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Role of PTHrP in human intestinal Caco-2 cell response to oxidative stress

Virginia Lezcano, Claudia Gentili, Ana Russo de Boland *

Dept. Biología, Bioquímica & Farmacia, Universidad Nacional del Sur, Bahía Blanca, Argentina

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ABSTRACT

We have previously demonstrated that parathyroid hormone (PTH) induces apoptosis in human colon adenocarcinoma Caco-2 cells but the effects of its tumoral analog PTH-related peptide (PTHrP) in this cell line are still unknown. In the present work we investigated whether PTHrP, as PTH, is able to induce Caco-2 cell apoptosis or if it exerts protective effects under apoptotic conditions. Using Caco-2 cells cultured under serum deprivation in the presence or absence of PTHrP we demonstrated that, differently to PTH, its analog employed at the same concentration (10^{-8} M) is not a pro-apoptotic hormone. Cells were exposed to an oxidative insult in the form of hydrogen peroxide to induce apoptosis, which leads to a 50% loss of cell viability determined by MTS assay, morphological changes observed under fluorescence microscopy and Western blot analysis. Herein we demonstrate, for the first time, that pre-treatment with PTHrP prior to H_2O_2 incubation, prevents cell death induced by the apoptotic inductor; and using specific inhibitors we evidenced that protein kinase B (AKT), extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase 1/2 (JNK1/2) and p38 mitogen-activated protein kinase (MAPK) mediate this anti-apoptotic effect. Also, we found that PTHrP decreases the pro-apoptotic protein BAX levels and increases the protein expression of the anti-apoptotic HSP27. Immunoblot analysis revealed that H_2O_2 increases the phosphorylation levels of AKT and MAPKs, exhibiting a cellular defense response; and consequently increases phospho-BAD levels. The H_2O_2-induced activation of protein kinases is reverted when cells are pre-treated with PTHrP. Altogether these results evidence a protective effect of PTHrP under apoptotic conditions in intestinal cells, which may be mediated by AKT and MAPKs.

1. Introduction

Colorectal adenocarcinoma accounts for over 90% of the malignant tumors of the large bowel, and is the second most common cause of death from malignant disease in the Western world [1,2]. Cellular migration and invasion are critical for the ability of tumor cells to metastasize locally and to distant sites [3]. These properties are dependent on inherent tumor cell characteristics [4–6] and on the presence of several growth factors in the metastatic microenvironment. One of these factors is parathyroid hormone-related protein (PTHrP), whose expression correlates with the severity of colon carcinoma [7]. PTHrP was originally described as the factor responsible for the humoral hypercalcemia of malignancy [8]. PTHrP has limited homology to PTH in its N-terminal region and can bind the same receptor as PTH with similar biological effects. The PTH receptor (PTH1R) is found in a variety of tissues not regarded as classical PTH target tissues, including intestinal cells [9,10]. PTHrP exerts a much broader spectrum of effects in both normal physiology and disease states. The protein regulates cell growth, cell survival, smooth muscle relaxation and development [11,12]. Multiple studies demonstrate that PTHrP plays a major role in tumors that metastasize to the bone, such as breast and prostate cancer [13–15]. There is now increasing evidence that PTHrP also plays a role in cancers that metastasize to other regions of the body [16–20], such as colon tumors which show a preference for liver metastasis [2].

Apoptosis is especially relevant in the gastrointestinal tract, as this tissue undergoes a continued process of cell turnover that is essential for its normal function [21]. Defective apoptosis may allow the progression of disease and maintain the resistance of colon cancer cells to cytotoxic therapy [22]. Thus, it is of interest to elucidate the mechanism as well as the various physiological/pathological apoptotic inductors in the intestinal epithelium. We have previously demonstrated that PTH, at long term exposure and in a serum-deprived medium, induces apoptosis in Caco-2 cells expressing PTH1R [23]. However, the effects of its tumoral analog PTHrP in this intestinal cell line are still unknown. Taking in account that the gastrointestinal epithelium is prone to cancer development, particularly in the colon, understanding the role of PTHrP in this system may provide important information for the diagnosis and treatment of colon cancer. Since PTHrP inhibits or promotes apoptosis depending on the cell type involved [24–26], in the present work we

* Corresponding author at: Dept. Biología, Bioquímica & Farmacia, Universidad Nacional del Sur, San Juan 670, (8000) Bahía Blanca, Argentina. Tel.: +54 291 4595100x2430; fax: +54 291 4595130.
E-mail address: aboland@criba.edu.ar (A.R. de Boland).

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investigate whether PTHrP, as PTH, is able to induce Caco-2 cell apoptosis or if it exerts protective effects under apoptotic conditions.

2. Materials and methods

2.1. Materials

DAPI and MitoTracker Red (MitoTracker Red CMXRos) dyes were from Molecular Probes (Eugene, OR, USA). High glucose Dulbecco's modified Eagle's medium (DMEM) and synthetic human PTHrP (1–34) were from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina). Antibodies were from the following sources: anti-phopho-ERK1/2(Thr202/Tyr204), anti-ERK1/2, anti-SAPK/JNK, anti-phospho-SAPK/JNK(Thr183/Tyr185), phospho-p38 MAPK, anti-phospho-AKT(Ser473), anti-phospho-Bad(Ser112), anti-phospho-Bad(Ser136) and HSP27 were from Cell Signaling Technology (Beverly, MA, USA). Anti-AKT1/2/3, anti-p38α MAPK, anti-αtubulin, goat anti-mouse and goat anti-rabbit peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-BAX antibody was from Thermo Fisher Scientific (Rockford, IL, USA). Anti-actin antibody and Trypan blue dye were from Sigma (Sigma Chemical Co., St. Louis, MO, USA). PD98059 was from Tocris (Ellisville, MO, USA), and SB203580 and LY294002 were from Calbiochem (San Diego, CA, USA). CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit was from Promega (Madison, WI, USA). Protein size markers were from Amersham Biosciences (Piscataway, NJ, USA), and PVDF (Immobilon polyvinylidene difluoride) membranes and ECL chemiluminescence detection kit were from Amersham (Little Chalfont, Buckinghamshire, England). All other reagents used were of analytical grade.

2.2. Cell culture

The human colon cell line Caco-2 (from the American Tissue Culture Bank (Bethesda, USA)) was cultured at 37 °C in DMEM containing 20% FBS, 1% non-essential acids, 100 UI/ml penicillin, 100 mg/ml streptomycin and 50 mg/ml gentamycin in a humid atmosphere of 5% CO_2 in air. The treatments were performed with sub-confluent cultures in serum free medium by adding vehicle or PTHrP (10^{-8} M) for 1 to 5 days. When apoptotic induction was carried out, 0.5 mM H_2O_2 for 4.5 h was added. Where indicated, cells were pretreated for 30 min with one of the following inhibitors: LY294002, PD98059, SP600125 or SB203580. The inhibitors were also present during subsequent exposure to the hormone or the apoptotic inductor.

2.3. Annexin V staining for apoptosis positive cells and flow cytometry

After treatment, Caco-2 cells were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml. As a marker of cell death, phosphatidylserine exposure was measured by the binding of Annexin V–fluorescein isothiocyanate (FITC), by using FITC Annexin V Apoptosis Detection Kit I (BD Bioscience) according to the instructions of the manufacturer. For differentiation of apoptosis and necrosis, cells were also stained with propidium iodide (PI) to detect membrane integrity and were immediately analyzed with a FACScan flow cytometer (FACSCalibur, BD Biosciences). A minimum of 5000 events were collected on each sample by duplicate.

2.4. Cell viability assay

To determine the number of viable cells a CellTiter 96® AQueous One Solution Cell Assay (MTS) from Promega was used, which is based on the ability of viable cells to bioreduce the tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS) into a colored formazan product that is soluble in tissue culture medium. The intensity of the product color is directly proportional to the number of living cells in the culture. Cells were seeded in quadruplicate at 3×10^4 cells/well in 96-well plates. After treatment, 20 μl of MTS was added per well, followed by incubation for ~30 min at 37 °C. Absorbance was then measured at 490 nm with a spectrophotometer. To determine background absorbance, wells without cells were used as negative controls.

2.5. Trypan blue uptake

The percentage of trypan blue-positive cells in each culture condition was used to calculate cell survival. Nonadherent cells combined with adherent cells were released from the cultured dish using trypsin–EDTA, resuspended in medium containing serum, and collected by centrifugation. Subsequently, 0.4% trypan blue was added and the percentage of cells exhibiting both nuclear and cytoplasmic staining was determined using a hemocytometer. At least 100 cells/condition performed by quadruplicate were counted.

2.6. Western blot analysis

Proteins in homogenate preparations obtained from Caco-2 cells were quantified by the Bradford method [27]. Proteins dissolved in 6× Laemmli sample buffer were separated (~25 μg/lane) using SDS-polyacrylamide gels (10% acrylamide) and electrotransferred to PVDF membranes. After blocking with 5% non-fat milk in TBST buffer (50 mM Tris pH 7.2–7.4, 200 mM NaCl, 0.1% Tween 20), the membranes were incubated overnight with the appropriate dilution of primary antibody in TBST plus 1% non-fat milk. After washing, the membranes were incubated with the appropriate dilution of horse radish peroxidase-conjugated secondary antibody in TBST plus 1% non-fat milk. Finally, the blots were developed by using a chemiluminescence substrate and digitalized with a GS-700 Imaging Densitomer (Bio-Rad, Hercules, CA, USA).

2.7. Stripping and reprobing membranes

The complete removal of primary and secondary antibodies from the membranes was achieved by incubating the membranes in stripping buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS and 50 mM β-mercaptoethanol) at 55 °C for 30 min with agitation. Then, membranes were washed for 10 min in TBST (1% Tween-20) and blocked, as indicated above, for 1 h at room temperature. After that, membranes were ready to reprobe with the corresponding antibodies.

2.8. Cytochemistry

Cells grown on glass coverslips were stained for 1 h at 37 °C with 100 nM MitoTracker Red CMXRos (Molecular Probes) before fixation to visualize mitochondria [28]. Then cells were washed with PBS and fixed in 100% methanol (10 min, −20 °C). After washing, fixed cells were DAPI-stained for 30 min at room temperature in the darkness. Finally the coverslips were analyzed using an epifluorescent microscope.

2.9. Statistical analysis

The statistical significance of the data was evaluated using Student's *t* test [29], and probability values below 0.05 ($P < 0.05$) or 0.01 ($P < 0.01$) were considered significant or highly significant, respectively. Quantitative data are expressed as means ± SD from the indicated set of experiments.

3. Results

3.1. PTHrP is unable to induce apoptosis in Caco-2 cells

In early stages of apoptosis, cells lose membrane asymmetry and translocate the membrane phospholipid, phosphatidylserine, to the outer leaflet of the plasma membrane where it can be detected by its high-affinity binding to Annexin V. To explore the possibility that PTHrP promotes apoptosis in Caco-2 cells, we analyzed whether hormone-treated cells have morphological alterations typical of apoptosis. To that end, Caco-2 cells were incubated in a free-serum media with vehicle (control) or PTHrP 10^{-8} M for 1 or 3 days, and then incubated with FITC Annexin V in a buffer containing propidium iodide (PI) and analyzed by two-parameter flow cytometry analysis, to evaluate early and late apoptosis. As positive control of apoptosis, cells were exposed to an oxidative insult in the form of hydrogen peroxide (H_2O_2, 1 mM) for 2 h. As shown in Fig. 1A a great percentage of control cells are FITC Annexin V and PI negative, indicating that they are viable and not undergoing apoptosis after 3 days with serum deprivation. As expected, after H_2O_2 treatment, a large population of cells are FITC Annexin V and PI positive, indicating that they are in an end stage of apoptosis or already dead. However, the treatment with PTHrP does not show changes in comparison with their respective controls demonstrating that, differently to PTH, the PTH analog is not a pro-apoptotic agent in Caco-2 cells

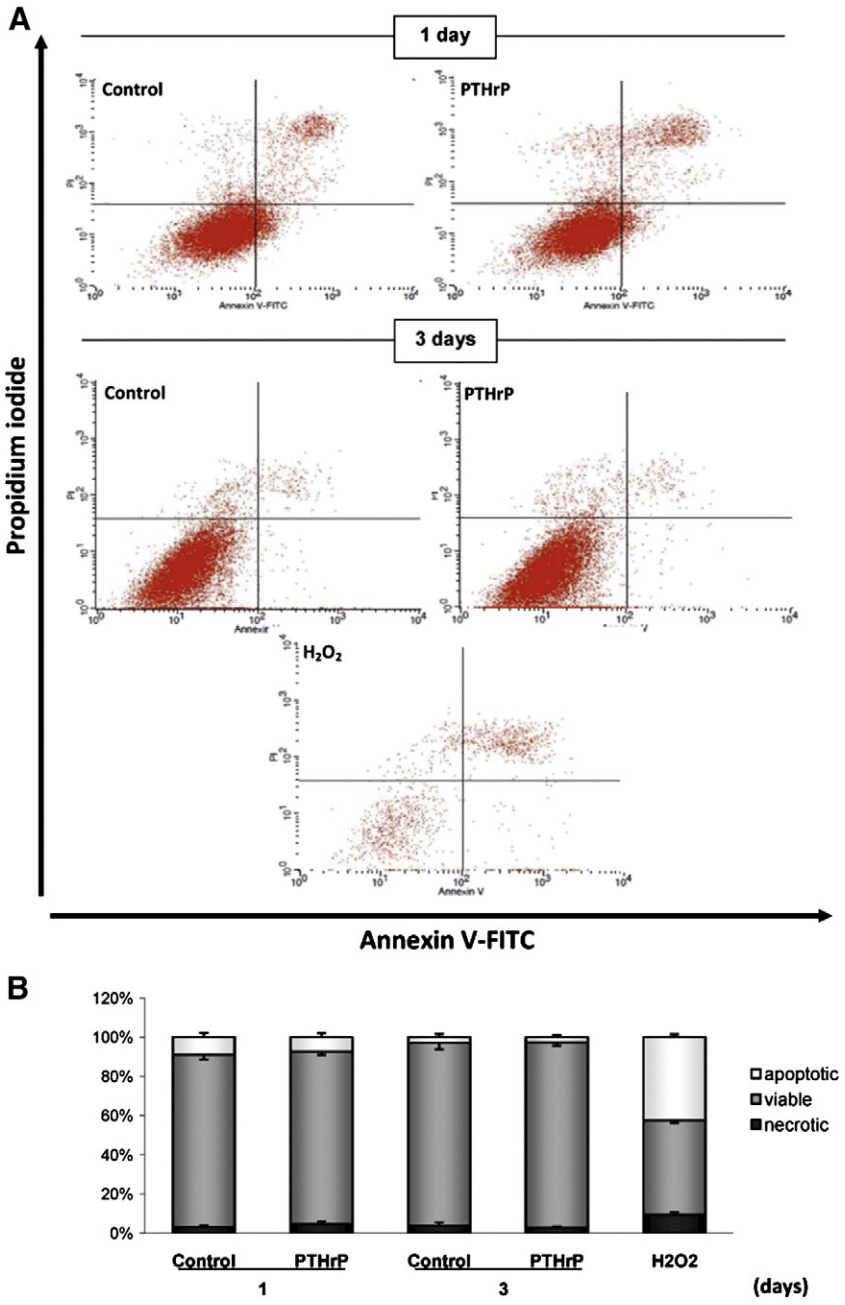

Fig. 1. Flow cytometry analysis of FITC-Annexin V staining. A) Caco-2 cells were treated with vehicle (control) or 10^{-8} M PTHrP for 1 and 3 days. Positive control cells were treated with 1 mM H_2O_2 for 2 h. Then were incubated with FITC-Annexin V in a buffer containing propidium iodide (PI) and analyzed by flow cytometry. The lower left quadrants of each panels show the viable cells, which exclude PI and are negative for FITC-Annexin V binding. The upper right quadrants contain the non-viable, necrotic cells, positive for FITC-Annexin V binding and for PI uptake. The lower right quadrants represent the apoptotic cells, FITC-Annexin V positive and PI negative, demonstrating cytoplasmic membrane integrity. One representative experiment out of three is shown. B) Quantification of each quadrant as percentage of total event collected ± SD.

under the conditions employed for this experiment. Fig. 1B shows the quantification of each quadrant as percentage of total event collected ± SD.

3.2. Loss of cell viability and activation of MAPKs and AKT induced by H_2O_2

In order to evaluate the probable anti-apoptotic effect of PTHrP in Caco-2 cells, we tested its action in the presence of an apoptotic stimulus. Reactive oxygen species such as H_2O_2 are involved in many cellular processes that positively and negatively regulate cell fate. We initially evaluated different concentrations and time points of incubation with H_2O_2 in Caco-2 cells to determine an optimum concentration to induce apoptosis. The results of Fig. 2A indicate that the effects of H_2O_2 on Caco-2 cells are both time and concentration dependent. Although a range of 2 to 8 h of treatment resulted in a significant decrease of cell viability, the studies reported in this work were performed with 4.5 h of incubation with 0.5 mM H_2O_2 for being a less aggressive apoptotic stimulus. The previous result is supported by microscopy images that show the typical mitochondrial and nucleus morphological changes observed in apoptotic cells. Control cells exhibit normal mitochondrial distribution and nucleus staining (Fig. 2B; upper panels), whereas H_2O_2 induced nuclear condensation and mitochondrial redistribution around the nucleus in apoptotic Caco-2 cells (Fig. 2B, lower panels).

Then, the proteins associated with survival events were studied by Western blot analysis to evaluate the response of Caco-2 cells to the apoptotic agent. As observed in Fig. 2C, using specific antibodies that recognize phospho-AKT, a downstream PI3-K effector, phospho-ERK1/2 and phospho-JNK MAPKs, we found that, after 2 h of 0.5 mM H_2O_2 treatment, cells exhibit a defense response with a significant increase in the activation/phosphorylation of ERK1/2 and AKT. However, at longer treatment times (4–8 h) with H_2O_2, a less pronounced effect in the phosphorylation of ERK1/2 and AKT is observed. Moreover, comparable effects on phospho-ERK1/2 were obtained when cells were treated with the apoptotic agent at 1 mM. JNK is also activated but with a different pattern, and in a time dependent way, being maximal between 6 and 8 h of hydrogen peroxide exposure.

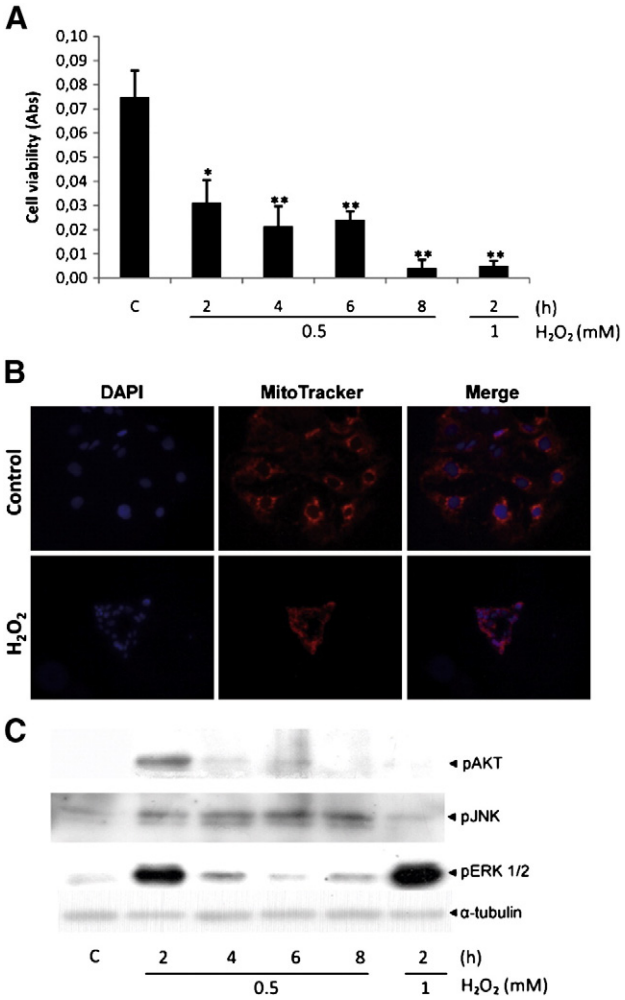

Fig. 2. Hydrogen peroxide (H_2O_2)-induced loss of cell viability. A) Caco-2 cells were exposed to 0.5 and 1 mM H_2O_2 and cell viability was measured by MTS assay over time. Values are expressed as mean ± SEM from 2 independent experiments performed in quadruplicate. Significance levels $^*p < 0.05$, $^{**}p < 0.01$ *vs.* control. B) Caco-2 cells grown on glass coverslips were incubated with vehicle (control) or H_2O_2 (0.5 mM) for 4.5 h and then stained with 100 nM MitoTracker Red and DAPI to evidentiate mitochondria and nucleus, respectively. Cell morphology was analyzed by epifluorescence microscopy. C) Caco-2 cell lysates were prepared and subjected to Western blot analysis using anti-phospho-AKT, anti-phospho-ERK and anti-phospho-JNK antibodies. The blots are representative of three independent experiments.

3.3. PTHrP prevents H_2O_2-induced apoptosis of Caco-2 cells

To investigate whether PTHrP exerts protective effects under apoptotic conditions, we performed the treatments as schematized in Fig. 3A for the subsequent experiments. After 1, 3 or 5 days of treatment with vehicle or 10^{-8} M PTHrP, the apoptosis inductor was added for an additional 4.5 h and then, the percentage of apoptotic cells was measured by means of trypan blue uptake as explained in Materials and methods. Pre-treatment of Caco-2 cells with the PTH analog for 3 and 5 days prevent by 40% and 48%, respectively, the apoptotic effect of hydrogen peroxide (Fig. 3C and D). In addition, and in agreement with our previous observation, these results demonstrate that PTHrP alone, in the absence of serum, is not able to induce apoptosis in Caco-2 cells after 1 to 5 days of exposure (Fig. 3B–D, 3rd lane).

3.4. Effect of PTHrP on BAX and HSP27 protein levels

Taking in account that there is a demonstrated correlation between the expression of heat shock proteins (HSPs) and increased cell survival, we evaluate through Western blot analysis the chaperone expression levels in response to PTHrP. As shown in Fig. 4, immunoblot analysis using an anti-HSP27 monoclonal antibody revealed that PTHrP increases the expression of HSP27 after 3 days of exposure (Fig. 4B). As expected, the incubation with the apoptotic stimulus (H_2O_2) decreases the expression of the anti-apoptotic protein HSP27 and, the pre-incubation of Caco-2 cells with PTHrP for 1 and 3 days prevents the decrease of HSP27 protein levels induced by H_2O_2 (Fig. 4A and B). After 5 days of pre-treatment with PTHrP, the levels of HSP27 are highly significant compared to both control and H_2O_2 conditions. The pro-apoptotic protein BAX, in physiological conditions is a cytosolic protein, however, upon apoptosis induction BAX inserts into the mitochondrial outer membrane, where it is thought to form supramolecular openings, alone or in association with other pro-apoptotic members, to permit the release of pro-apoptotic factors such as cytochrome *c*, the second mitochondrial activator of caspases SMAC/DIABLO and the apoptosis initiating factor (AIF), which activates caspases in the nucleus [30,31]. Employing a specific anti-BAX antibody we observed that PTHrP does not modify the protein expression levels after 1 day of exposure (Fig. 4A) but decrease BAX levels after 3 days of treatment (Fig. 4B, 3rd lane). Even more, under apoptotic conditions in the presence of H_2O_2, PTHrP exposure for 3 and 5 days significantly reduced BAX protein levels (Fig. 4B and C, 4th lane).

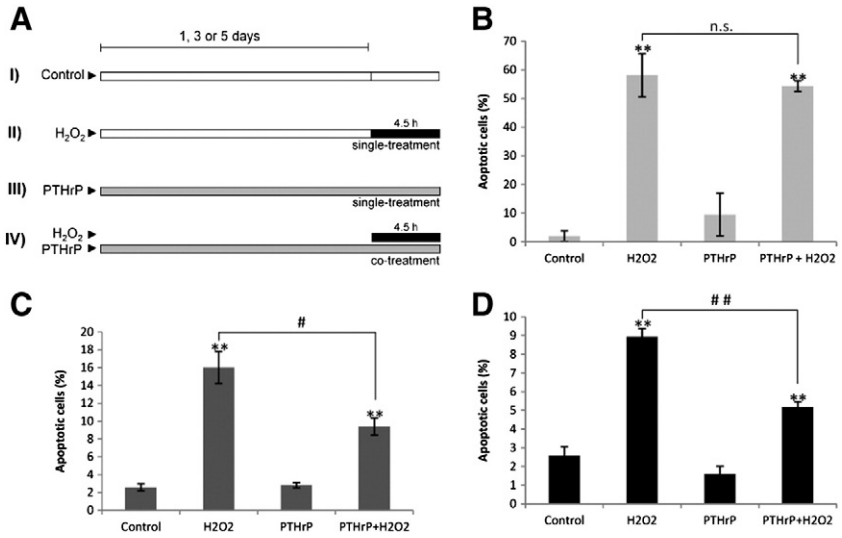

Fig. 3. PTHrP prevents H_2O_2-induced apoptosis of Caco-2 cells. A) Schematic diagram of the cell treatments for the following experiments. To investigate the effect of PTHrP in apoptotic conditions the assays were carried out in four groups: (I) Control; (II) H_2O_2 (0.5 mM–4.5 h); (III) PTHrP (10^{-8} M); (IV) H_2O_2 + PTHrP. Caco-2 cells were pretreated with vehicle or 10^{-8} M PTHrP for 1 (B), 3 (C) and 5 (D) days, and subsequently H_2O_2 was added for 4.5 h. The percentage of apoptotic cells was determined by trypan blue exclusion, as described in the Materials and methods. Bars represent the mean ± SEM of 3 independent experiments performed in quadruplicate. *P < 0.05 vs. control; **P < 0.01 vs. control; #P < 0.05 vs. H_2O_2. ##P < 0.01 vs. H_2O_2, n.s. = not significant.

3.5. PTHrP attenuates H_2O_2-dependent MAPKs and AKT activation

With the purpose of evaluating the signal transduction pathways involved in the anti-apoptotic effect of PTHrP on Caco-2 cells, we studied the phosphorylation/activation state of the pro-survival kinase AKT and the well known mitogen-activated protein kinases (MAPKs): ERK1/2, JNK1/2 and p38 MAPK. As observed in Fig. 5 (A–C), Caco-2 cells respond to the apoptotic agent (0.5 mM H_2O_2, 4.5 h) with a high increase in the phosphorylation levels of AKT and MAPKs. According to the previous results of this work that clearly demonstrate that H_2O_2 at the concentration employed is an apoptotic inductor, the activation of AKT and MAPKs suggests a cell defense response against the H_2O_2 injury. Differently to H_2O_2, PTHrP alone does not activate AKT and the MAPK pathways in Caco-2 cells, at none of the times probed (Fig. 5A–C, 3rd lane). Additionally, after 1 day of incubation with the hormone, no significant changes were observed in the expression levels of all the kinases studied among the different conditions (Fig. 5D). Similar results were obtained at 3 and 5 days of PTHrP exposure (data not shown).

Of relevance, under apoptotic conditions, when cells are pre-treated with the hormone for 3 days prior the exposure to H_2O_2, the levels of phosphorylation/activation of all these kinases decrease (Fig. 5B, 4th lane). As PTHrP exerts a protective effect, these conditions are less adverse than the cultures exposed to H_2O_2 alone and for this reason, it is not essential for Caco-2 cells to activate a defense mechanism. Thus, the activation of AKT and MAPKs seems to be unnecessary.

We next studied possible changes in the phosphorylation status of the pro-apoptotic protein BAD, a BH3 domain-containing protein, which forms heterodimers with Bcl-2 or Bcl-xl resulting in cytochrome *c* release from mitochondria [30]. As shown in Fig. 5 (E–G, 2nd lane) and, congruent with the above result, the Western blot analysis evidence that apoptosis induction by H_2O_2 increases the phospho-BAD levels at any time probed, while only the pre-incubation with PTHrP

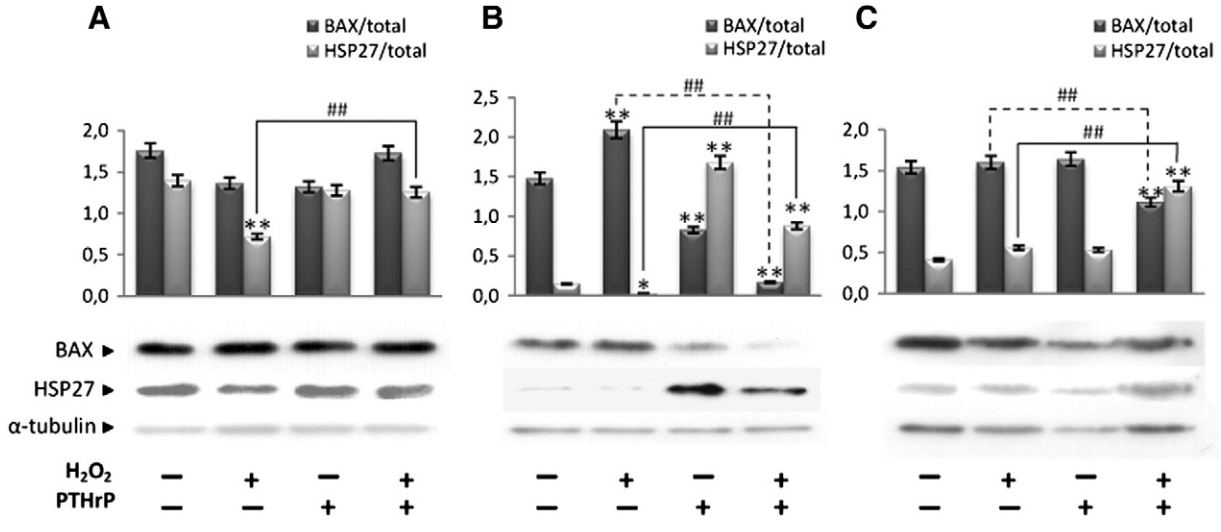

Fig. 4. Effect of PTHrP on Caco-2 cell BAX and HSP27 levels. Following the treatments explained in Fig. 3A Caco-2 cells were pre-treated with vehicle or 10^{-8} M PTHrP for 1 (A), 3 (B) and 5 (C) days, and subsequently H_2O_2 was added for 4.5 h. Cell lysates were prepared and subjected to Western blot analysis using antibodies that specifically recognizes the pro-apoptotic protein BAX, the heat shot protein of 27 kDa (HSP27), and alpha-tubulin as loading control. The blots are representative of three independent experiments. Densitometric quantification of each protein normalized with the loading control is shown in the corresponding bar graphics. *P < 0.05 vs. control; **P < 0.01 vs. control; ##P < 0.01 vs. H_2O_2.

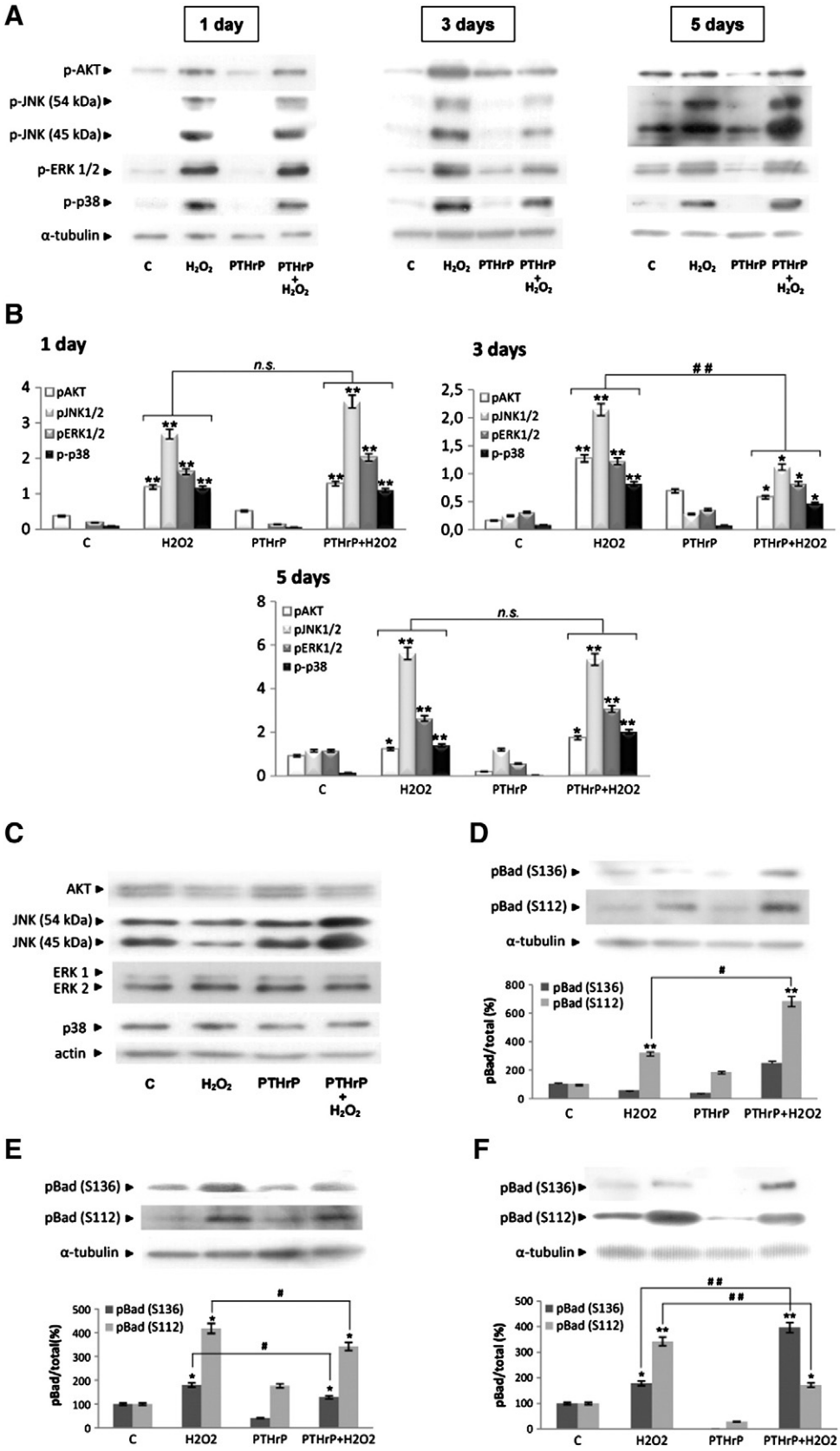

Fig. 5. PTHrP attenuates H_2O_2-dependent MAPK and AKT activation. Caco-2 cells were pre-treated with vehicle or PTHrP for 1 (A, D), 3 (A, E) or 5 days (A, F). Each condition was performed as explained in the scheme of Fig. 3A. Cell lysates were prepared and subjected to Western blot analysis using antibodies that specifically recognizes phospho-AKT, phospho-JNK1/2, phospho-ERK1/2, phospho-p38 MAPK (A), phospho-Bad (S112), phospho-Bad (S136) (D–F), and alpha-tubulin as loading control. The blots are representative of three independent experiments. Densitometric quantification of phosphorylated kinases (B) and phospho-Bad (D–F) normalized with loading control was performed and represented by the corresponding bar graphics. Total protein expression of kinases was evaluated with specific antibodies that recognize phosphorylated and not phosphorylated proteins (anti-AKT, anti-ERK1/2, anti-JNK1/2 and anti-p38); and anti-actin antibody was employed as loading control (C). *P < 0.05 vs. control; **P < 0.01 vs. control; #P < 0.05 vs. H_2O_2; ##P < 0.01 vs. H_2O_2; n.s. = not significant.

(3 days) showed a decrease in the phosphorylation levels at Ser136 and Ser112 (Fig. 5F, 4th lane), probably as a consequence of AKT inactivation. In addition, a longer PTHrP treatment (5 days) also decreases Bad phosphorylation at Ser112 but not at Ser136 (Fig. 5G, 4th lane).

3.6. Inhibition of apoptosis in Caco-2 cells by PTHrP is dependent on AKT and MAPK pathways

To examine the role of protein kinases as mediator of the anti-apoptotic effects of PTHrP, Caco-2 cells were pre-incubated with 50 μM LY294002, an inhibitor of AKT or 20 μM PD98059, SP600125 and SB203580, inhibitors of the MAP kinases ERK1/2, JNK1/2 and p38, respectively. Then, apoptosis was evaluated by trypan blue uptake, after challenging the colon adenocarcinoma human cells with H_2O_2 in the absence and presence of PTHrP. The effectiveness of the kinase inhibitors employed was confirmed by determining phosphorylated protein levels of AKT and MAPKs by Western blot (Fig. 6B–D). As shown in Fig. 6A (1st bar), as expected, H_2O_2 induces Caco-2 cell death (1.5-fold of control) and this effect is not modified by treatment with the inhibitors employed, except for SP600125 that allows a 0.7-fold increase of H_2O_2-induced apoptosis *vs.* control (Fig. 6A, 2nd bar), suggesting that AKT, ERK1/2, and p38 do not mediate the apoptosis induced by hydrogen peroxide, and that H_2O_2 may exert its apoptotic effects in Caco-2 cells *via* the JNK MAPK pathway [32]. Of relevance, the pre-treatment with AKT and MAPKs specific inhibitors completely reverses the anti-apoptotic effect of PTHrP, suggesting that these protein kinases participate in the mechanism of PTHrP necessary to prevent the apoptosis induced by oxidative stress in Caco-2 cells (Fig. 6A, 4th bar).

4. Discussion

Parathyroid hormone and PTH-related peptide share the same receptor (PTH receptor type 1, PTH1R); however they show a great variability in regard to target cells and intracellular signaling. Although PTHrP was initially characterized by its PTH-like activities, it must be considered as a peptide hormone on its own, with several newly discovered effects distinct from those exerted by PTH. We have previously reported the pro-apoptotic effect of PTH on Caco-2 cells [23] and herein, for the first time, we demonstrate that its tumoral analog PTHrP may exert a protective effect of Caco-2 cells under oxidative stress. PTH1R is a class b of G-protein coupled receptor (GPCR) with seven transmembrane domains (7TMR) that are bound and activated by PTH and PTHrP [33]. The PTH1R couples to Gs, Gq/11 and Gi/o and signals *via* adenylyl cyclase (AC) and phospholipase C (PLC) [34,35]. The fact that PTH and PTHrP exert opposing effects on apoptosis in Caco-2 cells even though they interact with the same receptor (PTH1R), could be explained because of cell-specific responses elicited by either ligand that are dependent of the complement of available G proteins and also, of cytosolic factors that are not G proteins but are

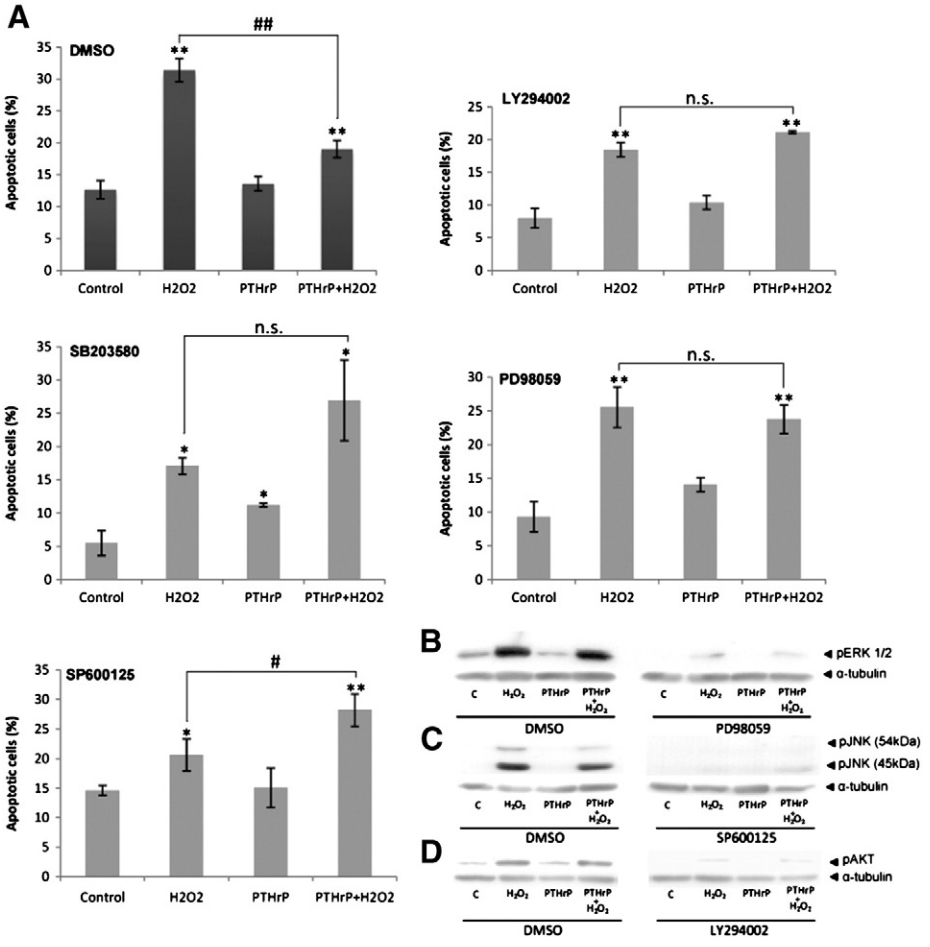

Fig. 6. Specific inhibition of MAPKs and AKT reverses the anti-apoptotic effect of PTHrP. A) Caco-2 cells were pre-treated with vehicle (DMSO) or kinase inhibitors (50 μM PI3-K inhibitor LY294002, 20 μM p38 MAPK inhibitor SB203580, 20 μM MEK-1 inhibitor PD98059 and 20 μM JNK inhibitor SP600125) for 30 min prior to treatment with vehicle (control) or PTHrP for 5 days, as explained in Fig. 3A. The percentage of apoptotic cells was estimated by Trypan blue uptake after 4.5 h of H_2O_2 incubation. Bars represent the mean ± SEM of 3 independent experiments performed in quadruplicate. *P < 0.05 vs. control; **P < 0.01 vs. control; #P < 0.05 vs. H_2O_2; ##P < 0.01 vs. H_2O_2; n.s. = not significant. The effectiveness of the kinase inhibitors employed was confirmed by Western blot analysis (B–D).

able to bind and regulate the activity of the PTH1R. The main signaling pathways by which GPCRs have normally been thought to function are by activation of heterotrimeric G proteins. Previous studies, however, have provided evidence for G-protein-independent signaling by PTH, the b2 adrenoceptor, vasopressin V2, and angiotensin AT1 receptors [36–43]. The potential for GPCRs to form complexes with signaling proteins other than G proteins also raises the possibility that agonists and antagonists can discriminate between these complexes in terms of both binding affinity and efficacy. Thus, each downstream signaling pathway measured in a particular cell type might have its own unique pharmacology depending on the pathway stimulated by each unique ligand–receptor conformation or complex involved.

Apoptotic cells can be recognized by its characteristic morphological changes, which are similar across cell type and species [44,45]. Assessment of cell death by detection of phosphatidylserine exposure and loss of membrane integrity, by combining Annexin V and PI staining with multiparameter flow cytometry, is a simple and reproducible method and one of the few techniques usually accepted as able to distinguish among viable, apoptotic, and necrotic cells [46]. In this work, we found that PTHrP, unlike PTH, and, in a serum-deprived medium, did not induce apoptosis in Caco-2 cells. It has been reported that apoptosis induced by serum deprivation is a late event in Caco-2 cells [47]. According to this, our data show that the percentage of Caco-2 apoptosis in control conditions after 1–3 days of serum deprivation is not significant. Apoptosis occurs in response to environmental or developmental cues, cellular stresses, and specific cell death signals. Reactive oxygen species such as hydrogen peroxide are involved in many cellular processes that positively and negatively regulate cell fate [48–51]. H_2O_2-mediated signaling alters the function of various proteins, including protein phosphatases, protein kinases, phospholipases, transcription factors, and ion channel proteins [52]. In this regard, through flow cytometry analysis we herein found that the response of Caco-2 cells to H_2O_2 is mainly apoptotic. Oxidative stress occurs when the production of oxidizing agents exceeds the antioxidant capacity of cellular antioxidants in a biological system. This imbalance between oxidants and antioxidants leads to tissue injuries and to the progression of degenerative diseases in humans, such as cancer [53,54]. Oxidative stress also leads to the development of other intestinal pathological conditions [55]. Therefore, it is important to study the damaging effects of oxidative stress on the intestinal epithelium and to understand protective mechanisms by which the cells respond to stress.

As we found that PTHrP is not a pro-apoptotic hormone in Caco-2 cells, we then investigated if it presents a protective effect under apoptotic conditions such as oxidative stress. Our results support other findings that show that the effects of H_2O_2 on Caco-2 cells are both time and concentration dependent [56,57]. In agreement with results obtained in other cell lines [57,58], our data suggest that stimulation with H_2O_2 activates MAPKs and AKT, signaling pathways that are thought to be a protective response against oxidative stress, in order to avoid apoptosis. Moreover, the apoptotic agent also triggers morphological features consistent with apoptosis. We found that PTHrP is capable of preventing Caco-2 cell apoptosis induced by H_2O_2 showing a highly significant response. In accordance with previous observations by flow cytometry, PTHrP alone did not induce apoptosis at any time of incubation probed, while H_2O_2 provoked 50% of Caco-2 cell apoptosis. Our data indicates that, even though PTH and PTHrP bind the same receptor, the long term exposure of these two hormones in Caco-2 cells cultured in serum-deprived medium, exert a totally opposite biological effect. PTH behaves as a pro-apoptotic hormone [23] and in this work we present evidence showing PTHrP-anti-apoptotic activity. PTHrP has been shown to have multiple and opposing roles in other circumstances, such as its ability to both protect and promote apoptosis in osteoblastic cells and pneumocytes [25,26,59]. This dual behavior may be based on novel 7 transmembrane receptor signaling mechanisms that are distinct from the classical G-protein second messenger-dependent pathways. The potential signaling diversity of 7TMRs also suggests the possible existence of multiple discrete "active" receptor conformations. This implies that specific ligands might direct distinct signaling responses by preferentially stabilizing one or more of these active conformations. The latter, may have significant implications both for understanding the molecular signaling mechanism of PTH/PTHrP as well as for the development of novel therapeutics for the treatment of various pathologies. For example, PTH stimulation can lead to anabolic or catabolic effects dependent upon intermittent or persistent exposure, respectively [36]. The regulatory mechanisms invoked in these contrary responses are incompletely understood.

Effects of PTHrP on apoptosis and survival have been noted in other cell lines not intestinal in origin. For example, PTHrP has been found to protect chondrocytes against apoptosis induced by serum depletion [60], protect cerebellar granule cells from kainic acid toxicity [61], and protect prostate cells against phorbol-induced apoptosis [62]. Even more, the peptide has been recently shown to reduce apoptosis mainly through the paracrine pathway in messangial cells [63]. However, this is the first report, to our knowledge, that demonstrates an anti-apoptotic effect of PTHrP on Caco-2 intestinal cells. The exact mechanisms underlying the ability of PTHrP to prevent apoptosis in Caco-2 cells remain to be clarified. Our initial attempts to address this question in the present study suggest that the hormone may alter the expression of HSP27 and BAX, an anti-apoptotic protein regulator and a pro-apoptotic regulator, respectively. A correlation between the expression of heat shock proteins and increased cell survival was already shown, pointing them as regulatory agents of components of apoptotic pathways [64–66]. HSPs are a group of evolutionarily conserved proteins with diverse functions, including anti-apoptosis [67], mitochondrial protection [68], prevention of Ca^{2+} disturbances [69], and protection against oxidant-induced injury [59]. HSPs have been demonstrated to exert these diverse cytoprotective effects in many tissues, including the colon [70,71]. In our cell system we demonstrated an increment of HSP27 protein expression in response to PTHrP treatment. It has been reported that HSP27 decreases apoptosis by inhibiting BAX [72]. In agreement with this observation, we found that PTHrP decreased BAX protein expression simultaneously with the increase of HSP27 expression. Interestingly, both anti-apoptosis and molecular changes occur since 3 days of PTHrP treatment, resulting in a more significant biological effect after a longer time of peptide exposure. Another important finding identified in our study, is that H_2O_2 induces phosphorylation of AKT, ERK1/2, JNK1/2 and p38 MAPK, indicating that Caco-2 cells exert a defense response against the apoptotic agent. The activation of AKT, ERK1/2 and p38 MAPK may not be involved in the H_2O_2-induced intestinal epithelial cell apoptosis since the inhibition of these pathways using specific inhibitors did not attenuate the apoptotic response. However, the inhibition of the JNK pathway decreased the apoptosis induced by H_2O_2, indicating that in these cells the apoptotic agent may be exerting its effects *via* JNK.

The function of the pro-apoptotic molecule BAD is regulated by the phosphorylation of two sites. It has been shown that phosphorylation/inactivation of BAD at Ser-136 is mediated by the serine/threonine protein kinase AKT-1 and that the signaling process leading to phosphorylation of BAD at Ser-112 is mediated by MAPKs [73]. In Caco-2 cells, we observed the phosphorylation of the pro-apoptotic protein BAD, as a consequence of AKT and MAPKs activation, which is in agreement with observations made in other cell lines [58]. Under the experimental conditions employed, we observed that PTHrP alone did not induce the activation of AKT and MAPKs but, interestingly, this hormone has the ability to revert the activation of the kinases induced by H_2O_2, without modifying its total protein expression. When Caco-2 cells are pretreated with PTHrP prior to the exposure to the apoptotic agent, the conditions are less adverse than the cells treated with H_2O_2 alone and thus, it is not essential for cells to activate a defense mechanism, because of the protecting effect of the peptide.

Altogether these results indicate that Caco-2 cells activate a defense response under oxidative stress, whereas the programmed cell death finally starts, and it is at 3 days previous of that effect that PTHrP carries out its protective action against apoptosis.

Finally, when PI3K/AKT and MAPKs pathways were blocked with specific pharmacological inhibitors, we found a complete reversion of the anti-apoptotic action of PTHrP, suggesting that these protein kinases are involved in the mechanism necessary for PTHrP to prevent H_2O_2-induced apoptosis.

These results suggest that PTHrP action might contribute to colon cancer progression. However, additional studies are necessary to further elucidate the signaling mechanisms which mediate the anti-apoptotic action of PTHrP in intestinal Caco-2 cells. Strategies aimed at decreasing PTHrP production or blocking PTHrP action in colon cancer may thus provide therapeutic benefits.

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