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BRIEF REPORT

Effect of Cyclic Adenosine 3′,5′-Monophosphate/Protein Kinase A Pathway on Markers of Cell Proliferation in Nonfunctioning Pituitary Adenomas

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Context: Alterations in cAMP signaling have been identified as a cause of endocrine neoplasia. In particular, activating mutations of the $G_s\alpha$ gene and protein kinase A (PKA) overactivity due to low expression of PKA regulatory subunit 1A (R1A) have been implicated in somatotroph proliferation.

Objective: The objective of this study was to evaluate the effects of cAMP-PKA cascade activation in nonfunctioning pituitary adenomas (NFPA).

Design and Methods: By immunohistochemistry, R1A, R2A, and R2B expression was evaluated in cells obtained from eight surgically removed NFPA positive for gonadotropins. Cyclin D1 expression and ERK1/2 activity were analyzed under basal conditions and after cAMP-PKA cascade activation.

Results: Immunohistochemistry studies demonstrated a low R1/R2 ratio in all NFPA. Additional unbalance of R1/R2 ratio by 8-chloroadenosine cAMP (8-Cl-cAMP) and direct adenylyl cyclase stimulation by forskolin did not increase cyclin D1 expression or ERK1/2 activity in five NFPA (group 1), but even caused 74 ± 15% and 85 ± 13% inhibitions of cyclin D1 and ERK1/2 activity, respectively, in the remaining NFPA (group 2). Moreover, in group 2, PKA blockade by the specific inhibitor PKI increased cyclin D1 expression (96 ± 25% over basal) and ERK1/2 activity (116 ± 28% over basal).

Conclusions: These data show that in contrast with what was previously observed in transformed somatotrophs, activation of the cAMP-PKA pathway did not generate proliferative signals in tumoral cells of the gonadotroph lineage, and in a subset of tumors even exerted a tonic inhibitory effect, thus confirming a different role for the cAMP-mediated pathway in promoting proliferation in the pituitary. **(*J Clin Endocrinol Metab* 90: 6721–6724, 2005)**

CYCLIC AMP is implicated in the regulation of a variety of cell functions that are mainly related to protein phosphorylation through the activation of protein kinase A (PKA). In particular, cAMP inhibits or stimulates cell proliferation depending on the cell type. In recent years, mutations of genes involved in cAMP signaling and resulting in the constitutive activation of cAMP formation have been identified as a cause of endocrine neoplasia. In particular, in addition to the presence of the *gsp* oncogene in about 30–40% of GH-secreting pituitary adenomas (1), genetic defects affecting the PKA complex have been identified in endocrine disorders associated with multiple neoplasia. In mammalian cells there are two types of PKA, PKA1 and PKA2, which share common catalytic subunits, but possess different regulatory subunits, R1 and R2 (2). Through gene cloning, four genes coding for different R isoforms, R1A, R1B, R2A, and R2B, have been identified. These subunits differ in tissue distribution, subcellular localization, and biological properties that have been mainly characterized for R1A, R2A, and R2B. Loss of function mutations in R1A gene (*PRKAR1A*) resulting in unrestrained catalytic activity have been identified in patients with the Carney complex (3). Accordingly, we have recently demonstrated that in the absence of *PRKAR1A* mutations, the expression of PKA R1A subunit protein was low or absent in transformed somatotrophs and that the resulting low R1/R2 ratio represented a mitogenic signal for these cells (4).

Although in the past the ERK1/2 pathway was thought to be specifically activated by growth factors, it is now well established that several G protein-coupled receptors are able to stimulate ERK1/2 by different mechanisms (5, 6). In particular, we have recently demonstrated that the ERK1/2 cascade can be activated by the PKA-dependent pathway in tumorous somatotrophs, probably contributing to the mitogenic potential of the cAMP pathway in this specific cell type (7).

The aim of this study was to evaluate the effects of the cAMP/PKA pathway on cell cycle proteins and ERK1/2 activity in nonfunctioning pituitary adenomas (NFPA), which are largely constituted by cells of the gonadotroph lineage.

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Abbreviations: 8-Cl-cAMP, 8-Chloroadenosine cAMP; IHC, immunohistochemistry; NFPA, nonfunctioning pituitary adenoma; PKA, protein kinase A; PKC, protein kinase C; PKI, PKA inhibitor; R1A, PKA regulatory subunit 1A.

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Materials and Methods

Pituitary tissue samples and cell cultures

The study included eight NFPAs surgically removed by the transsphenoidal route, and fragments were fixed for light microscopy as previously described (8). Tissues surgically removed were in part quickly frozen for subsequent molecular analysis and in part placed in sterile medium for cell culture to perform ERK1/2 activity and cyclin D1 expression after the exclusion of blood cell contamination, as previously described (9, 10). Local ethical approval was obtained for all studies.

PRKAR1A and GNAS1 sequencing analysis

Genomic DNA was extracted with the phenol-chloroform method from adenomatous tissues (Nucleon-Amersham Life Science, Little Chalfont, UK). *PRKAR1A* gene (GenBank accession no. NM 002734) analysis was performed as previously described (4). *GNAS1* gene analysis was performed as previously described (7), and no NFPAs were found positive for the *gsp* mutation.

PKA regulatory subunit expression

PKA regulatory subunit expression was evaluated by immunohistochemistry (IHC) and Western blot analysis using specific monoclonal antibodies for PKA R1A, R2A, and R2B (BD Transduction Laboratories, Lexington, KY). Sections from paraffin-embedded tissues from all NFPAs were processed for IHC, as previously reported (4). As a positive control, normal human adrenal cortex was used. At least two blinded readers graded the specimens for all stainings. Briefly, PKA regulatory subunit immunoreactivities were graded 0–3: 0, absence of immunoreactivity; 1, less than 10%; 2, 10–50%; and 3, more than 50% in at least 400 cells in the main representative high power field.

Tissue homogenates obtained from four NFPAs (10 μg) were then used for Western blot analysis, and densitometric reading of the resulting bands was evaluated using a Bio-Rad GS-670 imaging densitometer (Bio-Rad Laboratories, Hercules, CA). Experiments were repeated at least twice.

Cyclin D1 and cyclin E expression

After 24 h of serum starvation, cells obtained by enzymatic digestion were incubated with different agents [10–100 μM 8-chloroadenosine cAMP (8-Cl-cAMP), alone or in combination with 5 μM PKA inhibitor (PKI) or 20 μM H89, and 1 μM forskolin; all reagents from Sigma-Aldrich Corp., St. Louis, MO] for 8 h at 37 C. The determination of cyclin D1 and cyclin E was performed after immunoprecipitation of cell lysates with specific monoclonal antibodies (Novocastra, Newcastle, UK) and Western blotting as previously reported (7). Experiments were repeated at least twice.

Determination of ERK1/2 activity

For ERK1/2 activity, cell monolayers were treated with different agents after serum starvation, as previously described (7). Subsequently, active ERK was immunoprecipitated from cell lysates using an immobilized antiphospho-p44/42 ERK monoclonal antibody, and the immunoprecipitates were then incubated with an Elk-1 fusion protein (2 μg) in the presence of ATP (200 μM) and kinase buffer for 30 min at 37 C, which allowed immunoprecipitated active MAPK to phosphorylate Elk-1, as previously described (7). Phosphorylation of Elk-1 was measured by Western blotting using an antiphospho-Elk-1 (Ser383) antibody. All the antibodies were purchased from New England Biolabs (Beverly, MA).

Statistical analysis

The results are expressed as the mean ± SD. A paired or unpaired two-tailed Student's *t* test was used to detect the significance between two series of data. $P < 0.05$ was accepted as statistically significant.

Results

PRKAR1A sequencing analysis

Analysis of the 12 exons and flanking regions of *PRKAR1A* did not reveal mutations in any adenoma. Two known polymorphisms (11) in the noncoding sequence, *i.e.* a T insertion in intron 3 (exon 4 IVS–5) and a base substitution (A to C) in the 5′-untranslated region of exon 1A, were found in three and four tumors, respectively.

PKA R1A, R2A, and R2B protein expression

No immunoreactivity for R1A subunit was found in four pituitary tumors, whereas in the remaining four a low number of cells (<10%) showed weak cytoplasmic staining. Conversely, all tumors showed a strong positivity for both R2A and R2B subunits, which were detected in more than 50% of the total cell population in all the cases studied. Western blot analysis confirmed the IHC data (data not shown).

Effect of PKA activation on cyclin D1 expression

Contrary to what was previously observed in transformed somatotrophs (7), exposure to forskolin (1 μM) and 8-Cl cAMP (10–100 μM), a cAMP analog selective for PKA regulatory subunit R2, did not induce any significant change in cyclin D1 levels in NFPAs 1–5 (group 1; Fig. 1A). In the remaining three NFPAs (tumors 6–8; group 2), cyclin D1 expression was inhibited by forskolin (−74 ± 15% *vs.* basal; Fig. 1A) and increased by the PKA inhibitor PKI at 5 μM (+96 ± 25% *vs.* basal; Fig. 1A). Similar results were obtained using the selective PKA inhibitor H89 (20 μM) and evaluating the expression of cyclin E in three NFPAs (data not shown). No correlation between cAMP-induced cyclin D1 changes and tumor size and/or extension was observed.

ERK1/2 activity in NFPAs

In all NFPAs, GnRH (10 nM) caused a significant increase of ERK1/2 activity (+152.5 ± 67% *vs.* basal) that was abrogated by the protein kinase C (PKC) inhibitor calphostin C (Fig. 2). Forskolin did not modify ERK1/2 activity in group 1 and was inhibitory in group 2 (−85 ± 13% *vs.* basal), thus paralleling its effects on cyclin D1 expression observed in the two groups (Figs. 1B and 2). Consistent with a possible inhibitory effect of PKA on ERK1/2, PKA blockade by PKI or H89 in group 2 caused a marked increase in basal ERK1/2 activity (+116 ± 28% over basal; Figs. 1B and 2). Finally, in most tumors, PKC blockade by calphostin C was associated with a reduction in basal ERK1/2 activity (80 ± 15% *vs.* basal), consistent with a stimulatory effect of the PKC pathway on ERK1/2 in these cells (Fig. 2).

Discussion

This study shows that contrary to what was observed in somatotrophs (4), activation of the cAMP-PKA pathway does not generate proliferative signals in NFPA that are largely constituted by cells of the gonadotroph lineage. These data confirm that cAMP, through the activation of PKA, may not influence, induce cell arrest, or promote cell growth depending on the cell type (12). All NFPAs included in this study were characterized by a reduction or loss of PKA R1A protein. Because R2A and R2B were expressed at high levels, all tumors displayed a strong prevalence of R2 over R1 protein. A similar pattern has been demonstrated to result from either inactivating mutations of *PRKAR1A* gene in the Carney com-

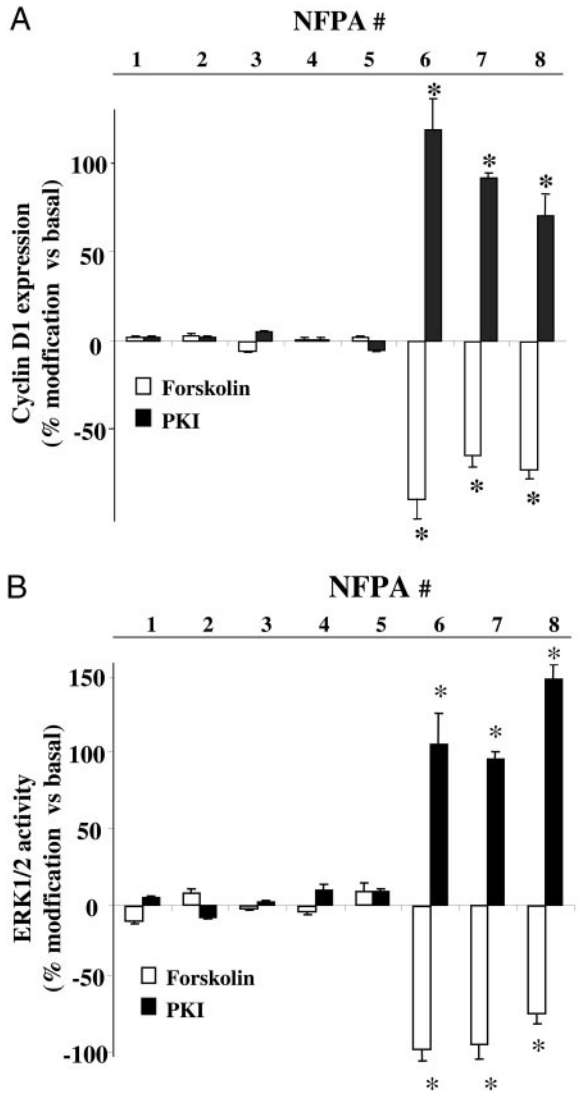

FIG. 1. Effect of cAMP pathway modulation on cyclin D1 expression and ERK1/2 activity in NFPAs. Activation of the cAMP cascade by forskolin (1 μM) and PKA blockade by PKI (5 μM) did not affect cyclin D1 expression (A) and ERK1/2 activity (B) in NFPAs 1–5. In NFPAs 6–8, forskolin induced a reduction of cyclin D1 (A) and ERK1/2 (B) activities, which were both increased by PKA blockade. The data represent the percent variation (mean ± SD) *vs.* basal values. *, $P <$ 0.05.

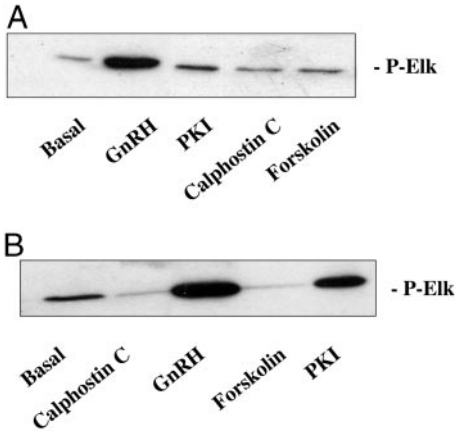

FIG. 2. Effects of forskolin, GnRH, PKI, and calphostin C on ERK1/2 activity in human NFPAs. A, Representative immunoblotting performed in NFPA 4. The exposure to GnRH (10 nM) induced the expected increase in ERK1/2 activity. Conversely, PKI and forskolin (1 μM) did not induce any significant change in this proliferative cascade. Similar data were obtained in tumors 1, 2, 3, and 5. B, In the remaining three tumors (NFPAs 6–8), PKA blockade (PKI, 5 μM) induced a significant increase in ERK1/2 activity, as shown in the representative immunoblotting performed in NFPA 6. The inhibitory role of the cAMP cascade was also confirmed in these tumorous cells by the inhibitory effect on ERK1/2 elicited by forskolin. Finally, PKC blockade by calphostin C (1 μM) induced a decrease in ERK1/2 activity.

plex or R1A protein instability in transformed somatotrophs; this phenomenon leads to PKA activation and cell proliferation (2, 4). The present study indicates that in NFPA the imbalance of the R1A/R2B ratio and activation of the cAMP-PKA pathway resulted in the generation of signals different from or even opposite to those triggered in somatotroph tumors. In agreement with previous reports (13), cyclin D1 and cyclin E, two key regulators of G_1 phase progression, were detectable in all NFPAs investigated. Increasing concentrations of 8-Cl-cAMP and forskolin, a cAMP analog able to selectively activate R2 subunits and an activator of all subunits, respectively, did not induce the expression of cyclin D1 and cyclin E in these tumors. The minor, if any, role of cAMP in the control of cell proliferation was confirmed by the lack of cAMP-induced activation of ERK1/2 cascade. Interestingly, in a subset of NFPAs additional imbalance of the R1/R2 ratio by 8-Cl-cAMP and direct adenylyl cyclase stimulation by forskolin resulted in a clear reduction of both cyclin D1 expression and ERK1/2 activity, a phenomenon that was confirmed by the increase in these proliferative markers observed in resting cells after PKA blockade. These data suggest that basal PKA activity may have a tonic inhibitory effect on NFPA cell proliferation, although no significant correlation with the clinical characteristics of the tumors was observed.

This study provides new evidence for the existence of different proliferative cascades specifically signaling in different pituitary cell lineages. In fact, in contrast to the lack of proliferative effect of the cAMP-PKA pathway on NFPA, the activation of PKC by specific neurohormones, *i.e.* GnRH, triggered the activation of mitogenic kinases, consistent with data previously obtained in transformed gonadotroph cells (7, 14). Moreover, PKC blockade by calphostin C in resting cells was associated with a significant decrease in basal ERK1/2 activity, confirming the stimulatory effect of PKC on this pathway in NFPAs.

Taken together, these data show that in contrast with what was observed in transformed somatotrophs, activation of the cAMP-PKA pathway does not generate proliferative signals in NFPAs, at least in those immunopositive for gonadotropins. In a subset of tumors, the activation of this pathway exerts a tonic inhibitory effect, thus confirming a different role for the cAMP-mediated pathway in promoting proliferation in the pituitary. These conclusions are consistent with the rare or absent occurrence of *gsp* mutations in NFPA and strongly suggest the involvement of other pathogenetic

mechanisms able to specifically confer a growth advantage to cells of the gonadotroph lineage (15, 16).

Acknowledgments

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