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PKA-dependent growth stimulation of cells derived from human pulmonary adenocarcinoma and small airway epithelium by dexamethasone

H.A.N. Al-Wadei [a], T. Takahasi [b], H.M. Schuller [c,*]

[a] *Experimental Oncology Laboratory, College of Veterinary Medicine, University of Tennessee, Knoxville, TH, USA*
[b] *Department of Molecular Carcinogenesis, Center for Neurological Diseases, Nagoya University, Graduate School of Medicine, Nagoya, Japan*
[c] *Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN 37996, USA*

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Abstract

Smoking is a risk factor for lung cancer, chronic obstructive pulmonary disease, chronic bronchitis and asthma. The chronic lung diseases are also a predisposing factor for the development of lung cancer. Glucocorticoids are used for the management of chronic lung diseases because of their anti-inflammatory activity. These drugs also have anti-tumourigenic effects in mouse models of lung cancer. Glucocorticoids are frequently used as co-treatment with cancer therapy. Using the human pulmonary adenocarcinoma (PAC) cell line NCI-H322 with features of bronchiolar Clara cells, and immortalised human small airway epithelial cells, our data show that the glucocorticoid dexamethasone increased cell proliferation in MTT assays in a PKA-dependent manner. Dexamethasone significantly increased intracellular cAMP in direct immunoassays. Immunoblot analysis revealed increased phosphorylation of ERK1/2 and of the transcription factor CREB in response to dexamethasone. These data suggest that glucocorticoids could have tumour promoting activity on a sub-set of human PAC.

Keywords: Lung adenocarcinoma; Dexamethasone; cAMP; PKA; CREB; ERK1/2; Cell proliferation

1. Introduction

Lung cancer ranks second as a cause of death after cardiovascular disease and the death rate for lung cancer exceeds the combined total for cancer of the breast, prostate and colon in developed countries [1]. Among the four major histological lung cancer types (adenocarcinoma, small-cell carcinoma, squamous cell carcinoma, large-cell carcinoma), peripheral adenocarcinoma (PAC) has increased dramatically over the last 20 years and is the leading type of lung cancer today in both male and female smokers and non-smokers [2–4].

Smoking is a documented risk factor for the development of all histological lung cancer types and is believed to account for 70–90% of all lung cancer cases [5,6]. Pre-existing non-neoplastic pulmonary diseases such as chronic obstructive pulmonary disease (COPD), bronchitis and asthma additionally increase the risk for the development of lung cancer in smokers and non-smokers [7–10]. However, few reports have addressed the effects of individual members of this chronic disease family on defined histological lung cancer types [11–13]. To our knowledge, the potential effects of therapeutics used for the long-term management of chronic non-neoplastic pulmonary disease on the development of lung cancer have also not been investigated to date.

Glucocorticoids are widely used for the long-term management of COPD, chronic bronchitis and asthma

* Corresponding author. Tel.: +1 865 974 8217; fax: +1 865 974 5616.
E-mail address: hmsch@utk.edu (H.M. Schuller).

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because of their anti-inflammatory actions and documented synergy with β_2-adrenergic receptor agonist bronchodilators [14,15]. They are also frequently prescribed to minimise the side effects of cancer chemotherapy and radiation therapy [16]. A significant population of lung cancer patients is therefore exposed to glucocorticoids either before the diagnosis of lung cancer or during cancer therapy. Studies in mouse models of lung cancer have shown significant cancer preventive effects of glucocorticoids [17], leading to recent strategies to propose this family of drugs for lung cancer prevention in smokers and ex-smokers. In addition, glucocorticoids have been shown to inhibit cell proliferation in a number of non-pulmonary cancers and embryonic fibroblasts, an effect involving the cyclin-dependent kinase inhibitors p21 and p27 [18,19].

Studies conducted in our laboratory have identified a novel β-adrenergic receptor-initiated growth-stimulating pathway in cell lines derived from human PAC of Clara cell phenotype [20]; whereas the same signalling pathway was growth-inhibiting in cell lines derived from human PAC with phenotypic features of alveolar type II cells [21]. Moreover, we have identified the tobacco-specific carcinogenic nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) as a high affinity agonist for β_1- and β_2-adrenergic receptors in Chinese hamster ovary (CHO) cells stably overexpressing the human β_1- or β_2-adrenergic receptor [20]. In cell lines derived from human PAC of Clara cell phenotype, NNK stimulated the release of arachidonic acid (AA) and DNA synthesis via binding to β_1- and β_2-adrenergic receptors [20], an observation in line with the documented overexpression of AA-metabolising enzymes in human PAC [22]. In support of these in vitro observations, bioassay experiments in a hamster model of NNK-induced PAC derived from bronchiolar Clara cells have documented strong tumour promoting effects of the β-adrenergic agonist epinephrine while the β-adrenergic antagonist propanolol inhibited tumour development [23]. More recently, we have also shown that theophylline, which increases the levels of the β-adrenergic receptor second messenger cAMP via inhibition of phosphodiesterase, significantly promoted the development of NNK- induced PAC in hamsters [24].

Because of the documented synergy of glucocorticoids with β-adrenergic agonists in the treatment of non-neoplastic pulmonary disease [15], our current study has tested the hypothesis that glucocorticoids may stimulate the β-adrenergic growth-regulating pathway in human PAC of Clara cell phenotype and in their putative cells of origin, small airway epithelial cells (SAECs). In support of this hypothesis, our data reported in this study, from NCI-H322 cells derived from a human PAC of Clara cell phenotype and in immortalised human small airway epithelial cells expressing the Clara cell specific CC10 antigen, demonstrates that dexamethasone caused PKA-dependent stimulation of cell proliferation. It was also accompanied by a significant increase in intracellular cAMP and activity of the cAMP-dependent protein kinase A (PKA), the transcription factor cAMP response element (CREB) and of the mitogen-activated protein kinases ERK1/2.

2. Materials and Methods

2.1. Cell lines and tissue culture

The human PAC cell line with characteristics of Clara cells, NCI-H322 (Center for Applied Microbiology and Research, ECACC, Salisbury, Wiltshire, UK) was maintained in RPMI-1640 medium, containing 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 4500 mg/l glucose, 1500 mg/l sodium bicarbonate and supplemented with 10% fetal bovine serum (American Type Culture Collection, Manassas, VA). The Simian virus 40 (SV40)-immortalised human peripheral airway cell line HPL1D was established and characterised by Dr. Takahashi's laboratory [25] and is referred to in this publication as small airway epithelial cells (SAECs). This cell line was maintained in F-12 nutrient mixture (HAM) with L-glutamine (Gibco) medium, buffered with 15 mM HEPES (Ph 7.3) and supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 10^{-7} M hydrocortisone, 2×10^{-10} M triiodothyronine (Cambrex, Walkersville, MD) and 1% fetal calf serum (ATCC).

2.2. Assessment of cell numbers by MTT assay

The effects of dexamethasone (Sigma, St. Louis, MO, USA) on cell proliferation were assessed by the colorimetric 3-(4,5-dimethyle thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (**2**) (Sigma). Briefly, the MTT test is based on the NADH-dependent enzymatic reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] in metabolically active cells but not in dead cells. Cells were seeded into 6-well tissue culture plates (Falcon, Franklin Lakes, NJ, USA) at a density of 50 000 cells per well for NCI-H322, and 50 000 cells per well for SAEC cells. The cells were left to grow in complete media at 37 °C with 5% CO_2 for 5 h to attach. The cells were then switched to fresh low serum media and dexamethasone was added at the concentrations specified in the figure legend and incubated for 24, 48, or 72 h. Fifty microliters of 3-(4,5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (0.5 mg/ml) was dissolved in phenol-free RPMI-1640 medium (Gibco) and then added to the cells for 2–3 h to allow metabolic conversion of the MTT substrate to blue formazan. After 2–3 h, the media were replaced with isopropanol, and optical density at 570 nm was determined using an ELISA reader. Data are expressed as mean values and standard errors of four

samples per treatment group. The assay was repeated twice. Cell counts by hemocytometer after trypan blue dye exclusion stain served as controls and yielded similar data as the MTT assay. Statistical analysis of data was by one-way ANOVA, followed by Tukey–Kramer multiple comparison test and two-tailed unpaired *t*-test.

2.3. cAMP immunoassay

Cells were plated at 400 000 cells per 6-well plate and grown until 65–70% confluence. The cells were then fed with low serum (0.1%) for NCI-H322 cells or 0.05% serum for SAECs for 24 h, washed twice with 1X PBS, preincubated for 30 min with 1 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma) and then incubated with dexamethasone (20 nM) for the time intervals specified in the figure legend in the appropriate medium for 10 or 30 min. After three washes with distilled water, cells were treated with 0.1 M HCl for 10 min, then lysed and sonicated. After vortexing, samples were analysed for cAMP levels using a direct cyclic AMP enzyme immunoassay kit according to the manufacturer's instructions (Assay Designs Inc, Ann Arbor, MI, USA). Reactions were stopped with trisodium phosphate and color intensity was measured at 405 nm.

Data are expressed as mean values and standard errors of triplicate samples per treatment group. The experiment was repeated twice. Statistical analysis of data was by one-way ANOVA, followed by Tukey–Kramer multiple comparison test and two-tailed unpaired *t*-test.

2.4. PKA activation assay

Following incubation of cells with dexamethasone (20 nM) for 5 min to 1 h as specified in the figure legends, PKA activity was assayed in cell lysates using a Pep Tag assay for non-radioactive detection of activated PKA (Promega Corperation, Madison, WI, USA), following the instruction of the manufacturer. This assay utilises fluorescent substrate for PKA that changes the peptide's net charge upon phosphorylation of PKA, thus allowing the phosphorylated peptide to migrate to the positive electrode (+), while the non-phosphorylated peptide migrates to the negative electrode (−). Briefly, reactions containing a brightly colored fluorescent Pep Tag A1 peptide (0.4 μg/μl), peptide protection, Pep Tag reaction and PKA activator solutions were incubated on ice for few min before 1 min incubation at 30 °C. After adding samples, reactions were incubated at room temperature for 30 min, boiled at 95 °C for 10 min, and separated on 0.8% agarose gel in 50 mM Tris–HCl (pH 8.0). At this point the qualitative assay is complete, and the protein kinase A activity in samples was determined by examining the gel under UV lights. Densitometric analysis of the bands was conducted using NIH Scion software for image quantitation.

Data are expressed as mean values and standard errors of five densitometric readings per band. The experiment was repeated once. Statistical analysis of data was by one-way ANOVA, followed by Tukey–Kramer multiple comparison test and two-tailed unpaired *t*-test.

2.5. Assessment of total proteins and phosphorylated proteins by immunoblotting

To assess the effects of dexamethasone (20 nM) on the expression and phosphorylation of the mitogen-activated protein kinases ERK1/2 or the cAMP response element binding protein CREB, 500 000 NCI-H322 or SAEC cells were seeded into culture vessels (100 cm^2) containing their respective growth media. When the cells had reached 60–65% confluence, they were rinsed once with 1XPBS and serum-starved for 24 h. Following removal of the media and replacement with fresh low-serum media, dexamethasone was added to the culture vessels and cells were incubated from 5 min to 1 h as detailed in the figure legends. The cultured cells were then washed once with cold PBS, lysed in 20 mM Tris-base, 200 mM NaCl, 1 M sodium fluoride. 0.5 M EDTA, 100 mM Na_3VO_4, 100 mM PMSF, 1 μl pepstatin, 1 μl leupeptin, 1 μl aprotinin, and 0.25% NP-40. Then, protein samples were denatured by boiling at 95 °C for 5 min, separated on 12% SDS–PAGE, and transferred to nitrocellulose. Membranes were blocked with 5% non-fat dry milk, probed with rabbit polyclonal CREB and Phosphor CREB antibodies, respectively, and developed by chemiluminescence with ECL reagents. Membranes were blocked in 10 ml of 5% none-fat dry milk (Kroger, Knoxville, TN, USA) in 1X TBST for 1 h. Membranes were then incubated over night at 4 °C with primary antibodies at a 1:1000 dilution (rabbit polyclonal for total ERK1/2, rabbit polyclonal for Thr202/Tyr204 phosphorylated ERK1/2; rabbit polyclonal for total CREB, mouse monoclonal for SER33 phosphorylated CREB; Cell Signalling Technology, Beverly, MA, USA). Incubation with secondary anti bodies (goat anti-rabbit or goat anti-mouse) at a dilution of 1:3000 was for 2 h at room temperature. Equal loading of lanes was confirmed by blotting for actin using mouse actin monoclonal antibody.

Data are expressed as mean values and standard errors of five densitometric readings per band. Each experiment was repeated twice. Statistical analysis of data was by one-way ANOVA, followed by Tukey–Kramer multiple comparison test and two-tailed unpaired *t*-test.

3. Results

We tested the effects of dexamethasone on cell numbers in the MTT assay after 24, 48 or 72 h of incubation

of SAECs and NCI-H322 cells. While the stimulating effect of dexamethasone was highly significant at all time intervals tested in both cell lines, the responses after 48 h of exposure was the most dramatic and illustrated in Fig. 1. Dexamethasone caused a highly significant ($P < 0.001$) increase in the numbers of SAECs particularly at the low concentrations, an effect that peaked with a 5.6-fold increase after incubation with a 100 pM dexamethasone concentration (Fig. 1). The cancer cell line NCI-H322 also responded with a highly significant ($P < 0.001$) increase in cell number at concentrations from 1 pM to 20 μM dexamethasone but was less responsive than SAECs as reflected by a maximum response at the 20 nM concentration (Fig. 1). Preexposure (20 min) of cells to the PKA inhibitor, H89 (30 μM), abrogated the proliferative response to dexamethasone (20 nM) in a highly significant ($P < 0.001$) manner (Fig. 2), suggesting that this effect was PKA dependent.

Immunoassays for the determination of intracellular cAMP revealed a time-dependent increase in response to 20 nM dexamethasone in both cell lines (Fig. 3). This response peaked after ten min of exposure (SAECs: 1.8-fold increase; NCI-H322: 2.2-fold increase) and was significant ($P < 0.01$) at this time point.

Assessment of PKA activation by a non-radioactive kit that measured the migration of the phosphorylated peptide to the positive electrode (+), while the non-phosphorylated peptide migrated to the negative electrode (−), revealed a time-dependent increase in activated PKA of SAECs exposed to dexamethasone (20 nM;

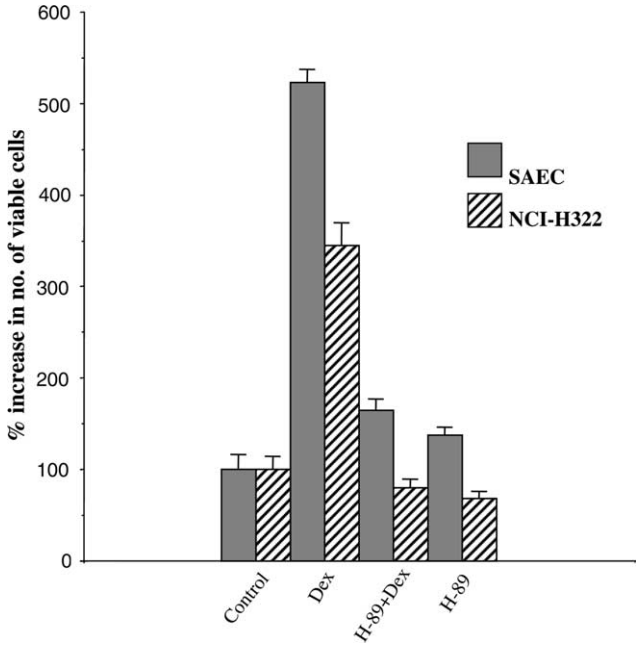

Fig. 2. Effects of the selective PKA inhibitor, H89 (30 μM), on dexamethasone-induced (20 nM) cell proliferation in SAECs and NCI-H322 cells as assessed by MTT assay. Following a 24 h starvation period, cells were pre-exposed for 20 min to H89 followed by the addition of dexamethasone and incubated for 48 h. Cells exposed to dexamethasone alone or H89 alone served as positive controls for each agent. Bars represent normalised (control values set at 100%) mean values and standard errors of three independent experiments, each with four samples per group. The response to dexamethasone was significantly inhibited in both cell lines ($P < 0.001$) by one way ANOVA, Tukey–Kramer multiple comparison test and two-tailed unpaired *t*-test.

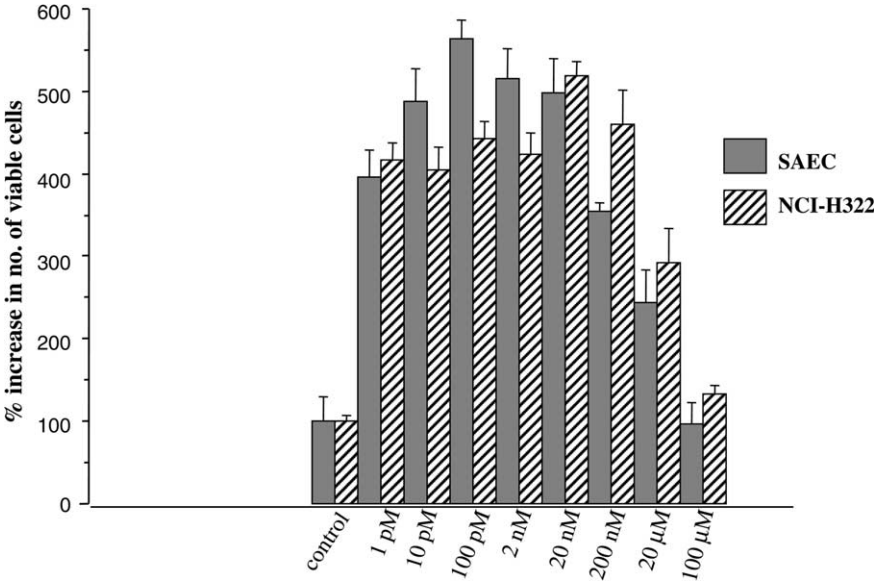

Fig. 1. Effects of dexamethasone (1 pM–100 μM) on cell number in SAECs and NCI-H322 cells as assessed by MTT assay. Following a 24-h starvation period, cells were exposed for 48 h to dexamethasone at the concentrations indicated. Bars represent normalised (control values set at 100%) mean values and standard errors of three independent experiments, each with four samples per group. Cell numbers were significantly increased at concentrations from 1 pM to 20 μM ($P < 0.001$) by one way ANOVA, Tukey–Kramer multiple comparison test and two-tailed unpaired *t*-test.

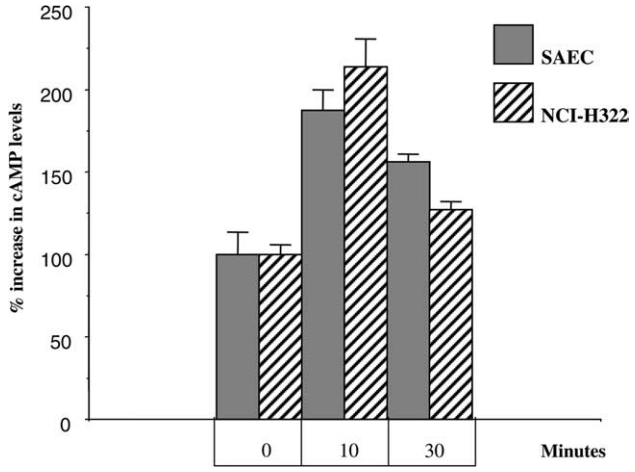

Fig. 3. Stimulation of intracellular cAMP by dexamethasone in SAECs and NCI-H322 cells was assessed by a direct enzyme immunoassay. Following a 24 h starvation period, the cells were incubated for 10 or 30 min with dexamethasone (20 nM). Bars represent mean values and standard errors of triplicate samples expressed as normalised data (controls were set as 100%). The experiment was repeated twice with similar results. Statistical analysis of data was by one-way ANOVA, Tukey–Kramer multiple comparison test and two-tailed unpaired *t*-test. The increase in intracellular cAMP was significant ($P < 0.01$) at both time intervals tested in each cell line.

Fig. 4) with a maximum 5.4-fold increase ($P < 0.01$) at the 5 min time interval (Fig. 4). Similarly, the human PAC cell line NCI-H322 responded to 20 nM dexamethasone with a time-dependent increase of PKA activity that peaked with a 4.8-fold increase ($P < 0.01$) at the 15 min time interval (Fig. 4).

Assessment of ERK1/2 and p-ERK1/2 expression by Western blot analysis showed no changes in expression levels of the unphosphorylated proteins in SAECs or NCI-H322 cells exposed to dexamethasone (20 nM; Fig. 5). By contrast, the levels of phosphorylated ERK1/2 increased in response to dexamethasone in a time-dependent manner in both cell lines (Fig. 5). Densitometric analysis of the bands revealed maximum density at the 5 and 15 min time intervals with both cell systems (SAEC: 4.3-fold and 3.8-fold; NCI-H322: 3.9-fold and 3.7-fold).

Determination of CREB and p-CREB expression by immunoblot analysis showed a highly significant ($P < 0.001$) increase in p-CREB expression at all time intervals measured after exposure to dexamethasone in both cell lines (Fig. 6). In SAECs, this response peaked after 30 min (3.0-fold) while maximum expression (6.8-fold) was observed in NCI-H322 cells after 60 min of exposure to dexamethasone (Fig. 6).

4. Discussion

Our data show that the glucocorticoid dexamethasone significantly stimulated the proliferation of NCI-H322 cells and human immortalised SAECs in a PKA-dependent manner and that this response involved stimulation of cAMP and activation of ERK1/2 and CREB. These findings suggest, for the first time, that dexamethasone may have tumour promoting activity on a subset of human lung cancers within the family of adenocarcinoma. This is in sharp contrast to reports on lung cancer preventive effects of dexamethasone and other glucocorticoids in mouse models of lung cancer [17]. Human PAC may express phenotypic features of bronchiolar Clara cells or of alveolar type II cells [26]. *In vitro* studies in human PAC cell lines have documented antagonistic growth regulation at the level of cAMP in these two PAC types [21]. Significant growth stimulation has thus been reported for agents that increase intracellular cAMP in PAC of Clara cell phenotype [27] whereas inhibition of DNA synthesis was observed with identical agents in PAC with features of alveolar type II cells [21]. It is well documented that chemically induced mouse PAC originates from alveolar type II cells and expresses phenotypic and functional features of this cell type [28]. The reported preventive effects of glucocorticoids on the development of mouse PAC is

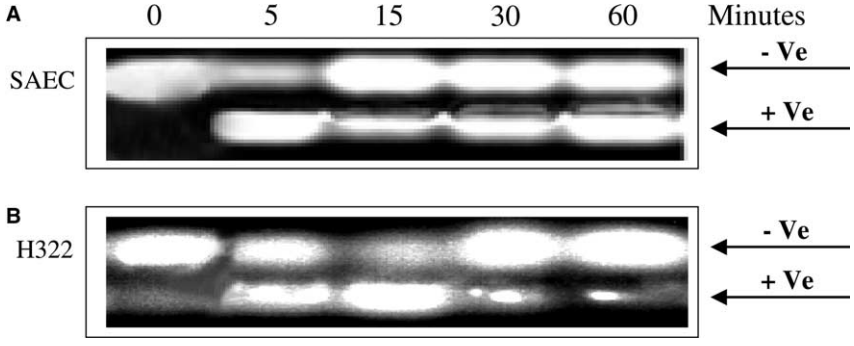

Fig. 4A and B. Agarose gels exemplifying the effects of dexamethasone (20 nM incubated for 5–60 min) on phosphorylation of PKA in SAECs and NCI-H322 cells. Following a 24 h starvation period, the cells were exposed to dexamethasone for the indicated times. PKA activity was then assayed in cell lysates using a Pep Tag assay for non-radioactive detection of PKA and the samples were separated on 0.8% agarose gel. Phosphorylated peptide migrated towards the positive electrode (+), while non-phosphorylated peptide migrated towards the negative electrode (−).

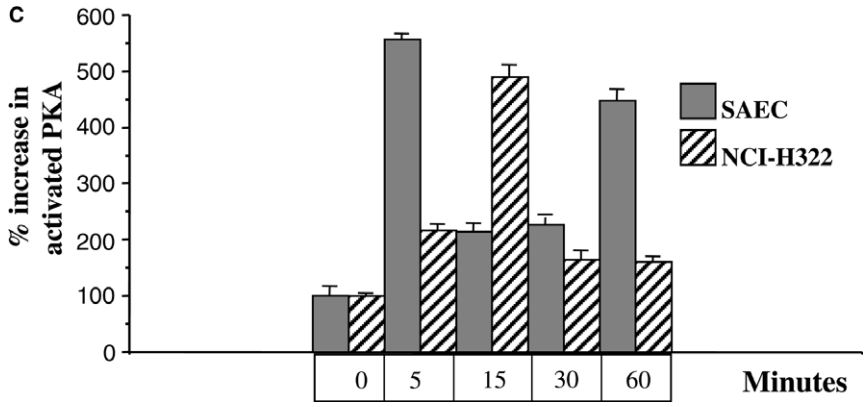

Fig. 4C. Bar graph illustrating densitometry values of the bands in Fig. 4A and B. Densitometric analysis was performed using the NIH Scion software for image quantitation. Bars represent normalised (control values set at 100%) mean values and standard errors of five densitometric readings per band. The experiment was repeated twice with similar results. Statistical analysis of data was by one-way ANOVA, Tukey–Kramer multiple comparison test and two-tailed unpaired *t*-test. The observed increases in phosphorylated PKA were significant ($P < 0.001$) at all time intervals tested in the SAECs and after 5 and 15 min of exposure in NCI-H322 cells.

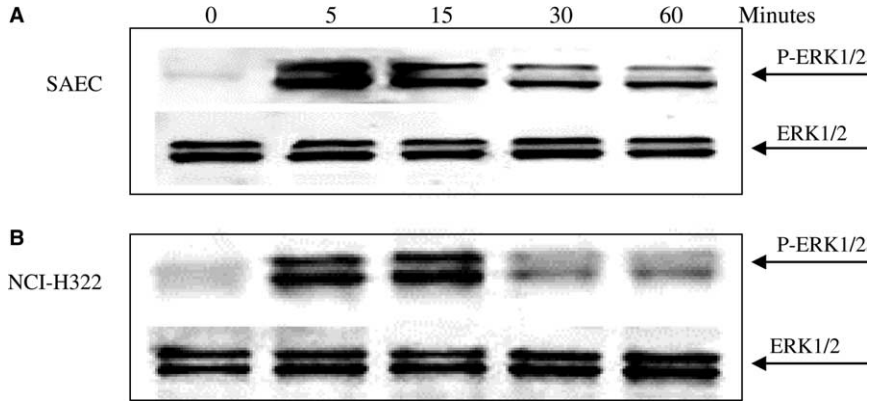

Fig. 5A and B. Western blots illustrating the effects of dexamethasone (exposures from 5 to 60 min, 20 nM) on the expression of ERK1/2 and its phosphorylated form in SAECs and NCI-H322 cells. Cells were exposed to dexamethasone for the time intervals indicated after a 24 h starvation period. The bands for p-ERK1/2 increased in size and intensity, an effect that peaked after 5 min in both cell lines whereas the expression of ERK1/2 remained unchanged.

therefore in accordance with the observation in human alveolar type II cell PAC that cAMP inhibits cell growth [21]. By contrast, experimentally induced PAC of documented Clara cell origin and phenotype in hamsters [29] responded with a significant promotion of tumour development to forskolin [23], which activates cAMP directly, or theophylline [24], which increases intracellular cAMP via inhibition of phosphodiesterase. The PKA-dependent growth stimulating effects of dexamethasone on NCI-H322 and SAECs in the current study are thus in line with these results generated in a hamster model of PAC.

The mechanisms how dexamethasone stimulates cAMP/PKA in our cell lines yet remain to be elucidated. The majority of biological responses to glucocorticoids are thought to be mediated through nuclear glucocorticoid receptors which function as transcriptional activators or repressors in a variety of cell types [18,19]. However, recent studies in human peripheral blood mononuclear cells and lymphoma cells have provided evidence for the presence of membrane-bound glucocorticoid receptors [30]. The rapid induction of cAMP/PKA observed in the current study is consistent with that concept.

Our findings raise serious concerns about strategies for the development of glucocorticoids as lung cancer preventive agents. Technology is not available to determine which cell type has been initiated to develop into lung cancer at a later time. The long-term exposure of populations at risk for the development of lung cancer to "chemopreventive" agents of the glucocorticoid family may therefore promote the development of lung cancer in a subset of such patients. In addition, the widely used practice to reduce side effects of chemo or radiation therapy in lung cancer patients by co-treatment with glucocorticoids should be limited to patients with

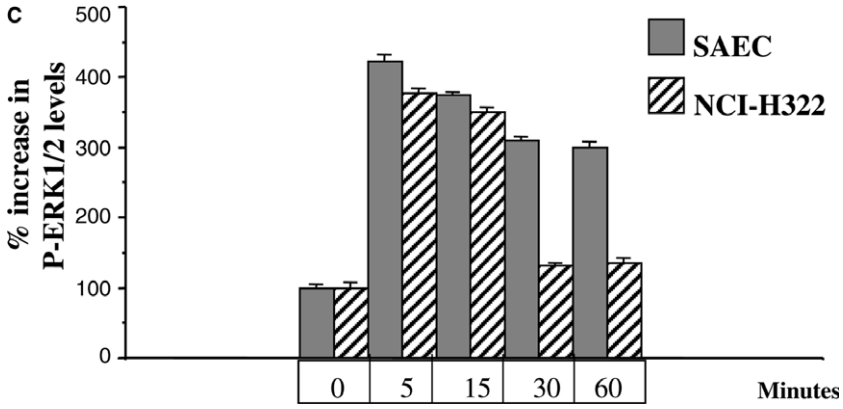

Fig. 5C. Bar graph illustrating densitometry values of the bands in Fig. 5A and B. Densitometric analysis was performed using the NIH Scion software for image quantitation. Bars represent normalised (control values set at 100%) mean values and standard errors of five densitometric readings per band. The experiment was repeated twice with similar results. Statistical analysis of data was by one-way ANOVA, Tukey–Kramer multiple comparison test and two-tailed unpaired *t*-test. The observed increases in p-ERK1/2 were significant ($P < 0.001$) in SAECs at all time intervals tested and in NCI-H322 cells after 5 and 15 min.

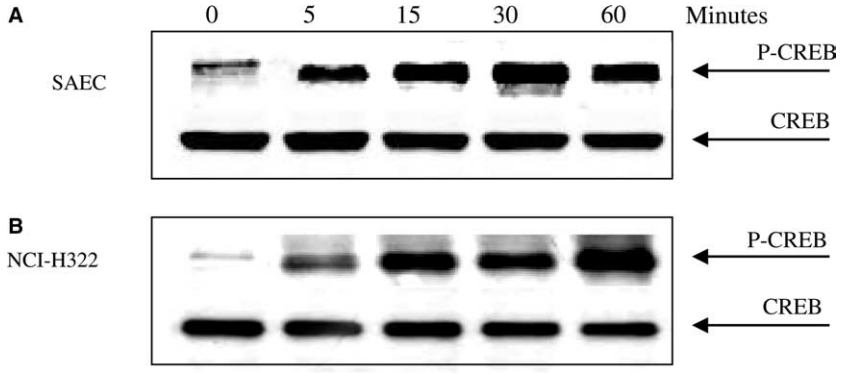

Fig. 6A and B. Western blots exemplifying the effects of dexamethasone (20 nM) on the expression of phosphorylated CREB and total CREB protein in SAECs or NCI-H322 cells. Following a 24 h starvation period, cells were exposed to dexamethasone for the time intervals indicated. The bands for p-CREB increased in size and density over time whereas no increase was observed in the bands for total CREb protein.

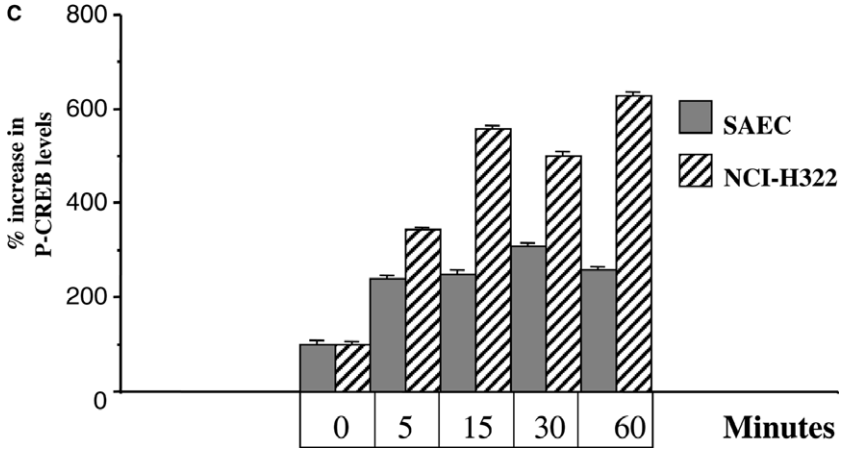

Fig. 6C. Bar graph illustrating densitometry values of the bands in Fig. 6A and B. Densitometric analysis was performed using the NIH Scion software for image quantitation. Bars represent normalised (control values set at 100%) mean values and standard errors of five densitometric readings per band. The experiment was repeated twice with similar results. Statistical analysis of data was by one-way ANOVA, Tukey–Kramer multiple comparison test and two-tailed unpaired *t*-test. The observed increases in p-CREB were significant ($P < 0.001$) in both cell lines at all time intervals tested.

documented non-Clara cell type lung cancers. Moreover, studies are urgently needed to assess the potential promoting effects of glucocorticoids used for the long-term management of COPD, bronchitis and asthma on defined histological lung cancer types.

The observed activation of ERK1/2 and CREB downstream of cAMP/PKA in response to dexamethasone is a particularly interesting finding. The activation of ERK1/2 has been widely studied as downstream effectors of the epidermal growth factor receptor (EGFR) pathway in PAC [31,32]. By contrast, activation of the transcription factor CREB has only been associated with endocrine tumours and tumours of the thyroid gland [33] and has not been considered as a potential contributor to lung cancer to date. Studies by our laboratory have recently shown that PAC of Clara cell phenotype in hamsters induced by the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) selectively overexpressed PKA and phosphorlyated CREB simultaneously with phosphorylated ERK1/2 [34]. Collectively, these findings point to a potential transactivation of the EGFR/ERK1/2 pathway by cAMP/PKA similar to recent findings in fibroblasts [35]. While CREB is a classical downstream effector of cAMP/PKA in many cell types, PKA-dependent transactivation of ERK1/2 has recently been described in response to activation of the β_1-adrenergic receptor [36], a member of the membrane-bound G-protein coupled receptor family. Further studies are necessary to dissect the complex signalling events associated with the observed stimulatory actions of dexamethasone on PAC and SAE cells.

Conflict of interest statement

None declared.

Acknowledgments

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