

Activation of the receptor for parathyroid hormone and parathyroid hormone related protein induces apoptosis via the extrinsic and intrinsic signaling pathway

MARTINA MÜLLER¹, SARAH GAGIANNIS², PETER P. NAWROTH², MAIK BRUNE² and TOBIAS SCHILLING²

Departments of ¹Internal Medicine IV, Hepatology and Gastroenterology; ²Internal Medicine I and Clinical Chemistry, University Hospital, Im Neuenheimer Feld 410, D-69120 Heidelberg, Germany

Received February 19, 2009; Accepted March 26, 2009

DOI: 10.3892/ijmm_00000242

Abstract. Parathyroid hormone (PTH) is the primary regulator of serum calcium homeostasis and bone metabolism. PTH acts primarily by binding to its receptor, PTH1R, in the bone and kidney. In addition to PTH, PTH1R also recognizes PTH-related peptide (PTHrP), a paracrine/autocrine factor originally described as the hormone responsible for hypercalcemia of malignancy. PTHrP is developmentally regulated and expressed, and it has been shown to play a physiological role in development, differentiation, cell proliferation and survival. We investigated the effects of PTH1R activation on the apoptosis signaling programs of human embryonic kidney (HEK) cells. Stimulation experiments of the CD95, TNF-R and TRAIL-R death receptor systems revealed that activation of PTH1R in HEK cells triggers signaling via each of these death receptors. Furthermore, our findings demonstrate a link between activation of PTH1R and the mitochondrial apoptosis pathway. PTH1R overexpression led to an alteration of the mitochondrial membrane potential and activation of the intrinsic apoptosis signaling pathway. Our data indicate that activation of PTH1R engages major apoptosis signaling pathways by inducing signaling via death receptors and mitochondria in HEK cells. Thus, beyond its importance in development and differentiation, we describe an important role for the PTH/PTHrP receptor system in apoptosis of differentiating/embryonic cells.

Introduction

Parathyroid hormone (PTH) is central to calcium homeostasis and bone maintenance in vertebrates. PTH is a classic endocrine hormone that was identified more than 80 years ago as a key regulator of blood calcium levels (1). In response to either low calcium or high phosphate levels in the circulation, PTH is secreted as an 84-amino acid polypeptide from the parathyroid glands and acts primarily on the bone and kidney. In the bone, PTH increases osteoclastic bone resorption as part of calcium homeostasis, and the stimulatory effects of PTH on osteoblasts increase bone mass (2). In the kidney, PTH promotes renal tubular calcium resorption and synthesis of 1,25-dihydroxyvitamin D₃ but prevents resorption of phosphate (3). The effects of PTH are mediated primarily through activation of the PTH receptor (PTH1R) (4), which is highly expressed in the PTH target tissues of the bone and kidney (5). PTH also binds to a second receptor (PTH2R) (6). Both PTH receptors belong to the class B G protein-coupled receptors (GPCRs) (7).

In addition to PTH, PTH1R also recognizes PTH-related peptide (PTHrP) (4,8). PTHrP is a secretory protein that was first described as the hormone responsible for hypercalcemia of malignancy and was subsequently described as having a role in both cell proliferation and differentiation (9-11). Its partial homology to PTH allows PTHrP to activate PTH1R. Although other PTH receptors have been identified, the PTH1R is the major receptor responsible for skeletal actions of PTH and PTHrP, as evidenced by similar phenotypes in the PTHrP and PTH1R null mouse models (12). Ablation of the PTH1R gene in mice results in a neonatal-lethal phenotype with severe abnormalities in development of cartilage and bone (13). The perinatal lethality of PTHrP or PTH1R knockout mice emphasizes the biological importance of this peptide system.

PTH1R is also expressed in many fetal and adult tissues, in which it mediates an array of paracrine and autocrine functions in response to locally produced PTHrP. Thus, the PTHrP/PTH1R system acts as a local regulator of cell proliferation, differentiation and apoptosis. Besides the PTH-like domain, which lies within the N-terminal, part of the PTHrP protein, PTHrP contains two other functional domains,

Correspondence to: Dr Tobias Schilling, Department of Internal Medicine I and Clinical Chemistry, University Hospital, Im Neuenheimer Feld 410, D-69120 Heidelberg, Germany
E-mail: tobias_schilling@med.uni-heidelberg.de

Abbreviations: ANOVA, analysis of variance; FACS, fluorescence-activated cell sorting; MANOVA, multivariate analysis of variance; TNF, tumor necrosis factor

Key words: PTH1 receptor, parathyroid-hormone related protein, parathyroid-hormone, apoptosis, CD95, TRAIL-R

the mid-region and C-terminal domains. Post-translational cleavage of the PTHrP protein allows these domains to function independently (14-17). By activation of PTH1R, the N-terminal parathyroid hormone-like domain stimulates protein kinase A (PKA), C (PKC), and/or the calcium-dependent pathways, respectively (17-20). The mid-region domain, containing a bipartite nuclear localization sequence at residues 88-91 and 102-106 and an importin β -binding site at 66-94, can enter the nucleus and interfere with gene expression (17,21-25). The C-terminal domain physically interacts with β -arrestin, which regulates internalization and desensitization of ligand-stimulated G-protein-coupled receptors (26-30). The physiological function of PTHrP is of a developmental regulatory molecule that controls the rate of programmed differentiation during organogenesis. Thus, although PTHrP was discovered as a tumor-derived hypercalcemic factor, its primary physiological role is as a local regulator of many physiological processes (reviewed in ref 5). Previous studies suggest that apoptosis is initiated by activation of the PTH1R, and that this is likely to contribute to the spectrum of physiological responses to PTH and/or PTHrP (31,32).

So far, the role of PTH1R in apoptosis has not been mechanistically investigated. Therefore, this study takes advantage of an embryonic cell line stably expressing PTH1R to explore the impact of PTH1R on apoptosis signaling pathways. Here we report that PTHrP induces apoptosis in human embryonic kidney (HEK) 293 cells stably expressing the PTH1R via the induction of both, the extrinsic/death receptor- and the intrinsic/mitochondria-mediated apoptosis pathways.

Materials and methods

Cell lines and culture. HEK cells, and HEK cells stably transfected with wild-type full-length opossum PTH/PTHrP receptor (HEK1R) (33,34) were cultured in Dulbecco's modified Eagle's medium (DMEM, PAA, Cölbe, Germany) supplemented with 10% FCS, 0.4% Fungizone, 1 M Hepes buffer and 1.2% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany).

Treatment with parathyroid hormone related protein. N- (1-34) or C-terminal fragments (107-139) of PTHrP and human N-terminal PTH (1-34) (Bachem, Weil, Germany) were added to HEK or HEK1R cells in concentrations of 300-900 pMol.

Cyclic AMP immunoassay. cAMP production was assessed using a cAMP Immunoassay Kit (R&D Systems, Wiesbaden, Germany).

Adenoviral constructs and transduction. Replication-deficient adenoviral vectors (35,36), each under control of the cytomegalovirus immediate/early gene (CMV) promoter, encoding the complete human wild-type p53 cDNA (rAd-p53) together with GFP, or GFP alone (rAd-GFP), were added to cells and incubated for 4 h. At a multiplicity of infection (moi) of 10-15 IU/cell, an infection rate of 80-90% of the cells was obtained.

Western blot analysis. Cells were lysed in RIPA-buffer (50 mM TrisCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium desoxycholat, 1x protease Inhibitor Cocktail

(#11836 153 001 [Roche, Germany]) for 15 min on ice and centrifuged for 30 min at 12,000 x g. The supernatant was resolved by electrophoresis in a 12% SDS-polyacrylamide gel, transferred to a Hybond ECL Nitrocellulose (Amersham, Buckinghamshire, UK) and probed with antibodies against PTH1R (Santa Cruz, Heidelberg, Germany) and against PTHrP (Aviva Systems Biology, San Diego, USA). To ensure equivalent loading and transfer, membranes were stripped and re-probed with anti-human actin (Oncogene Research Products, Boston, USA).

Detection of apoptosis. Quantification of DNA fragmentation was performed by FACS analysis of propidium iodide-stained nuclei according to Nicoletti (37,38), carried out in a FACScan® flow cytometer (Becton-Dickinson) using the CELLQuest® software system.

To induce apoptosis, cells were treated with irinotecan at 5 μ g/ml (Pfizer, Karlsruhe, Germany), adenoviral transfer of wild-type p53 (35,36) or thapsigargin (Sigma-Aldrich, Taufkirchen, Germany) at 10-20 nMol.

To induce CD95 receptor-mediated apoptosis, we used the monoclonal antibody anti-APO-1 IgG3, at 1 μ g/ml added to 20 ng/ml Protein A (39-41). TNF- α (Immunotools, Friesoythe, Germany) was added at a concentration of 100 ng/ml, together with 10 μ g/ml cycloheximide (Sigma) 16 h prior to harvesting. Killer-TRAIL (Alexis Biochemicals, Lausen, Switzerland) was applied at a concentration of 1 μ g/ml 16 h before harvesting.

For caspase activation assays, cells were harvested 48 h after treatment (caspase-2, -3, -6, -8 and -9 fluorometric assay; R&D Systems, Wiesbaden, Germany).

Detection of death receptors. Cells were treated for 48 h, harvested by 0.2 g/l EDTA-incubation, centrifuged and distributed on 96-well plates. Then, cells were incubated on ice for 30 min with 10 μ g/ml mouse IgG1 antibodies against death receptors CD95 (kindly provided by H. Walczak), TNF-R1 (clone 16803, R&D Systems), TRAIL-R1 and 2 (clone HS101 and HS201, Alexis Biochemicals) or an unspecific isotype control (R&D Systems), followed by a biotinylated F(ab)'₂-fragment goat anti-mouse IgG Fc antibody (Dianova GmbH, Hamburg, Germany). Streptavidin-Allophycocyanin (APC) (Becton-Dickinson) was added for an additional 20 min. Cells were washed with FACS-buffer (PBS containing 2% FCS) following each incubation step. Finally, cells were resuspended in FACS-buffer containing 1 μ g/ml propidium iodide. Cell surface expression of the death receptors was assessed by FACScalibur® at an excitation of 633 nm.

Determination of mitochondrial membrane potential. Cells were incubated with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine-iodide (JC-1, 5 μ g/ml; Sigma) or with 3,3 dihexylocarbocyanine-iodide (DiOC; Molecular Probes) at room temperature for 20 min (JC-1) or at 37°C in the dark for 15 min (DiOC), then washed and analyzed by FACScan (42,43).

Statistical analysis. We applied ANOVA or MANOVA to test for statistical significance. Statistical analysis was carried out using the SAS software system (SAS Institute Inc., Cary, USA).

Results

Activation of PTH1R induces apoptosis. HEK cells stably transfected with wild-type PTH/PTHrP receptor (HEK1R) showed increased expression of PTH1R as shown by Western blot analysis, while endogenous PTHrP levels remained unchanged (Fig. 1A and B). We used two PTHrP fragments, an N-terminal residue (1-34), known to signal through PTH1R and a C-terminal residue (107-139), which acts through endocytosis or an uncharacterized receptor. Consequently, a cAMP immunoassay showed activation of the cAMP/PKA pathway through human N-terminal PTH and PTHrP, but not through the C-terminal PTHrP fragment in HEK1R cells (Fig. 1C). Thus, the wild-type PTH1 receptors expressed in human embryonic kidney cells (HEK1R) are functional and activate the PKA pathway. PTH1R responds to binding of PTH or PTHrP by activation of the PKA and PKC pathways. The PKA pathway is mediated by the G_s protein that activates adenylylcyclase and leads to cAMP production and PKA activation. The PKC pathway is mediated by the G_q protein that activates phospholipase C, resulting in an increase in intracellular Ca^{2+} levels and activation of protein kinase C. Untransfected HEK cells did not respond towards N-terminal PTH- or N-terminal PTHrP-stimulation of the PTH1R (Fig. 1C).

The purpose of this study was to determine the effects of overexpressing wild-type PTH1R on the cellular apoptosis program of HEK cells. Therefore, we treated HEK1R cells with thapsigargin. Thapsigargin is a tight-binding inhibitor of a class of enzymes known as sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA). Thapsigargin is a Ca^{2+} ATPase inhibitor that elevates intracellular calcium concentration by promoting its release from intracellular stores, and induces apoptosis in certain cells (44). Thapsigargin is a strong inducer of apoptosis in HEK1R cells but not in control HEK cells (Fig. 2A). In addition, adenoviral transfer of the p53 gene into HEK1R cells led to an increased rate of wild-type p53-dependent apoptosis in HEK1R cells (Fig. 2B). Concomitant treatment of HEK1R cells with adenoviral transfer of the p53 gene and the cytotoxic drug irinotecan led to an additive effect on wild-type p53-induced apoptosis (Fig. 2C). These findings indicate that upregulation and activation of PTH1R leads to increased sensitivity of HEK cells towards apoptosis.

Addition of N-terminal PTHrP or PTH following adenoviral transfer of wild-type p53 into HEK1R cells potentiated the effects of PTH1R expression on induction of apoptosis (Fig. 2D). This indicates that G_s -mediated cAMP/PKA- and/or G_q -mediated PKC- (triggered by N-terminal PTHrP) are involved in induction of PTH1R-mediated apoptosis.

PTH1R-induced apoptosis involves activation of caspases. Involvement of caspases in the induction of PTH1R-mediated apoptosis was shown by fluorometric determination of the increased enzymatic activity of caspase-2, -3, -6, -8 and -9 class of proteases in HEK and HEK1R cells following treatment with thapsigargin (Fig. 3A). Of note, caspase activation was significantly enhanced in HEK1R cells due to expression of PTH1R. Addition of N-terminal PTHrP further augmented PTH1R-dependent caspase activation following treatment of HEK1R cells with thapsigargin (Fig. 3B). Thus,

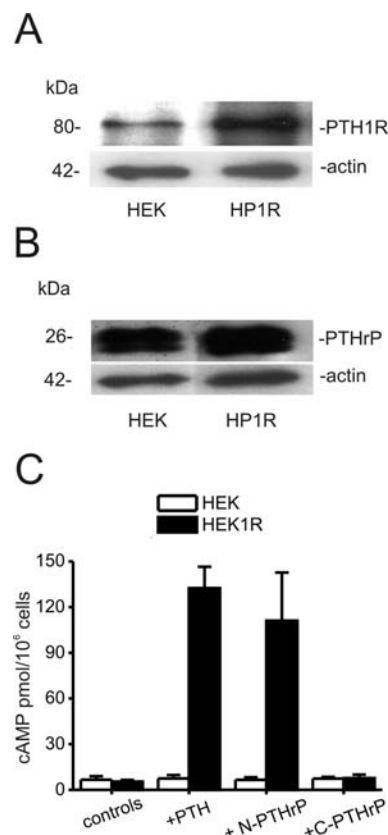


Figure 1. Expression of PTH1R and parathyroid hormone-related protein (PTHrP) in HEK cells. (A) Western blot analysis of PTH1R protein expression. HEK1R cells, stably transfected with wild-type PTH1R show increased PTH1R protein levels. (B) Western blot analysis of PTHrP protein expression. Both, HEK and HEK1R cells show constitutive PTHrP protein expression. (C) cAMP immunoassay demonstrates PTH1R-dependent activation of the cAMP/PKA pathway in HEK1R but not in HEK cells by ligand-binding of human N-terminal PTH 1-34 and PTHrP 1-34, but not by C-terminal PTHrP 107-139. Cells were treated with N-PTH, N- and C-terminal PTHrP (each 900 pMol) for 15 min before harvesting. Three independent experiments were performed in triplicates, a representative result is shown, mean \pm SD, n=3.

the proapoptotic action of PTH1R stimulation on caspases occurs at least in part via the PKA- and/or the PKC pathways.

Activation of the PTH1R triggers the extrinsic apoptosis pathway via activation of death receptors. We next investigated if activation of the PTH1R triggers the extrinsic apoptosis pathway. Addition of the specific agonistic antibodies and ligands (agonistic anti-APO-1 antibody, TNF- α , or Killer-TRAIL) led to a further increase of thapsigargin-mediated apoptosis in HEK1R cells. In contrast these agonistic antibodies and ligands did not induce apoptosis via the death-receptor pathway in HEK cells (Fig. 4A). Thus, PTH1R-induced apoptosis is mediated by a set of death receptors including the CD95, TNF and TRAIL receptor systems.

Activation of PTH1R engages the mitochondrial apoptosis pathway. In order to further characterize the molecular mechanisms of PTH1R-mediated apoptosis, we investigated the influence of PTH1R activation on mitochondrial apoptosis signaling pathways. FACSscan analysis following DiOC staining revealed an alteration of the mitochondrial membrane

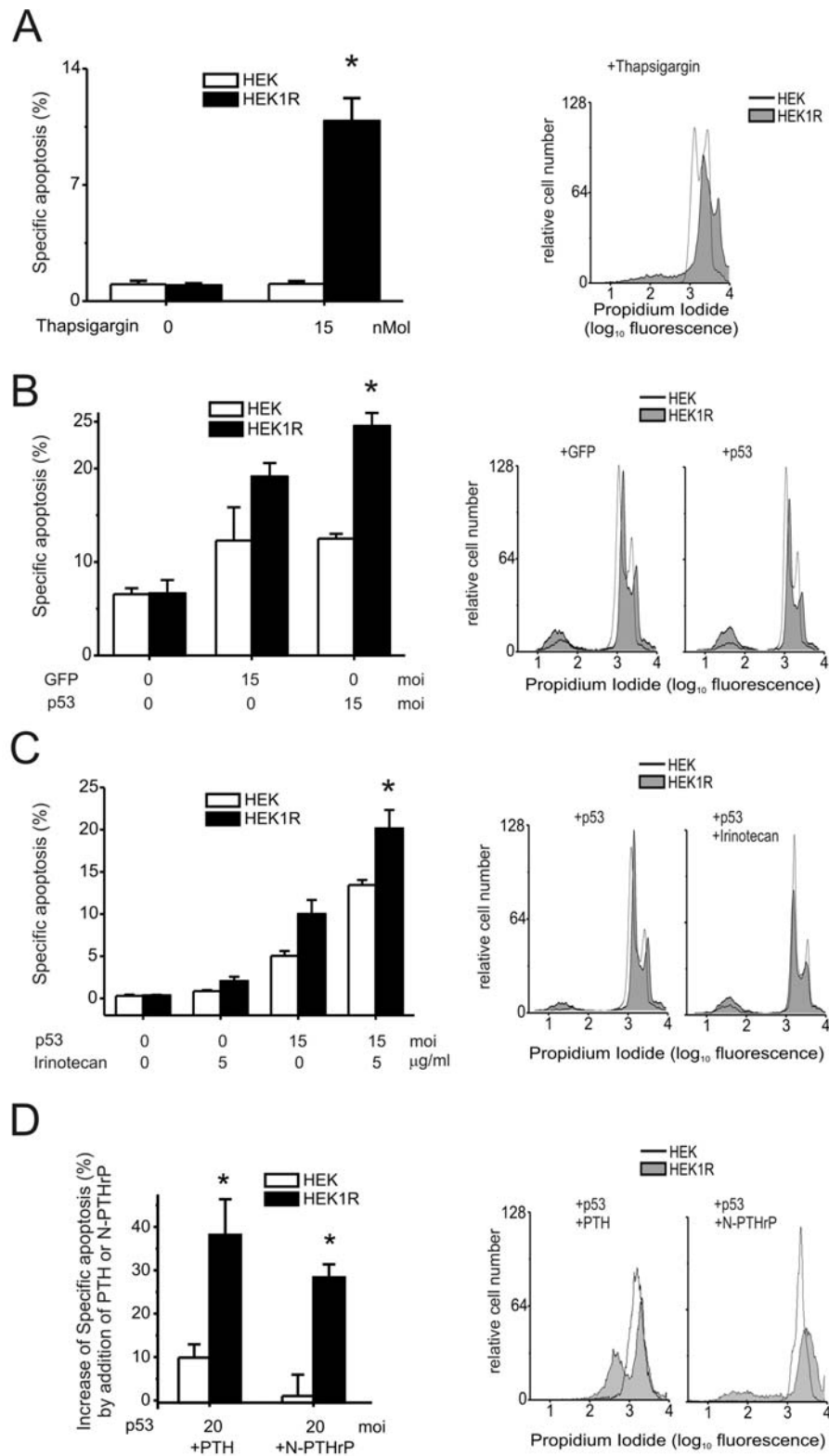


Figure 2. Activation of the receptor for parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP) induces apoptosis. (A) FACSscan analysis of propidium iodide-stained nuclei of HEK and HEK1R cells following treatment with thapsigargin. Cells were treated with thapsigargin (48 h). Expression of the PTH1R led to an increased rate of apoptosis in HEK1R cells. Three independent experiments were performed, and a representative result is shown, mean \pm SD, $n=3$. * $p<0.05$, Wilcoxon test, compared to HEK cells. (B) FACSscan analysis of propidium iodide-stained nuclei of HEK and HEK1R cells following adenoviral transfer of wild-type p53. Wild-type p53 transfer induced apoptosis in HEK1R cells. Two independent experiments were performed; a representative result is shown, mean \pm SD, $n=3$. * $p<0.008$, MANOVA between subject effect (HEK1R compared to HEK cells), GFP and p53 were considered as repeated measurements. (C) FACSscan analysis of propidium iodide-stained nuclei of HEK- and HEK1R cells following combined treatment with adenoviral transfer of wild-type p53 and irinotecan. HEK1R cells were more susceptible towards a combinatory gene- and chemotherapy compared to HEK cells. Two independent experiments were performed, and a representative result is shown, mean \pm SD, $n=3$. * $p<0.001$, MANOVA between subject effect (HEK1R compared to HEK cells), irinotecan, p53 and irinotecan+p53 were considered as repeated measurements. (D) FACSscan analysis of propidium iodide-stained nuclei of HEK and HEK1R cells following treatment with p53 with or without addition of PTH or N-terminal PTHrP (each 900 pMol). Addition of the specific ligands of the PTH1R led to a further enhancement of wild-type p53-dependent apoptosis in HEK1R cells. Three independent experiments were performed, and a representative result is shown, mean \pm SD, $n=3$. * $p<0.05$, Wilcoxon test, compared to HEK cells.

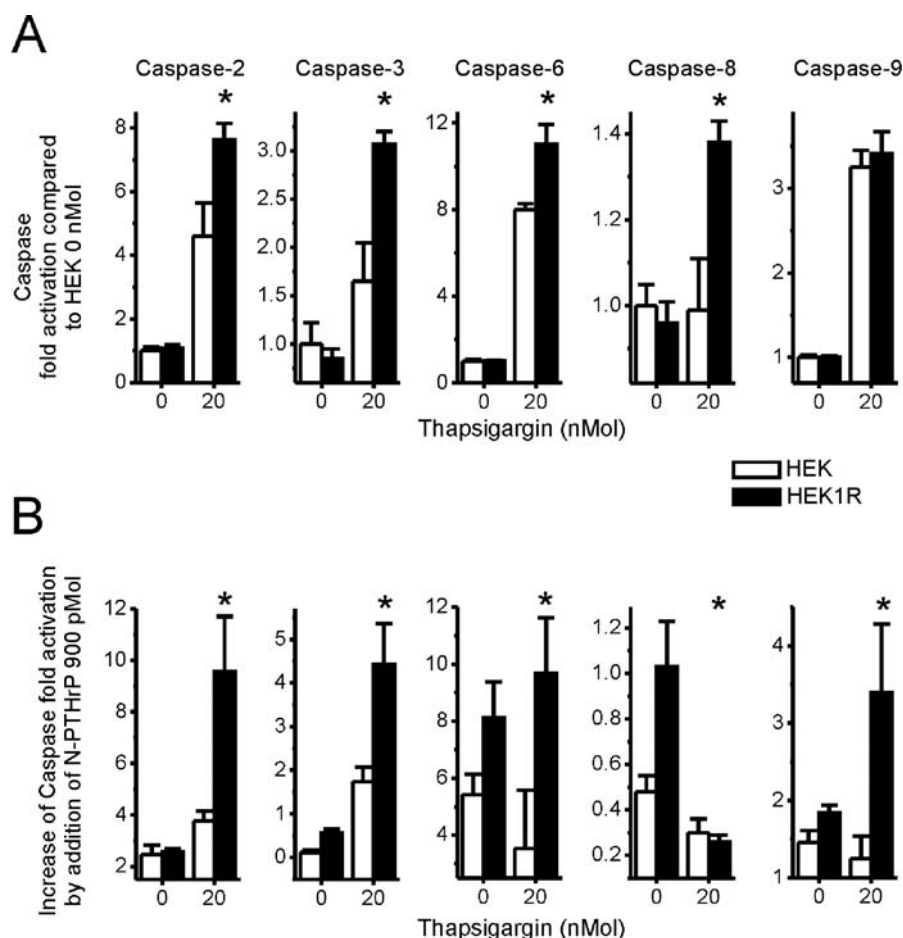


Figure 3. PTH1R-induced apoptosis involves activation of caspases. (A) Determination of the enzymatic activity of caspase-2, -3, -6, -8, and -9 class of proteases was performed by fluorometric assays 24 h following treatment of HEK and HEK1R cells with thapsigargin with or without addition of N-PTHrP (PTHrP 1-34). Fold activation represents the VDVADase- (consistent with caspase-2), DEVDase- (consistent with caspase-3), VEIDase- (consistent with caspase-6), IETDase- (consistent with caspase-8) and LEHDase- (consistent with caspase-9) activity of thapsigargin-treated HEK1R cells compared to control HEK cells. Caspase activation was significantly enhanced in HEK1R cells due to expression of PTH1R. Assays were performed in triplicate, and two independent experiments were performed; a representative result is shown (mean \pm SD, n=3). * $p < 0.0001$, MANOVA, between subject effect (HEK1R-compared to HEK cells), thapsigargin 0 and 20 nMol were considered as repeated measurements. (B) PTHrP augments PTH1R-dependent caspase activation following treatment of HEK1R cells with thapsigargin. Addition of N-PTHrP significantly potentiated activation of caspases triggered by PTH1R. Shown here is the increase in caspase activity through addition of N-PTHrP(1-34), 900 pMol. * $p < 0.0001$, MANOVA, between subject effect (HEK1R compared to HEK cells), thapsigargin 0 and 20 nMol were considered as repeated measurements.

potential of HEK1R cells following treatment with thapsigargin (Fig. 4B). Thus, PTH1R activation by thapsigargin contributes to apoptosis by inducing the mitochondrial apoptosis signaling pathway. Thapsigargin-dependent alteration of the mitochondrial membrane potential was further enhanced by addition of N-terminal PTHrP (Fig. 4C). This suggests that G_s -mediated cAMP signaling and G_q -mediated phospholipase C/ Ca^{2+} signaling is involved in induction of PTH1R-mediated apoptosis.

Discussion

The purpose of this study was to determine the effects of PTH/PTHrP receptor (PTH1R) activation on the apoptosis signaling programs of human embryonic kidney (HEK) cells. Data obtained in the present study indicate a model for interference of PTH1R with key regulators of apoptosis signaling. Our data suggest that apoptosis is initiated by activation of PTH1R in HEK cells, and that this is likely to contribute to the spectrum of physiological responses to PTH and/or PTHrP. Overexpression of wild-type PTH1R induced

apoptosis in HEK cells and activated apoptosis signaling via death receptors and mitochondria.

Stimulation experiments of the CD95, TNF-R and TRAIL-R death receptor systems revealed that activation of PTH1R in HEK cells triggers signaling via each of these death receptors and consequently sensitize HEK 293 cells stably expressing PTH1R towards CD95-, TNF-R- and TRAIL-R-mediated apoptosis. Furthermore, our findings demonstrate a link between activation of the PTH1R and the mitochondrial apoptosis pathway. Stimulation of the PTH1R induces mitochondrial-mediated apoptosis signaling.

The effects of PTH1R-activation on apoptosis likely depend on the cell type and the differentiation state of the cells. PTH was reported to be antiapoptotic in osteoblasts (32,45), whereas it was shown before to promote apoptosis in HEK 293 cells (31). In addition, findings of a bi-directional effect of PTH on cells of differing maturity states, were reported in mesenchymal cell lines. PTH was shown to be antiapoptotic in preconfluent cells and proapoptotic in more differentiated postconfluent cells. In accordance with the bi-directional

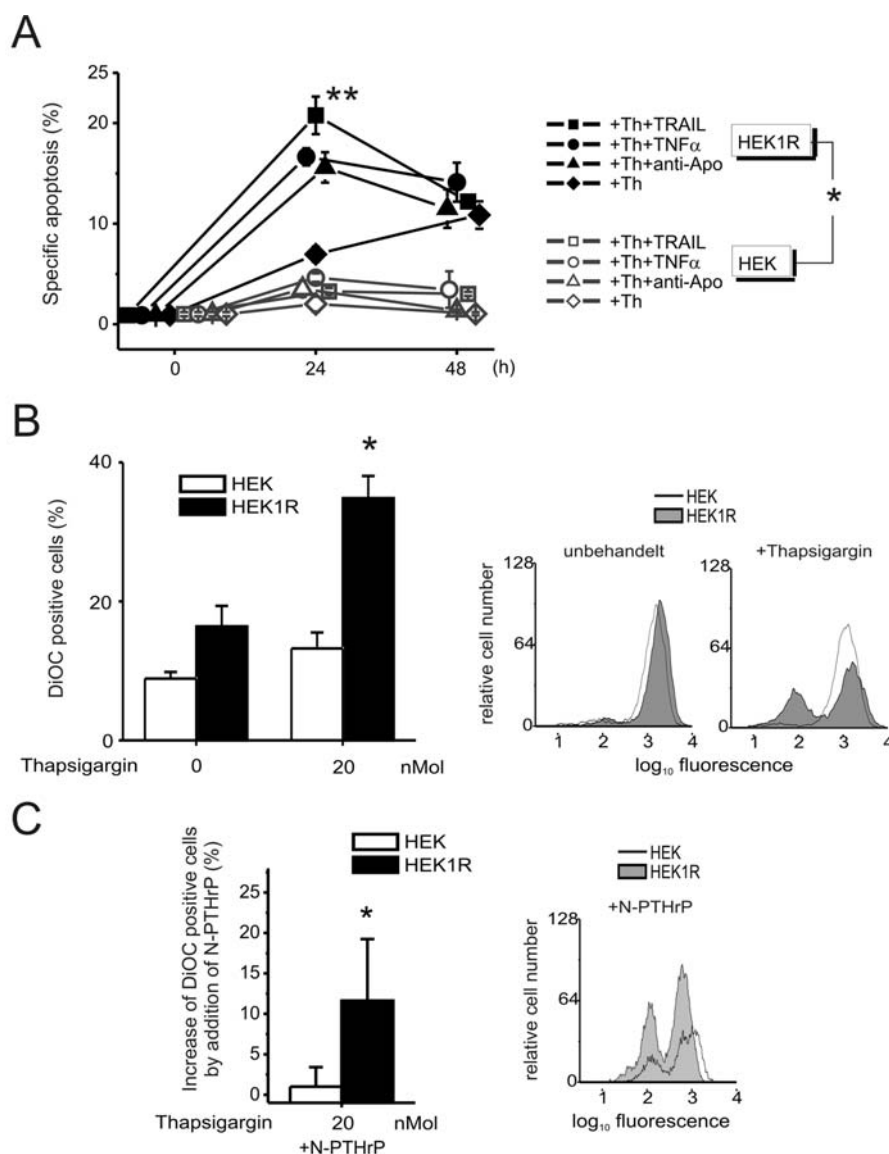


Figure 4. Activation of the PTH1R triggers the extrinsic apoptosis pathway via activation of death receptors and the intrinsic apoptosis pathway via activation of mitochondria. (A) Activation of the PTH1R engages the death receptor-triggered apoptosis signaling pathway. Addition of the specific agonistic antibodies to and ligands of CD95, TRAIL-R and TNF-R following treatment of HEK1R or HEK cells with thapsigargin led to a further increase of thapsigargin-induced apoptosis of HEK1R cells and to a sensitization towards CD95-, TRAIL-R- and TNF-R-mediated apoptosis. Following thapsigargin treatment for 24/48 h, specific ligands and antibodies (Killer-TRAIL, agonistic anti-APO-1 antibody or TNF- α) were added 24 h before harvesting. Three independent experiments were performed, and a representative result is shown, mean \pm SD, $n=3$. * $p<0.0001$, MANOVA between subject effect comparing HEK1R vs. HEK cells for all treatment groups (time points 0, 24, 48 h were considered as repeated measurements); ** $p<0.0001$, MANOVA between subject effect comparing thapsigargin + ligand/agonistic antibody vs. thapsigargin alone (ligand/agonistic antibody effect), for all ligands/antibodies (time points 2, 24, 48 h were considered as repeated measurements). (B) Activation of PTH1R engages the mitochondrial apoptosis signaling pathway. FACSscan analysis of HEK and HEK1R cells following DiOC staining showed alteration of the mitochondrial membrane potential after treatment with thapsigargin. Alteration of the mitochondrial membrane potential was significantly enhanced in HEK1R cells. Shown is one representative out of three experiments performed. Presented is mean \pm SD, $n=3$. * $p<0.0001$, MANOVA, between subject effect (comparing HEK1R vs. HEK cells), considering thapsigargin 0 and 20 nMol as repeated measurements. (C) N-terminal PTHrP augment mitochondrial-dependent apoptosis triggered by PTH1R. FACSscan analysis of HEK and HEK1R cells following DiOC staining showed alteration of the mitochondrial membrane potential after treatment with thapsigargin, which was significantly enhanced by N-terminal PTHrP in HEK1R cells. Two independent experiments were performed, and a representative result is shown, mean \pm SD, $n=6$. * $p<0.005$, Wilcoxon test, compared to HEK cells.

effect of PTH1R triggering on apoptosis of mesenchymal cells, we previously showed that in Saos-2 cells, triggering the PTHrP/PTH1R axis inhibits major apoptosis signaling pathways by blocking signaling via p53, death receptors and mitochondria and, consequently, confers chemoresistance of cancer cells (Gagiannis *et al*, Int J Cancer, in press). This strongly argues in favour of both, pro- and antiapoptotic effects of the PTH/PTHrP/PTH1R ligand/receptor system. Furthermore, the effects of the PTH/PTHrP/PTH1R system on

apoptosis are dependent on the cell type and differentiation status. Tumor cells may benefit from an antiapoptotic action of PTHrP, which confers a selective growth advantage upon tumor cells. Embryonic cells, on the other hand, may benefit from a proapoptotic PTH/PTHrP/PTH1R system, which promotes cell departure from the differentiation program later in the developmental scheme.

The underlying mechanisms of this bi-directional effect of PTH1R signaling on apoptosis pathways are not clarified

yet but were reported to be dependent on the PKA pathway (32) in mesenchymal cells. The PTH1R responds to binding of PTH or PTHrP by activation of the PKA and PKC pathways. The PKA pathway is mediated by the G_s protein that activates adenylylcyclase and leads to cAMP production and PKA activation. The PKC pathway is mediated by the G_q protein that activates phospholipase C, resulting in an increase in intracellular Ca^{2+} levels and activation of PKC. In contrast, the proapoptotic effects of PTH reported in HEK cells were found to be dependent on the PKC pathway (31). In our cell system, HEK cells, activation of the cloned PTH1R produced concurrent signaling through both pathways. Our results suggest that G_s -mediated cAMP signaling and G_q -mediated phospholipase C/ Ca^{2+} signaling are involved in apoptosis signaling triggered by activation of PTH1R.

In summary, activation of the PTH/PTHrP/PTH1R ligand/receptor system in HEK cells activated multiple apoptosis signaling pathways by stimulating both, death receptor- and mitochondria-mediated apoptosis signaling. Interfering with apoptosis signaling pathways may be a key mechanism through which the PTH/PTHrP/PTH1R system exerts its physiological and developmental functions in cell proliferation, differentiation and survival.

Acknowledgements

This work was supported by the Tumorzentrum Heidelberg/Mannheim to M.M. and T.S.; the Helmholtz Alliance of Immunotherapy to M.M.; the Forschungsschwerpunkt program Baden-Württemberg to M.M.; the Hopp-Stiftung 'Altern - Mobilität erhalten, Regeneration ermöglichen' to T.S. and P.P.N. We thank Eberhard Blind for providing the HEK1R cell line and we thank Gisela Schwahn, Katja Lorenz and Petra Hill for expert technical assistance.

References

- Collip JB: The internal secretion of the parathyroid glands. *Proc Natl Acad Sci USA* 11: 484-485, 1925.
- Barnicot NA: The local action of the parathyroid and other tissues on bone in intracerebral grafts. *J Anat* 82: 233-248, 1948.
- Pullman TN, Lavender AR, Aho I and Rasmussen H: Direct renal action of a purified parathyroid extract. *Endocrinology* 67: 570-582, 1960.
- Juppner H, Abou SA, Freeman M, *et al*: A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science* 254: 1024-1026, 1991.
- Clemens TL, Cormier S, Eichinger A, *et al*: Parathyroid hormone-related protein and its receptors: nuclear functions and roles in the renal and cardiovascular systems, the placental trophoblasts and the pancreatic islets. *Br J Pharmacol* 134: 1113-1136, 2001.
- Gensure RC, Gardella TJ and Juppner H: Parathyroid hormone and parathyroid hormone-related peptide, and their receptors. *Biochem Biophys Res Commun* 328: 666-678, 2005.
- Pioszak AA and Xu HE: Molecular recognition of parathyroid hormone by its G protein-coupled receptor. *Proc Natl Acad Sci USA* 105: 5034-5039, 2008.
- Juppner H, Abou SA, Ueno S, Gu WX, Potts JT Jr and Segre GV: The parathyroid hormone-like peptide associated with humoral hypercalcemia of malignancy and parathyroid hormone bind to the same receptor on the plasma membrane of ROS 17/2.8 cells. *J Biol Chem* 263: 8557-8560, 1988.
- Moseley JM, Kubota M, Diefenbach-Jagger H, *et al*: Parathyroid hormone-related protein purified from a human lung cancer cell line. *Proc Natl Acad Sci USA* 84: 5048-5052, 1987.
- Suva LJ, Winslow GA, Wettenhall REH, *et al*: A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. *Science* 237: 893-896, 1987.
- Burtis WJ, Wu T, Bunch C, *et al*: Identification of a novel 17,000-dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumour associated with humoral hypercalcemia of malignancy. *J Biol Chem* 262: 7151-7156, 1987.
- Lanske B, Amling M, Neff L, Guiducci J, Baron R and Kronenberg HM: Ablation of the PTHrP gene or the PTH/PTHrP receptor gene leads to distinct abnormalities in bone development. *J Clin Invest* 104: 399-407, 1999.
- Lanske B, Karaplis AC, Lee K, *et al*: PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* 273: 663-666, 1996.
- Diefenbach JH, Brenner C, Kemp BE, *et al*: Arg21 is the preferred kexin cleavage site in parathyroid-hormone-related protein. *Eur J Biochem* 229: 91-98, 1995.
- Ditmer LS, Burton DW and Deftos LJ: Elimination of the carboxy-terminal sequences of parathyroid hormone-related protein 1-173 increases production and secretion of the truncated forms. *Endocrinology* 137: 1608-1617, 1996.
- Wu TL, Vasavada RC, Yang K, *et al*: Structural and physiologic characterization of the mid-region secretory species of parathyroid hormone-related protein. *J Biol Chem* 271: 24371-24381, 1996.
- Dittmer A, Vetter M, Schunke D, *et al*: Parathyroid hormone-related protein regulates tumor-relevant genes in breast cancer cells. *J Biol Chem* 281: 14563-14572, 2006.
- Mannstadt M, Juppner H and Gardella TJ: Receptors for PTH and PTHrP: their biological importance and functional properties. *Am J Physiol* 277: F665-F675, 1999.
- Maioli E and Fortino V: The complexity of parathyroid hormone-related protein signalling. *Cell Mol Life Sci* 61: 257-262, 2004.
- Cataisson C, Lieberherr M, Cros M, *et al*: Parathyroid hormone-related peptide stimulates proliferation of highly tumorigenic human SV40-immortalized breast epithelial cells. *J Bone Miner Res* 15: 2129-2139, 2000.
- Massfelder T, Dann P, Wu TL, Vasavada R, Helwig JJ and Stewart AF: Opposing mitogenic and anti-mitogenic actions of parathyroid hormone-related protein in vascular smooth muscle cells: a critical role for nuclear targeting. *Proc Natl Acad Sci USA* 94: 13630-13635, 1997.
- Lam MH, Briggs LJ, Hu W, Martin TJ, Gillespie MT and Jans DA: Importin beta recognizes parathyroid hormone-related protein with high affinity and mediates its nuclear import in the absence of importin alpha. *J Biol Chem* 274: 7391-7398, 1999.
- Henderson JE, Amizuka N, Warshawsky H, *et al*: Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. *Mol Cell Biol* 15: 4064-4075, 1995.
- Shen X and Falzon M: Parathyroid hormone-related protein upregulates integrin expression via an intracrine pathway in PC-3 prostate cancer cells. *Regul Pept* 113: 17-29, 2003.
- Pickard BW, Hodsman AB, Fraher LJ and Watson PH: Type 1 parathyroid hormone receptor (PTH1R) nuclear trafficking: regulation of PTH1R nuclear-cytoplasmic shuttling by importin-alpha/beta and chromosomal region maintenance 1/exportin 1. *Endocrinology* 148: 2282-2289, 2007.
- Ferrari SL, Behar V, Chorev M, Rosenblatt M and Bisello A: Endocytosis of ligand-human parathyroid hormone receptor 1 complexes is protein kinase C-dependent and involves beta-arrestin2. Real-time monitoring by fluorescence microscopy. *J Biol Chem* 274: 29968-29975, 1999.
- Conlan LA, Martin TJ and Gillespie MT: The COOH-terminus of parathyroid hormone-related protein (PTHrP) interacts with beta-arrestin 1B. *FEBS Lett* 527: 71-75, 2002.
- Rey A, Manen D, Rizzoli R, Caverzasio J and Ferrari SL: Proline-rich motifs in the parathyroid hormone (PTH)/PTH-related protein receptor C terminus mediate scaffolding of c-Src with beta-arrestin2 for ERK1/2 activation. *J Biol Chem* 281: 38181-38188, 2006.
- Gesty-Palmer D, Chen M, Reiter E, *et al*: Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *J Biol Chem* 281: 10856-10864, 2006.
- Sneddon WB and Friedman PA: Beta-arrestin-dependent parathyroid hormone-stimulated extracellular signal-regulated kinase activation and parathyroid hormone type 1 receptor internalization. *Endocrinology* 148: 4073-4079, 2007.

31. Turner PR, Mefford S, Christakos S and Nissenson RA: Apoptosis mediated by activation of the G protein-coupled receptor for parathyroid hormone (PTH)/PTH-related protein (PTHrP). *Mol Endocrinol* 14: 241-254, 2000.
32. Chen HL, Demiralp B, Schneider A, *et al*: Parathyroid hormone and parathyroid hormone-related protein exert both pro- and anti-apoptotic effects in mesenchymal cells. *J Biol Chem* 277: 19374-19381, 2002.
33. Blind E, Bambino T and Nissenson RA: Agonist-stimulated phosphorylation of the G protein-coupled receptor for parathyroid hormone (PTH) and PTH-related protein. *Endocrinology* 136: 4271-4277, 1995.
34. John MR, Bosel J, Breit S, Wickert H, Ziegler R and Blind E: Changing serine-485 to alanine in the opossum parathyroid hormone (PTH)/PTH-related peptide receptor enhances PTH stimulation of phospholipase C in a stably transfected human kidney cell line: a useful model for PTH-analog screening? *Bone* 28: 182-186, 2001.
35. Gressner O, Schilling T, Lorenz K, *et al*: TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria. *EMBO J* 24: 2458-2471, 2005.
36. Müller M, Schilling T, Sayan AE, *et al*: TAp73/DeltaNp73 influences apoptotic response, chemosensitivity and prognosis in hepatocellular carcinoma. *Cell Death Differ* 12: 1564-1577, 2005.
37. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F and Riccardi C: A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 139: 271-279, 1991.
38. Müller M, Wilder S, Bannasch D, *et al*: p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. *J Exp Med* 188: 2033-2045, 1998.
39. Trauth BC, Klas C, Peters AJM, *et al*: Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* 245: 301-305, 1989.
40. Dhein J, Daniel PT, Trauth BC, Oehm A, Möller P and Krammer PH: Induction of apoptosis by monoclonal antibody anti-APO-1 class switch variants is dependent on cross-linking of APO-1 cell surface antigens. *J Immunol* 149: 3166-3173, 1992.
41. Müller M, Strand S, Hug H, *et al*: Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. *J Clin Invest* 99: 403-413, 1997.
42. Lund PK, Westvik AB, Joo GB, Ovstebo R, Haug KB and Kierulf P: Flow cytometric evaluation of apoptosis, necrosis and recovery when culturing monocytes. *J Immunol Methods* 252: 45-55, 2001.
43. Zuliani T, Duval R, Jayat C, *et al*: Sensitive and reliable JC-1 and TOTO-3 double staining to assess mitochondrial transmembrane potential and plasma membrane integrity: Interest for cell death investigations. *Cytometry* 54: A100-A108, 2003.
44. Lin XS, Denmeade SR, Cisek L and Isaacs JT: Mechanism and role of growth arrest in programmed (apoptotic) death of prostatic cancer cells induced by thapsigargin. *Prostate* 33: 201-207, 1997.
45. Calvi LM, Sims NA, Hunzelman JL, *et al*: Activated parathyroid hormone/parathyroid hormone-related protein receptor in osteoblastic cells differentially affects cortical and trabecular bone. *J Clin Invest* 107: 277-286, 2001.