cAMP-dependent protein kinase activation inhibits proliferation and enhances apoptotic effect of tumor necrosis factor-α in NCI-H295R adrenocortical cells

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Abstract

Adrenocorticotropin is the major regulator of adrenocortical development and function. It acts mainly through the cAMP-dependent protein kinase A (PKA) pathway. Our aim was to study the interaction of tumor necrosis factor-α (TNFα) and the PKA pathway in adrenocortical cell proliferation and apoptosis. The PKA activator Dibutyryl cAMP ((Bu)2cAMP) strongly induced differentiation and inhibited proliferation in the human adrenocortical cell line NCI-H295R (H295R). TNFα induced apoptosis of H295R cells. Interestingly, (Bu)2cAMP treatment clearly enhanced TNFα-induced apoptosis in H295R cells, but not in another human adrenocortical cell line SW-13, the mouse adrenocortical Y-1 cell line or the human HeLa cell line. This synergistic effect was not due to the (Bu)2cAMP-induced glucocorticoid secretion since dexamethasone had no significant effect on the TNFα-induced apoptosis. (Bu)2cAMP treatment rapidly increased the expression of the proto-oncogene c-myc in H295R cells, but not in SW-13, Y-1 or HeLa cells. In transient c-myc transfection assay, c-myc expression associated with decreased expression of the proliferation marker Ki-67 in H295R cells. In conclusion, cAMP-dependent protein kinase activation reduced proliferation and augmented TNFα-induced apoptosis in adrenocortical H295R cells, and these effects were associated with increased c-myc expression.

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Introduction

The adrenal glands are organs that undergo dynamic changes in cell growth and death in physiological and pathophysiological conditions (Keegan & Hammer 2002, Kirschner 2002). The essential regulator adrenocorticotropic hormone (ACTH) affects adrenocortical cell differentiation, proliferation and cell death mainly by increasing the intracellular concentration of cyclic adenosine monophosphate (cAMP), which serves as a second messenger. The ubiquitous regulatory molecule cAMP binds to protein kinase A (PKA), leading to bi-directional effects on the proliferation of mammalian cells: inhibition in several cell types but stimulation in others (Keegan & Hammer 2002, Kirschner 2002, Langlois et al. 2002). Meticulously regulated differentiation, proliferation and apoptosis play fundamental roles in the development of multicellular organisms and the maintenance of tissue homeostasis (Abrams 2002, Corn & El-Deiry 2002). We previously found that ACTH is able to induce differentiation and apoptosis, but it inhibits proliferation of rat fetal adrenocortical cells in primary cultures (Arola et al. 1993). Expression of the proto-oncogene c-myc gene is lost in human adrenocortical carcinomas (Liu et al. 1997) and induction of c-myc expression by PKA activation is observed during ACTH treatment in primary cultures of human adrenocortical cells (Liu et al. 1996b). In many cell types, induction of c-myc expression puts the cells on the crossroads of coupled pathways: it promotes cell cycle progression, inhibits cell differentiation and
sensitizes the cells to apoptosis (Pelengaris et al. 2002). The situation is different in adrenocortical cells, where c-myc expression is associated with ACTH-induced differentiation and inhibited proliferation (Arola et al. 1993, Heikkilä et al. 1995, Liu et al. 1996b), leaving the mechanisms underlying the physiological actions of ACTH and PKA unclear.

A number of stimuli, including tumor necrosis factor-α (TNFα), Fas, TNF-related apoptosis-inducing ligand and radiation, have been demonstrated to induce apoptosis in different tissues (Zimmermann & Green 2001, Karin & Lin 2002). The intensively studied potent cytokine TNFα is produced by many cell types, and functions through its two receptors (TNF-R1 and TNF-R2). Although TNF-R2 can have an important contribution in a number of responses (Heyninck & Beyaert 2001). TNFα binding to its receptors results in recruitment of signal transducers that can simultaneously activate at least two signaling pathways. One leads to apoptosis through the activation of the caspase cascade, whereas the other leads to cell survival by inhibition of the death signal. For the latter, evidence for an important role of nuclear factor-κB-mediated anti-apoptotic gene expression has already been obtained. When both pathways are activated by TNFα, the final decision to survive or die is mostly dependent on the activity ratio of these two pathways in individual cells (Baud & Karin 2001).

There is increasing evidence that TNFα is involved in the regulation of adrenocortical function. TNFα and its receptors are present in human adrenal steroidogenic cells, as well as in resident macrophages. The release of the cytokine from adrenal cells is regulated by factors that alter adrenal function (Jäättelä et al. 1990, González-Hernández et al. 1996, Judd et al. 2000). TNFα decreases steroidogenic P450 enzyme gene expression and cortisol release from human fetal adrenal cells, but increases cortisol release from adult human adrenal cells (Darling et al. 1989, Jää tälä et al. 1991, Ivesmäki et al. 1993). Regardless of these studies on the effects of TNFα on adrenal steroid production, the apoptosis-inducing effect of TNFα in adrenals and its mechanisms are still largely unknown. Our goal was therefore to study the interaction of TNFα and the PKA pathway in adrenocortical cell proliferation and apoptosis.

Materials and methods

Cell cultures

Human adrenocortical carcinoma cell line NCI-H295R (H295R) from the American Type Culture Collection (ATCC; Rockville, MD, USA) was grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (DMEM-F-12; Sigma Chemical Company, St Louis, MO, USA) supplemented with 1% ITS+1 liquid media supplement (Sigma), 2% Ultroser SF (Bioprese, Marlborough, MA, USA), 2 mM l-glutamine (Gibco-BRL, Paisley, Strathclyde, UK) and antibiotics (125 µg/ml streptomycin and 125 IU/ml penicillin; Orion Pharmaceutical Co., Espoo, Finland) at 37 °C in a 5% CO2 atmosphere. Human adrenocortical carcinoma SW-13, mouse adrenal carcinoma Y-1 and human cervical adenocarcinoma HeLa cells (ATCC) were cultured in DMEM-F-12 medium supplemented with 10% fetal calf serum, glutamine and antibiotics. The medium was refreshed every other day, and the cells were split at a ratio of 1:3 to 1:6 with trypsin after reaching confluence. Treatment or transfection was initiated on the second day after reseeding of the cells. Triplicate dishes were used for the experiments which were repeated at least three times. Cortisol was routinely measured from the conditioned cell culture media with the Technicon Immuno 1 System (Bayer Corporation, Tarrytown, NY, USA).

RNA analysis

Cytoplasmic RNA was extracted from the cultured cells (Voutilainen et al. 1986). Northern blotting, probe sequences, labeling and hybridizations were the same as previously described (Liu et al. 1996a,b). The relative intensities of autoradiographic signals were quantified by densitometric scanning. All Northern blotting data shown here were normalized with the respective 28S ribosomal RNA values. For detecting TNFα mRNA expression, RT-PCR was performed. Briefly, 1 µg RNA was used for cDNA synthesis with the First Strand
cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). The PCR reaction was run as denaturing at 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s; and then final extension at 72 °C for 10 min. The reaction volume was 50 µl containing 0·2 mM each dNTP, 0·5 µM each primer, 1× reaction buffer, 2·5 mM MgCl₂, 1·5 U Taq DNA polymerase (Fermentas Tamro Corporation, Vantaa, Finland) and template cDNA with respect to 50 ng cytoplasmic RNA. The primer set was 5′-TCA GAT CAT CTT CTC GAA CC-3′ (sense) and 5′-CCA GAC TCG GCA AAG TCG AG-3′ (antisense), corresponding to the human TNF mRNA (GenBank Accession Number XM 041847). Ten microliters of the PCR products were resolved on 1·8% agarose gels and Southern blotted onto nylon membrane. The membrane was hybridized with a32P-labeled internal probe. The sequence of this 30-mer oligonucleotide probe was 5′-GGC AAG GGC TCT TGA TGG CAG AGA GGA GGC-3′.

**Western blot**

Cells grown on 10 cm plates were collected and washed with ice-cold phosphate-buffered saline (PBS) twice. Then, 160 µl protein sample buffer (63·5 mM Tris–HCl, pH 6·8, 10% glycerol, 2% SDS, 0·01% bromophenol blue) without β-mercaptoethanol was added to the cell pellets. The mixture was sonicated briefly on ice and centrifuged at 4 °C for 10 min. The supernatant was collected and the total protein concentration was determined by the standard BCA protein assay kit (Pierce Boston Technology, Woburn, MA, USA). Subsequently, 4 µl β-mercaptoethanol was added to each sample and mixed. Samples were incubated at 95 °C for 5 min. Total protein (25 µg) was loaded onto each lane and separated in 10% SDS-PAGE, and then transferred onto nitrocellulose membrane. Non-specific antibody binding was minimized by blocking with 5% non-fat milk in PBS–0·05% Tween 20. The primary antibodies diluted at 1:100 in PBS were added onto the adrenal cells and incubation was performed overnight at 4 °C. The primary antibodies were detected using biotin-conjugated goat anti-rabbit IgG from the corresponding ABC-Elite kit (Vector Laboratories, Burlingame, CA, USA), followed by incubation with ABC solution according to the manufacturer’s instructions. Finally, light counterstaining was performed with hematoxylin, and the slides were dehydrated and mounted. For the negative controls, the primary antibodies were replaced with normal rabbit serum or PBS alone. To exclude the effect of possible endogenous biotin, biotin blocking (avidin-biotin blocking kit; Vector Laboratories) was performed for each antigen detection before the addition of the primary antibodies. The immunoreactivity was quantified by calculating the ratio of positively stained cells to the total number of cells.

**Analyses of apoptotic cells**

Terminal transferase-mediated fluorescent-dUTP nick end-labeling (TUNEL) was performed with the in situ cell death detection kit (1684795; Roche Diagnostics GmbH, Penzberg, Germany). Monoclonal anti-β-tubulin antibody (Sigma) was used for the loading control.

**Immunocytochemical staining**

The staining was performed on H295R cells cultured on two-well chamber plastic slides (Nalge Nunc International, Naperville, IL, USA). The cells were permeabilized by microwaving at 600 W for 3×5 min in citrate buffer (10 mM Na-citrate, pH 6·0). The slides were then washed in PBS and non-specific staining was blocked with normal goat serum for at least 30 min at room temperature. Endogenous peroxidase activities were blocked by 1% H₂O₂ for 30 min. The TNF-R1 protein was detected with two affinity-purified polyclonal antibodies (sc-7895 from Santa Cruz Biotechnology, Santa Cruz, CA, USA and CSA-810 from StressGen Biotechnologies, Victoria, BC, Canada). Expression of the TNF-R2 was also studied using two polyclonal antibodies (sc-7862 and sc-1072 from Santa Cruz Biotechnology). The proliferation marker Ki-67 antigen was detected with a monoclonal antibody MIB-1 (Immunotech, Marseille, France). The primary antibodies diluted at 1:100 in PBS were added onto the adrenal cells and incubation was performed overnight at 4 °C. The primary antibodies were detected using biotin-conjugated goat anti-rabbit IgG from the corresponding ABC-Elite kit (Vector Laboratories, Burlingame, CA, USA), followed by incubation with ABC solution according to the manufacturer’s instructions. Finally, light counterstaining was performed with hematoxylin, and the slides were dehydrated and mounted. For the negative controls, the primary antibodies were replaced with normal rabbit serum or PBS alone. To exclude the effect of possible endogenous biotin, biotin blocking (avidin-biotin blocking kit; Vector Laboratories) was performed for each antigen detection before the addition of the primary antibodies. The immunoreactivity was quantified by calculating the ratio of positively stained cells to the total number of cells.
according to the producer’s instruction manual. Briefly, the cells were cultured on two-well chamber plastic slides. After treatment with different agents, the cells were fixed with 4% paraformaldehyde and permeabilized with 0·1% Triton X-100. Then 50 µl label solution was added onto each chamber and the slides were incubated in a humidified atmosphere for 60 min at 37 °C in the dark. The apoptotic cells were analyzed by Zeiss Axiophot fluorescence microscopy. For the morphological analysis of apoptotic cells, the cultured cells were fixed with methanol and stained with DAPI (or Hoechst 33258; Sigma) dye. Apoptotic cells were examined for nuclear morphology using a fluorescence microscope. End-stage apoptotic cells were also detected by trypan blue staining.

**Proliferation assays**

H295R cell proliferation was evaluated immuno-cytochemically with a cell proliferation kit (Amersham Pharmacia Biotech, Amersham, Bucks, UK) according to the manufacturer’s instructions. In this assay, 5-bromo-2’-deoxyuridine (BrdU) is incorporated into replicating DNA during 15 h of incubation, localized with a specific monoclonal antibody and stained with a peroxidase-based detection system. The culture area was photographed and the nuclei were counted from each culture dish. The proliferation percentage was calculated on the basis of the positively stained nuclei in relation to all nuclei. Flow cytometric analysis was performed on cells treated with Dibutyryl cAMP ((Bu)2cAMP). The cells were trypsinized, collected into 70% ethanol, and stained with propidium iodide. DNA content was analyzed with a flow cytometer. Cell cycle analysis was performed using the Sync Wizard Model of ModFit LT software (Becton Dickinson, Franklin Lakes, NJ, USA). Ki-67 immunocytochemistry was also used to estimate the proliferation of H295R cells.

**Transfection of c-myc into H295R cells**

Semi-confluent cells were transfected with the pSVT c-myc construct (Mäkelä et al. 1992) using FuGene 6 (Roche) transfection reagents. Cells were plated (1:5) on cover slips in a 35 mm culture dish 1 day before the transfection. The ratio of transfection reagent/vector DNA was 4:1 (1 µg DNA per dish) in all experiments. Otherwise the transfection procedures were performed in serum-free medium according to the manufacturer’s instructions. After 36 h the cells were fixed with 3·7% paraformaldehyde in PBS and permeabilized with 0·1% Triton X-100. The cells were washed three times in PBS, and the cover slips were then immunostained with c-myc and Ki-67 antibodies for an hour at room temperature and followed by three washes with PBS. To visualize the localization of antibodies, we used FITC- or TRITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). After incubation, the cover slips were washed with PBS three times for 5 min each. Finally, the cover slips were mounted onto Anti-FADE media (Molecular Probes, Eugene, OR, USA) and analyzed with a Zeiss inverted fluorescence microscope (Axophot 2) equipped with a cooled CCD camera (SensiCam; PCO Computer Optics GmbH, Kelheim, Germany). The data presented in this paper are from the total pool of the transfection experiments.

**Statistical analyses**

All experiments were repeated at least three times. Non-parametric Kruskal–Wallis test was used to detect differences among the different experimental groups. If significant differences were found, Mann–Whitney U test was subsequently used for statistical evaluation in two-group comparisons. Chi-square test was used for analyzing the correlation between Ki-67 and transfected c-myc expression. The level of significance was chosen as P<0·05.

**Results**

Activation of PKA stimulates differentiation but inhibits proliferation of adrenocortical cells

To find out if the ACTH-induced apoptosis in primary cultures of rat fetal adrenal cells (Kahri et al. 1996) can be reproduced in human adrenocortical cells and to further study its mechanisms, we used the human adrenocortical carcinoma cell line H295R as a model. As reported previously in primary cultures of normal rat and human adrenocortical cells (Arola et al. 1993, Liu et al. 1996a) and in H295 cells (the parent cell line of the H295R cells; Staels et al. 1993), (Bu)2cAMP
induced dose- and time-dependent differentiation of H295R adrenocortical cells (measured by morphological change, steroidogenic gene expression and cortisol secretion) (Fig. 1; other data not shown). Using the BrdU incorporation assay, inhibition of proliferation was found at the 72 h time-point and maintained at least for 1 week in (Bu)2cAMP (1 mM)-treated H295R cell cultures. The proliferation percentage of the (Bu)2cAMP (1 mM for 7 days)-treated cells was approximately 60% that of the control group (means ± S.E. proliferation percentage 20·6 ± 8% for the treated cells and 32·7 ± 16% for the controls, n=5, P<0·01). Direct counting of the cells also showed a decrease in cell number in (Bu)2cAMP-treated cultures down to approximately 65% of the control (Fig. 2A). Immunostaining of Ki-67 showed similar results to the BrdU staining (data not shown). We further analyzed at what stage in the cell cycle the cells had been arrested by measuring their DNA content with propidium iodide staining and flow cytometric analysis (Fig. 2B). Under normal growth conditions, more than 60% of the H295R cells were in the G0/G1 phase and only 18% were in the G2/M phase. In contrast, over 30% of the cells were in the G2/M phase after 3 days of sustained treatment with (Bu)2cAMP (1 mM) and the fraction of the G0/G1 cells decreased down to 45%. The average DNA content of the cells increased during (Bu)2cAMP treatment in the G0/G1, G2/M and S phases. (Bu)2cAMP treatment for 7 days showed a similar result (data not shown). Immunocytochemical staining of BrdU and Ki-67 also showed enlarged nuclei after (Bu)2cAMP treatment. Another analog of cAMP, 8-bromo-cAMP, also induced differentiation and reduced proliferation of H295R cells at the same concentration range as did (Bu)2cAMP (data not shown). The effect of (Bu)2cAMP was significantly blocked by the PKA inhibitor H-89 (data not shown). ACTH had no significant effect on either the differentiation markers or proliferation rate, probably due to the lack of functional ACTH receptors in this cell line (data not shown).
TNFα induces apoptosis of H295R adrenocortical cells and the effect is enhanced by (Bu)2cAMP

In contrast to the situation in primary rat adrenocortical cultures (Kahri et al. 1996), ACTH did not induce apoptosis in H295R cells. Trypan blue exclusion test and TUNEL analysis showed about 2% of the cells to be apoptotic. The apoptotic cells were usually localized in the peripheral areas of the cell colonies. (Bu)2cAMP increased apoptosis slightly after long-term (more than 24 h) treatment as analyzed by flow cytometry (the sub-G1 hypodiploid fraction in the histogram of Fig. 2B), trypan blue exclusion and TUNEL (Fig. 3). Since the dramatic effect of ACTH on apoptosis was observed in the primary rat adrenal cell cultures with heterogeneous population of cells (Kahri et al. 1996), we hypothesized that the apoptotic effect might involve some factors secreted by macrophages present in those cultures. We focused on the effect of the well-known apoptotic factor TNFα, and found as expected that TNFα induced apoptosis five- to tenfold in H295R cells after 24 h treatment, as analyzed by both trypan blue exclusion test and TUNEL analysis (Fig. 3). Interestingly, this effect was increased two- to fivefold by co-treatment with (Bu)2cAMP (1 mM) (Fig. 3). This synergistic effect of TNFα and (Bu)2cAMP on apoptosis was specific for H295R cells, since it was not detectable in HeLa, mouse adrenocortical Y-1 or in another human adrenocortical cell line, SW-13 cells, although TNFα alone increased apoptosis in these cells (data not shown). Since glucocorticoids are able to regulate apoptosis in a tissue- and cell-specific manner (Amsterdam et al. 2002), and cortisol concentration was very high in the conditioned media of the (Bu)2cAMP-treated H295R cells, but not in HeLa and SW-13 cells (data not shown), we used the well-known apoptosis inducer dexamethasone and steroidogenesis-inhibitor genistein treatment to clarify whether the secreted glucocorticoids affect the TNFα-induced apoptosis in H295R cells. Dexamethasone at the concentration of 500 µg/l for 24 h had no effect on the TNFα-induced apoptosis in H295R cells. Treatment with genistein
(at the concentration of 10 µg/ml) for 24 h reduced cortisol secretion by more than 80%, flattened the cells and prevented the contracting effect of (Bu)$_2$cAMP on the cells. Trypan blue assay showed that genistein alone slightly increased cell death (about 5–20%) compared with the control, and this effect was not affected by co-incubation with (Bu)$_2$cAMP. Genistein treatment did not significantly augment the apoptotic effect of TNFα. In addition, the absence of the synergistic effect of (Bu)$_2$cAMP and TNFα in Y-1 cells also excluded the effect of glucocorticoids, since (Bu)$_2$cAMP strongly stimulated steroidogenesis in this cell line (data not shown).

Expression of the c-myc gene does not correlate with proliferation of H295R cells

For clarifying the potential mechanisms underlying the synergistic effects of TNFα and (Bu)$_2$cAMP on apoptosis, we compared c-myc gene expression in H295R, SW-13, Y-1 and HeLa cells, since our previous studies showed that c-myc expression was upregulated by (Bu)$_2$cAMP in primary cultures of normal human adrenal cells and was lost in adrenocortical carcinomas (Liu et al. 1996b, 1997). Preceding the decreased cell proliferation, c-myc expression was increased by (Bu)$_2$cAMP in a dose- and time-dependent manner in H295R cells. Interestingly, this effect was not detected in SW-13, Y-1 or HeLa cells (Fig. 4). For analyzing further c-myc functions, we transfected c-myc cDNA into H295R cells in five different experiments (Fig. 5). Interestingly, overexpression of c-myc gene was not associated with stronger Ki-67 staining in the transfected than in the untransfected cells (Chi square=0.229, Phi=0.071, P=0.632). Approximately 7% of the Ki-67-positive cells expressed the c-myc gene simultaneously and only 35% of the c-myc-overexpressing cells were Ki-67 positive, suggesting that induction of c-myc expression alone in this cell line may not be coupled to cell proliferation.

Figure 3  Synergistic effect of (Bu)$_2$cAMP (1 mM) and TNFα (100 ng/ml) treatments (24 h) on apoptosis in H295R cells. The cell culture conditions and apoptosis measurements were as described in the Materials and methods. (A) Trypan blue exclusion analysis. Each bar represents the mean percentage of positively staining cells (±S.E. of three experiments in triplicate dishes). *P<0.01. (B) TUNEL analysis. (a and b) Control cells without stimulation; (c and d) (Bu)$_2$cAMP alone; (e and f) TNFα alone; (g and h) combined TNFα and (Bu)$_2$cAMP treatment. (a, c, e and g) Phase contrast and (b, d, f and h) fluorescence microscopic images.
Effect of PKA and TNFα on adrenocortical cells

A

Relative c-myc mRNA levels

<table>
<thead>
<tr>
<th>Time for (Bu)2cAMP treatment</th>
<th>2 h</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative c-myc mRNA levels</td>
<td>*</td>
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</tbody>
</table>

B

(Bu)2cAMP (μmol/L)

control 1000 100 10 1 0.1

-- 28S

-- c-myc

-- 18S

-- 28S

C

Control (Bu)2cAMP

-- c-myc

-- β-tubulin

D

c control (Bu)2cAMP

-- 28S

-- c-myc

-- 18S

-- 28S

SW-13 cells

E

c control ACTH (Bu)2cAMP

-- 28S

-- c-myc

-- 18S

-- 28S

Y-1 cells

F

c control (Bu)2cAMP

-- 28S

-- c-myc

-- 18S

-- 28S

HeLa cells
H295R cells express TNFα receptors

Although TNFα mRNA was not detectable in H295R cells with RT-PCR analysis (data not shown), weak staining of both TNF-R1 and TNF-R2 (Fig. 6) was detected in these cells, with occasional strong expression for TNF-R2 in some cells (Fig. 6B). The positive staining was mainly in the cytoplasm, with occasional weak staining in the nuclei. When the primary antibodies were replaced with non-specific rabbit IgG or PBS, there was no specific staining. These results indicated that the components for TNFα signal transduction are available in H295R cells. Treatment with (Bu)2cAMP had no significant effect on the expression of TNFα receptors (data not shown).

Discussion

This study was undertaken to characterize the interaction of PKA activation and TNFα on the apoptotic process in cultured H295R adrenocortical cells. (Bu)2cAMP induced H295R cell differentiation, reduced proliferation and increased the sensitivity of the cells to TNFα-induced apoptosis. This finding is similar to the previous report that cAMP inducers, such as forskolin and 8-bromo-cAMP, inhibited cell growth but also induced cell death in some other cell types (Chen et al. 1998). Nevertheless, the H295R cell line originated from a human adrenocortical carcinoma (Rainey et al. 1994), giving the cells particular characteristics. Previous reports have described that adrenocortical cell apoptosis is affected by the in vitro culture system, species and differentiation status (Negoescu et al. 1995, Carsia et al. 1998, Spencer et al. 1999).

Although this cell line was recently reported to respond to ACTH (Suzuki et al. 2004), previous studies showed no response in either the H295R cell line or in its parental H295 cells, probably because of low ACTH receptor expression (Staels et al. 1993, Rainey et al. 1994, Denner et al. 1996). Similarly, our H295R cells did not show significant response to ACTH treatment. We therefore used (Bu)2cAMP to mimic the ACTH signal pathway. In the present study, (Bu)2cAMP reduced H295R cell proliferation by arresting the cell cycle mainly to the G2/M phase. (Bu)2cAMP treatment seems to override the cell-cycle inhibition in the G1 checkpoint and promote cells into the cell cycle,
possibly by inducing mitogenic factors, such as c-myc. Obviously the induction of these mitogenic factors by (Bu)2cAMP is unable to overcome the inhibition in the G2/M checkpoint, ultimately leading the cells to arrest in the G2/M phase. After long-term treatment, the pool of cells in the G0/G1 phase and subsequent re-entering into the cell cycle could decrease, resulting in reduced DNA synthesis measured by BrdU incorporation. c-myc gene expression was rapidly induced by (Bu)2cAMP treatment in H295R cells, as reported previously in primary cultures of rat fetal and normal human adult adrenocortical cells (Heikkilä et al. 1995, Liu et al. 1996b). The increased c-myc expression may initially promote the cells into the cell cycle through the G1 to G2/M phase. However, overexpression of c-myc gene in the transfected cells was not associated with increased Ki-67 staining, suggesting that the proliferation pathway was blocked in these c-myc-overexpressing cells. The previously reported putative tumor suppressor H19 and cyclin-dependent kinase inhibitor p57KIP2, stimulated by (Bu)2cAMP treatment in adrenocortical cells (Voutilainen 1998), may function as proliferation blocking factors to stop the cells within the cell cycle. During treatment with (Bu)2cAMP for 3–7 days, the percentage of cells in the G2/M phase and the average cellular DNA content increased, but the total cell number and the late-stage BrdU incorporation decreased. These findings also suggested an ultimate inhibition of DNA synthesis and cell division after initial entering into the cell cycle. It was previously reported that induction of c-myc alone is not sufficient to induce DNA synthesis in Y-1 cells (Lepique et al. 2000). While blocking cell proliferation, (Bu)2cAMP-induced PKA activation may set H295R cells to a low threshold for apoptosis in the G2/M phase, probably by increasing the expression of apoptosis-related genes (such as c-myc). Interestingly, PKA activation did not induce c-myc expression in another human adrenocortical cell line SW-13, in the mouse adrenocortical cell line Y-1 or in the human cervical adenocarcinoma cell line HeLa. In these cell lines, (Bu)2cAMP treatment did not enhance cellular sensitivity to TNF. In the fetal zone of the human fetal adrenals, ACTH apparently causes differentiation and hypertrophy of the cells migrating inwardly from the definitive zone. The enlarged senescent cells in the fetal zone go into apoptosis more easily than the smaller definitive zone cells (Mesiano & Jaffe 1997). The synergistic effect of PKA activation and TNF on apoptosis seems to be tissue specific. It was previously reported that cAMP-elevating reagents were able to suppress TNF or Fas/APO-1 (CD95)-induced apoptosis in neutrophils and hepatocytes (Fladmark et al. 1997, Niwa et al. 1999), whereas multidrug-resistant leukemic cells with higher constitutive levels of PKA activity were more susceptible to TNF-induced apoptosis and growth inhibition (Yin et al. 2000).

Glucocorticoids are tissue- and cell-specific regulators of apoptosis (Amsterdam et al. 2002). We therefore used dexamethasone and genistein to clarify whether the high concentration of cortisol in the conditioned media could affect the TNF-induced apoptosis. Our results showed that treatment with dexamethasone had no effect on basal or TNF-induced apoptosis. Inhibition of cortisol synthesis by genistein, a strong suppressor of the steroidogenic enzymes steroid 21-hydroxylase and 3β-hydroxysteroid dehydrogenase in H295R cells (Mesiano et al. 1999, Ohno et al. 2002), could not protect the cells from apoptosis. These results suggested that the enhancing effect of (Bu)2cAMP on TNF-induced apoptosis in H295R cells is not due to the increased secretion of cortisol into the culture medium.

In the human adult adrenal gland, TNF-secreting cells are predominantly localized in the zona reticularis of the cortex. ACTH reduces TNF release from cultured rat and bovine adrenocortical cells, probably via increased intracellular cAMP (Judd et al. 2000). In addition, the ACTH-induced secretion of glucocorticoids may also suppress TNF release from monocytes/macrophages surrounding the adrenal cells (Joyce et al. 1997). In physiological conditions, ACTH stimulation increases intracellular cAMP, which could then predispose adrenocortical cells to the TNF-induced apoptotic effect; at the same time ACTH reduces TNF production directly or indirectly. Thus, normal adrenocortical cells differentiate without apoptosis in the absence of TNF ligand. Hormonally regulated TNF levels together with cellular PKA activity may be at least one mechanism maintaining the normal dynamics of adrenocortical cells. However, in exceptional conditions, ACTH stimulated cells may meet high levels of TNF from the circulation or from the monocytes/macrophages concentrated locally in
the adrenals. In these conditions, enhanced apoptosis could occur, similarly to that in primary cultures of fetal rat adrenocortical tissues (Kahri et al. 1996). In that tissue culture system, plenty of macrophages attach on adrenocortical cells (authors’ unpublished data). This phenomenon does not occur in primary cultures of human fetal or adult adrenocortical cells (Ilvesmäki et al. 1993, Liu et al. 1996b), suggesting that the enzymatic dispersion and washing process during the preparation of human adrenal cell cultures may eliminate the macrophages. The ACTH/PKA-induced enhanced sensitivity of adrenocortical cells to TNFα-induced apoptosis may have a role in fetal zone regression after birth, since human fetal adrenal cells secrete more TNFα than do adult adrenal cells (Jaätelä et al. 1990). H295R cells are characterized by a loss of cAMP response element (CRE)-binding protein (CREB) expression and compensatory overexpression of CRE modulator τ (CREMt) (Groussin et al. 2000). The role of this shifted signal transduction pathway of PKA in the sensitizing effect of (Bu)2cAMP in H295R cells has not been ruled out, as the normal adrenal cortex expresses high levels of CREB and no or low levels of CREMt (Groussin et al. 2000).

In summary, in human adrenocortical H295R cells, activation of cAMP-dependent PKA inhibited cell proliferation and increased the sensitivity of the cells to TNFα-induced apoptosis, associated with the induction of c-myc gene expression. The tightly regulated proliferation and apoptosis probably contribute to the dynamic changes in cell growth and death in adrenal glands.

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