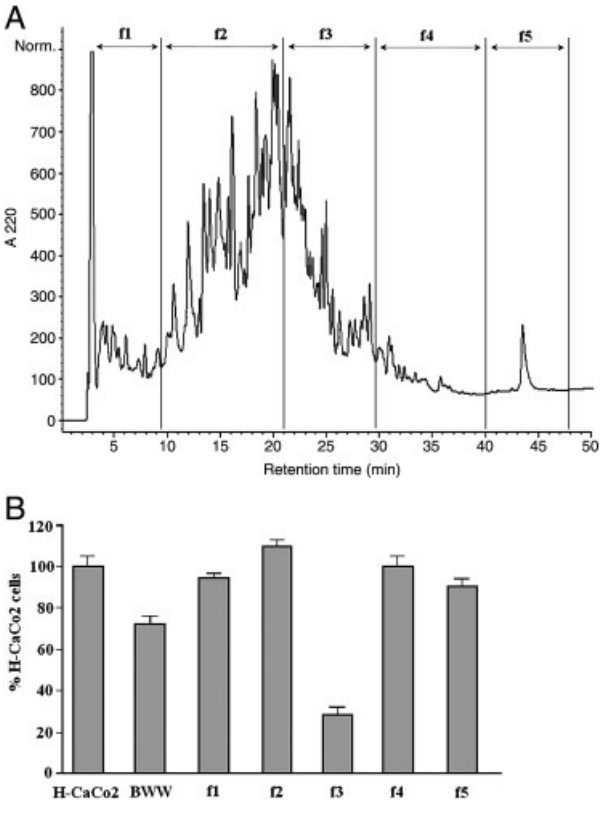

Figure 1. (A) RP-HPLC fractionation of the 3 kDa-permeate obtained from BWW. Each peptide fraction was collected at 10 min time intervals and termed with f followed by a number. (B) H-CaCo2 cell proliferation assay. The H-CaCo2 were treated for 24 h with different RP-HPLC sub-fractions (f1–f5) of BWW. Cell proliferation was expressed as percent compared with the control. Each experimental value is the mean of three different determinations.

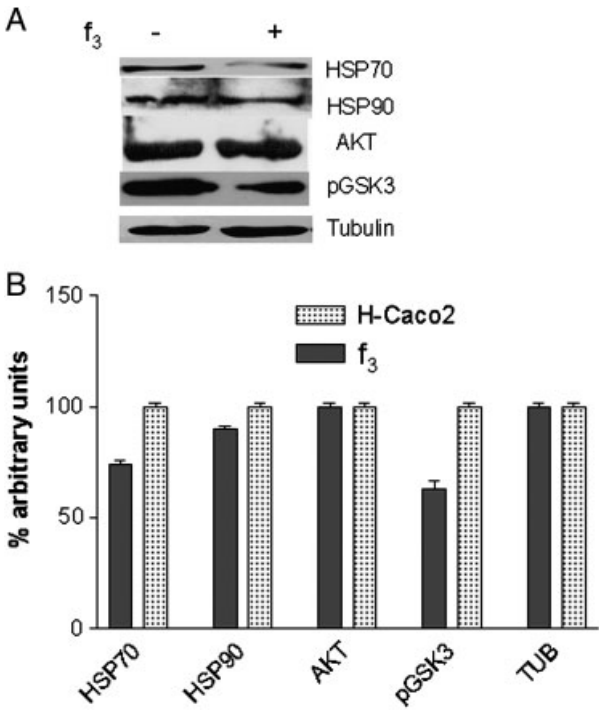

Figure 2. Western blot analysis of H-CaCo2 without (−) and with (+) f_3 treatment. (A) The bands associated with expression of Hsp 70, Hsp 90, house-keeping γ-tubulin, Akt, and phospho-GSK-3a/b(Ser21/9) after 24 h of treatment with f_3 are visualized. The expression of the house-keeping protein γ-tubulin was used as loading control. (B) The expression levels of the above proteins are reported as percentage respect to the level of the protein in untreated cells, used as control. Bars show the mean values ± SD. Each experimental value reported is the mean of three different determinations.

Hsp 70 and Hsp 90 expression of 36 and 10%, respectively (Fig. 2B).

Hsps have a profound impact on the activities of several prosurvival signaling cascades, including those mediated by Akt, a downstream effector of the PI3-K pathway. For this reason, we compared the expression and activity of Akt in H-CaCo2 treated with f3 and control cells (Fig. 2A). The treatment with f3 induced no effect on Akt expression, whereas the level of p-glycogen synthase kinase was decreased of 37% with respect to the control cells (Fig. 2B).

3.3 Effect of f3 on lipid synthesis and secretion

We examined whether the Hsp decrease by f3 treatment in preconfluent H-CaCo2 was correlated with production of ceramides. In Fig. 3, the MALDI-TOF mass spectra of the chloroform extracts from the cell growth medium without (Fig. 3A) and with (Fig. 3B) f3 incubation are reported. A series of sphingolipid components in the mass range 700–1200 Da were observed in the cells treated with f3, whereas a single component at m/z 1031 Da was found in the control sample. Identified sphingolipid classes in the sample treated with f3 include ceramides EOH, EOS, AH, and cerebrosides EOS, all molecules involved in the process of cell differentiation [35]. To denominate families of sphingolipids, we followed the nomenclature of Motta *et al.* [36] who designated the three types of fatty acids in sphingolipids as nonhydroxy acids, α-hydroxy acids, and ω-hydroxy acids esters linked to linoleate as N, A, and EO, respectively. The three types of sphingoid bases, sphingosine, phytosphingosine, and 6-hydroxysphingosine found in ceramides and cerebrosides were indicated as S, P, and H. For example, a ceramide consisting of a ω-hydroxyacid ester-linked to a molecule of phytosphingosine would be designated as CER EOP, according to Motta *et al.* [36].

3.4 f3 induced quiescence, differentiation and senescence in H-CaCo2 cells

In order to elucidate if the ceramide productions were involved in the regulation of the cell cycle, we analyzed by FACS the percentage of G1/G0, G2/M, and S-phase cells. In Fig. 4A, the percentage of G1, G2, and S-phase untreated and treated cells with f3 after 24 h is reported. The treatment resulted in accumulation of cells in the G1/G0 phases (66%), while concomitantly the S-phase populations decreased (25%). These results suggested that f3 arrest the cell cycle in G1 phase as further confirmed by a fivefold decrease of cyclin A expression [37] as shown in Fig. 4A. These results are associated with increased expression of ALP activity (Fig. 4B), a marker of enterocytic differentiation correlated to post-confluent phase. Moreover, cell cycle arrest is accompanied by an increased senescence-associated (β)-galactosidase (1.5-fold), compared with control (Fig. 4C) [38].

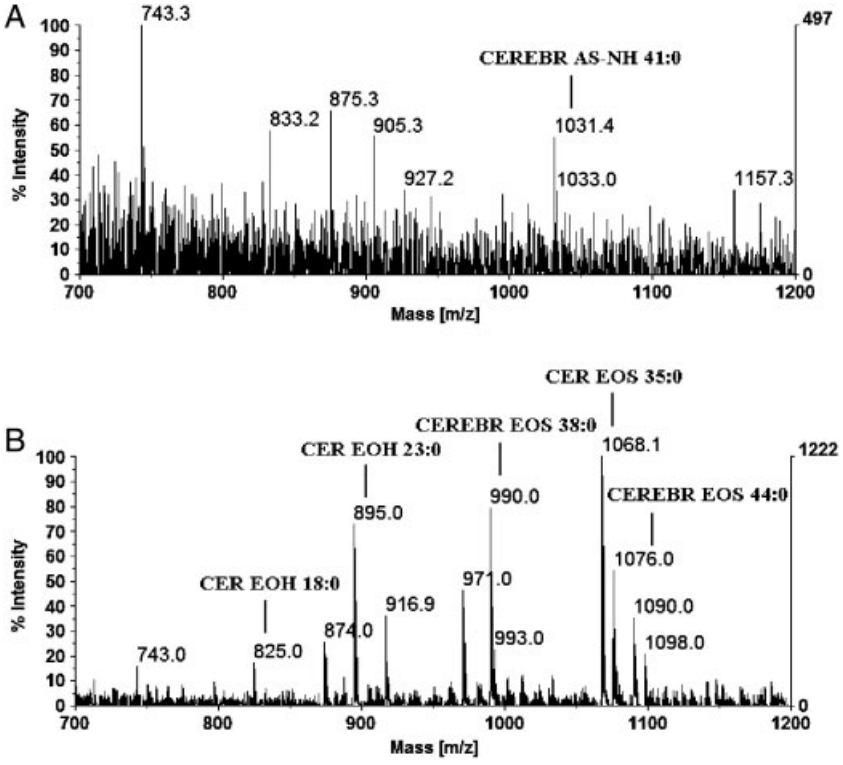

Figure 3. MALDI-TOF mass spectra of chloroform extracts from the growth serum of preconfluent cells without (A) and with (B) f3 incubation. A series of sphingolipid components in the mass range 700–1200 Da were observed in the cells treated with f3. CER, ceramides; CERBR, cerebrosides.

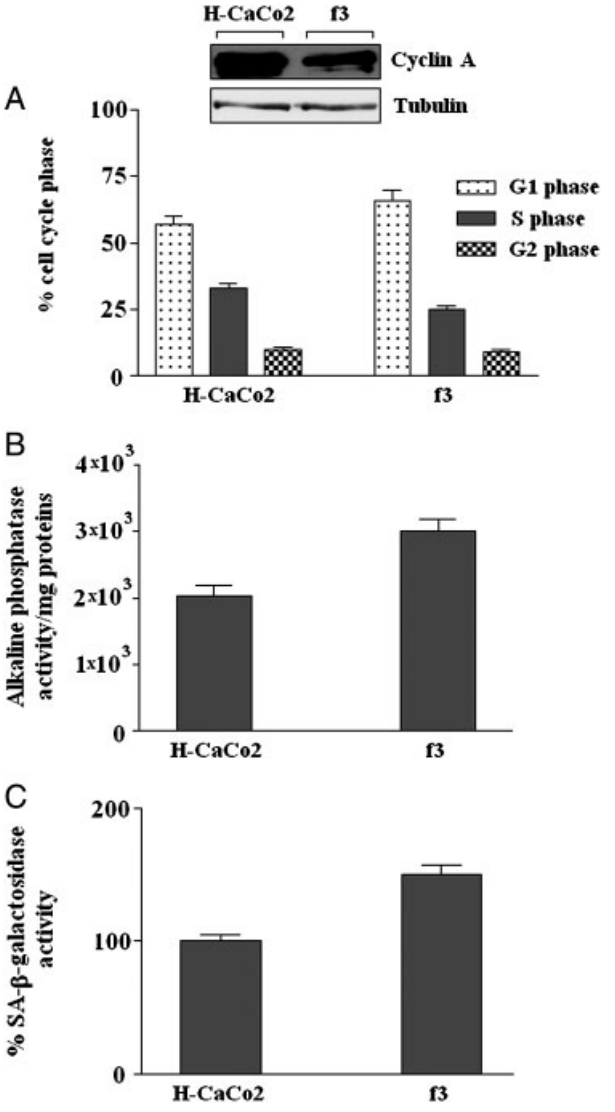

Figure 4. (A) G1, G2, and S-phase cell cycle and cyclin A expression and (B) ALP of H-CaCo2 treated with f3. To evaluate cell cycle, PI fluorescence was collected as FL2 (linear scale). In the inset Western blot analysis with Anti-cyclin A antibody is reported. (B) ALP/mg proteins are shown. ALP activity was measured in homogenates of H-CaCo2 cells (without and with f3 treatment) and expressed as activity *per* cell proteins. (C) Senescence-associated (β)-galactosidase activity. Bars show mean values ± SD; each experimental value reported is the mean of three different determinations.

4 Discussion

Adenocarcinoma cells are remarkably resistant to injury by radiation and systemic, immunological, and chemotherapeutic agents. CaCo2 cells derived from human intestinal epithelial adenocarcinoma extensively used in several studies of intestinal functions, nutrient absorption, and vectorial transport [39], were used in this study because of their ability to reproduce several of the normal physiological responses to various modulatory agents, in a model which mimics the exposition to oxidants produced from ingested foods. In a previous study [32], we showed the antioxidant and antiproliferative effects of the peptide extract from Mozzarella cheese waste whey. Here, we identified a purified peptide fraction from cheese whey (f3) which maintained the antioxidant and antiproliferative action, and investigated its functional effect against H-CaCo2 cells. After 24 h of f3 treatment, we observed a decrease of mitochondrial superoxide anion level and a subsequent reduction of Hsp 70 and 90 expression in survived H-CaCo2 cells. It is well known that CaCo2 cell lines constitutively express high levels of Hsp 70 and 90 even under nonstress conditions, which is at the basis of their high resistance to chemical agents. The lowered Hsp expression induced by f3 treatment made these cells significantly more sensitive to the injurious effects of the oxidant agents. This finding might also envisage future applications of this active peptide fraction for the development of nutraceuticals in diets aimed to increase the efficacy of drugs for treatment of gastrointestinal cancer.

Akt is a downstream effector of PI3-K, a ubiquitous lipid kinase involved in receptor signal transduction by tyrosine kinase receptors [8]. There is increasing evidence that the activation of PI3-K/Akt is associated with colorectal carcinoma, and can convert differentiated human gastric or colon mucosa to a less differentiated and more malignant phenotype [40–42]. Akt is overexpressed in several cancers, including those of the colon, pancreas, ovary, and breast [43]. Moreover, Akt phosphorylation in human colon carcinomas is correlated with cell proliferation and inhibition of apoptosis, as well as different clinico-pathological parameters such as invasive grade, vessel infiltration, lymph node metastasis, and tumor stage [44, 45]. Thus, the inhibition effects of f3 on H-CaCo2 cell proliferation may be a consequence of the decrease of Akt activity.

As a further point, several inductors of cell death, including TNFα [35], anthracyclines [46], or irradiation [47] involve ceramide signalling. Administration of exogenous ceramide also causes cell death in various cancer cell lines [48]. It is noteworthy that many cancer cells have a specific ''sphingolipid-phenotype,'' including lower endogenous ceramide levels [49] and a higher sensitivity to the effects of exogenous ceramides [15]. It is well accepted that ceramides function as a second messenger in cells. The increase in their intracellular concentration in response to extracellular signals induces cell arrest and inhibition of growth in cancer cells [50–52]. We found that f3 treatment induces increased secretion of ceramides which resulted in cell cycle arrest, differentiation, and in subsequent ''accelerated senescence'' cell death [53] in H-CaCo2.

The presence of bioactive peptides in milk and milk-derived products is well documented [54–56]. The range of bioactivities so far reported includes opioid, antihypertehensive, immunomodulating, antimicrobial, metal transport, and several others. Only a few studies describe

positive and/or negative effects of milk peptides on cell growth [57–59]. The structural analyses carried out on f3 sub-fraction showed the presence of peptides with already known potential bioactivity, for instance β-cn f(60–68) and β-cn f(57–68) (Supporting Information Table S1) which are precursors of the agonist opioid β-casomorphin 7 and β-casomorphin 5 with opioid-like activity. These peptides have been already identified in peptide extract from BWW in a previous study [32]. Because of their ability to decrease cell proliferation interacting with both opioid [60, 61] and somatostatin receptors present in the intestinal tract of mammals [62], including CaCo2, we hypothesized that the proliferation decrease observed in colon cells could be mediated by opioid peptides. This hypothesis is now further confirmed by the antiproliferative effect observed upon f3 incubation. An intriguing finding was that peptides characterized in f3 are spontaneously produced during the cheese-making process of buffalo Mozzarella cheese, and released by action of the lactic acid bacteria into the waste whey, a natural source with low or null cost, available in large amounts, from which peptides could be easily purified once the appropriate industrial methods has been set up.

It is known that the intestine undergoes a continuous remodelling process in which ceramides play an important role as messenger molecules. Thus, as f3 increased ceramides secretion, with subsequent cell differentiation and accelerated senescence, it is possible to envisage an application in the development of nutriaceuticals for prevention of gut disfunctions, for recovery in cases of acute injuries such as damage occurring in celiac disease or other intestinal pathologies.

The authors have declared no conflict of interest.

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