Molecular and Cellular Endocrinology 371 (2013) 166–173

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

N-POMC$_{1-28}$ increases cyclin D expression and inhibits P27^{kip1} in the adrenal cortex

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ARTICLE INFO

Article history:
Available online 5 December 2012

Keywords:
Adrenal cortex
N-POMC$_{1-28}$
ACTH
Cyclin D
P27^{kip}
Proliferation

ABSTRACT

The Adrenocorticotropic hormone (ACTH) and Pro-opimelanocortin (POMC) 1–28 N-terminal peptide (N-POMC$_{1-28}$) have been shown to act as an adrenal mitogen *in vivo*. A possible role for cyclin E in the zona glomerulosa (ZG) proliferation, following ACTH and/or N-POMC$_{1-28}$ administration, has been previously demonstrated. In this study, we investigated the effect of ACTH and N-POMC$_{1-28}$ on the expression of adrenal cortex proteins related to cell cycle control such as cyclins D and P27^{kip1}. The administration of N-POMC upregulated cyclin D1 and D2 expression in the outer zone of the adrenal cortex; cyclin D3 expression was upregulated in the cortex inner zone even after administration of ACTH. Both ACTH and N-POMC peptides induced a decrease in the P27^{kip1} expression in the ZG. These novel findings suggest that the POMC-derivate peptides, ACTH and N-POMC, promote proliferation in the adrenal cortex by upregulating the D2 and D3 cyclins and downregulating the P27^{kip1} expression.

1. Introduction

The adrenal gland is an organ of dynamic growth that requires stimuli from peptides derived from the pituitary gland in order to maintain the proliferating rate of its basal cells. Fragments of the N-terminal portion of POMC, known as N-POMC, have been shown to stimulate adrenal cell proliferation (Estivariz et al., 1982; Lowry et al., 1983). Among these fragments, the N-POMC$_{1-28}$ and N-POMC$_{2-54}$ peptides were found to be potent mitogens, both *in vitro* (Estivariz et al., 1982) and *in vivo* (Estivariz et al., 1988). Previous findings from our group showed that the administration of modified synthetic N-POMC$_{1-28}$ peptides induced *in vivo* and *in vitro* proliferation in the adrenal cortex (Torres et al., 2010; Mendonca and Lotfi, 2011; Mattos et al., 2011). It has also been shown that cyclin E is over-expressed in the zone glomerulosa (ZG) after mitogenic stimuli suggesting a mitogenic role for cyclin E in the adrenocortex (Mendonca and Lotfi, 2011). However, the potential effect of N-POMC or ACTH stimuli on the expression of other proteins of the cyclin D family, which like cyclin E also regulate the cell cycle progression from the G1 through the S phase in the rat adrenal (for review, Pestell et al., 1999), has not been investigated.

This study evaluated the expression pattern of cyclin D (D1, D2, and D3) and Cdk-inhibitory protein P27^{kip1} in the adrenal cortex of dexamethasone-treated rats after administration of ACTH and/or modified N-POMC$_{1-28}$ peptides, with cysteine correctly aligned disulfide bonds (N-POMC^{Cys}) or with methionine (N-POMC^{Met}). Our results strongly suggest the involvement of specific cyclins and Cdk-inhibitors in the proliferative response of POMC peptides in a zone-dependent pattern.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, at average weights of 250 ± 30 g, were obtained from the Biomedical Sciences Institute of the University of São Paulo and maintained in a temperature-controlled environment and 12-h light/dark cycle. The Animal Experimentation Ethics Committee from the Biomedical Sciences Institute approved the study. The animals were fed with standard rat feed and received water *ad libitum*. All experimental procedures were conducted between 09 and 11 am; the rats were euthanized by decapitation and adrenal glands were harvested.

2.2. N-POMC$_{1-28}$ peptides synthesis

Two types of rat N-POMC$_{1-28}$ peptides were synthesized: with cysteine (N-POMC^{Cys}) and with methionine (N-POMC^{Met}) as described in Mendonca and Lotfi (2011). The identities of the peptides were confirmed by analysis of their amino acid composition. The analyses were performed in the Peptide Chemis-

Abbreviations: ACTH, Adrenocorticotropic hormone; DEX, dexamethasone; POMC, pro-opiomelanocortin; N-POMC, N-terminal fragments of POMC; POMC, pro-opiomelanocortin; ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis.

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http://dx.doi.org/10.1016/j.mce.2012.11.017

try Laboratory from the Department of Biochemistry, Institute of Chemistry, at the University of São Paulo.

2.3. Dexamethasone, ACTH, and N-POMC$_{1-28}$ treatments

The concentration of 50 μg/100 g of body weight (BW) of Dexamethasone (DEX) (Aché Laboratórios Farmacêuticos, Campinas, SP, Brazil) was administered intraperitoneally, once a day at 9 am for 2 days, with the purpose of inhibiting the HPA axis. The circulating ACTH levels were measured in the plasma from trunk blood samples collected at the time of euthanasia (at 24, 32, and 48 h after the second DEX) with an immunochemiluminescent assay (Immulite; Diagnostic Products, Los Angeles, Calif., USA). The control animals received the same treatment schedule applied to the experimental animals, however with saline injections. The rats were divided into five groups ($n = 4–5$) 28 h after the last DEX injection and were given a single intraperitoneal injection of ACTH (3.3×10^{-2} μg/100 g BW), N-POMC^{Cys} (3.2×10^{-2} μg/100 g BW), N-POMC^{Met} (4.5×10^{-2} μg/100 g B.W.), ACTH + N-POMC^{Met} or saline (100 μl/100 g B.W.). The levels of the cyclins D and P27^{kip1} were measured at 4, 6, or 8 h after treatment with the peptides, based on the pattern of cyclins expression observed in adrenocortical cells *in vitro* after a mitogenic stimuli (Lotfi et al., 2000; Schwindt et al., 2003).

2.4. Preparation of lysates

The preparation of protein lysates was performed as described in Mendonca and Lotfi (2011). Briefly, at 4, 6, or 8 h after the final treatments, the adrenal glands were removed and gently decapsulated to separate the capsule/zona glomerulosa (ZG + Capsule) from the zona fasciculata/reticularis and medulla (ZF + ZR). The medulla was removed from the ZF + ZR and the samples were lysed in ice-cold RIPA and protease inhibitors. The protein concentrations were quantified by the Bradford assay.

2.5. Detection of cyclins D and P27^{kip1} protein expression

Immunoblotting was performed as described in Mendonca and Lotfi, 2011. Briefly, protein samples were sorted in SDS–PAGE and membranes incubated with the following antibodies: anti-cyclin D1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cyclin D2 (1:1000; Santa Cruz), anti-cyclin D3 (1:3000, Cell Signaling Inc., Danvers, MA, USA), anti-P27 (1:1000; Santa Cruz), or β-actin (1:2000, Santa Cruz) for two hours at room temperature or overnight at 4 °C. Proteins were detected using chemiluminescent secondary peroxidase-conjugated anti-rabbit or anti-mouse polyclonal antibodies (ECL-Amersham-Pharmacia, Piscataway, NJ, USA). The immunoblot results were densitometrically quantified using the Gel-Pro Imager and Gel-Pro Imager kit Version 1.0 quantification program for Windows.

2.6. Analysis of cyclin D3 and P27^{kip1} through immunohistochemistry

The analysis through immunohistochemistry was performed as described in Mendonca and Lotfi (2011). Briefly, rats were euthanized by decapitation 4 h (for cyclin D3) or 6 h (for P27^{kip1}) after the final treatments; adrenal glands were removed, fixed in 4% paraformaldehyde in a solution of 0.1 M phosphate buffered saline (PBS; pH 7.4) and deparaffinized sections were rehydrated. Sections were incubated for antigen retrieval, background staining was blocked, and incubated in PBS with 1:200 anti-P27 (C-19) antibody (Santa Cruz) or 1:200 anti-cyclin D3 (Cell Signaling). The immune complexes were detected by immunoperoxidase followed by counterstaining with Harris' hematoxylin and a saturated solution of lithium carbonate. The adrenal sections incubated in non-immune primary sera tested negative (data not shown).

2.7. Statistical analysis

The results were representative of three independent experiments. Data were presented as the mean ± standard error of the mean (SEM). Statistical significance was determined using Tukey–Kramer Multiple Comparisons Test after an analysis of variance (ANOVA) except where otherwise noted. Results were considered statistically significant when $p < 0.05$.

3. Results

In this study, the expression of another important G0–G1 cell cycle transition protein family, the D cyclins and the P27^{kip1} cell cycle inhibitor protein, a G1 arrest cell cycle protein, was evaluated. The potential role of cyclin E in the proliferation of the adrenal cortex when triggered by ACTH and N-POMC synthetic peptides has been demonstrated in a previous study (Mendonca and Lotfi, 2011).

3.1. HPA axis inhibition after DEX administration

A significant reduction of 44% in the circulating levels of ACTH was observed 24 h after the last DEX administration. No difference in the circulating levels of ACTH was observed at 32 and 48 h after the last DEX injection (Table 1). Based on these findings and based on previous results reported in Mendonca and Lotfi (2011), injections of the mitogenic peptides were initiated 28 h after the last treatment with DEX.

3.2. HPA axis inhibition upregulated P27^{Kip1} and downregulated cyclins D2 and D3 expression in the adrenal cortex

A 2.2-fold increase in the P27^{kip1} protein expression was observed in the inner part of the adrenal cortex, ZF/ZR, 4 h after the last DEX injection. However, no changes in the expression level of cyclin D proteins were observed (Fig. 1A).

Cyclin D2 and P27^{kip1} protein expression levels significantly decreased after 6 h in the ZF/ZR (0.3-fold and 0.4-fold decrease, respectively), when compared to controls, whereas a 0.3-fold decrease in cyclin D3 was observed in the entire cortex (Fig. 1B).

A lower expression level (0.2-fold decrease) of cyclin D2 was kept in the ZF/ZR, 8 h after the DEX treatment, whereas the cyclin D3 expression was lower in the entire cortex (0.7-fold and 0.3-fold decrease in ZF/ZR and ZG, respectively). At this same time point in the treatment, the P27^{kip1} protein expression presented a 0.6-fold decrease and no modulation effect in ZF/ZR and ZG, respectively (Fig. 1C1–C4).

Table 1
ACTH plasma concentration (pg/ml) at 24, 32, and 48 h after the last dexamethasone (DEX) injection.

Time after the second injection of DEX	Saline	DEX (50 μg/100 g B.W.)
24 h	100 (±25)	51 (± 20)[a]
32 h	128 (±52)	175 (±49)
48 h	55 (± 23)	50 (±17)

Statistical significance was evaluated using unpaired *T*-test.
[a] $p < 0.05$; $n = 3$.