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Antiproliferative Effects of Palmitoylethanolamide on Human Cervical Cancer Cells

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Abstract

Palmitoylethanolamide (PEA) is a bioactive fatty acid amide that accumulates during certain types of inflammation. PEA has been identified as a potent analgesic and anti-inflammatory agent, thus representing a promising molecule in the treatment of chronic pain and inflammation. Several studies evidenced the coexistence of multiple mechanisms of action of PEA. For example, PEA may act as an enhancer of the anti-inflammatory and antinociceptive activity exerted by other endogenous compounds (i.e. anandamide), either by increasing their affinity for receptors or inhibiting their metabolic degradation. The ubiquitin-proteasome system is the main extra-lysosomal proteolytic pathways and it is involved in the removal of inflammatory proteins, cell cycle regulators, oxidized and misfolded proteins. The modulation of the proteasome functionality represents an established target in a number of pathologic conditions such as cancer, neurodegenerations and inflammation.

In the present study, we investigated the ability of PEA to modulate the enzymatic activity of the proteasome. At first, we studied the effect of different concentrations of PEA on isolated constitutive and immuno-proteasomes. Successively, human cervical carcinoma cells were used as a model to measure PEA effect on tumour cell viability. The effect of PEA on the proteolytic activities of cellular proteasomes has been evaluated and the expression level of several proteasome substrates were determined. Our data propose an additional mechanism of action of PEA, precisely the modulation of proteasome-mediated proteolysis and demonstrate that PEA can affect tumour cells survival through the activation of apoptosis. These findings could represent an important preliminary step in considering PEA as a possible anti-cancer tool.

Keywords:

Palmitoylethanolamide;

Proteasome,

Anticancer.

1. Introduction

N-palmitoylethanolamine (PEA) is a naturally occurring fatty acid amide belonging to the class of endocannabinoids and it is present in most mammalian tissues. PEA is known for its anti-inflammatory properties which has been observed in vitro and in clinically relevant animal models of inflammatory pain [1, 2]. PEA is a signalling molecule, which can inhibit mast cell activation and microglial cell activities [3-5] thus reducing central pain hypersensitization [6]. The exact mechanisms of action of PEA are still under discussion, although it seems that the antinociceptive effects are mediated by multimodal mechanisms [7]. Membrane receptors (i.e., cannabinoid receptors) [4], nuclear receptors (i.e. PPAR, perossisome proliferator receptor) [8, 9], neurosteroid synthesis [10], mast cell down-modulation [3, 11, 12] and control of microglial activation [13] are all supposed mechanisms of action of PEA. These may coexist, depending upon the physiological and pathophysiological circumstances [14]. Additionally, a distinct entourage-effect has been hypothesized to explain PEA biological activities [15] with PEA acting as enhancer of the anti-inflammatory and antinociceptive activity exerted by other endogenous compounds and increasing their affinity for receptors or inhibiting their metabolic degradation. One of these endogenous compounds is anandamide, whose activity may be potentiated by PEA [7]. PEA antinociceptive effects are prevented by the cannabinoid receptor antagonists [16] although its affinity for cannabinoid receptors (CB1 and CB2) is very low [17]. Although PEA has affinity for other PPAR isoforms, G

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coupled receptors and novel cannabinoid receptors, PPAR-α can be considered the main biological target of PEA [18]. PEA up-regulation of PPAR-α results in decreased output of inflammatory mediators like tumor necrosis factor-α and interleukins, thus supporting PEA's role as a modulator of inflammation and pain [12]. Additionally, PEA reduced neurological deficits in a spinal trauma model, via reduction of mast cell infiltration and activation [12].
Considering PEA affinity for PPAR-α and the widespread presence of this receptor in CNS microglia and astrocytes, it is reasonable to think to PEA possible applications in the treatment of neuropathic pain [18].
The increase of the local synthesis of a compound with documented anti-inflammatory properties, during certain types of inflammation, raises the possibility that compounds affecting the metabolism of PEA may be therapeutically useful. In this work, the hypothesis that PEA can induce apoptosis in cancer cells through proteasome modulation has been investigated.

2. Material and methods

2.1. Reagents and chemicals

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) were all purchased from Sigma-Aldrich (Italy) while PEA was from EPITECH (Italy). The substrate Z-Gly-Pro-Ala-Phe-Gly-pAB and the inhibitor Z-Gly-Pro-Phe-Leu-CHO to test the BrAAP activity were the kind gift of Prof. Cardozo (Department of Medicine, Mount Sinai School of Medicine, New York). Media and reagents for cell cultures were purchased from Euroclone (Italy). Antibodies used in western blotting were purchased from Santa Cruz Biotechnology (CA. USA). The western blot stripping buffer was from Pierce (USA). All chemicals and solvents were of the highest analytical grade available. Human cervical carcinoma cells (HeLa) and the non-tumorigenic epithelial cell line MCF10A were from ATCC (Manassas, VA, USA).

2.2 20S proteasome in vitro assays

The effects of increasing concentrations of PEA on constitutive and immuno- 20S proteasomes functionality were evaluated through fluorimetric assays. The following synthetic substrates were used: Suc-Leu-Leu-Val-Tyr-AMC for ChT-L, Z-Leu-Ser-Thr-Arg-AMC for T-L, Z-Leu-Leu-Glu-AMC for PGPH, and Z-Gly-Pro-Ala-Phe-Gly-pAB for BrAAP. The tests for the BrAAP activity were performed in the presence of aminopeptidase-N. 20S proteasome and immunoproteasome were isolated from bovine brain and thymus respectively. The incubation mixture contained increasing concentrations of PEA (from 0 to 20 μM), 1 μg of isolated 20S proteasome or immunoproteasome, the appropriate substrate and 50 mM Tris-HCl pH 8.0 up 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: λexc=365 nm, λem=449 nm; PABA λexc=304 nm, λem=664 nm) on a SpectraMax® Gemini™ XPS Microplate spectrofluorometer (Molecular Device®). Control assays were performed to evaluate a possible effect of the molecules on the proteolytic activity of aminopeptidase-N.

2.3 Cell viability assay

Cell viability upon PEA treatment was evaluated by MTT assay, an index of mitochondrial activity and cell viability. It is based on viable cells' ability to convert a soluble yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, to a purple–blue formazan crystal by mitochondrial succinate dehydrogenase. In detail, cells were treated with increasing concentrations (0-50μM) of PEA for 24-72 hours. After experimental treatment, media were replaced with serum-free media containing 0.5 mg/mL MTT. Plates were incubated at 37 °C for 2 h. After discarding the supernatant, the formazan product was solubilized in 100 μL of dimethyl sulfoxide and absorbance was measured at 550 nm. The MTT assay was also carried out in the
presence and in the absence of the caspase inhibitor zVADfmk (10 μM concentration in the assay; Sigma-Aldrich, St Louis, MO, USA). Each time point was performed in sixtuplicate.

2.4 Cells treatment

HeLa cells were grown in DMEM medium supplemented with 10% FBS, antibiotic, antimycotic and L-glutamine at 37°C. MCF10A cells (an in vitro model for studying normal epithelial cell function) were cultured in a DMEM/F12 Ham's mixture supplemented with 5% equine serum, 20 $ng \cdot mL^{-1}$ epidermal growth factor, 10 $\mu g \cdot mL^{-1}$ insulin, 0.5 $mg \cdot mL^{-1}$ hydrocortisone, antibiotics and antimycotic. Cells were incubated with growth medium at 37°C equilibrated with 95% air and 5% CO_2 in flasks or 96- or 6-well plates depending on the assay. Cells were treated with 10 μM palmitoylethanolamide (PEA) for 6 and 24 h. Control cells were treated with DMSO. Upon treatment, cells were harvested in PBS, centrifuged and the pellet was resuspended in lysis buffer (20 mM Tris, pH 7.4, 250 mM sucrose, 1 mM EDTA and 5 mM β-mercaptoethanol). Lysates were centrifuged at 12000xg for 15 min and the supernatants were stocked at -80 °C. Protein concentration in cell lysates was determined by the method of Bradford using bovine serum albumin as a standard.

2.5 Proteasomal activities on cell lysates

20S proteasome activity assays in cell lysates (1 μg of total proteins in the mixture) were performed using the substrates listed above; control experiments were done in the presence of specific proteasome 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 measurements of the release AMC and PABA groups were detected on a SpectraMax® Gemini™ XPS Microplate spectrofluorometer (AMC: λexc = 365 nm, λem= 449 nm; PABA: λexc= 304 nm, λem= 664 nm). 26S proteasome ChT-L activity was measured including 10 mM $MgCl_2$, 1 mM DTT and 2 mM ATP in the reaction mixture. Incubation was carried out at 37°C and, after 60 min, the AMC release was detected.

2.6 Caspase 3 activity

Caspase 3 activity was measured in cell lysates (20 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. Incubation was carried out at 37 °C for 60 min, then the fluorophore hydrolysis product was detected (AMC: λexc= 365 nm, λem= 449 nm) on a spectrofluorimetric microplate reader Gemini XPS.

2.7 Western blotting analyses

Intracellular levels of ubiquitinated proteins, IkBα and p27 after PEA treatment were analyzed through western blotting assays. Cell lysates (15 μg) were loaded on sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) and electroblotted onto PVDF membranes. After incubation with specific primary and secondary 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 with MW from 6.5 to 205 kDa (SigmaMarker Wide Molecular Weight Range, Sigma-Aldrich S.r.l., Milano, Italy). As a control for equal protein loading glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized: membranes were stripped and reprobed for GAPDH using a monoclonal antibody diluted 1:500. A densitometric algorithm has been developed to quantitate the Western Blot results. Each Western Blot film has been scanned (16 bits greyscale) and the obtained digital data were processed to calculate the background mean value and its standard deviation. The background-free image was then obtained subtracting the background intensity mean value from the original digital data. The integrated densitometric value associated to each band was then calculated as the sum of the density values over all the pixels belonging to the considered band having a density value higher than the background standard deviation. The band densitometric value was then normalized to the relative GAPDH signal intensity. The ratios of band intensities were calculated within the same Western Blot. All the calculations were carried out using the Matlab environment (The MathWorks Inc., MA, USA) [19].

2.8 Statistical analysis

Values are expressed as mean values and standard deviation of results obtained from separate experiments. Student's t-test was used to compare differences between the means of control and treated groups. Statistical tests were performed with Sigma-stat 3.1 software (SPSS, Chicago, IL, USA). P-values < 0.05 and < 0.01 were considered to be significant.

3. Results

3.1 Effect of PEA on isolated 20S proteasomes

The ability of PEA to inhibit 20S proteasomes was assayed by incubating both constitutive proteasome and immunoproteasome with increasing concentrations of PEA. All proteasomal activities were significantly inhibited. In particular, a strong inhibition of the BrAAP activity of the constitutive 20S proteasome was observed (60% at 10 μM). ChT-L, T-L and PGPH activities of constitutive proteasome showed a 30-40% inhibition upon incubation with the highest PEA concentration (20 μM). When the immunoproteasome was incubated with 20 μM PEA, the BrAAP component resulted inhibited of a 60%, while the ChT-L, T-L and PGPH showed a 30-40% inhibition (Figure 1). Interestingly, relatively low concentrations of PEA (1-2.5 μM) were already able to significantly affect only the BrAAP component of both enzymes (Fig. 1)

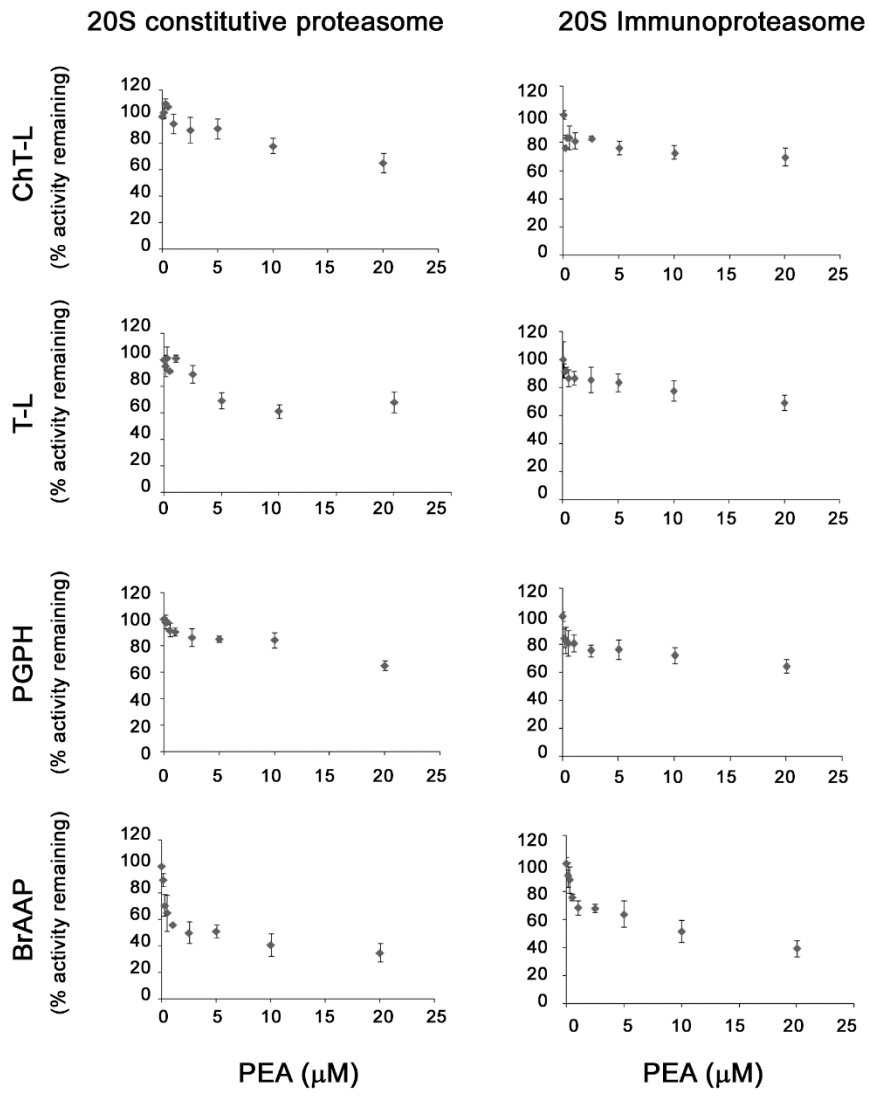

Fig. 1 Effects of PEA on isolated 20S proteasomes. Effect of increasing concentration of PEA on the ChT-L, T-L, PGPH and BrAAP proteolytic components of constitutive (Left) and immuno- (Right) 20S proteasomes, isolated from bovine brain and thymus respectively. Data shown are expressed as mean values ± SE obtained from six distinct determinations.

Interestingly, relatively low concentrations of PEA (1-2.5 μM) were already able to significantly affect only the BrAAP component of both enzymes (Fig. 1).

3.2. HeLa cells viability upon PEA exposure

Upon cell treatment with increasing concentrations (0-50μM) of PEA for 24-72 hours a concentration-dependent effect on HeLa cells viability has been observed. Upon 24h treatment with 15 μM PEA a significant decrease of HeLa cell viability occurred, whereas 5 μM PEA significantly decreased HeLa cell survival at 48 and 72 h. Conversely, MCF10A viability was not influenced by the treatment, suggesting a selective effect on cancer cells (Fig 2).

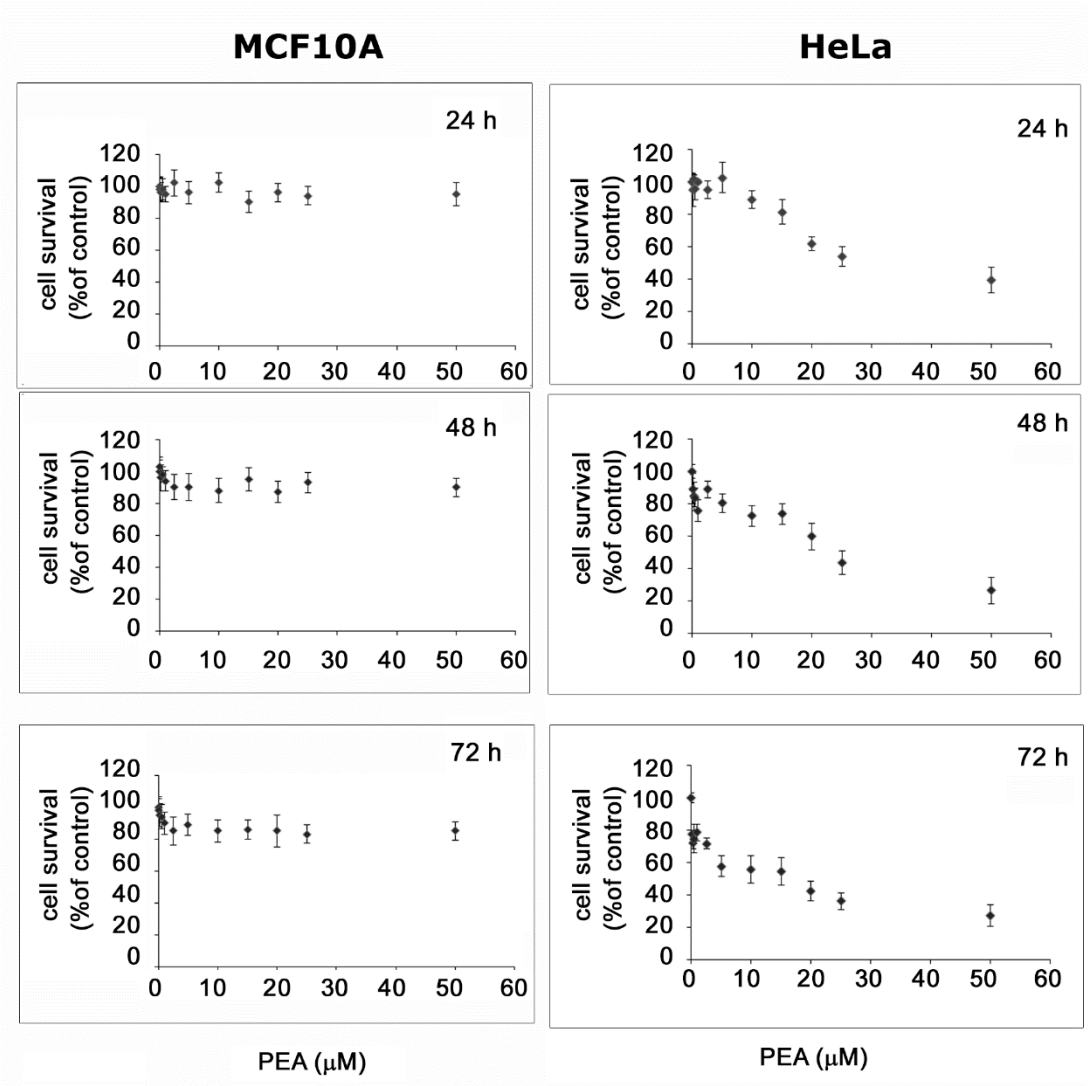

Fig 2. Cell survival analyzed with the MTT assay. Cell viability plots of MCF10A (left) and HeLa (right) cells treated with increasing concentrations (0-50μM) of PEA for 24, 48 and 72 h as described in Materials and methods section. Data are reported as % of cell viability. Data shown are expressed as mean values ± SE obtained from six distinct determinations.

3.3 Effects of PEA on cellular proteasomes

20S proteasomal activities were measured in cells treated with 10 μM PEA for 6 and 24 h. PEA significantly inhibited 20S proteolytic components, mainly the ChT-L (25% inhibition at each time point) and T-L activities (50% and 35% at 6 and 24 h respectively) in HeLa cells. BrAAP activity decreased of 15% and 35% at 6 and 24 h, respectively, and PGPH activity was inhibited of 10% at 24h (Figure 3).

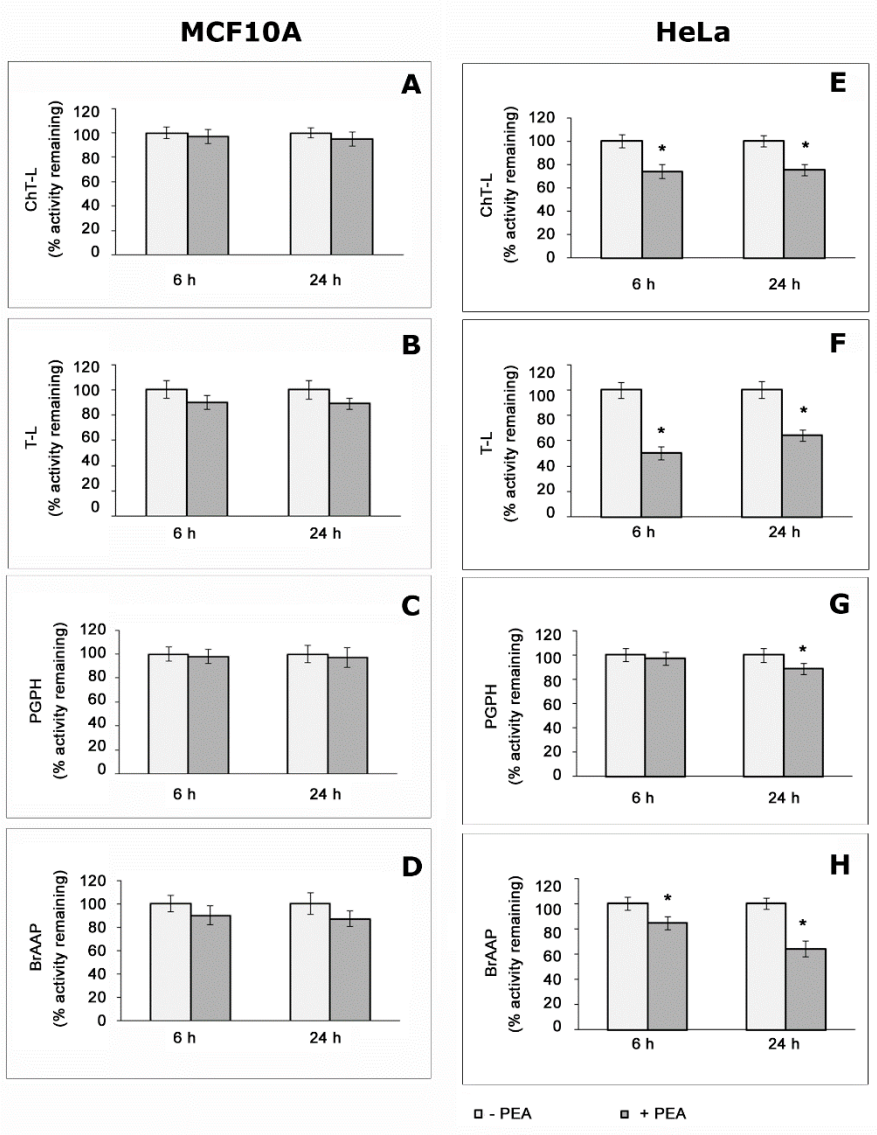

Fig 3 Proteasomal activities measured in cell lysates. Upon treatment with PEA, MCF10A (left) and HeLa (right) cells lysates were tested for the ChT-L (panel A), T-L (panel B), PGPH (panel C) and BrAAP (panel D) activities of 20S proteasomes, using fluorescent substrates. Data are reported as % of cell viability. Data shown are expressed as mean values ± SE obtained from six distinct determinations.

The 26S ChT-L activity was measured to explore the functionality of the ubiquitin-proteasome system. The activity resulted inhibited of 35% upon 6 and 24 h treatment exclusively in Hela cells (Figure 4)

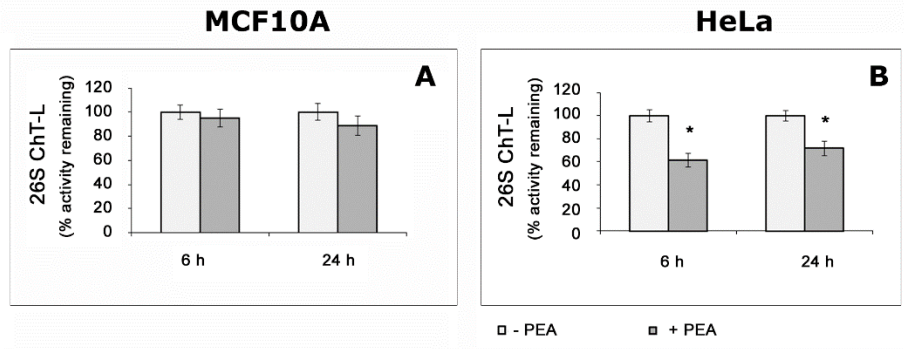

Fig 4 26S proteasome ChT-L activity. MCF10A and HeLa cells were treated with PEA for 6 and 24 h, as described in the Materials and methods section. 26S ChT-L activity was measured in cell lysates including in the

reaction mixture 10 mM MgCl2, 1 mM DTT and 2 mM ATP. Data are expressed as % activity remaining compared to their respective control in each time set (*p <0.05).

PEA-dependent proteasome inhibition was also confirmed by the accumulation of ubiquitinated proteins and increase in the levels of other proteasomal substrates, such as IkBα and p27 in HeLa cells (Fig 5).

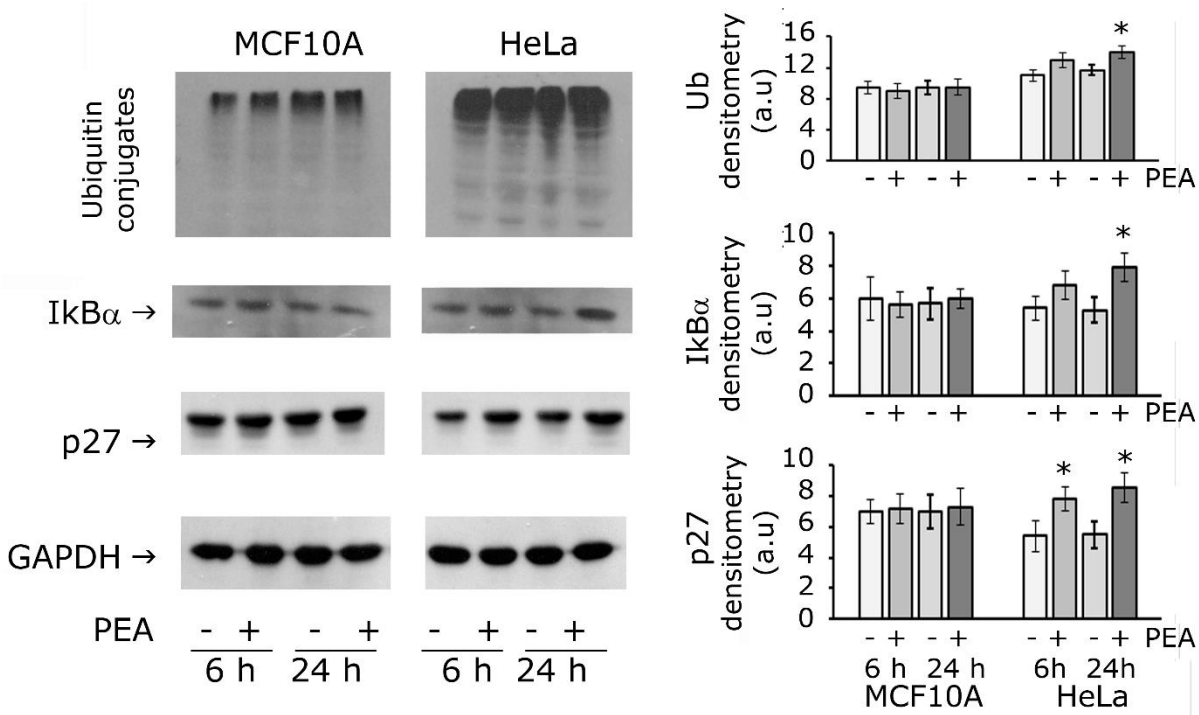

Fig. 5. Proteasome substrates accumulation in HeLa cells treated with PEA. Representative western blotting of proteasome substrates levels in MCF10 and HeLa cells treated with 10 μM PEA for 6 and 24 h. The densitometric analyses from six separate blots provided for quantitative analysis are presented. 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).

3.4 Caspase-3 activity

Caspase 3 is a crucial enzyme in apoptosis, since it catalyzes the proteolysis of many cellular regulatory proteins, finally resulting in DNA fragmentation. Caspase 3 activity was measured to elucidate the basis for the observed cellular death. Its activity was significantly enhanced selectively in HeLa cells at 6 and 24 h in the presence of 10 μM PEA (Fig. 6).

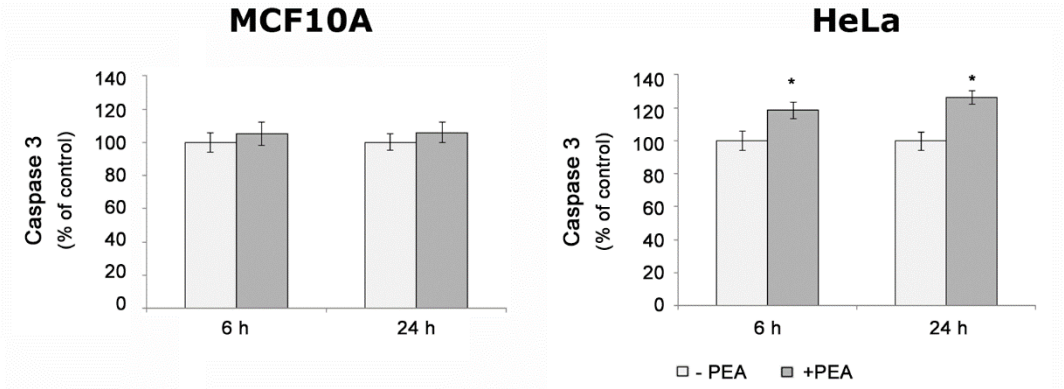

Fig. 6. Caspase 3 activity. Caspase-3 activity measured using the fluorogenic substrate Ac-Asp-Glu-Val-Asp-AMC. Data are expressed as percentage of the respective untreated control cells (100%) in both MCF10A (left) and HeLa (right) cell lines.

4. Discussion

The endogenous fatty acid amide palmitoylethanolamide (PEA) was initially considered acting mainly as an anti-inflammatory agent. In fact, recent data have demonstrated that PEA may play an important key role in the regulation of complex systems involved in the inflammatory response, pruritus, neurogenic and neuropathic pain [7]. PEA was suggested to exert its actions via several molecular mechanisms, including direct activation of peroxisome proliferator-activated receptor-α (PPAR-α), or indirect activation of cannabinoid receptors and transient receptor potential vanilloid type-1 (TRPV1) channels following potentiation of AEA activity at these targets [20]. However, its precise mechanism of action remains debated. The aim of the present study was to evaluate if PEA effects could also be mediated by the modulation of the proteasome activity, the major complex in charge of the removal of misfolded and oxidized proteins with a pivotal role in cell proliferation, apoptosis, inflammation and diseases onset and progression.

In a first phase, the investigation was conducted on isolated constitutive and immuno-proteasomes and successively on human cervical carcinoma cells (HeLa) and a normal counterpart (MCF10A). In detail, PEA was able to significantly modulate isolated proteasome activities in both a subunit- and concentration-dependent manner, exerting an inhibitory effect mainly on the BrAAP component of both complexes. HeLa cells viability upon PEA treatment was evaluated exposing cells to increasing concentration of PEA for 24, 72 and 48h. The amide was able to decrease cell survival in a concentration dependent way, 15 μM of PEA being the lowest concentration able to affect cell survival at 24h exposure. Tests on cell cultures were then performed using a concentration of PEA of 10 μM, as this treatment did not caused significant changes in cell survival.

Cellular proteasomes functionality, both 20 and 26S, resulted significantly inhibited upon PEA treatment. Comparing these results with those obtained treating isolated enzymes, we once more detected a subunit-dependent inhibition of the complex, but in this test the most affected activities resulted the T-L of the 20Sproteasome and 26S-ChT-L activity. The inhibition of the complex was further confirmed by the finding of accumulated proteasomal substrates including Ub-protein conjugates, IκBα and p27. Together, such results demonstrate for the first time that PEA mechanisms of action also include the modulation of the proteasome functionality.

In addition, the anti-inflammatory properties of PEA may also be mediated by its direct effects on the proteasome as it results from IκB-α accumulation.

As abovementioned, the proteasome plays a role in numerous cellular pathways, including apoptosis. In this study, we show that proteasome inhibition is accompanied by increased levels of p27 and IκBα, which are recognized apoptosis markers [21]. To better explore this pathway, we tested PEA effects on caspase-3 activity, an enzyme involved in the last step of the apoptotic cascade. As expected, a significant enhancement of the enzyme activity in HeLa cells at 6 and 24 h in the presence of 10 μM of the amide was observed. This result confirms that PEA, at the concentration used in the present study, is able to promote the activation of the apoptotic pathway selectively in tumor cells.

Concluding, in the present study we identify an additional mechanism of PEA action, precisely the induction of proteasome inhibition. Moreover, we demonstrate that PEA can affect tumor cells survival through the activation of apoptosis. This finding could represent an important preliminary step in considering PEA as a possible anti-cancer drug, however further studies are needed to better explore the involved pathways.

5. Acknowledgements

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