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The Early Phase of Programmed Cell Death in Caco-2 Intestinal Cells Exposed to PTH

Natalia G. Calvo, Claudia R. Gentili, and Ana Russo de Boland*

Department Biología, Bioquímica & Farmacia, Universidad Nacional del Sur, Bahia Blanca, Argentina

ABSTRACT

The regulation of apoptosis is critical for ensuring the homeostasis of an organism. As such, the cell has derived various mechanisms to precisely control the balance between survival and apoptotic signaling. Parathyroid hormone (PTH) function as a major mediator of bone remodeling and as an essential regulator of calcium homeostasis. Depending on the cell type involved, PTH also inhibits or promotes the apoptosis. In a previous work we found that PTH promotes the apoptosis of human Caco-2 intestinal cells. In the current study, we demonstrate, for the first time, that stimulation of Caco-2 cells with PTH (10^{-8} M) results in the dephosphorylation and translocation of pro-apoptotic protein Bad from the cytosol to mitochondria and release of cytochrome *c* and Smac/Diablo. The hormone also triggers mitochondria cellular distribution to the perinuclear region, morphological features consistent with apoptosis. PTH increases the enzymatic activity of caspase-3 (48 h) that is also evidenced from the appearance of its cleaved fragments in western blot experiments. Moreover, active caspase-3 is present in nucleus after PTH treatment. In addition, a caspase-3 substrate, poly (ADP-ribose) polymerase (PARP), is degraded by 48 h of PTH treatment. Taken together, our results suggest that, in Caco-2 cells, the induction of apoptosis in response to PTH 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. J. Cell. Biochem. 105: 989–997, 2008.

KEY WORDS: PTH; Caco-2 CELLS; APOPTOSIS

Parathyroid hormone (PTH) is a major mediator of bone remodeling and an essential regulator of calcium homeostasis. Very small decrements in serum calcium levels induce the secretion of PTH from the parathyroid glands initiating a rapid response to raise serum calcium levels by acting directly on kidney and bone or indirectly on intestine (via $1,25(OH)_2$ vitamin D_3) facilitating calcium absorption [Potts et al., 1997; Silverberg et al., 1999]. Mammalian PTH is an 84-amino acid single-chain polypeptide, although only the first 34 amino acids are required for most biological effects [Gensure et al., 2005; Murray et al., 2005]. Detailed structure-function analysis of the PTH(1–34) ligand demonstrated the importance of its extreme N terminus for activation of adenylyl cyclase [Gardella et al., 1991] and of its C terminus for high-affinity receptor binding [Jüppner et al., 1994]. PTH(1–34), as well as PTH(1–84), could activate other signal transduction pathways, independently of adenylyl cyclase, including those involving phospholipase C (PLC), protein kinase C (PKC)(s), cytosolic free Ca^{2+}, phospholipase D, and phospholipase A2 [Hruska et al., 1987; Yamaguchi et al., 1987; Fujimori et al., 1992; Derrickson and Mandel, 1997; Singh et al., 1999]. PTH also regulates MAP Kinases, including p42/p44 ERKs, p38 and c-Jun N-terminal kinase subtypes, although the direction of this regulation and its mediation by more proximal effectors such as cAMP/PKA and PKC, appears to depend on cell type [Cole, 1999; Swarthout et al., 2001; Zhen et al., 2001; Doggett et al., 2002; Kaiser and Chandrasekhar, 2003]. The PTH receptor (PTH1R) is highly expressed in bone and kidney, but is found also in a variety of tissues not regarded as classical PTH target tissues, including intestinal cells [Urena et al., 1993; Gentili et al., 2003]. In intestinal cells, the PTH1R, 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 [Massheimer et al., 2000], PLC [Gentili et al., 2000], cytoplasmic Ca^{2+} [Picotto et al., 1997] and the MAP kinases ERK1/2 [Gentili et al., 2001] and p38 MAPK [Buzzi et al., 2007].

Apoptosis is genetically programmed cell death that is essential for development, the maintenance of tissue homeostasis, and the

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*Correspondence to: Dr. Ana Russo de Boland, Department Biologia, Bioquimica & Farmacia, Universidad Nacional del Sur, San Juan 670, (8000) Bahia Blanca, Argentina. E-mail: aboland@criba.edu.ar

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elimination of unwanted or damaged cells from multicellular organisms. It is characterized by distinct morphological changes including plasma membrane blebbing, cell shrinkage, depolarization of the mitochondria, chromatin condensation, and DNA fragmentation. The mammalian signal transduction pathways that mediate apoptosis, although under intense scrutiny, remain incompletely understood. Recently, it has become apparent that apoptosis is a crucial process in skeletal development and homeostasis. The reported effects of PTH on apoptosis appear to be dependent upon the cell culture model [Zerega et al., 1999; Turner et al., 2000] and the differentiation state of the cells [Chen et al., 2002]. PTH has been found to be anti-apoptotic in pre-confluent mesenchymal cells as opposed to its pro-apoptotic effects in more differentiated post-confluent cells [Chen et al., 2002].

The intestine is lined by a single-layered epithelium and represents one of the most rapidly proliferating tissues in the body. Stem cells at the base of the crypts generate a population of mitotically active crypt cells. As those cells migrate up the adjacent villus, they undergo cell cycle arrest and subsequent differentiation [Potten and Loeffler, 1990]. Under normal conditions, the majority of these differentiated enterocytes are removed by apoptosis occurring throughout the villus and by shedding at the tip of the villus [Hall et al., 1994; Potten et al., 1997; Potten, 1997]. A dynamic balance between cell proliferation at the crypt and cell loss through apoptosis or exfoliation maintains the integrity of the gut epithelium.

Using the Caco-2 cell line, as an in vitro model of human small intestinal enterocytes, we previously obtained evidence that PTH treatment, in a serum-free medium, diminishes the number of viable cells, induces disruption of actin filaments with changes of cellular shape, alteration of cell-to-cell junctions, externalization of membrane phosphatidylserine and also chromatin condensation and DNA fragmentation of the nucleus, morphological features consistent with apoptosis (Calvo et al., submitted). In this study, we further explored the mechanism involved in PTH-induced apoptosis in Caco-2 intestinal cells.

MATERIALS AND METHODS

MATERIALS

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

Antibodies were from the following sources: Bad, phospho-specific Bad (S112, S136), phospho-Akt (S473) and anti-Smac/Diablo were from Cell Signaling Technology (Beverly, MA). Polyclonal antibody against cytocrhome *c*, anti-caspase-3, anti-Akt, anti-Bcl-2 and to the C-terminus of poly-(ADP-ribose) polymerase (PARP), goat anti-rabbit and anti-mouse peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488 conjugated-anti-rabbit and Alexa Fluor 647 conjugated-anti-mouse antibodies were from Molecular Probes. Anti-actin antibody was from Sigma.

Protein size markers were from Amersham Biosciences (Piscataway, NJ), 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.

CELL CULTURE AND TREATMENT

The human colon cell line Caco-2 (from the American Tissue Culture Bank (Bethesda)) was cultured at 37°C in DMEM containing 10% FBS, 1% non-essential acids, penicillin 100 UI/ml, streptomycin 100 mg/ml and gentamycin 50 mg/ml 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 and 72 h.

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 g/ml aprotinin, 20 g/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 [Bradford, 1976]. Lysate proteins dissolved in 6× Laemmli sample buffer were separated (25 g/lane) using SDS–PAGE (10–15% 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 plus 1% non-fat milk. After washing, 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 ECL with the use of Kodak BioMax Light film and digitalized with a GS-700 Imaging Densitomer (Bio-Rad, Hercules, CA).

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 -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 re-probe with the corresponding antibodies.

INMUNOCYTOCHEMISTRY

Caco-2 cells grown onto glass coverslips were fixed in methanol (at −20°C) for 15 min. After washing with PBS, non-specific sites were blocked with PBS, 5% BSA. Samples were then incubated with the appropriate primary antibody prepared in PBS, 2% BSA (1:50, overnight). After washing with PBS, the samples were incubated with secondary Alexa Fluor 488 conjugated antibody (1:200, 1 h, room temperature) or Alexa Fluor 647 conjugated antibody (1:250, 1 h, room temperature). Cells were washed with PBS and mounted.

Where indicated, cells were stained with MitoTracker Red CMXRos, (Molecular Probes) before fixation to visualize mitochondria [Minamikawa et al., 1999]. It can be used to stain active mitochondria and is retained after fixation. The samples were examined using a Leica TCS SP2 AOBS confocal laser microscope.

CASPASE ACTIVITY ASSAY

The activity of caspase-3 was measured using the substrate DEVD-aminoluciferin from Caspase-Glo^{TM} 3/7 assay kit (Promega) following the manufacturer's instructions. The luminescence signal was detected by a Wallac 1420 Multilabel counter.

SUBCELLULAR FRACTIONATION

Cells were washed with PBS, resuspended in ice-cold TES buffer (50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose containing protease inhibitors) homogenized in a Teflon-glass hand homogenizer (30 stokes) and then centrifuged at 3,500 rpm for 20 min (4°C) to pellet the nuclei and unbroken cells. The supernatant was centrifuged at 12,000 rpm for 20 min (4°C) to pellet mitochondria. The resulting supernatant is the cytosolic fraction. The purity of each isolated fraction was assessed by assaying for proteins known to be associated with cellular components. Equal proteins from each fraction were subjected to SDS–PAGE.

STATISTICAL EVALUATION

Statistical significance of data was evaluated using Student's *t*-test and probability values below 0.050 ($P < 0.050$) were considered significant. Quantitave data are expressed as the means ± SD from the indicated set of experiments.

RESULTS

In a previous study (Calvo et al., submitted) we found that PTH promotes the apoptosis of human Caco-2 intestinal cells. In the present work we studied the mechanism by which PTH induces apoptosis in these cells. Bad is a pro-apoptotic factor that interacts with and inhibits the anti-apoptotic protein Bcl-2. MAPK as well as other kinases target Bad and phosphorylation of Ser 112 by ERK1/2 inhibits the pro-apoptotic functions of Bad. To investigate the role of this protein in PTH-induced pro-apoptotic effects, we first evaluated whether the hormone induces changes in serine phosphorylation of Bad. Caco-2 cells were exposed to PTH 10^{-8} M (48 and 72 h) followed by Western blot analysis of cell lysates with phospho-specific antibodies against Bad- Ser 112 and -Ser 136 residues. As shown in Figure 1A,B, PTH induces the dephosphorylation of

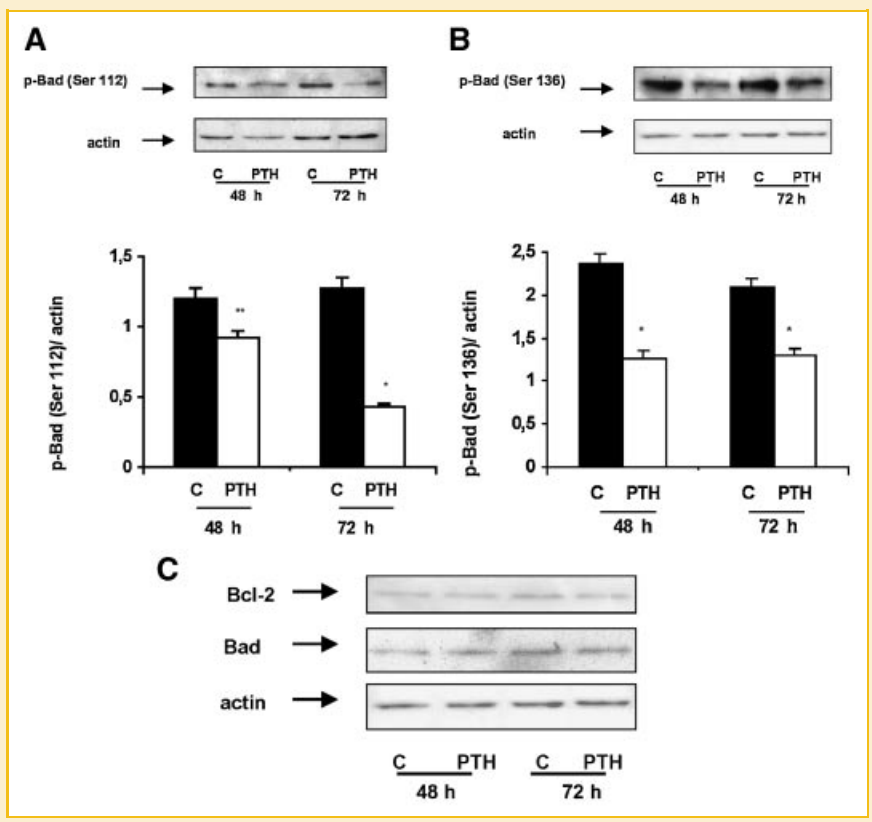

Fig. 1. PTH induces the dephosphorylation of Bad. Caco-2 cells were exposed to PTH 10^{-8} M (48 and 72 h) followed by Western blot analysis of cell lysates with phospho-specific antibodies against Ser 112 and Ser 136 residues. In order to evaluate the equivalence of protein content among the different experimental conditions, blotted membranes were re-probed with anti- actin antibody. Representative image and bar graph of phospho ser 112 Bad (A) and phospho ser 136 Bad (B) quantified by scanning densitometry are shown. $^{*}P < 0.025$, $^{**}P < 0.05$ with respect to the corresponding control. C: Expression levels of Bad and Bcl-2 proteins in Caco-2 cells. Cell lysates from control and PTH treated cells were immunoblotted with anti-total Bad antibody and the same immunoblot was re-probed with anti-Bcl-2 antibody. -actin was used as the loading control. A representative immunoblot from three independent experiments is shown.

both Ser residues at 48 h, with maximal effects at 72 h. In order to evaluate the equivalence of protein content among the different experimental conditions, blotted membranes were re-probed with anti-actin antibody. In the presence of serum we found that, after 48 and 72 h of treatment, PTH (10^{-8} M) does not modify the phosphorylation of Bad (data not shown). The amount of protein expression determined by Western blot analysis for total Bad was no different in the absence or presence of PTH (48 and 72 h). Furthermore, no changes were observed when total Bcl-2 was measured in the same immunoblot by stripping the membranes and reincubating with anti-Bcl-2 antibody (Fig. 1C). Bad is cytosolic but translocates to mitochondria during apoptosis. Translocation of this protein is triggered by specific post-transcripcional modifications such as dephosphorylation. Therefore, in an attempt to further investigate whether PTH dephosphorylation of Bad stimulates its mitochondrial localization, Caco-2 cells were treated with the hormone (10^{-8} M) for 48 and 72 h and after treatment, cells were stained with anti-total Bad antibody and with a mitochondrion-selective dye, MitoTracker Red CMX Ros. The fields shown were analyzed independently by confocal microscopy at the appropriate wavelength for Alexa 488 (anti-total Bad antibody) and MitoTracker Red CMX Ros and the two images were overlaid. Upon treatment, we found that Bad colocalized with MitoTracker Red CMX Ros in cells treated with the hormone (Fig. 2A). These results were confirmed when the presence of Bad in mitochondria isolated from PTH treated cells was detected by Western blot analysis (Fig. 2B). In view of these findings, studies were carried out to explore the possibility that PTH activates the mitochondrial pathway of apoptosis. To that end, Caco-2 cells treated with the hormone (10^{-8} M, 48–72 h) were incubated with MitoTracker Red CMX Ros and analyzed in a confocal microscope. As shown in Figure 3, PTH triggers mitochondria cellular distribution to the perinuclear region, morphological features consistent with apoptosis, and this abnormality was more evident at 72 h. Caco-2 cells exposed to hydrogen peroxide (0.5 mM; 8 h) were used as a positive control since there is evidence that H_2O_2-treated cells show the typical morphological characteristics of apoptosis caused by exposing to hydrogen peroxide at concentrations above 250 M [Wijeratne et al., 2005]. Cytocrhome *c* and Smac/Diablo are pro-apoptotic factors released from mitochondria into the cytosol upon apoptosis induction. Therefore, we evaluate the localization of both proteins in Caco-2 cells by fluorescence immunostaining and microscopic analysis using anti-cytocrhome *c* and anti-Smac/Diablo antibodies and MitoTracker Red CMX Ros as a dye to stain mitochondria. As shown in

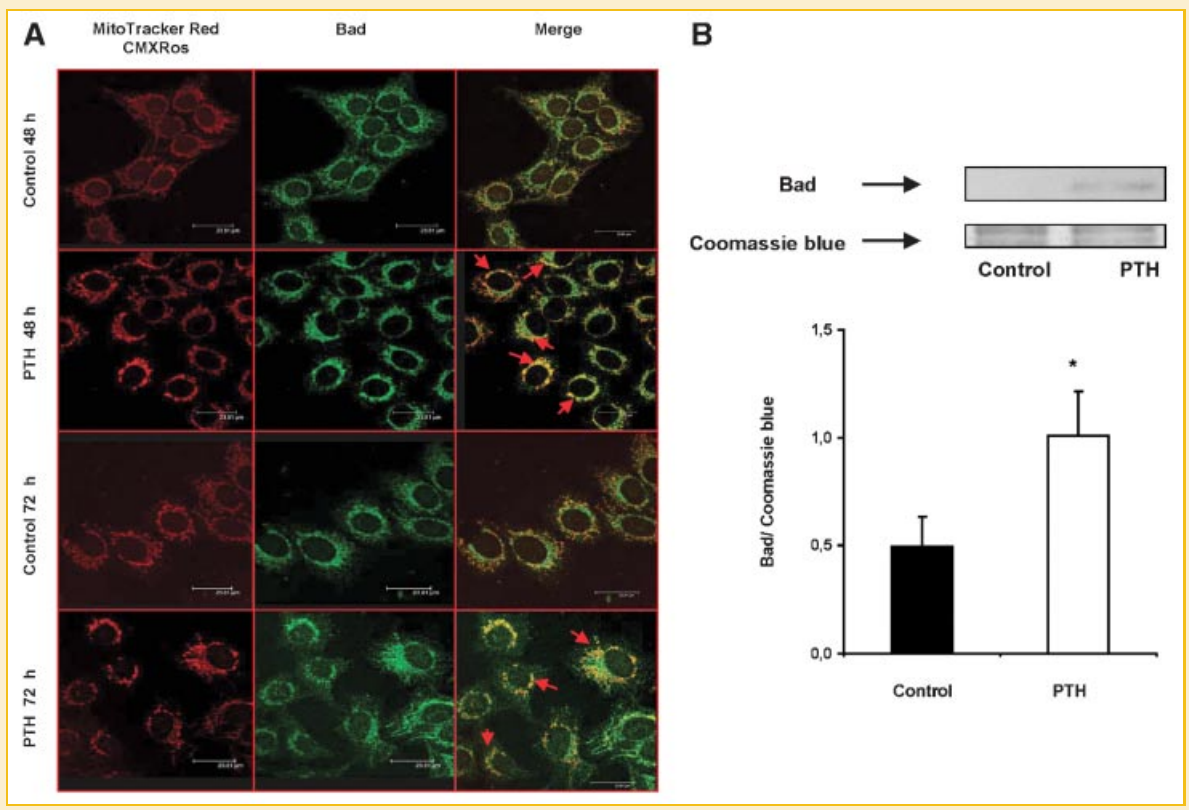

Fig. 2. A: PTH induces mitochondrial localization of Bad. Caco-2 cells were treated with or without PTH (10^{-8} M) during 48 and 72 h. The cells were then fixed with methanol and incubated with a rabbit Polyclonal antibody against Bad. Fluorescence-conjugated secondary antibody and MitoTracker Red CMX Ros were used to visualize Bad (green) and mitochondria (red) localization patterns under a confocal microscope. Red- and green-stained images were merged. Areas of co-localization appear yellow. B: Protein levels of Bad in mitochondrial fraction. Upon PTH treatment, the mitochondrial fraction was isolated and the proteins from this organelle were analyzed by Western blotting with an anti-Bad antibody. The membranes were dyed using Coomassie brilliant blue to demonstrate equal loading. A representative immunoblot and the quantification by scanning densitometry of three independent experiments are shown; means ± SD are given. $^{*}P < 0.025$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

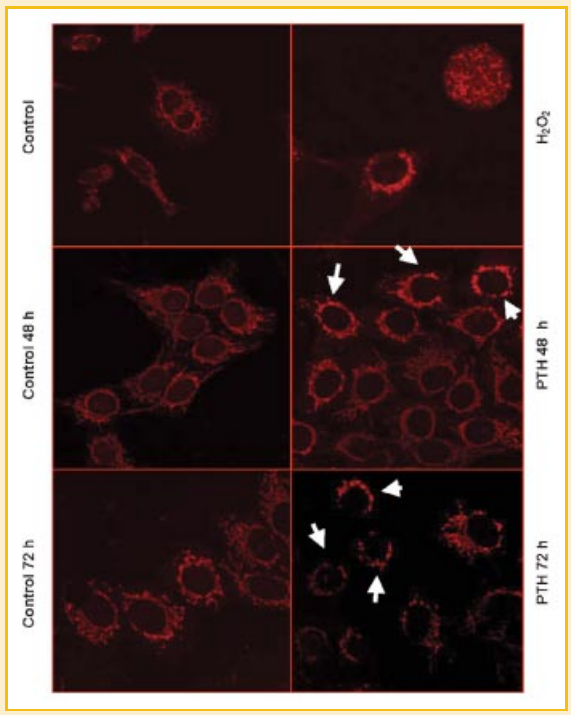

Fig. 3. PTH triggers mitochondria cellular distribution to the perinuclear region. Caco-2 cells were treated with or without PTH (10^{-8} M) during 48 and 72 h. The cells were stained with a mitochondrion-selective dye MitoTracker Red CMXRos before fixation and were visualized in a confocal microscope. Caco-2 cells exposed to hydrogen peroxide (0.5 mM; 8 h) were used as a positive control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 4A,B, in control cells cytochrome *c* and Smac/Diablo were localized in mitochondria. However, PTH treatment (48 and 72 h) revealed a cytosolic staining pattern of both proteins with the maximum response achieved at 72 h. Consistent with these results, subcellular fractionation revealed that PTH induced cytoplasmic localization of cytochrome *c* and Smac/Diablo in Caco-2 cells, as assessed by Western blot analysis (Fig. 4C).

Additional studies were designed to investigate if PTH modulates activation of the caspase-3 pathway. The processing of caspase-3 was analyzed after incubation of Caco-2 cells with 10^{-8} M PTH for 48 h and caspase-3 activity assay was performed using a specific luminescent activity assay kit. As shown in Figure 5A, PTH increased the enzymatic activity of caspase-3. In agreement with these results, Western blot analysis with an anti-caspase-3 antibody specific for active caspase-3 (p11, p17, and p20 subunits) and full length precursor showed the appearance of all cleaved fragments of caspase-3 in Caco-2 cells stimulated with the hormone (Fig. 5B). Although the precursor form of caspase-3 is localized in the cytoplasm, caspase-3 plays essential roles in the nuclear changes in apoptotic cells [Kamada et al., 2005]. Therefore, caspase-3 seems to translocate from cytoplasm into the nucleus after apoptosis induction. To determine whether active caspase-3 is present in nucleus after PTH treatment, Caco-2 cells were stained with anti-caspase-3 antibody (that recognize active caspase-3 and the inactive precursor) and analyzed by confocal microscopy. Figure 5C shows cytoplasmic localization of caspase-3 in control cells while in PTH-treated cells the enzyme localizes in nucleus and cytoplasm. We also analyzed the cleavage status of its substrate, PARP, by western blotting with an anti-PARP antibody that recognizes the 116 kDa substrate. As shown in Figure 6, PARP-degradation was observed in Caco-2 cells stimulated with PTH. Actin immunoblotting was performed as an internal control of equal loading. Taken together, these results suggest that PTH activates caspase-3 and this activation was followed by cleavage of its substrate PARP.

Akt controls the exquisite balance between cell survival and apoptosis. It is a well-known downstream effector of PI3K, and its activation is mainly induced by the phosphorylation of residue Ser-473 or Thr-308 [Brazil and Hemmings, 2001]. To investigate whether PTH affects the activation of PI3K/Akt signaling, the phosphorylation status of Akt was measured using a phospho-specific antibody against the Ser-473 residue. Treatment with PTH (10^{-8} M, 48 h) caused a decrease in Akt basal phosphorylation at Ser-473 (Fig. 7, top panel). By contrast, there was no change in the total amounts of these proteins (bottom panel), demonstrating a true decrease in their phosphorylation status. In the presence of serum, the hormone does not modify the phosphorylation of AKT and neither promotes PARP cleavage in Caco-2 cells (data not shown).

DISCUSSION

The results of the present investigation provide, to our knowledge, the first direct evidence demonstrating that Bad, Akt, cytochrome *c*, Smac/Diablo and caspase-3 are part of the mechanism by which PTH induces apoptosis in the intestinal Caco-2 cells. The proteins of the Bcl-2 family are key regulators of apoptosis and their main function is to control mitochondrial permeability and particularly, the release of apoptogenic proteins from this organelle [Adams and Cory, 2001]. We found that stimulation of cells with PTH resulted in the dephosphorylation and translocation of pro-apoptotic protein Bad from the cytosol to mitochondria. Bad, a BH3-only protein induces apoptosis by activating pro-apoptotic proteins like Bax or by inhibiting anti-apoptotic proteins like Bcl-2 [Huang and Strasser, 2000; Moreau et al., 2003]. Cytochrome *c* is normally bound to the inner mitochondrial membrane by an association with the anionic phospholipid cardiolipin, where it can reversibly interact with complexes III and IV of the respiratory chain. Smac/Diablo is a mitochondrial protein that is released along with cytochrome *c* during apoptosis and promotes cytochrome *c*-dependent caspase activation by neutralizing IAP family of apoptosis inhibitory proteins [Verhagen et al., 2000]. Confocal microscopy and western analysis demonstrated that PTH triggered cytochrome *c* and Smac/Diablo release from mitochondria to cytosol in Caco-2 cells. Mitochondria play a key role in the regulation of apoptotic cell death [Orrenius, 2004]. Different pro-apoptotic proteins, such as cytochrome *c* and Smac/Diablo, which are normally present in the intermembrane space of these organelles are released during the early stages of apoptosis [Cai et al., 1998; Green and Reed, 1998]. Once in the cytosol, Cytochrome *c* participates in the formation of the apoptosome complex together with its adaptor molecule,

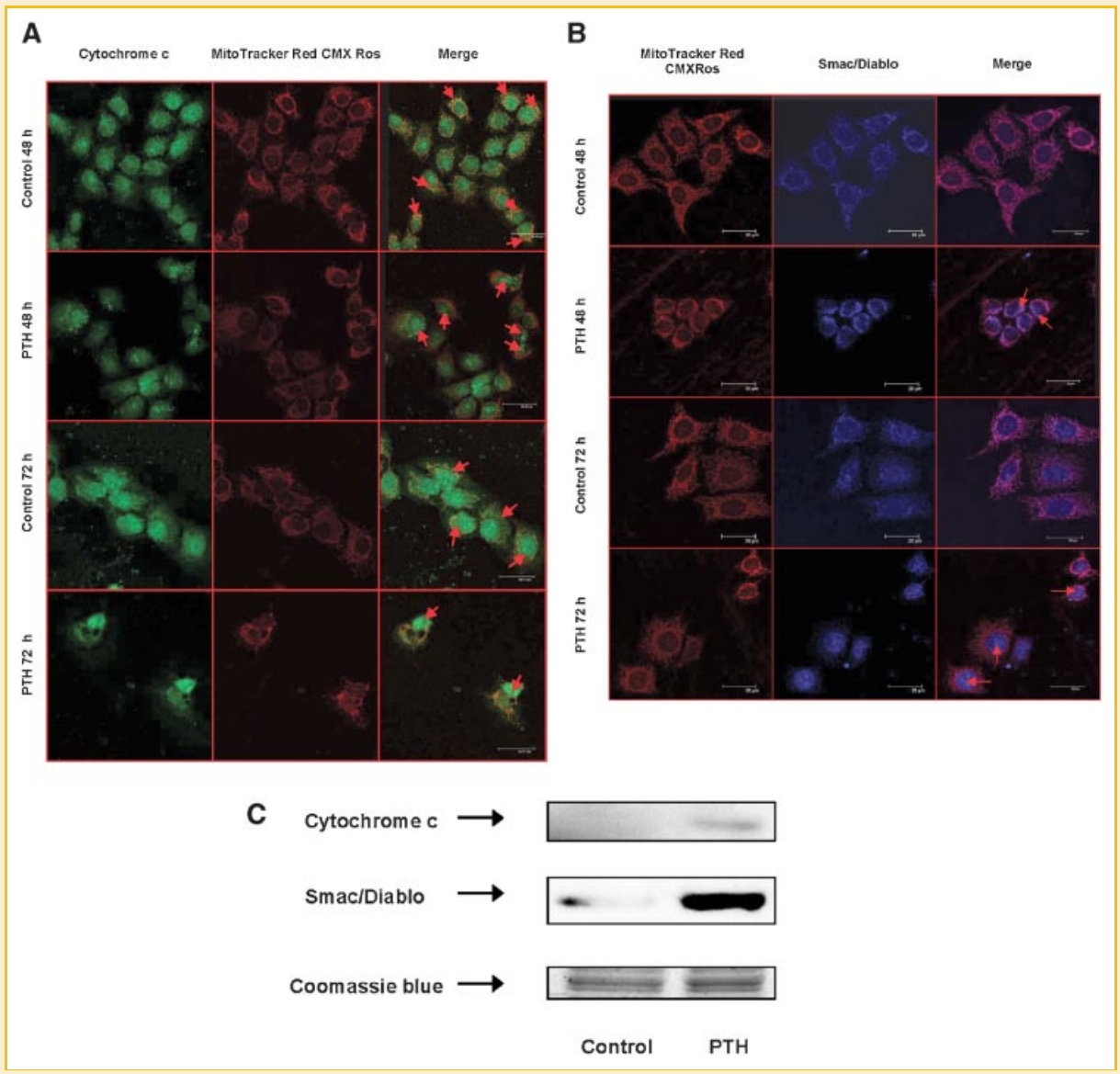

Fig. 4. PTH induces the release of cytochrome *c* and Smac/Diablo from mitochondria into the cytosol. Caco-2 cells were treated with or without PTH during 48 and 72 h. The cells were then fixed with methanol and incubated with a rabbit Polyclonal antibody against cytochrome *c* or with a mouse monoclonal antibody against Smac/Diablo. Fluorescence-conjugated secondary antibody and MitoTracker Red CMX Ros were used to visualize cytochrome c (green), Smac/Diablo (blue) and mitochondria (red) localization patterns under a confocal microscope. A: Red- and green-stained images were merged. Areas of co-localization appear yellow. B: Red- and blue-stained images were merged. Areas of co-localization appear purple. C: Cytochrome *c* and Smac/Diablo levels in cytosolic fraction. Equal aliquots of the cytosolic fraction from control and PTH treated cells were analyzed by Western blotting with an anti-cytochrome *c* antibody. The same immunoblot was re-probed with an anti- Smac/Diablo antibody. The membranes were dyed using Coomassie brilliant blue to demonstrate equal loading. A representative immunoblot from three independent experiments is shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Apaf-1, resulting in the recruitment, processing and activation of procaspase-9 [Zou et al., 1999]. Subsequently, caspase-9 cleaves and activates pro-caspase-3 and -7; these effector caspases are responsible for the cleavage of various proteins leading to biochemical and morphological features characteristic of apoptosis [Robertson et al., 2000]. Caspases are a family of cysteine proteases that are ubiquitously expressed as inactive pro-enzymes [Boatright and Salvesen, 2003]. Although the pro-enzymes contain a small amount of catalytic activity, they are kept in check by a variety of regulatory molecules. Upon receiving an apoptotic signal, they undergo proteolytic processing to generate two subunits that comprise the active enzyme. The cleavage of the pro-enzyme is not always an obligatory requirement for caspase activation, but all activated caspases can be detected as cleaved fragments in apoptotic cells [Degterev et al., 2003; Fuentes-Prior and Salvesen, 2004]. In intestinal Caco-2 cells, we found that PTH increased the enzymatic activity of caspase-3 that was also evidenced from the appearance of its cleaved fragments in western blot experiments. The hormone also triggered mitochondria cellular distribution to the perinuclear region, morphological features consistent with apoptosis. In

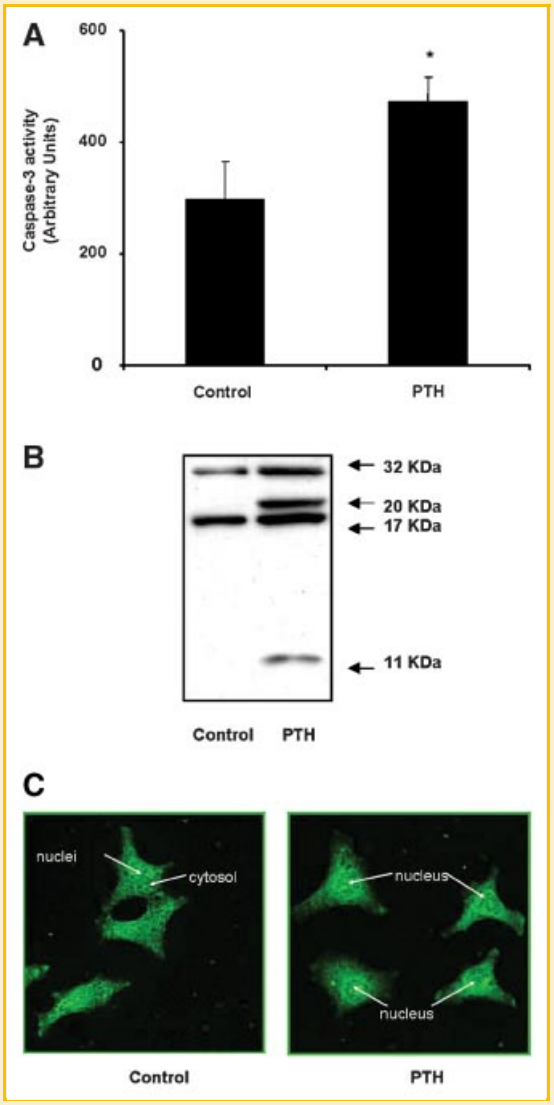

Fig. 5. A: PTH stimulates caspase-3 activity. Caco-2 cells were incubated in presence of PTH 10^{-8} M for 48 h and assay of caspase-3 activity using a specific luminescent activity assay kit was carried out as detailed in Materials and Methods Section. Results are the average of three independent experiments. $^{*}P < 0.025$ with respect to the control. B: PTH induces the appearance of all cleaved fragments of caspase-3. Cleavage of pro-caspase-3 was analyzed by Western blotting with an anti-caspase-3 antibody specific for active caspase-3 (p11, p17 and p20 subunits) and full length precursor. A representative immunoblot is shown. C: PTH induces nuclear localization of caspase-3. After PTH treatment, Caco-2 cells were fixed with methanol and incubated with a rabbit Polyclonal antibody against caspase-3 Fluorescence-conjugated secondary antibody was used to visualize caspase-3 (green) localization patterns under a confocal microscope. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

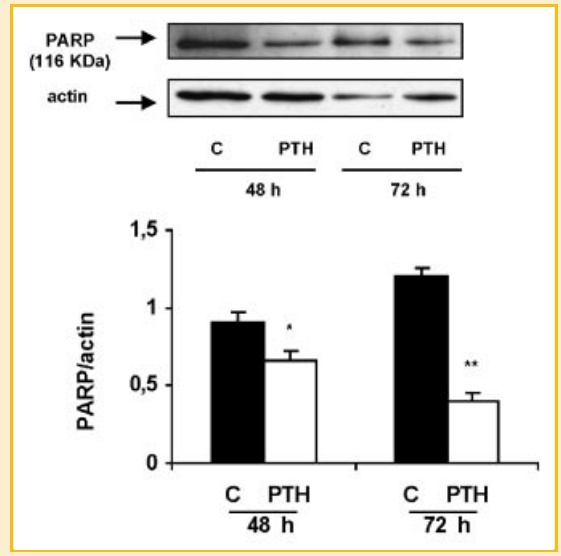

Fig. 6. PTH induces PARP-degradation in Caco-2 cells. Cleavage of poly (ADP-ribose) polymerase was analyzed by Western blotting with an anti-PARP antibody that recognizes pro-PARP (116 kDa). In order to evaluate the equivalence of protein content among the different experimental conditions, blotted membranes were re-probed with anti- actin antibody. A representative immunoblot and the quantification by scanning densitometry of three independent experiments are shown; means ± SD are given. $^{*}P < 0.05$, $^{**}P < 0.025$ with respect to the corresponding control.

mammals, the function and regulation of caspases in cell death is complex. The caspases that get activated via recruitment to signaling complexes are known as the initiator caspases, as they provide a link between cell signaling and apoptotic execution. The main initiator caspases are caspase-2, -8, -9, and -10 whereas caspase-3, and to a lesser extent caspase-6 and -7, serve as effector caspases [Degterev et al., 2003; Fuentes-Prior and Salvesen, 2004]. Caspase-3 is the main downstream effector caspase that cleaves the majority of the cellular substrates in apoptotic cells. It is activated following cleavage by caspase-8 or -9, but not by caspase-2 [Porter and Janicke, 1999].

Our study shows that PTH activation of caspase-3 was followed by cleavage of its substrate PARP. During apoptosis, a precocious and transient stimulation of PARP-1 causes accumulation in early apoptotic cells. Excessive NAD consumption is prevented by the cleavage of PARP-1 by caspases [Soldani and Scovassi, 2002]. PARP-1 cleavage generates two inactive fragments of 24 and 89 kDa. The N-terminal fragment (p24) remains in the nucleolus, retains its DNA-binding activity and inhibits the catalytic activity of uncleaved PARP-1, and also impairs DNA repair [D'Amours et al., 2001]. The p89 fragment migrates from the nucleus to the cytoplasm in late apoptotic cells with advanced nuclear fragmentation [Soldani et al., 2001]. Finally, ours results demonstrates that PTH mediated inhibition of Akt occurs concomitantly with dephosphorylation of downstream target Bad and the activation of caspase 3, suggesting that inhibition of PI3K/Akt signaling is part of the mechanism for PTH-induced apoptosis in Caco-2 cells. Future studies will therefore be needed to test the mechanism underlying the inhibition of Akt signaling by PTH in serum deprived Caco-2 cells. In the presence of serum we found that, after 48 and 72 h of treatment, PTH does not modify the phosphorylation of Bad, AKT and neither promote PARP cleavage.

Depending on the cell type involved, PTH also inhibits or promotes the apoptosis. In agreement with our findings, PTH has been shown to induce apoptosis in human embryonic kidney cells stably expressing the PTH1R. Moreover, these effects require the

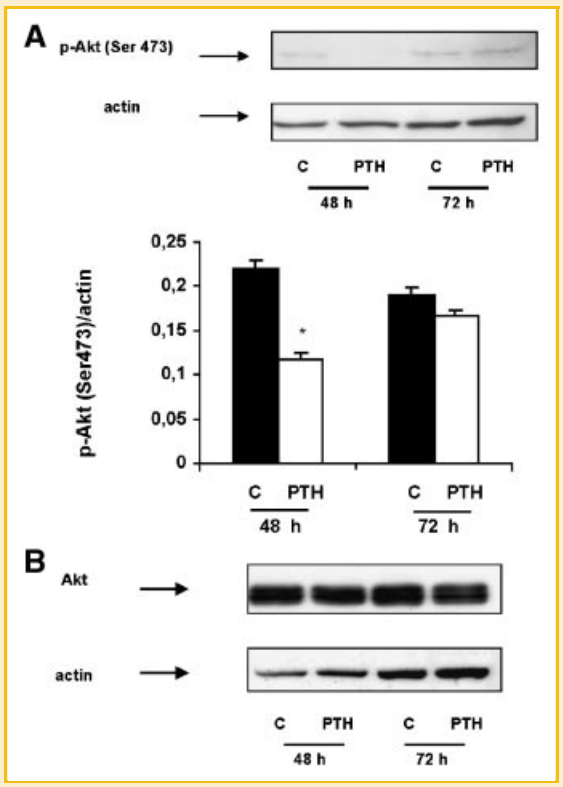

Fig. 7. PTH induces the dephosphorylation of Akt. Caco-2 cells were exposed to PTH 10^{-8} M (48 and 72 h) followed by Western blot analysis of cell lysates with anti-Akt phospho Ser 473. Blotted membranes were re-probed with anti- actin and anti-Akt antibody. A representative immunoblot and the quantification by scanning densitometry of three independent experiments are shown; means ± SD are given. $^{*}P < 0.05$, with respect to the control (C).

second messenger products of PLC signaling, but are independent of adenylyl cyclase signaling [Turner et al., 2000]. In bone and mesenchymal cells, it has become apparent that signaling by the PTH1R can either promote or suppress apoptosis depending on the cellular context [Amling et al., 1997; Stanislaus et al., 2000; Chen et al., 2002]. Pretreatment of osteoblasts with PTH decreases dexamethasone- and etoposide-induced apoptosis [Sowa et al., 2003]. Although the "PTH-Smad3 axis," was reported to be essential in PTH-induced anti-apoptotic effects, it is still unclear what signal transduction pathways are used in apoptosis of osteoblasts and what steps in apoptosis are targeted by PTH in its rescue effects. The mechanism by which PTH induces colonic Caco-2 cells apoptosis has not been elucidated yet. The small intestine is considered more prone to apoptotic effect than the large intestine due to its higher level of spontaneous apoptosis [Potten, 1997]. In the colon, on the other hand, a low apoptotic rate protects a potentially more vulnerable stem cell population from initiating programmed cell death [Merritt et al., 1995].

In conclusion, the induction of apoptosis, as evidenced by translocation of mitochondria to the perinuclear region, dephosphorylation of Akt, dephosphorylation and movement of Bad to the mitochondria and subsequent release of cytochrome *c* and Smac/Diablo which result in activation of downstream caspase-3, is a novel effect in response to PTH and presents a new perspective in understanding the function of the PTH1R in Caco-2 intestinal cells. Further studies are under progress to further characterize the molecular mechanisms of PTH-mediated apoptotic signaling pathway in these cells.

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