PARATHYROID HORMONE-RELATED protein (PTHrP) is best known as the mediator of humoral hypercalcemia of malignancy, but several lines of evidence suggest that it could also play an important role in lung cancer progression. PTHrP demonstrates widespread distribution in lung carcinomas, being present in a high percentage of lung tumors (13) of all histological types (4). In addition, PTHrP is an autocrine growth factor for lung cancer cells (5) and protects them from apoptosis (11), characteristics that could contribute to a cancer’s capacity for unregulated growth. Furthermore, PTHrP induces expression of metalloproteinases in carcinoma cells and may stimulate angiogenesis in tumors (3, 17). These factors are important in providing a carcinoma with the means to develop its blood supply, to invade into adjacent tissues, and to metastasize. Finally, several reports suggest that PTHrP expression may contribute to morbidity and mortality of patients with lung carcinoma. For instance, expression increases ranging from early stage, indolent tumors to more advanced, aggressive tumors (13) and elevated serum PTHrP levels in cancer patients portend a shorter life expectancy (14, 20). Accordingly, PTHrP could contribute to carcinogenesis, angiogenesis, progression, resistance to therapy, or metastasis of lung carcinomas.

A recent study (11) reported that the PTHrP-derived peptide PTHrP-(140–173) exerts regulatory effects on lung cancer cell apoptosis. Pretreatment with 100 nM PTHrP-(140–173) for 24 h protected BEN human squamous lung carcinoma cells from the apoptotic effects of ultraviolet (UV)-B irradiation or activating Fas antibody (11). The protective effects were marked by decreased cell death, decreased activation of caspase-3, -8, and -9, decreased nuclear condensation and apoptotic body formation, and increased survival in a clonogenic survival assay. Thus the peptide protected the lung cancer cells against caspase-8-mediated apoptosis, although mechanisms were not established. PTHrP-(1–34) also reduced caspase-3 activation and DNA fragmentation after UV irradiation. However, the amino-terminal peptide did not reduce caspase-8 activity or increase clonogenic survival, casting doubt on its role as an antiapoptotic agent for the lung cancer cells. The finding that PTHrP-(140–173) had antiapoptotic effects was novel. Aside from preliminary observations in prostate cancer (2), the only other known biological function of PTHrP-(140–173) is to decrease pyrophosphate levels and inhibit collagen synthesis in cultured rabbit articular chondrocytes (8). Because recognition of physiological activity is so recent, comprehensive investigation has not yet occurred and a receptor for this particular PTHrP peptide has not been identified.

The signaling pathways mediating the antiapoptotic effects of PTHrP-(140–173) in lung cancer cells are unknown, but either protein kinase A (PKA) or protein kinase C (PKC) can protect cells from apoptosis (9, 10, 24, 26). Thus this study investigated the role of signaling pathways in the antiapoptotic effects of PTHrP peptides in lung cancer cells. Our aims were to compare PTHrP-(1–34) and PTHrP-(140–173) for activation of PKA and PKC, to test whether second messenger pathway inhibitors block the effects of PTHrP-(140–173), and to determine the effects of signal transduction pathway activation on sensitivity of the cells to apoptosis.


Parathyroid hormone-related protein regulates apoptosis in lung cancer cells through protein kinase A

Randolph H. Hastings,1,2,4 Flavio Araiza,1 Douglas W. Burton,1,3,5 Maxwell Bedley,1 and Leonard J. Deftos1,3,5
1Research, 2Anesthesiology, and 3Medicine Services, Department of Veterans Affairs
San Diego Healthcare System, San Diego 92161; and Departments of 4Anesthesiology and 5Medicine, University of California-San Diego, La Jolla, California 92093

Submitted 24 June 2004; accepted in final form 24 July 2004

Hastings, Randolph H., Flavio Araiza, Douglas W. Burton, Maxwell Bedley, and Leonard J. Deftos. Parathyroid hormone-related protein regulates apoptosis in lung cancer cells through protein kinase A. Am J Physiol Cell Physiol 287: C1616–C1622, 2004. First published July 28, 2004; doi:10.1152/ajpcell.00300.2004.—Parathyroid hormone-related protein (PTHrP)-(1–34) and PTHrP-(140–173) protect lung cancer cells from apoptosis after ultraviolet (UV) irradiation. This study evaluated upstream signaling in PTHrP-mediated alteration of lung cancer cell sensitivity to apoptosis. The two peptides increased cAMP levels in BEN lung cancer cells by 15–35% in a dose-dependent fashion, suggesting signaling through protein kinase A (PKA). In line with this view, the PKA inhibitor H89 abrogated the protective effects of PTHrP-(1–34) and PTHrP-(140–173) against caspase activation and DNA loss. PKA activation by forskolin, 3-isobutyl-1-methylxanthine (IBMX), or 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate attenuated and H89 augmented apoptosis after UV exposure as indicated by caspase-3 activation, cell DNA loss, and morphological criteria. Studies with IBMX and varying doses of forskolin indicated that small increases in cAMP, on the order of those generated by IBMX alone and the PTHrP peptides, were sufficient to protect lung cancer cells from apoptosis. In summary, PTHrP-(1–34) and PTHrP-(140–173) stimulate PKA in lung carcinoma cells and protect cells against UV-induced caspase-3 activation and DNA fragmentation. PKA activation by other means also induces resistance to apoptosis, and the protective effect of the PTHrP peptide is blocked by PKA inhibition. Thus PKA appears to have a role in the regulatory effects of PTHrP on lung cancer cell survival.
METHODS

Chemicals

The PTHrP peptides were purchased from Bachem (Torrance, CA). All other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified.

Cell Culture

BEN squamous lung cancer cells (a gift of T. J. Martin, University of Melbourne) were plated in 100-mm dishes and grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine at 37°C in an atmosphere of 5% CO2-95% air.

Cell Treatments

Experiments were conducted with cells at 60–70% confluence. The PKA inhibitor H89 (0.05–5 μM) was incubated with cells for 30 min before other treatments or exposure to UV irradiation. Agonists, including 0.1–10 μM forskolin, 1 μM 3-isobutyl-1-methylxanthine (IBMX), 10 μM 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CPT-cAMP), 100 nM phorbol 12-myristate 13-acetate (PMA), 0.1 μM ATP, 100 nM PTHrP-(1–34), and 100 nM PTHrP-(140–173), were applied to cells 10 min before UV irradiation.

Second Messenger Assays

cAMP and inositol phosphates were measured with protocols described previously (12). Cells were washed twice with PBS and incubated for 30 min in serum-free medium before application of agonists. Cells were incubated for 15 min with 100 nM PTHrP-(1–34) or 100 nM PTHrP-(140–173) to assess effects on cAMP production. Cells were lysed and extracts were assayed for cAMP and inositol phosphates were measured with protocols described previously (27). Data are reported as means ± SE.

UV Irradiation

Apoptosis experiments used cells in growth medium with serum. Apoptosis was induced by irradiation with 0.9 J/cm2 UV-B administered over 30 min as previously described (11). Cells were studied after 24 h recovery in growth medium.

Caspase-3 Assays

Adherent cells were washed once in PBS, scraped from the plate, pooled with the nonadherent cells from the same wells, lysed by sonication in caspase lysis buffer [50 mM PIPES-KOH, 2 mM EDTA, 0.1% (wt/vol) 3-[3-(Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM dithiothreitol, 1 μM leupeptin and pepstatin A], and frozen at −20°C until time of assay. Caspase-3 activity was measured in cell lysates by a fluorescent substrate assay in a 96-well plate format as described previously (11). The mass of cell protein for each sample was adjusted to 20 μg, measured by the Pierce bicinchoninic acid (BCA) protein assay. The substrate was acetyl-aspartyl-glutamyl-valyl-aspartyl-7-amino-4-methyl-coumarin (AMC). Activities were calculated as the slope of the plot of relative fluorescent units (fluorescence – background in arbitrary units) vs. time over the first 1–2 h, the linear portion of the curve.

Flow Cytometry

Pellets containing 3 × 106 cells were fixed in an equal volume of 100% ethanol, resuspended in a solution of 50 μg/ml propidium iodide and 1.3 mg/ml RNase in PBS, and analyzed on a Coulter Elite flow cytometer (Beckman Coulter Electronics, Miami, FL). Cell fractions in G0/G1, S, and G2/M phase were determined from histograms of propidium iodide fluorescence with Multicycle software (Phoenix Flow Systems, San Diego, CA). Cells whose propidium iodide fluorescence was less than the lower limit of the G0/G1 peak, assessed in cells that were not irradiated, were considered apoptotic on the basis of their subdiploid DNA content.

Fluorescent Microscopy

BEN cells in 60-mm dishes were fixed with 100% methanol, stained with 4 μM Hoescht 33342 dye (Calbiochem, San Diego, CA) to label nuclei fluorescently, and coverslipped. Cells were imaged with a Nikon Eclipse TE300 inverted microscope (Nikon USA, Melville, NY) at ×400 magnification with a Plan Fluorite objective lens, numerical aperture 0.75. Hoescht 33342 was excited with light from a 100-W mercury lamp passing through a UV-2EC filter, giving wavelengths of 340–380 nm, and the emitted light was collected through a 435- to 485-nm barrier filter. Digital images were captured with a Spot Slider2 Camera, model 1.4.0, operated with Spot Software Version 2.2.2 (Diagnostic Instruments, Sterling Heights, MI) and saved as 12-bit (the standard format for the Spot camera) grayscale TIFF files at 1,600 × 1,200 pixels. The optical path, lamp intensity, and exposure time were held constant for all images. Exposure time was set to avoid saturating the pixel intensities in the brightest image.

Image Analysis

An observer blinded to the experimental group counted total nuclei and fragmented nuclei in each image. Four separate images each in two independent cell preparations were evaluated for each experimental group. At least 300 cells were counted for each preparation.

Statistical Analysis

Values were compared among experimental groups by analysis of variance, and the Tukey test was used for post hoc pairwise comparisons (27). Data are reported as means ± SE. Significance was accepted if the probability of a type I error was <0.05.

RESULTS

Secondary Messenger Production from PTHrP Peptides

PTHrP-(1–34) and PTHrP-(140–173) both stimulated production of cAMP by lung cancer cells in a dose-dependent fashion (Fig. 1). Between the lowest and highest doses of PTHrP-(1–34), cAMP levels rose by ~15% (P < 0.05 vs. lowest dose). PTHrP-(140–173) caused greater increases in secondary messenger production, ~35% (P < 0.05). These experiments were performed in the presence of 1 μM IBMX. IBMX by itself increased cAMP by 15 ± 5% vs. cells treated with the DMSO vehicle, an increase roughly the same as that caused by PTHrP-(1–34) treatment. In contrast, cAMP levels were increased almost 30-fold in cells treated with forskolin (data not shown). PTHrP peptides did not alter inositol phosphate levels in the lung cancer cells. Treatment with ATP caused a 70% increase in inositol phosphates (n = 4; P < 0.05), demonstrating that the cells were able to augment their phospholipase C activity in response to a paracrine stimulus. We performed additional studies with PTHrP-(140–173) because its signaling mechanisms have not been previously investigated. We also screened a series of prostate carcinoma cell lines to investigate whether PTHrP-(140–173) activates PKA or phospholipase C in other cell types. In Dupro-1 and
LNCaP prostate carcinoma cells, 100 nM PTHrP-(1–34) stimulated inositol phosphate levels by 29 ± 10% and 24 ± 2%, respectively, compared with untreated cells (P < 0.05).

Interactive Effects of PTHrP-(1–34), PTHrP-(140–173), and PKA on UV-Induced Apoptosis

The PTHrP peptides protected lung cancer cells against UV-induced apoptosis as previously described (11). Pretreating BEN cells with 100 nM PTHrP-(140–173) reduced caspase-3 activity after UV by 15–25% (Fig. 2; P < 0.05 vs. untreated irradiated cells). In addition, the amino-terminal and carboxy-terminal peptides reduced the size of the population of cells with less than a diploid complement of DNA by 22% and 14%, respectively, compared with untreated irradiated cells (P < 0.05). The percentage of cells with subdiploid DNA content was 27 ± 2% in untreated irradiated cells vs. 20.8 ± 0.9% and 20.9 ± 1.0% in cells treated with PTHrP-(1–34) and PTHrP-(140–173), respectively (Fig. 3). The protective effects were blocked when PKA was pharmacologically inhibited. When cells were treated with 5 μM H89 before PTHrP-(140–173) exposure, the peptide had no significant effect on caspase-3 activity or the incidence of DNA loss (Figs. 2 and 3).

Effect of PKA or PKC Activation on UV-Induced Apoptosis

Effects on caspase activities. UV irradiation caused a three-fold increase in caspase-3 activity in lung cancer cell lysates (Fig. 4). Pretreating cells with IBMX or forskolin plus IBMX reduced caspase activities in irradiated cells to levels close to control values. PMA caused a small decrease in caspase-3 activity, but the effect was not statistically significant. None of the treatments had a significant effect on caspase-3 in the absence of irradiation. We observed that IBMX ameliorated UV-induced caspase activation to approximately the same extent as forskolin plus IBMX. To expand on this finding, we also investigated whether forskolin would have a protective effect by itself. Forskolin treatment alone reduced caspase activity in irradiated cells to 28 ± 0.3% of levels in irradiated cells that were untreated (n = 3 replicates per experimental treatment; P < 0.001), similar to the effect of forskolin plus IBMX. We also tested the effect of another PKA activator, CPT-cAMP. It, too, protected against caspase-3 activation, reducing levels to 59 ± 1% of activity in untreated but irradiated cells (P < 0.001; n = 4 replicates/group).

Effects on DNA fragmentation and loss. The incidence of apoptotic cells with a subdiploid DNA content increased after exposure to UV radiation from 6 ± 1% in control cells to 35 ± 4% after UV exposure and recovery (Fig. 5A; P < 0.001). IBMX and forskolin plus IBMX reduced the percentage of apoptotic cells after UV irradiation to 11 ± 1% and 5 ± 2%, respectively, not significantly different than values in nonirradiated control cells (P < 0.001 vs. irradiated untreated cells). Forskolin and IBMX had no effect on the size of the subdiploid peak in nonirradiated cells. To gain an appreciation for the relationship between cAMP generation and apoptosis protection, we examined the dose dependence for the effects of forskolin on the size of the subdiploid peak and on cAMP production (Fig. 6). Production of cAMP had a steep dose dependence, with minimal change after treatment with 0.1 μM forskolin and only a near-maximal effect at 1 μM (Fig. 6B). Similarly, 0.1 μM forskolin caused a small, not statistically significant decline in apoptosis whereas 1 μM forskolin had a near-maximal effect. IBMX stimulated cAMP production to a significantly lesser degree than 1 μM forskolin and only slightly greater than 0.1 μM forskolin (Fig. 6B) but had the
same inhibitory effect on apoptosis as the higher concentrations of forskolin (Fig. 6A).

Effects of PKA Inhibition on UV-Induced Apoptosis

Effects on caspase activity. BEN lung cancer cells that were pretreated for 30 min with H89, a PKA inhibitor, demonstrated a nearly 50% increase in caspase-3 activity after UV compared with untreated irradiated cells (P < 0.05; Fig. 7). H89 had no effect on caspase-3 activity in nonirradiated cells.

Effect on DNA fragmentation and loss. H89 caused a dose-dependent increase in the percentage of cells with subdiploid DNA content after UV irradiation (Fig. 8). H89 had no effect on subdiploid DNA content in nonirradiated cells (data not shown).

DISCUSSION

Our work has provided evidence that PTHrP-(1–34) and PTHrP-(140–173) both activate PKA in lung cancer cells. It was already known that PTHrP-(1–34) acts through a known G protein-coupled receptor and can activate PKA, PKC, intracellular calcium, and nitric oxide pathways in different cell types (1, 16, 19). It was reasonable to surmise that the two PTHrP peptides might share signaling mechanisms, because both reduced caspase-3 activation after UV irradiation in our previous study (11). In fact, the two peptides augmented cAMP levels in cultured lung cancer cells, with PTHrP-(140–173) having a somewhat larger effect than its amino-terminal counterpart. The extent of the increase was similar to that observed following phosphodiesterase inhibition with IBMX. Our results for amino-terminal PTHrP are in agreement with Piziozzi and coworkers (21), who also found that PTHrP-(1–34) and purified PTHrP-(1–141) stimulated cAMP production in BEN cells. Neither peptide affected inositol phosphate levels in our study, arguing against a role for phospholipase C in downstream signaling in lung cancer cells. Parathyroid hormone can activate PKC through the action of phospholipase D (23, 25), but we did not examine this pathway and we did not measure PKC activation directly. Because PTHrP-(140–173) augments cAMP levels, the peptide may interact with a G protein-coupled receptor, similar to the receptor for PTHrP-(1–34) (1, 16, 19). In addition to effects in lung cancer, our laboratory has found (2) that PTHrP-(140–173) also exerts cell surface effects in prostate carcinoma cells, where it stimulated increases in inositol phosphates. Thus a receptor for PTHrP-(140–173) may be found in various tissues, including at least lung cancer and prostate cancer.

Studies with our well-established model using UV irradiation as an apoptosis-producing stimulus provided evidence that the antipapoptotic effects of the PTHrP peptides were linked to PKA. PTHrP-(1–34) and PTHrP-(140–173) reduced caspase-3 activation and attenuated DNA loss after UV irradiation in lung cancer cells, consistent with our previous results (11). PKA blockade with H89 prevented these protective effects, indicating that PKA acts downstream of the peptides and that PKA-independent antipapoptotic effects are unlikely to be important. Investigating the effects of activating drugs bolstered the evidence in favor of PKA-mediated resistance to apoptosis. Agents that augmented cAMP levels or stimulated PKA, such as forskolin, IBMX and CPT-cAMP, reduced lung cancer apoptosis after UV irradiation. The experimental design utilized several measures of apoptosis to endow the results with greater confidence. The measures included evaluation of caspase activation, DNA loss, and morphological criteria. The changes in caspase activation and DNA loss with IBMX and low-dose forskolin shown in Fig. 6 indicate that very small changes in cAMP were sufficient to protect cells from programmed cell death. Indeed, the efficacy of the PTHrP peptides appeared to be dependent on the potent relationship between cAMP and apoptosis resistance. PTHrP-(1–34), PTHrP-(140–173), and IBMX each increased cAMP by small increments, on the order of the changes with low-dose forskolin. Nonetheless, the changes induced by each were sufficient to alter the apoptotic process. BEN cells make PTHrP, so the endogenous
protein would confer some resistance against apoptotic stimuli. BEN cell PTHrP production would not affect our results because it is constant throughout the experimental groups. The endogenous effect must not be maximal, because exogenous PTHrP accords additional protection.

Our observations on PTHrP, PKA, and programmed cell death are consistent with a large body of studies on lung cancer and other tissues. It is well established that cAMP can have pro- or antiapoptotic effects depending on cell type and context. For example, increases in cAMP induce apoptosis in S49 T lymphoma cells (28) but protect intestinal crypt cells and hepatocytes against apoptosis (9, 24). PKA is involved in apoptosis protection for a variety of cancers, including non-small cell carcinoma and small cell carcinoma (7, 15, 18, 22). An example of this type of effect in lung cancer is the ability of nicotine to suppress apoptosis by PKA-mediated phosphorylation of Bad (15). Other investigators have reported that PTHrP regulates apoptosis through PKA. For instance, PTHrP-(1–34) blocks dexamethasone-mediated apoptosis of preconfluent mesenchymal cells in a cAMP-dependent manner (6). Thus our discovery that PTHrP confers apoptosis resistance in lung cancer utilizing a PKA-dependent mechanism is in concert with results in previous studies.

In summary, we have demonstrated that PTHrP-(140–173) augments lung cancer cell cAMP levels, that PKA activation protects lung cancer cells from UV-induced apoptosis, and that PKA inhibition blocks the antiapoptotic effects of the PTHrP peptide. The observations on PTHrP-(140–173) are among the first to demonstrate activity of this peptide and to suggest that it could act through a G protein-coupled receptor, similar to the receptor for PTHrP-(1–34). Further studies are indicated to explore downstream mechanisms for the actions of PTHrP on

Fig. 5. DNA distributions of lung cancer cells after UV irradiation and PKA-related treatments. The effects of PKA activation on UV-induced apoptosis were also assessed with flow cytometry. A: means ± SE from 3 or 4 replicates for each experimental group. UV irradiation increased the percentage of subdiploid cells by almost 6-fold in untreated cells (*P < 0.001 vs. all other groups), but DNA loss did not occur in cells that were treated with IBMX or forskolin + IBMX before being irradiated. The data represent results from 3 independent experiments including cell wells from each experimental group. B: representative DNA distributions. These graphs are histogram plots of cell number vs. DNA content for nonirradiated (a, b, c) and irradiated (d, e, f) cells treated with growth medium (a, d), IBMX (b, e), or forskolin + IBMX (c, f) for 10 min before UV irradiation. Apoptotic cells with less than the diploid complement of DNA are located left of the G0/G1 peak and are marked by double-headed arrows. In the cell well shown here that was exposed to UV radiation but otherwise untreated (d), 32% had subdiploid DNA content, indicative of apoptosis, much higher than the incidence in nonirradiated cells. In contrast, the number of apoptotic cells did not increase substantially after UV irradiation in the cells pretreated with IBMX or forskolin + IBMX (e and f). IBMX and forskolin had minimal effects on the subdiploid shoulder of the distribution in nonirradiated cells (b and c), as verified by the average numbers in the plot in A.

Fig. 6. Dose-dependent effects of forskolin on UV-induced apoptosis and cAMP production. Pretreatment with 0.1–10 μM forskolin before UV exposure caused a progressive reduction in the % of BEN lung cancer cells with subdiploid DNA content (A). The maximal antiapoptotic effect occurred at concentrations ≥1 μM. The dose-dependent increase in cAMP production was also maximal at 1 μM forskolin (B). IBMX at a concentration of 1 μM caused a decrease in UV-induced apoptosis similar to that with 1 μM forskolin but stimulated cAMP production to a much lesser effect (*P < 0.01 vs. cells not treated with forskolin or IBMX).
Fig. 7. Effects of PKA inhibition on measures of apoptosis in irradiated cells. H89 augmented caspase-3 activity after UV irradiation by almost 50% (A) and increased the appearance of apoptotic cells with subdiploid DNA content (B). The effects of H89 on apoptosis were dose dependent, as shown in C (*P < 0.01 vs. nonirradiated cells; **P < 0.05 vs. untreated irradiated cells; +P < 0.01 vs. untreated irradiated cells).

Fig. 8. PKA effects on nuclear fragmentation and condensation after UV irradiation. Nuclei in BEN cells were stained with Hoescht 33342 and evaluated by fluorescent microscopy. A: representative fields of control, nonirradiated, and irradiated cells treated with growth medium alone, 10 μM forskolin + 1 μM IBMX, or 5 μM H89. Arrows mark representative fragmented nuclei. An observer unaware of the experimental conditions counted fragmented nuclei and total nuclei, producing the quantitative results shown in B. Fewer than 5% of nuclei were fragmented under control conditions. UV exposure increased this figure to 15%. Both forskolin + IBMX and IBMX alone reduced nuclear fragmentation to levels comparable to those seen in nonirradiated cells. In contrast, pretreatment with H89 augmented nuclear fragmentation by >10%. Data in B represent results from 3 separate experiments. Five fields at ×400 magnification were evaluated per cell preparation (*P < 0.01 vs. nonirradiated control; **P < 0.01 vs. untreated irradiated cells).
apoptosis in lung cancer and to determine the significance of these effects on progression of lung carcinoma.

**REFERENCES**


