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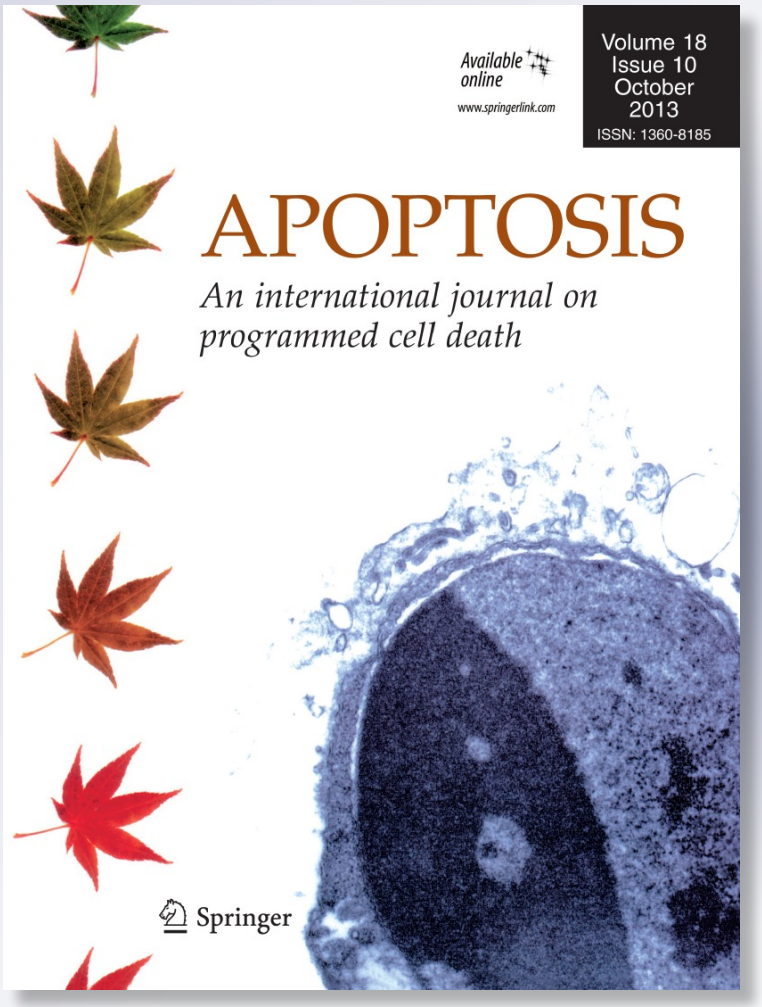

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ORIGINAL PAPER

Ghrelin induces apoptosis in colon adenocarcinoma cells via proteasome inhibition and autophagy induction

Laura Bonfili · Massimiliano Cuccioloni · Valentina Cecarini · Matteo Mozzicafreddo · Francesco Alessandro Palermo · Paolo Cocci · Mauro Angeletti · Anna Maria Eleuteri

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Abstract Ghrelin is a metabolism-regulating hormone recently investigated for its role in cancer survival and progression. Controversially, ghrelin may act as either anti-apoptotic or pro-apoptotic factor in different cancer cells, suggesting that the effects are cell type dependent. Limited data are currently available on the effects exerted by ghrelin on intracellular proteolytic pathways in cancer. Both the lysosomal and the proteasomal systems are fundamental in cellular proliferation and apoptosis regulation. With the aim of exploring if the proteasome and autophagy may be possible targets of ghrelin in cancer, we exposed human colorectal adenocarcinoma cells to ghrelin. Preliminary in vitro fluorimetric assays evidenced for the first time a direct inhibition of 20S proteasomes by ghrelin, particularly evident for the trypsin-like activity. Moreover, 1 μM ghrelin induced apoptosis in colorectal adenocarcinoma cells by inhibiting the ubiquitin–proteasome system and by activating autophagy, with p53 having an "interactive" role.

Keywords Ghrelin · Apoptosis · Proteasome · Autophagy · Colon adenocarcinoma cells

Introduction

Ghrelin is a 28-amino acid orexigenic peptide primarily produced by the gastrointestinal tract. It is the endogenous ligand for growth hormone secretagogue receptor (GHS-R) [1]. Aside from being a strong stimulator of growth hormone release, it also acts as an appetite-stimulating hormone [2]. Two major forms of ghrelin are found in tissues and plasma: *n*-octanoyl-modified and desacyl ghrelin, the latter being the major circulating form [3]. Since serine-3 ester bond of ghrelin is chemically unstable, the elimination of the octanoyl of ghrelin can occur during storage, handling, and/or dissolution in culture medium. Two forms of the ghrelin membrane receptor exist: GHS-R1a and GHS-R1b [4]. Desacyl ghrelin, devoid of any endocrine activity through the GHS-R, stimulates cell proliferation in prostate carcinoma cell lines and adipogenesis, induces cardiovascular effects, and inhibits apoptosis in cardiomyocytes and in endothelial cells [5]. These effects were proposed to be mediated by an unidentified ghrelin receptor, distinct from the GHS-R [6–8].

Ghrelin is expressed in a wide range of cancer tissues and plays a role in cell proliferation, cell migration and apoptosis [9, 10]. As a number of conflicting reports exists, it is currently unclear whether ghrelin promotes cancer or inhibits its development. It is possible that ghrelin could have both stimulatory and inhibitory effects. In most cell lines, ghrelin stimulates cell proliferation and is anti-apoptotic, thus promoting cancer progression [11]. As ghrelin is synthesized locally in many tissues, it could act as an autocrine/paracrine growth factor in normal and cancer tissues [12]. In normal cell lines ghrelin stimulates cell proliferation [13–18]. However, the effect of ghrelin in cancer cell lines is controversial. Ghrelin increases cell proliferation in a range of cancers [19–21]. For example, it stimulates proliferation in colorectal [22], gastric [23], breast [24] and endometrial [25] cancer cell lines. Other authors observed that ghrelin could promote ovarian cell proliferation [26] whereas ghrelin alone did not affect

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apoptosis in primary cultures of neurons [27]. Nevertheless, anti-proliferative effects of ghrelin or its synthetic analogs (GHSs) have been also described in other cell types, such as thyroid, breast, lung and prostatic cancer cells [7, 28, 29], indicating that ghrelin effect on viability is cell type specific and concentration dependent. It has been demonstrated in vivo that ghrelin and its receptor agonist GHRP-2 are able to decrease the arthritis-induced skeletal muscle proteolysis by affecting the E3 ubiquitin-ligating enzymes *MuRF1* and *MAFbx* gene expression. In fact, cachexia and muscular wasting is secondary to protein degradation by the ubiquitin–proteasome pathway [30, 31].

However, limited data are currently available on the effect exerted by ghrelin on intracellular proteolytic pathways in cancer. Both the lysosomal and the proteasomal systems besides playing a central role in intracellular protein degradation are also fundamental in cellular proliferation and apoptosis regulation. The proteasomal system is localized both in the cytoplasm and the nucleus of all eukaryotic cells where it principally carries out most of the intracellular proteolytic processes, being implicated in the degradation of short-lived and oxidized proteins, in the regulation of signal transduction, in the immune response, in cell division, growth and differentiation, in DNA repair, in the biogenesis of organelles and in the apoptosis [32, 33]. Eukaryotic 20S proteasomes are composed by four heptameric rings ($\alpha_{1-7}\beta_{1-7}$ β_{1-7} α_{1-7}) stacked to form a cylinder shaped structure. Seven distinct α subunits constitute the outer rings and regulate the access of extended polypeptides, whereas β subunits, assembled in the inner rings, are associated with the proteolytic activities: chymotrypsin-like (ChT-L, which cleaves after hydrophobic residues), trypsin-like (T-L, which cleaves after basic residues) and peptidylglutamyl peptide-hydrolyzing (PGPH, which cleaves after acidic residues), associated with β5, β2 and β1 subunits respectively. Two other catalytic activities have been described in eukaryotes: the branched chain amino acid preferring activity (BrAAP, associated to β5 and β1 subunits) and the small neutral amino acid preferring activity (SNAAP) [34]. In the presence of interferon-γ, the 20S proteasome is converted in an immune complex involved in the generation of antigenic peptides, namely the immunoproteasome, in which the constitutive subunits β5, β1, and β2 are replaced by the inducible subunits β5i, β1i, and β2i [35]. The proteasome is a highly attractive target for cancer therapy, and the identification of synthetic and natural modulators of this proteolytic pathway represents a primary goal of medicinal chemistry [36, 37].

Mediated via the lysosomal degradation pathway, autophagy is responsible for degrading cellular long-lived proteins [38] and it is currently the only known process for degrading cellular organelles, recycling them to ensure cell survival [39–41]. Recently, autophagy emerged as a multifunctional pathway activated in response to microenvironmental stress or intracellular damage caused by hypoxia, chemotherapeutic agents, viruses, and toxins. Autophagy may also have a role in cell death, as cancer cells often develop mutations that confer resistance to apoptosis [42, 43]. The autophagy response has been described in various patho-physiological situations, including neurobiology, cancer and more recently cardiovascular disease. A number of tumour-suppressor proteins control autophagy (e.g. Beclin-1 and PTEN), so it would seem reasonable to assume that a decrease in autophagy would lead to tumour progression [44–46]. It must be considered that whilst during tumour establishment autophagy may be a mechanism through which targeting tumour cells, once the tumour is established autophagy may provide a way for cancer cells to overcome nutrient-limiting conditions especially within the internal mass of the tumour which is poorly vascularised [44, 47].

With the aim of exploring if the proteasome and autophagy may be possible target for ghrelin in cancer, human colorectal carcinoma cells (HCT116 cells) were used as experimental model and exposed to ghrelin in order to evaluate its effects on the functionality of cellular proteolysis.

Materials and methods

Materials

Fluorogenic substrates for assaying the ChT-L, T-L, PGPH activities (Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Ser-Thr-Arg-AMC, Z-Leu-Leu-Glu-AMC, respectively), caspase-3 substrate (Ac-Asp-Glu-Val-Asp-AMC), Hoechst reagent, ethidium bromide and 6.5–205 kDa molecular weight markers were all purchased from Sigma–Aldrich (Italy). Human ghrelin was purchased from Ana Spec, Fremont (CA, USA). The inhibitor Z-Gly-Pro-Phe-Leu-CHO and the substrate Z-Gly-Pro-Ala-Phe-Gly-pAB to test the BrAAP activity were kindly donated by Prof. Cardozo (Department of Medicine, Mount Sinai School of Medicine, New York). All the reagents and media for cell cultures were bought from Euroclone (Italy). Polyvinylidene fluoride (PVDF) membranes were obtained from Millipore (Italy). All the antibodies used in western blots were purchased from Santa Cruz Biotechnology (CA, USA). The western blot stripping buffer was obtained from Pierce (USA). All chemicals and solvents were of the highest analytical grade available. Trizol Reagent and Superscript TM III reverse transcriptase were purchased from Invitrogen (Italy). All chemicals and solvents were of the highest analytical grade available. The constitutive proteasome and the immunoproteasome were isolated from bovine brain

and thymus, respectively [48]. Human colorectal carcinoma (HCT116) cell line was acquired from ATCC (Manassas, VA, USA).

20S proteasome in vitro assays

The effects of ghrelin on constitutive and immuno- 20S proteasomes functionality were evaluated according to fluorimetric activity assays. The tests for the BrAAP activity were performed in the presence of aminopeptidase N. Specifically, increasing concentrations of ghrelin in the range 1–10 μM were added to the incubation mixture, consisting of 1 μg of isolated 20S proteasome or immunoproteasome, the appropriate substrate and 50 mM Tris–HCl pH 8.0 to a final volume of 100 μL. Incubation was carried out at 37 °C and, after 60 min, the measurements of the hydrolyzed 7-amino-4-methyl-coumarin (AMC) and 4-aminobenzoic acid (PABA) were detected (AMC: $\lambda_{exc} = 365$ nm, $\lambda_{em} = 449$ nm; PABA $\lambda_{exc} = 304$ nm, $\lambda_{em} = 664$ nm) on a spectrofluorimetric microplate reader Gemini XPS. Control assays were also performed in order to evaluate a possible effect of ghrelin on the proteolytic activity of aminopeptidase N. To dissect the contribution of the deacylation products of ghrelin to the modulation of proteasome activities, control assays with the octanoic acid were conducted.

Determination of casein degradation by the 20S proteasomes

β-casein was used as macromolecular substrate in order to test the proteolytic activity of isolated 20S proteasomal complexes in the presence of ghrelin. β-casein was dissolved in 50 mM Tris–HCl, 20 mM $MgCl_2$, pH 7.5 to a final concentration of 1 mg/mL. 20S proteasome was pre-incubated for 30 min at room temperature with 10 μM of ghrelin and then 1 mg/mL of β-casein in 50 mM Tris–HCl, 20 mM $MgCl_2$, pH 7.5 up to a final volume of 90 μL was added. The mixture was kept at 37 °C, and 20 μL aliquots were withdrawn at different times (2, 15, 30, 60 and 90 min), acidified with 2 μL of TCA 10 % and analyzed on an Akta HPLC system (GE Healthcare Biosciences, Sweden) using a Hamilton PRP-3 column (4.1 × 150 mm). The elution of samples and the analysis of casein degradation products were carried out as previously reported [49, 50]. The rate of casein degradation was determined by monitoring the decrease in the peak area of casein ($\lambda = 210$ nm). Control experiments were performed in the absence of ghrelin.

mRNA expression of ghrelin and its receptors

HCT116 cells were grown in RPMI medium supplemented with 10 % fetal bovine serum (FBS), antibiotic, antimycotic and L-glutamine at 37 °C. HCT116 cells were treated with 1 μM ghrelin for 6, 12, 18, and 24 h, to evaluate the effects of exogenous ghrelin on mRNA levels of ghrelin and its receptors (namely, GHS-R1a and GHS-R1b). Total RNA was extracted from HCT116 cells using Trizol Reagent according to the manufacturer's instructions. RNA concentration and purity were monitored by 260 nm/280 nm absorbance ratios, and the integrity was confirmed by electrophoresis through 1 % agarose gels stained with ethidium bromide. Complementary DNA (cDNA) was synthesized from total RNA (4 μg of in 20 μL of total volume reaction) using random hexamers (50 ng/μL) and Superscript TM III reverse transcriptase (200 U) according to manufacturer's instructions. Amplification of cDNA was performed using the gene specific primers previously used by Duxbury et al. [51]. Cycling parameters for ghrelin cDNA amplification were as follows: 95 °C for 3 min, followed by 37 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s with a final extension step of 72 °C for 10 min. Glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was used as an internal control [10]. cDNA of human ghrelin receptors subtypes GHS-R1a and GHS-R1b were amplified as follows: 95 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min with a final extension step of 72 °C for 10 min according to Duxbury et al. [51] with slight modifications. PCR conditions were obtained in a volume of 25 μL containing the following: 2.5 μL 10× DreamTaq buffer, 1 μL dNTP mix (5 mM), 1 μL of each primers (10 μM), 1 μL cDNA and 0.625 U DreamTaq DNA Polymerase. Amplification products gel images were subsequently captured using a digital camera EDAS 290 (Kodak).

HCT116 cells treatment

HCT116 cells were grown in RPMI medium supplemented with 10 % FBS, antibiotic, antimycotic and L-glutamine at 37 °C. Cells were treated with 1 μM ghrelin for 6 and 24 h. Control cells were treated with DMSO. Upon treatment, cells were harvested in PBS, centrifuged and the pellet was dissolved in lysis buffer (20 mM Tris, pH 7.4, 250 mM sucrose, 1 mM EDTA and 5 mM β-mercaptoethanol). Lysates were centrifuged at 12,000×*g* for 15 min and the supernatants were stocked at −80 °C. Cell lysates protein concentration was determined through the method of Bradford [52] using bovine serum albumin as a standard.

Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) [53]. After experimental treatment, MTT was added to the

culture medium to a final concentration of 0.5 mg/mL and incubated for 2 h at 37 °C. The medium was replaced with 100 μL DMSO and the optical density was measured at 550 nm in a microtiter plate reader. At least six cultures were utilized for each time point.

Proteasomal activities on cell lysates

Proteasomal activities on HCT116 cell lysates (1 μg of total proteins in the mixture) were performed using the above listed substrates; control experiments were done in the presence of specific inhibitors: Z-Gly-Pro-Phe-Leu-CHO and lactacystin (5 μM in the reaction mixture). Incubation was carried out at 37 °C for 60 min, then the hydrolyses of AMC and PABA were monitored (AMC: $\lambda_{exc} = 365$ nm, $\lambda_{em} = 449$ nm; PABA: $\lambda_{exc} = 304$ nm, $\lambda_{em} = 664$ nm) on a spectrofluorimetric microplate reader Gemini XPS. Also the 26S ChT-L activity was assayed including 10 mM $MgCl_2$, 1 mM DTT and 2 mM ATP in the reaction mixture. Incubation was done at 37 °C, and the fluorescence of hydrolyzed AMC was measured after 60 min.

Western blotting analyses

Western blotting assays were performed to analyze intracellular levels of ubiquitinated proteins, poly(ADP-ribose) polymerase (PARP), caspase-9, the proteasomal substrates p27, IkBα, and p53, and the autophagy related proteins p62 and LC3, following ghrelin treatment. Cell lysates (15 μg) were loaded on 12 % SDS-PAGE (10 % for PARP, 15 % for LC3) and electroblotted onto PVDF membranes. After incubation with primary antibodies, the immunoblot detections were carried out with Enhanced ChemiLuminescence western blotting analysis system (Amersham-Pharmacia-Biotech). Every gel was loaded with molecular weight markers including proteins from 6.5 to 205 kDa. As a control for equal protein loading glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was utilized: membranes were stripped and reprobed for GAPDH using a monoclonal antibody diluted 1:500. A previously described densitometric algorithm was used to quantitate the Western Blot results [54].

Visualization of monodansylcadaverine-labelled vacuoles

Upon exposure to ghrelin, the formation of autophagic vacuoles was monitored with monodansylcadaverine (MDC) [55]. In detail, 1 μM MDC was added to cell medium. After 10 min incubation at 37 °C cells were washed three times with phosphate buffered solution (PBS) and immediately analysed with a fluorescence microscope (Olympus IX71) equipped with a filter system.

Caspase-3 activity

Caspase-3 activity assays were performed in cell lysates (5 μg of total proteins in the mixture) using the Ac-Asp-Glu-Val-Asp-AMC substrate in 50 mM Tris–HCl, 50 mM NaCl, 5 mM $CaCl_2$, 1 mM EDTA, 0.1 % CHAPS, 5 mM β-mercaptoethanol, pH 7.5. The incubation was carried out at 37 °C for 60 min, and the hydrolysis product was detected (AMC: $\lambda_{exc} = 365$ nm, $\lambda_{em} = 449$ nm) on a Shimadzu RF5301 spectrofluorimeter.

DNA fragmentation assay

The assay was conducted as described by Buonanno et al. [56]. Briefly, 1×10^6 cells were grown in microtiter plates and, upon treatments, were collected and suspended in lysis buffer (50 mM Tris–HCl, pH8, 10 mM EDTA, 0.5 % SDS, and 0.5 mg/ml proteinase K). After 1 h incubation at 50 °C, 10 mg/ml RNase was added to the lysates and incubated for 1 h at 50 °C and for 10 min at 70 °C. DNA was precipitated by addition of sodium acetate (pH 5.2) and ice-cold 100 % ethanol, incubated on ice for 10 min and centrifuged at $10{,}000 \times g$ for 10 min. Pellets were suspended in sterile water. Samples were resolved on a 1.8 % agarose gel stained with ethidium bromide.

Hoechst nuclear staining

Upon the abovementioned treatments, cells (1×10^6 cells/mL) were fixed in methanol/acetic acid (3:1), incubated with 0.05 mg/ml Hoechst 33258 nuclear stain for 15 min, and observed using an inverted fluorescence microscope.

Statistical analysis

Values are expressed throughout as mean values ± standard error of results obtained from six independent experiments. Student's t test was used to compare differences of means between control and treated groups. Statistical tests were performed with Sigma-Stat 3.1 software (SPSS, Chicago, IL). A value of $p < 0.05$ was considered significant.

Results

Ghrelin mainly inhibited the 20S proteasome trypsin-like activity

Ghrelin effects on the 20S proteasome were assayed by incubating both constitutive and immuno- proteasomes with increasing concentrations of the hormone. Interestingly, the most potent inhibition was observed for the T-L activity of the constitutive 20S proteasome. Conversely,

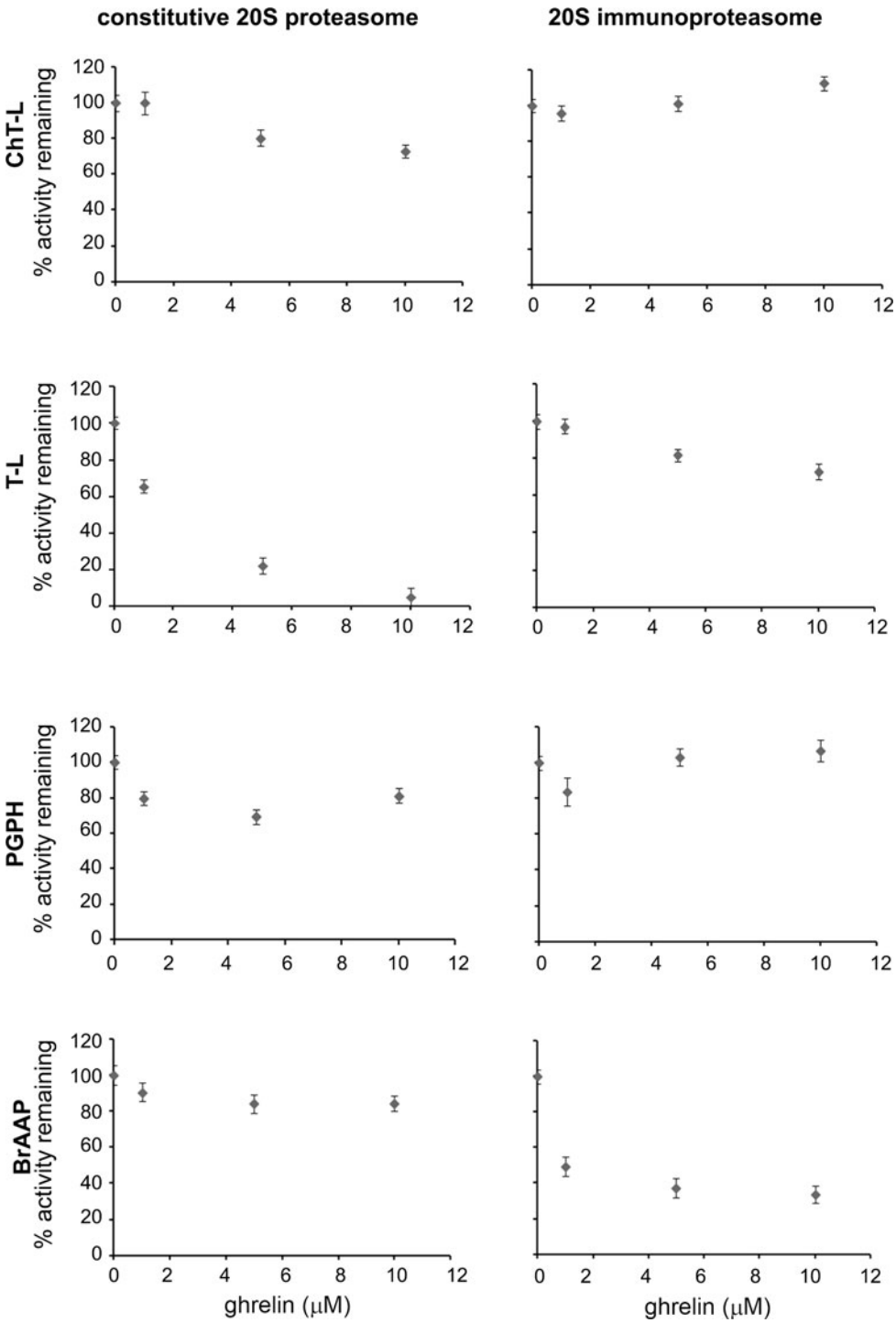


Fig. 1 Effects of ghrelin on isolated proteasomes. Effect of increasing concentrations of ghrelin on the ChT-L, T-L, PGPH and BrAAP proteolytic components of constitutive (*left plots*) and immuno- (*right plots*) 20S proteasomes, isolated from bovine brain and thymus respectively. Data shown are expressed as mean values ± SE obtained from six independent determinations

the ChT-L, PGPH and BrAAP activities of constitutive proteasome showed a lower inhibition, reaching the maximal inhibitory effect around 20–30 % upon incubation with the highest concentration of ghrelin tested (10 μM) (Fig. 1, left plots). On the other side the incubation of the immunoproteasome with 10 μM ghrelin resulted in 35 and 60 % inhibition of the T-L and BrAAP components, respectively (Fig. 1, right plots). Lower concentrations of ghrelin (1 μM) were already able to significantly affect the T-L and PGPH constitutive components and the BrAAP and PGPH activities of the immunoproteasome (Fig. 1). The contribution of deacylated form of ghrelin on the modulation of proteasome activities was also evaluated and control assays with n-octanoic acid group were conducted. No significant inhibition was observed (data not shown).

Besides possessing a partially unfolded structure that renders it particularly prone to degradation by the 20S proteasome, β-casein presents a number of potential

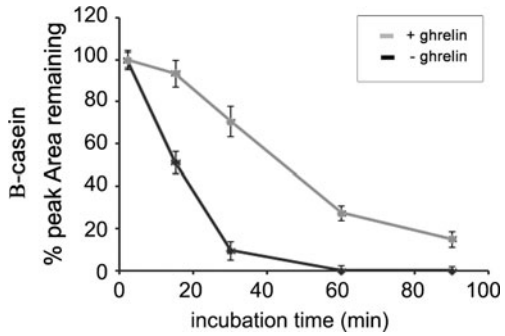


Fig. 2 Effect of ghrelin on the degradation of a macromolecular substrate by constitutive 20S proteasome. β-casein degradation rate was determined by measuring the peak area of casein at $\lambda = 210$ nm in the absence (*dark grey lines*) and in the presence (*light grey lines*) of ghrelin at different time points. Data shown are expressed as mean values ± SE obtained from six independent determinations

specific sites of cleavage for T-L activity; therefore it was chosen as protein substrate to evaluate the effective inhibition of that proteasomal component. The β-casein degradation rate by the constitutive 20S proteasome was clearly lower in the presence of 10 μM ghrelin, particularly upon short incubation times (Fig. 2), whereas no relevant changes on the chromatographic degradation pattern were observed (data not shown).

mRNA expression of ghrelin and its receptors upon exogenous ghrelin administration

Many studies proposed a possible role of gut peptides, including ghrelin, in the pathogenesis and natural history of gastrointestinal malignancies. Ghrelin production mainly occurs in the gastrointestinal tract, and it could affect cancer onset and progression. We investigated whether the administration of exogenous ghrelin could influence the endogenous ghrelin production. Thus, human colon adenocarcinoma cells (HCT116) were treated with 1 μM ghrelin for 6, 12, 18 and 24 h. A ghrelin transcript product corresponding to the predicted 327 bp size was present in all samples tested. Single RT-PCR products were detected for the ghrelin GHS-R1a (65 bp) and GHS-R1b (66 bp) receptors. Interestingly, the RT-PCR products of ghrelin and its functional receptor GHS-R1a significantly decreased at 18 and 24 h treatment (Fig. 3), suggesting a negative feedback triggered by exogenous ghrelin in the cell.

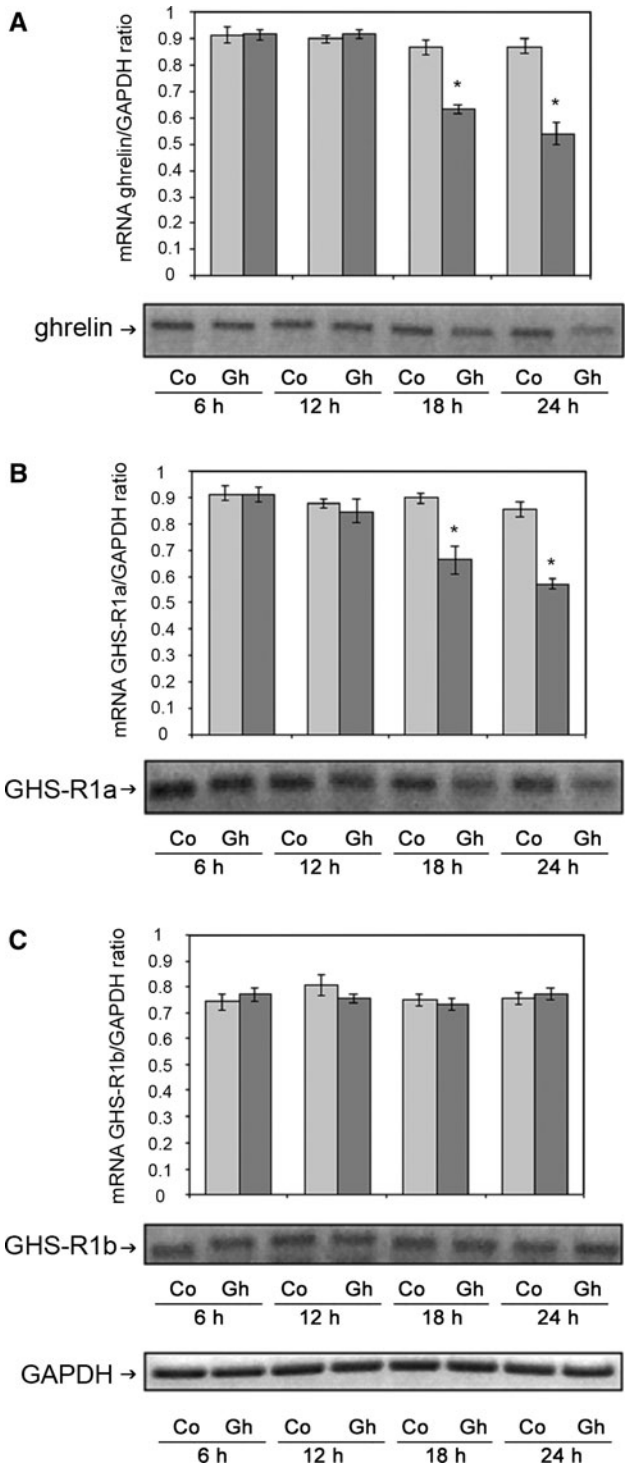
Ghrelin inhibits proteasomes and activates autophagy in colon adenocarcinoma cells

Colon adenocarcinoma cells (HCT116) were treated with 1 μM ghrelin to evaluate its effect on cell proteolytic pathways. Firstly, we observed a 20 % decrease in cell viability upon 24 h treatment with 1 μM ghrelin, monitored through MTT assay (data not shown). Successively, 20S proteasomal activities were measured in cells treated with 1 μM ghrelin for 6 and 24 h. Ghrelin induced a significant inhibition of the proteolytic components, mainly of the ChT-L (25 % inhibition at 24 h), T-L activities (around 35 % at 24 h) and BrAAP activities (40 % at 24 h) (Fig. 4, panels A, B and D). Then, the 26S ChT-L activity was measured to explore the ubiquitin–proteasome system functionality. A 20 and 40 % inhibition of 26S ChT-L activity was measured upon 6 and 24 h treatment, respectively (Fig. 4, panel E). The inhibition of the UPS in HCT116 cells was further confirmed by the accumulation of ubiquitinated proteins upon 6 and 24 h treatment with ghrelin (Fig. 4, panel F).

Additionally, the intracellular levels of the proteasome substrates p27 and IkBα were measured through western blotting. In detail, p27 is involved in the apoptotic cascade and its increase leads to the activation of pro-apoptotic mechanisms [57, 58]. IkBα is a regulatory protein that inhibits NF-kB by complexing with it in the cytoplasm. IkBα modulates transcriptional responses to NF-kB, including cell adhesion, apoptosis, cell growth, differentiation, immune and pro-inflammatory responses [59]. The accumulation of both proteins confirmed that the proteasome functionality was impaired by exposure to ghrelin, and suggested that pro-apoptotic events were in progress (Fig. 5, panels A, B).

Among numerous proteasome substrates, p53 is an important regulator of cell cycle and cell death [60]. p53 increased levels may either trigger or alter the autophagy pathway, causing the cell to react accordingly [61]. It has been demonstrated that p53 signalling plays an essential role in autophagy activation and cell death, following NF-kB inhibition [62]. As expected, we observed the accumulation of p53 upon ghrelin administration, in line with both proteasome inhibition and apoptosis activation (Fig. 5, panel C).

Considering that UPS and autophagy represent the main cellular proteolytic pathways and that a crosstalk between these two systems exists [63, 64], we detected the intracellular levels of autophagy related proteins such as LC3 and p62. LC3 has been identified as an autophagosomal marker in mammalian autophagy, and the levels of LC3 may also reflect the status of autophagy [65]. During the activation of autophagy, LC3-I (the cytosolic form) is processed and recruited to autophagosome, where LC3-II (the membrane-bound form) is generated by site-specific proteolysis and lipidation near the C-terminus. Immunoblotting assessment of LC3 expression was performed to estimate the autophagic activity of mammalian cells, because the amount of LC3-II correlates with the number



◀**Fig. 3** Time-related effects of exogenous ghrelin treatment on the expression of ghrelin, GHS-R1a, and GHS-R1b genes. Representative electrophoretic analysis pattern of the RT-PCR products of ghrelin, GHS-R1a, GHS-R1b mRNAs in HCT116 cells. Cells were left untreated (Co) or treated with 1 μM exogenous ghrelin (Gh). After 6, 12, 18, and 24 h, total RNA was extracted, retrotranscribed, and amplified as described in "Materials and methods". Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. Representative ratios of ghrelin/GAPDH (*panel A*), GHSR1a/GAPDH (*panel B*) and GHS-R1b/GAPDH (*panel C*) from four independent replicates are reported. Data points marked with an *asterisk* are statistically significant compared to their respective not treated control cells in each time set (* $p < 0.05$)

of autophagosomes. p62 is an autophagy substrate that accumulates in autophagy-deficient cells and decreases upon autophagy activation [66]. In response to ghrelin treatment, we observed an increase in LC3-II levels and a decrease in p62 expression, suggesting that the autophagic cascade was effectively activated in HCT116 cells (Fig. 6, panels A and B). To further dissect the autophagy pathway, we conducted the monodansylcadaverine (MDC) assay. In fact, MDC accumulates as a selective fluorescent marker for autophagic vacuoles [55]. The results summarized in Fig. 6, panel C clearly confirmed the activation of autophagy in the presence of 1 μM exogenous ghrelin.

Caspase-3 is a crucial enzyme in apoptosis, since it catalyzes the proteolysis of many cellular regulatory proteins, finally resulting in DNA fragmentation [67]. Caspase-3 activity was measured to elucidate the basis for the observed cellular death. Its activity was significantly enhanced upon ghrelin administration (Fig. 7, panel A). These data are consistent with the increased cleavage of poly (ADP-ribose) polymerase protein (PARP). PARP is a nuclear enzyme involved in several processes such as cell death pathways, DNA damage detection and repair. During apoptosis PARP is cleaved by caspase-3, generating an 89 kDa C-terminal fragment [68–70]. Ghrelin treatment caused an increase in the levels of the 89 kDa PARP fragment (Fig. 7, panel B). Additionally, DNA fragmentation assay revealed the presence of DNA ladder upon treatment, particularly evident at 24 h (Fig. 7, panel C). To evaluate cell morphology and further confirm the occurrence of apoptotic events, a Hoechst staining assay was performed. Hoechst 33342 is a fluorescent dye which permeates plasma membranes and binds to the dsDNA, with a resulting blue fluorescence signal. In apoptotic cells chromatin is condensed and nuclei are fragmented with respect to normal nuclear size. The assay revealed morphological changes in HCT116 cells nuclei upon 6 and 24 h treatment with ghrelin, such as a reduction in the volume and nuclear chromatin condensation (Fig. 7, panel D).

The activation of caspase-3 can be promoted by the initiator caspase-9 in the intrinsic pathway or by caspase-8 in the extrinsic pathway [71]. Here, caspase-3 is activated from caspase-9, as demonstrated by the increased expression of the cleaved caspase-9 at 3 h, suggesting that a caspase-9-dependent intrinsic apoptotic pathway was triggered upon ghrelin treatment (Fig. 8).

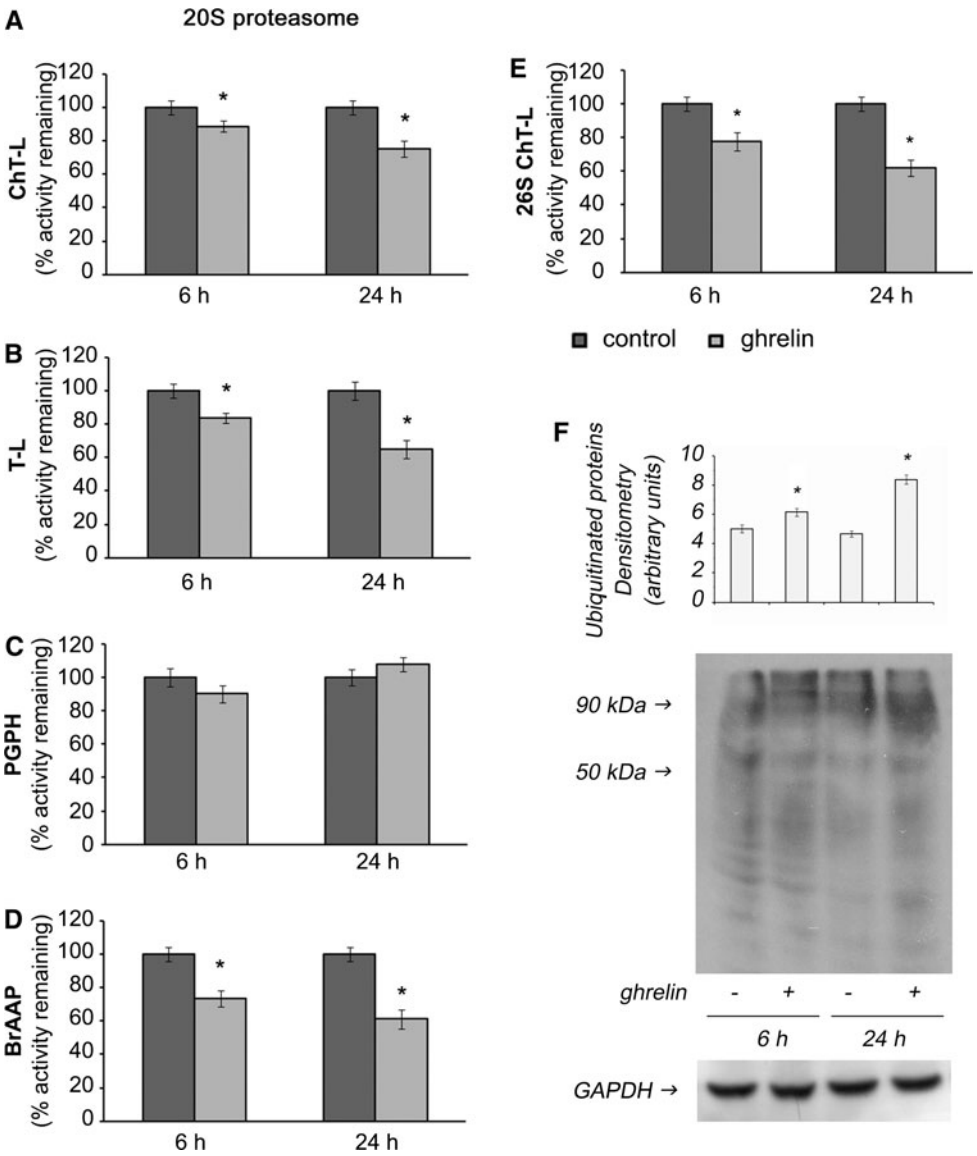


Fig. 4 *Panels A–D*: 20S proteasomal activities measured in cell lysates. Upon treatment with 1 μM ghrelin, HCT116 cell lysates were tested for the ChT-L, T-L, PGPH and BrAAP activities of 20S proteasomes. Fluorescence units were subtracted of the values of control assays in the presence of specific inhibitors. Results derived from six independent experiments. *Panel E*: 26S proteasome ChT-L activity. 26S ChT-L activity was measured including in the reaction mixture 10 mM $MgCl_2$, 1 mM DTT and 2 mM ATP. Data are expressed as % activity remaining compared to their respective control (*filled square*) in each time set (*$p < 0.05$). *Panel F*: Ubiquitinated proteins in treated cells. Representative western blotting of ubiquitin conjugates levels in treated cells. The densitometric analyses from six separate blots provided for quantitative analysis are presented. Equal protein loading was verified by using an antibody directed against GAPDH. Data points marked with an *asterisk* are statistically significant compared to their respective not treated control cells in each time set (*$p < 0.05$)

Discussion

Ghrelin is expressed in several cancer tissues where it influences cell proliferation, cell migration and invasion, and apoptosis [11, 14]. Nevertheless, its effects on cancer models are controversial since divergent reports indicate that ghrelin exerts either stimulatory or inhibitory effects on cell viability depending on the cell type and on the levels of the treatment [26–29]. In the present study we chose the human colon adenocarcinoma cell line HCT116 as a model to explore the ability of ghrelin to modulate the main proteolytic cellular pathways: UPS and autophagy. In fact, the proteasome is involved in the proteolysis of cell cycle regulators, oxidized and damaged proteins, therefore the identification of proteasome modulators is a promising approach in a number of pathologies, such as cancer, neurodegenerative, cardiovascular, and metabolic diseases [72]. On the other side, autophagy can be involved in cell defense mechanisms or in type II programmed cell death, in response to cellular stress conditions [73, 74].

Interestingly, our in vitro experiments evidence, for the first time, a direct inhibition of proteasome functionality: upon incubation of isolated proteasomes with increasing concentrations of ghrelin, all the proteolytic components of constitutive and immuno- proteasomes are differently inhibited, suggesting that ghrelin exerts a subunit-dependent inhibition on 20S proteasome, with T-L activity being the most affected. Additionally, the degradation rate of the proteasome macromolecular substrate β-casein decreases in the presence of ghrelin (in fact β-casein contains a

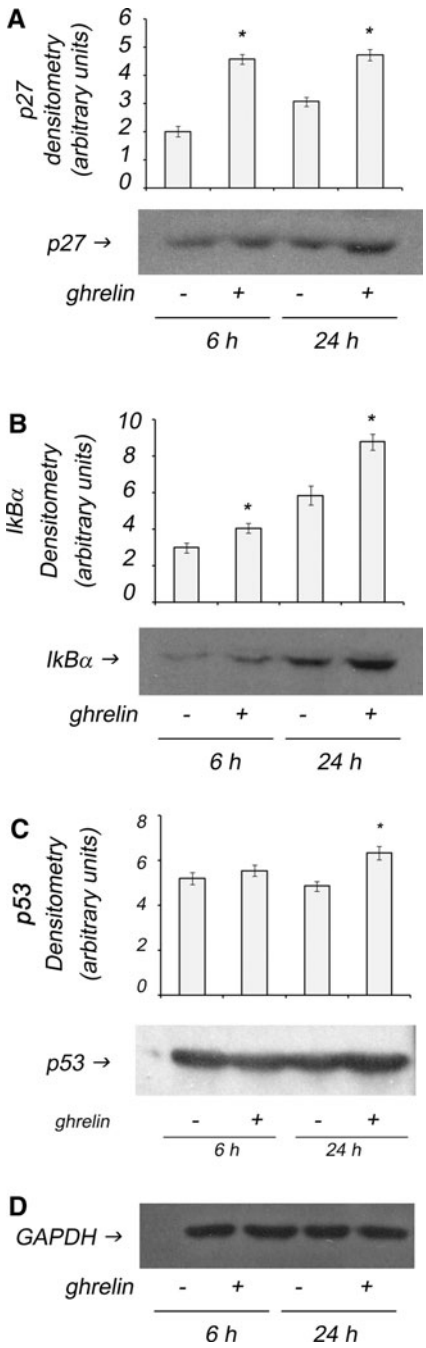


Fig. 5 Analysis of intracellular levels of proteasomal substrates p27, IkBα and p53 in cell lysates. HCT116 cells were treated with ghrelin for 6 and 24 h. Western blotting assays were carried out to evaluate the intracellular levels of proteasomal substrates p27 (*panel A*), IkBα (*panel B*) and p53 (*panel C*). Equal protein loading was verified by using an antibody directed against GAPDH (*panel D*). The densitometric analyses from six separate blots provided for quantitative analysis are presented. Data points marked with an asterisk are statistically significant compared to their respective not treated control cells in each time set ($*p < 0.05$)

number of potential sites of attack/cleavage by the T-L component). These data stimulated the need for studying cellular proteasomes modulation as a possible further ghrelin mechanism of action in cancer cells. Based on in vitro preliminary results, we exposed cells to 1 μM ghrelin, because at this concentration most of the proteasome proteolytic components resulted significantly inhibited, being aware that the normal physiological level is in the nanomolar range [11, 75]. Anyway, ghrelin is a hormone produced upon specific stimuli (such as fasting conditions); therefore it is not present at a constant level. Moreover, no data are available on ghrelin concentration in colon cancer cells. However, tumour cells, in order to meet the increased requirements of proliferation, often display fundamental changes in pathways of energy metabolism and nutrient uptake [76]. In this scenario it would not surprise to find increased ghrelin levels with respect to normal tissues. As shown in Fig. 3, the administration of exogenous ghrelin induced a time dependent compensatory mechanism, being evident a difference, between control and treated cells, in the expression levels of mRNAs for both ghrelin and its functional receptor at 18 and 24 h.

MTT assay revealed a weak anti-proliferative effect (20 % decrease in cell viability upon 24 h treatment), according with a recent work showing that ghrelin inhibited the growth of murine colon cancer cells upon 72 h treatment [77]. Other studies focused on different cell types and long-term treatments (48–96 h). Differently, we performed our measurements at 6–24 h with the aim to explore earlier events, such as the alteration of proteolytic enzymes activities or the induction of apoptosis.

As expected, the cellular 20S proteasome ChT-L, T-L and BrAAP components, as well as 26S ChT-L activity were inhibited by ghrelin. Proteasome inhibition was further confirmed by the cytosolic accumulation of poly-ubiquitinated proteins. Also the intracellular levels of the proteasome substrates p27 and IkBα accumulated upon 6 and 24 h treatment with ghrelin. These data suggest that, following ghrelin exposure, cellular homeostasis is influenced by proteasome modulation and apoptosis is triggered. In fact, p27 is a pro-apoptotic protein and IkBα increase leads to the nuclear accumulation of inactive NF-kB/IkBα complexes with a concomitant inhibition of NF-kB-dependent survival genes expression, thus contributing to the onset of apoptosis [57, 59]. We also observed an increase in the levels of p53, which is another proteasome substrate and a key regulator of cell cycle and apoptosis. On this regard, the accumulation of p53 protein in response to proteasome inhibition may mediate autophagy activation, corroborating the previously published evidence of the "interactive role" of p53 in the proteasomal inhibition-mediated activation of autophagy [78]. In fact, the accumulation of LC3-II protein, the p62 diminished levels, and the increased number of autophagic vacuoles demonstrated the activation of the autophagic cascade, which is strictly coordinated with apoptosis induction in cancer cells [79].

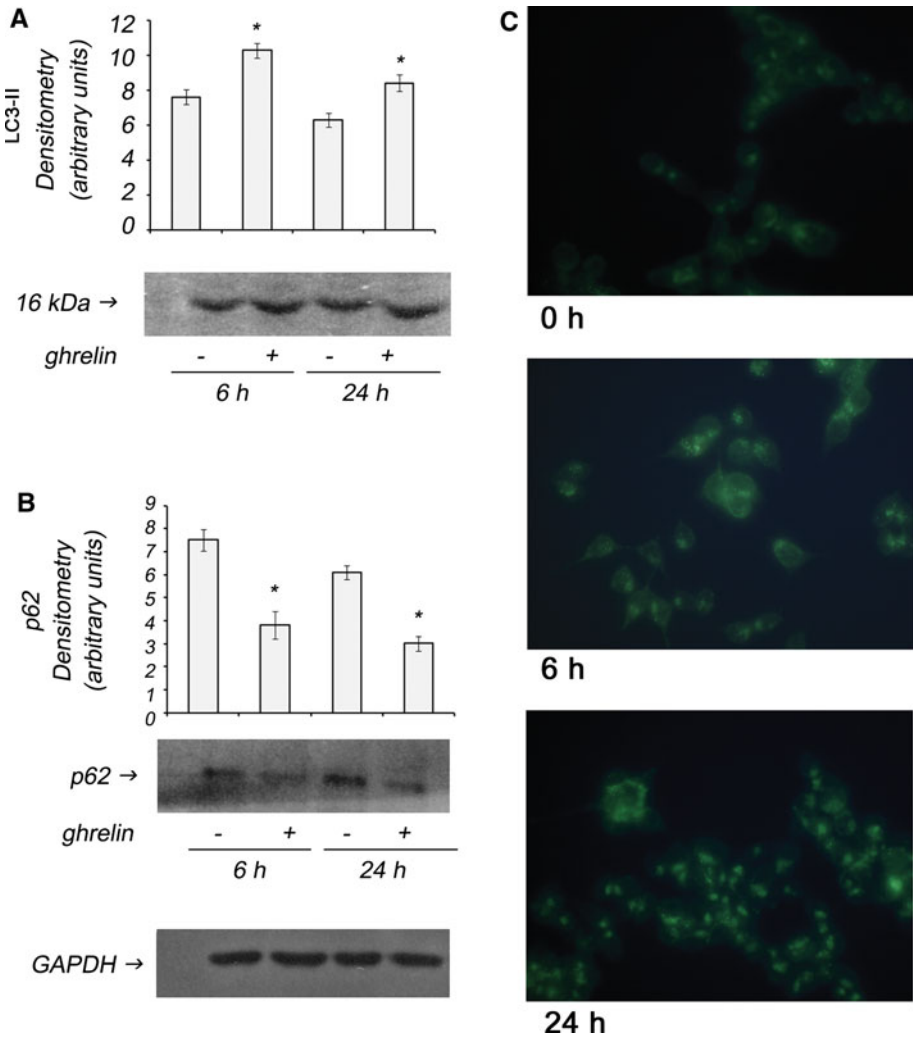


Fig. 6 Autophagy activation. Representative western blotting of LC3-II (*panel A*) and p62 (*panel B*) levels in HCT116 cells treated with 1 μM ghrelin for 6 and 24 h. The densitometric analyses from six separate blots provided for quantitative analysis are presented. Equal protein loading was verified by using an antibody directed against GAPDH. Data points marked with an asterisk are statistically significant compared to their respective not treated control cells in each time set ($*p < 0.05$). Autophagic vacuoles (*panel C*) were stained with monodansylcadaverine as described in "Materials and methods" section

Considering the accumulation of the above-described pro-apoptotic proteins, we further explored the apoptotic pathway. The activation of caspase-3 activity is consistent with the increased cleavage of PARP, hydrolysed by caspase-3 during apoptosis. These data are also in agreement with DNA fragmentation at 6 and 24 h and alterations in nuclei morphology assessed through Hoechst staining. Also the early increase in the expression of the cleaved caspase-9 suggests that the administration of exogenous ghrelin triggers the intrinsic apoptotic pathway in HCT116 cells.

It is important to stress that the acyl group of ghrelin can be easily hydrolyzed at neutral pH [80]. Consequently, desacyl ghrelin is the predominant form in our experimental conditions. Recently, some authors proposed that the inhibitory effect of human desacyl ghrelin on the growth of colon and prostate cancer lines is mediated via a different receptor than GHS-R1a, because this form of ghrelin does not bind to this receptor subtype [77]. Here, for the first time, we demonstrate that ghrelin directly inhibits isolated and cellular proteasomes and we propose the direct proteasome inhibition together with the activation of autophagy as a novel mechanism for the pro-apoptotic effect of ghrelin in colon cancer cells.

Moreover, it has been previously demonstrated that ghrelin and its synthetic analogue GHRP-2 are able to decrease the muscle proteolysis by affecting the ubiquitin-ligating enzymes in rats with burn injury [30, 31]. Here we demonstrate that ghrelin is able to affect UPS also by directly inhibiting the proteasomal complex.

Concluding, we show evidences that the intrinsic apoptosis is triggered via proteasome inhibition with concomitant autophagy induction, thus contributing to clarify the growing and debated literature on ghrelin effect on cancer cells. Future studies should not ignore the impairment of the two main proteolytic pathways, such as UPS and autophagy, being strictly related to cell cycle regulation and cell death.

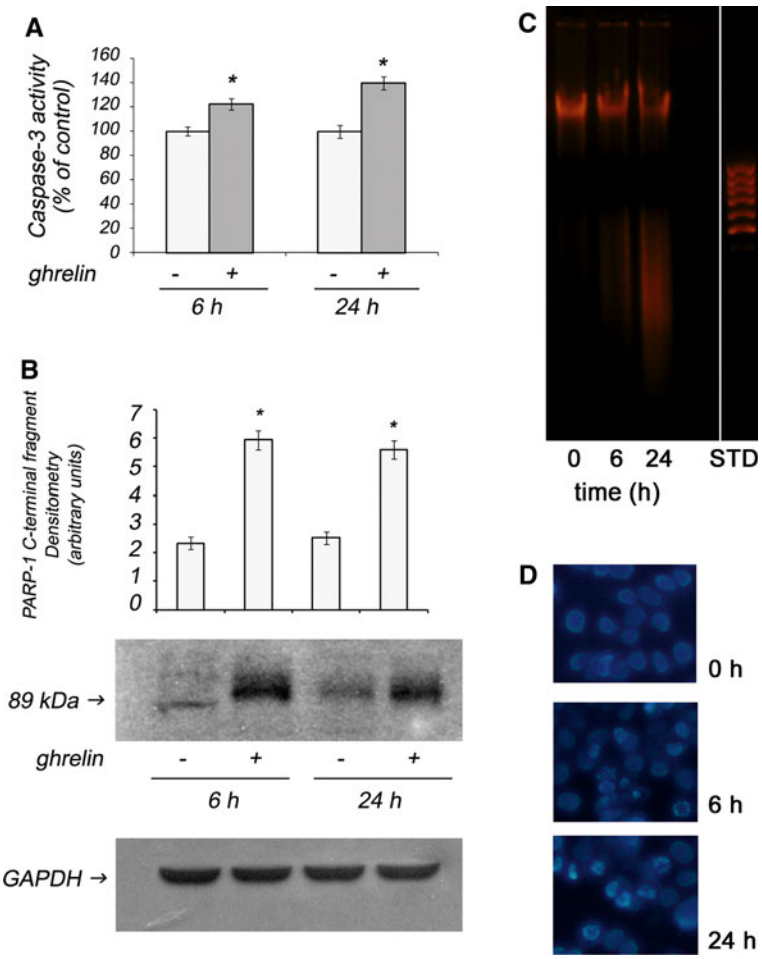


Fig. 7 Apoptotic events. *Panel A*: Caspase 3 assay. The specific activity percentage of the respective control cells is reported. Results are representative of six distinct experiments. Data points marked with an asterisk are statistically significant compared to their respective non-treated control cells (*$p < 0.05$). *Panel B*: Representative western blotting of 89 kDa PARP fragment. The densitometric analyses from six separate blots are presented. Equal protein loading was verified by using an antibody directed against GAPDH. Data points marked with an asterisk are statistically significant compared to their respective not treated control cells in each time set (*$p < 0.05$). *Panel C*: DNA fragmentation assay. Samples were resolved on a 1.8 % agarose gel electrophoresis and stained with ethidium bromide. *Panel D*: morphological changes of HCT116 cells nuclei (Hoechst staining)

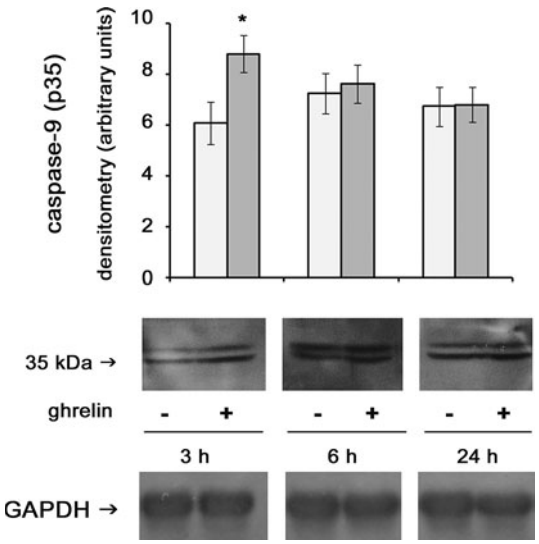


Fig. 8 Increased expression of 35 kDa cleaved caspase-9. The densitometric analyses from six separate blots are presented. Equal protein loading was verified by using an antibody directed against GAPDH. Data points marked with an *asterisk* are statistically significant compared to their respective not treated control cells in each time set (*$p < 0.05$)

Acknowledgments The authors declare that they have no conflict of interest.

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