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PTH inactivates the AKT survival pathway in the colonic cell line Caco-2

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ABSTRACT

In previous works, we found that PTH promotes the apoptosis of human Caco-2 intestinal cells, through the mitochondrial pathway. This study was conducted to investigate the modulation of different players implicated in the AKT survival pathway in PTH-induced intestinal cell apoptosis. We demonstrate, for the first time, that PTH modulates AKT phosphorylation in response to apoptosis via the serine/threonine phosphatase PP2A. PTH treatment induces an association of AKT with the catalytic subunit of PP2A and increases its phosphatase activity. PTH also promotes the translocation of PP2Ac from the cytosol to the mitochondria. Furthermore, our results suggest that PP2A plays a role in hormone-dependent Caco-2 cells viability and in the cleavage of caspase-3 and its substrate PARP. The cAMP pathway also contributes to PTH-mediated AKT dephosphorylation while PKC and p38 MAPK do not participate in this event. Finally, we show that PTH induces the dissociation between 14-3-3 and AKT, but the significance of this response remains unknown. In correlation with PTH-induced Bad dephosphorylation, the hormone also decreases the basal association of 14-3-3 and Bad. Overall, our data suggest that in Caco-2 cells, PP2A and the cAMP pathway act in concert to inactivate the AKT survival pathway in PTH-induced intestinal cell apoptosis.
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1. Introduction

Apoptosis is a form of programmed cell death that is characterized by specific morphological and biochemical features, and is tightly regulated by extracellular stimuli and intracellular signaling pathways [1]. There are two distinct signaling pathways of apoptosis in mammals. One is initiated by death receptors (death receptor pathways) [2], and the other is regulated by anti- and pro-apoptotic Bcl-2 family members and involves release of cytochrome *c* from mitochondria into the cytoplasm (mitochondrial pathways) [3,4]. It has been suggested that the balance of pro- and anti-apoptotic members of the Bcl-2 family determines life or death in a cell [5]. Indeed, the regulation of mitochondrial cytochrome *c* release and mitochondrial function by several Bcl-2 family members plays a large part in controlling cell death [3]. Bad, one of the pro-apoptotic family members, is the first target of AKT, a major inhibitor of apoptosis [6]. By phosphorylating Bad, AKT precludes its binding to the Bcl-xL and Bcl-2 anti-apoptotic family members leading to increased cell survival. AKT, a serine/threonine protein kinase, is a key component of cell survival pathways and is a major downstream target of phosphoinositide 3-kinase (PI3K) [7]. Activation of PI3K leads to AKT activation by both phosphoinositide-dependent protein kinase-1 (PDK1), which phosphorylates AKT at Thr-308, and PDK1-independent mechanisms, which result in AKT phosphorylation at Ser-473 [7].

AKT phosphorylation is tightly regulated, representing a balance between kinase-activating and phosphatase-inactivating events. Several protein phosphatases, including the dual-action phosphatase PTEN, which transforms PtdIns(3,4,5)P_3 into PtdIns(4,5)P_2 [8], canonical PP1 [9,10] and PP2A [11–13] as well as newly identified AKT phosphatases [14,15] were recently reported to bind and dephosphorylate AKT in an agonist-dependent manner.

Mammalian PTH is an 84-amino acid single-chain polypeptide, although only the first 34 amino acids are required for most biological effects [16,17]. The PTH receptor (PTH1R) is found in a variety of tissues not regarded as classical PTH target tissues, including intestinal cells [18,19]. In intestinal cells, PTH initiates its effects by interacting with its PTHR1 that, like other members of the class II family of GPCRs, is capable of coupling to several different G proteins, thereby activating multiple signaling pathways, including adenylyl cyclase/cAMP [20], PLC [21], cytoplasmic Ca^{2+} [22] and the MAP kinases ERK1/2 [23] and p38 MAPK [24].

Apoptosis is especially relevant in the gastrointestinal tract, as the mammalian intestinal mucosa undergoes a process of continual cell turnover that is essential for maintenance of normal function [25]. Defective apoptosis may allow the progression of disease and maintain the resistance of colon cancer cells to cytotoxic therapy [26], again illustrating the importance of apoptosis in the gastrointestinal tract. Thus, it is of interest to know the mechanism as well as the various physiological inducers of apoptosis in the intestinal epithelium. Since PTH, depending on the cell type involved, inhibits or promotes the apoptosis [27–29], we therefore used Caco-2 cells, a cell line derived from human colorectal adenocarcinoma, as an in vitro model for studying the effects of PTH on gut cell apoptosis. In a

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previous work we found that PTH promotes the apoptosis of these intestinal cells [30], which is mediated by translocation of mitochondria to the perinuclear region, dephosphorylation of AKT, dephosphorylation of Bad and its movement to the mitochondria and subsequent release of cytochrome *c* and Smac/Diablo which result in activation of downstream caspase-3 and degradation of its substrate PARP [30,31].

However, relatively little is known about how specific protein tyrosine and serine/threonine phosphatases function to regulate PTH-mediated apoptosis.

This study was conducted to investigate the modulation of different players implicated in the AKT survival pathway in PTH-induced intestinal cell apoptosis.

2. Materials and methods

2.1. Materials

Human PTH (1–34) was obtained from Calbiochem (San Diego, CA, USA). High glucose Dubelcco's modified Eagle's medium (DMEM) was from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina).

Antibodies were from the following sources: anti-Bad, anti-phospho-AKT (S473) anti-PP2Ac and anti-PARP were from Cell Signaling Technology (Beverly, MA, USA). Anti-AKT1/2/3, anti-σ14-3-3, anti-caspase-3, anti-cytochrome *c*, anti-lamin B and goat anti-rabbit peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 488 conjugated-anti-rabbit antibody was from Molecular Probes. Anti-actin antibody, okadaic acid sodium and *p*-nitrophenyl phosphate (*p*-NPP) were from Sigma (Sigma Chemical Co. St. Louis, MO, USA). Ro-31-8220, Rp-cAMP (cyclic adenosine 3′,5′-mono-phosphorothioate, Rp diastereomer) and SB 203580 were from Calbiochem (San Diego, CA, 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 and treatment

The human colon cell line Caco-2 (from the American Tissue Culture Bank (Bethesda, USA)) was cultured at 37 °C in DMEM containing 10% 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. Cultures were passaged every 2 days with fresh medium. The treatments were performed with 70% confluent cultures in serum free medium by adding PTH (10^{-8} M) for 48 h. Where indicated, cells were pretreated for 30 min with one of the following inhibitors: okadaic acid sodium, LY 294002, Ro-31-8220, Rp-cAMP or SB 203580. The inhibitors were also present during subsequent exposure to the hormone.

2.3. Western blot analysis

Caco-2 cells were washed with PBS buffer plus 25 mM NaF and 1 mM Na_3VO_4, and lysed in buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 3 mM KCl, 1 mM EDTA, 1% Tween-20, 1% Nonidet P-40, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM NaF and 1 mM Na_3VO_4. The lysates were incubated on ice for 10 min, vortexed for 45 s and maintained on ice for another 10 min. After centrifugation at 14,000×g and 4 °C during 15 min the supernatant was collected and proteins were quantified by the Bradford method [32]. Lysate proteins dissolved in 6× Laemmli sample buffer were separated (25 μg/lane) using SDS–polyacrylamide gels (10% or 15% 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 with 1% non-fat milk. After washing, membranes were incubated with the appropriate dilution of horse-radish peroxidase-conjugated secondary antibody in TBST with 1% non-fat milk. Finally, the blots were developed by ECL with the use of Kodak BioMax Light film and digitalized with a GS-700 Imaging Densitomer (Bio-Rad, Hercules, CA, USA).

2.4. 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.

2.5. Co-immunoprecipitation

Co-immunoprecipitation assays were performed under native conditions in order to preserve protein–protein associations. To that end, after hormone treatment, the cells were lysed in buffer A (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 3 mM KCl, 0.5 mM EDTA, 0.2 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 6 μg/ml leupeptin, 8 μg/ml aprotinin, and 1% Tween-20). After centrifugation at 14,000×*g* (4 °C, 15 min) the supernatant was collected and proteins were quantified by the Bradford method [32]. Lysate aliquots (200–500 μg protein) were incubated overnight at 4 °C with anti-Bad, anti-14-3-3, anti-AKT or anti-PP2Ac antibodies, followed by precipitation of the complexes with protein A conjugated with Sepharose. The immune complexes were washed three times with buffer A (14,000×g, 4 °C, 5 min) and then subjected to Western blot analysis. To confirm the association of both proteins, immunoprecipitation and immunoblotting were performed with the same antibodies used in reverse order.

2.6. Protein phosphatase 2A (PP2A) activity assay

Cell lysates (200 μg protein) were incubated overnight at 4 °C with anti-PP2Ac antibody, followed by precipitation of the complexes with protein A conjugated with Sepharose. After three washes with lysis buffer and two washes with a serine/threonine assay buffer (50 mM Tris–HCl, pH 7.0, 100 μM $CaCl_2$), the immune complexes were incubated at 37 °C for 10 min in Ser/Thr assay buffer (45 μl/sample) containing *p*-nitrophenyl phosphate as an exogenous substrate for PP2A (5 μl/sample). The reaction was terminated by the addition of NaOH 1N and the color developed was quantified by measuring the absorbance with a spectrophotometer at 405 nm against a reagent blank. The enzyme activity was expressed as percentage of the activity respect of control.

2.7. Cell proliferation assay

The MTS assay (Promega) 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)-2*H*-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 96-well plates at a density of 2×10^3 cells per well. After each experiment, 20 μl MTS was added to each well, followed by incubation for 45 min at 37 °C. Absorbance was then measured with a spectrophotometer at 490 nm. The results were expressed as a percentage of the mean

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