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The proliferative effect of synthetic N-POMC$_{1-28}$ peptides in rat adrenal cortex: A possible role for cyclin E

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ABSTRACT

Modified synthetic N-POMC$_{1-28}$ without disulfide bridges has been shown to act as an adrenal mitogen. Cyclins and their inhibitors are the major cell cycle controls, but in the adrenal cortex the effect of ACTH and N-POMC on the expression of these proteins remains unclear. In this work, we evaluate the effect of different synthetic N-POMC peptides on the S-phase of the cell cycle. In addition, we examine the cyclin E expression in rat adrenal cortex. Rats treated with dexamethasone were injected with ACTH and/or synthetic modified N-POMC and/or synthetic N-POMC with disulfide bridges. DNA synthesis was determined by BrdU incorporation and protein expression was analyzed by immunoblotting and immunohistochemistry. The results showed that similarly to modified N-POMC without disulfide bridges, administration of synthetic N-POMC with disulfide bridges and the combination of ACTH and N-POMC promoted an increase of BrdU-positive nuclei in adrenal cortex. However, the proliferative effect of N-POMC was comparable to that of ACTH only in the zona glomerulosa. An increase in cyclin E expression was observed 6 h after N-POMC treatment in the outer fraction of the adrenal cortex, in agreement with immunohistochemical findings in the zona glomerulosa. In summary, the effect of synthetic N-POMC with disulfide bridges was similar to modified synthetic N-POMC, increasing proliferation in the adrenal cortex, confirming previous evidence that disulfide bridges are not essential to the N-POMC mitogenic effect. Moreover, cyclin E appears to be involved in the N-POMC- and ACTH-stimulated proliferation in the zona glomerulosa of the adrenal cortex.

1. Introduction

Among pro-opiomelanocortin (POMC)-derived peptides, the adrenocorticotropic hormone (ACTH) is considered one of the main mitogenic stimuli for the adrenal cortex (New, 1998). However, other POMC peptides different from ACTH seem also appear to have a role in adrenal cell proliferation. The N-terminal fragments of POMC, known as N-POMC, have been shown to stimulate adrenal cell proliferation (Estivariz et al., 1982; Lowry et al., 1983). 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). Furthermore, previous findings from our lab showed that administration of modified synthetic N-POMC$_{1-28}$, without disulfide bridges, to dexamethasone (DEX)-treated rats induces S-phase entry in all zones of the adrenal cortex (Torres et al., 2010).

To promote tissue growth proliferating mammalian cells pass through an orderly sequence of phases that comprise the cell cycle (Pestell et al., 1999, for review). Progression through G1 to S phase is promoted by cyclin D1 and cyclin E, which heterodimerize with catalytic subunits, the cyclin-dependent kinases (Cdks), to form active holoenzymes. These active holoenzymes phosphorylate substrates essential for progression through the restriction point of the cell cycle.

The aim of this work was to compare the effects of synthetic peptide N-POMC$_{1-28}$ with disulfide brides (N-POMC^{w}), and a modified peptide without disulfide bridges (N-POMC$^{w/o}$) on cell proliferation in the adrenal cortex using the well-known in vivo model of dexamethasone (DEX)-treated rats. In addition, the pattern of cyclin E expression was analyzed 6 h after administration of ACTH and/or N-POMC. Our results show that in contrast with synthetic modified N-POMC$^{w/o}$, which increases DNA synthesis in all zones of the adrenal cortex, synthetic N-POMC^{w} has a mitogenic effect only in the zona glomerulosa and zona reticularis of the adrenal cortex. In addition, within 6 h of N-POMC and ACTH treatment cyclin E expression in the zona glomerulosa was increased, suggesting the

Abbreviations: ACTH, adrenocorticotropic hormone; BrdU, 5-bromo-2′-deoxyuridine; DEX, dexamethasone; N-POMC, N-terminal fragment of POMC; POMC, pro-opiomelanocortin; ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis.

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involvement of this protein in the mitogenic effect on the adrenal cortex.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats, weighing 250 ± 30 g, were obtained from the Biomedical Sciences Institute of the University of São Paulo and maintained in a temperature-controlled environment on a 12-h light/dark cycle. All protocols were approved by the Animal Experimentation Ethics Committee of the Biomedical Sciences Institute. The animals had free access to tap water and standard rat chow. All experiments were conducted between 9:00 am and 11:00 am. At the indicated time, rats were killed by decapitation, and the adrenal glands were harvested. Trunk blood was collected in chilled tubes containing 3.8% EDTA. The blood was separated into cellular and plasma fractions (10,000 rpm for 15 min at 4 °C), and the plasma was stored at −80 °C until analysis by chemiluminescent immunoassay for ACTH (Immulite; Diagnostic Products, Los Angeles, CA, USA).

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

Two forms of rat N-POMC$_{1-28}$ peptides were synthesized, with and without disulfide bonds, and at least two separate synthetic reactions were performed for the same peptide sequence. The rat N-POMC$_{1-28}$ peptide without disulfide bonds (N-POMC$^{w/o}$) was synthesized by the Department of Biochemistry and Biophysics of the Federal University of São Paulo (UNIFESP). To avoid undefined disulfide bonds the peptide was synthesized with the replacement of cysteine 2, 8, 20 and 24 residues by methionine. Meanwhile, the rat N-POMC$_{1-28}$ peptide with disulfide bonds (N-POMC^{w}) in the cysteine 2–24 and 8–20 positions was synthesized by Bachem America Inc. (Torrance, CA, USA).

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

The dexamethasone (Dex) (Aché Laboratórios Farmacêuticos, Campinas, SP, Brazil) treatment was performed as described in Baccaro et al. (2007). Briefly, male Sprague-Dawley rats were divided into seven groups (n = 4–5): Dex or Dex + ACTH or Dex + N-POMC$^{w/o}$ or Dex + ACTH + N-POMC$^{w/o}$ or Dex + N-POMC^{w} or Dex + ACTH + N-POMC^{w}, and control. Dex was administered intraperitoneally at a dose of 50 μg/100 g B.W. once a day at 9:00 am for 2 days. ACTH, N-POMC^{w} and N-POMC$^{w/o}$ treatments were administered at a concentration of 10^{-7} M in a dose of 100 μl/100 g B.W. Control rats received identical injections of saline only.

2.4. Preparation of fractions and protein lysates

At 6 and 8 h after final treatments, rats were decapitated and the adrenal glands were promptly removed. Adherent adipose tissue was cleaned from the adrenal glands, which were then gently decapsulated to separate capsule/zona glomerulosa (outer fraction; OF) from zona fasciculata/reticularis and medulla (inner fraction; IF). Fractions were rapidly frozen in liquid nitrogen and the medulla was removed from IF. All samples were lysed in ice-cold RIPA (50 mM of Tris–HCl (pH 7.5), 150 mM of NaCl, 1% (v/v) Nonidet P-40, 1% (w/v) sodium deoxycholate, 0.1% SDS) and protease inhibitors (1 mM of phenylmethylsulfonyl fluoride, 2 μg/ml of leupeptin, 2 μg/ml of aprotinin and 2 μg/ml of pepstatin). Samples were clarified by centrifugation and the protein concentration was quantified by the Bradford assay.

2.5. Characterization of fractions

Total lysate protein samples from IF and OF (30 μg) were resolved by 10% SDS-PAGE. After electrophoresis, the gel was electroblotted onto Hybond-C nitrocellulose membranes using a semi-dry Bio-Rad apparatus. Non-specific sites were blocked overnight at 4 °C with 5% non-fat dried milk dissolved in TBS (150 mM NaCl, 10 mM Tris, 1% Tween 20, pH 7.5). Membranes were then incubated with one of the following antibodies: anti-cytochrome P450 aldosterone synthase (CYP11B2, 1:100; Chemicon-Millipore Corporate, Billerica, MA, USA), anti-cytochrome P450 11β-hydroxylase (CYP11B1, 1:100; Chemicon), anti-tyrosine hydroxylase (TH, 1:5000; Immunostar Inc. Hudson, WI, USA), β-actin (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or α-actinin (1:2000; Santa Cruz) for 2 h at room temperature. Proteins were then visualized by chemiluminescent detection with secondary peroxidase-conjugated anti-rabbit polyclonal antibodies (ECL-Amersham-Pharmacia, Piscataway, NJ, USA).

2.6. BrdU incorporation

The protocol followed was that described in Torres and Lotfi (2007). Briefly, at 12 h after final treatments, all animals received a single intraperitoneal injection of 100 μg BrdU (Sigma, St. Louis, MO, USA) per 100 g BW. At 24 h after final treatments the rats were killed by decapitation, and adrenal glands removed, fixed and embedded in paraffin. Sections (3 μm) were deparaffinized, rehydrated, endogenous peroxidase activity blocked with 0.3% hydrogen peroxide in methanol, hydrolyzed with 1.5 N HCl, and then incubated with monoclonal anti-mouse-BrdU antibody (Amersham Pharmacia Biotech, Uppsala, Sweden). Data obtained from each zone were averaged for each experimental group, and the standard error of the mean was calculated. After an analysis of variance (ANOVA), statistical comparisons were made using the Tukey–Kramer multiple comparisons test. Values of $p \leq 0.05$ were considered statistically significant.

2.7. Immunoprecipitation of cyclin E

Cyclin E protein expression was analyzed by immunoprecipitation due to lack of specificity of the antibody when performing immunoblotting only. Total protein lysates (300 μg) were incubated with 20 μl of A-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 4 °C. The supernatant obtained after centrifugation was incubated with 1 μg of anti-cyclin E Sc-481 (Santa Cruz) or β-actin (Santa Cruz) overnight at 4 °C. Samples were immunoprecipitated with A-Agarose protein, and the pellet washed with RIPA buffer and boiled. Protein samples were resolved as described before and membranes were incubated with anti-cyclin E (1:1000; Santa Cruz) or β-actin (1:2000; Santa Cruz) for 2 h 30 min at room temperature. Chemiluminescent detection was performed as described above and immunoblot results were quantified by densitometric analysis using the Gel-Pro Imager and the quantification program Gel-Pro Imager kit-Version 1.0 for Windows. The results given are representative of three independent experiments. Ponceau staining was used to monitor protein transfer and total protein loaded.

2.8. Immunohistochemistry for cyclin E

At 6 h after final treatments rats were killed by decapitation; adrenal glands were removed, fixed in 4% paraformaldehyde in a solution of 0.1 M phosphate buffered saline (PBS; pH 7.4) for 8 h at room temperature, and then immersed in PBS plus 6% sucrose for 12 h. After being fixed and deparaffinized, the sections were rehydrated through a graded ethanol series, before being washed with sterile Milli-Q water,

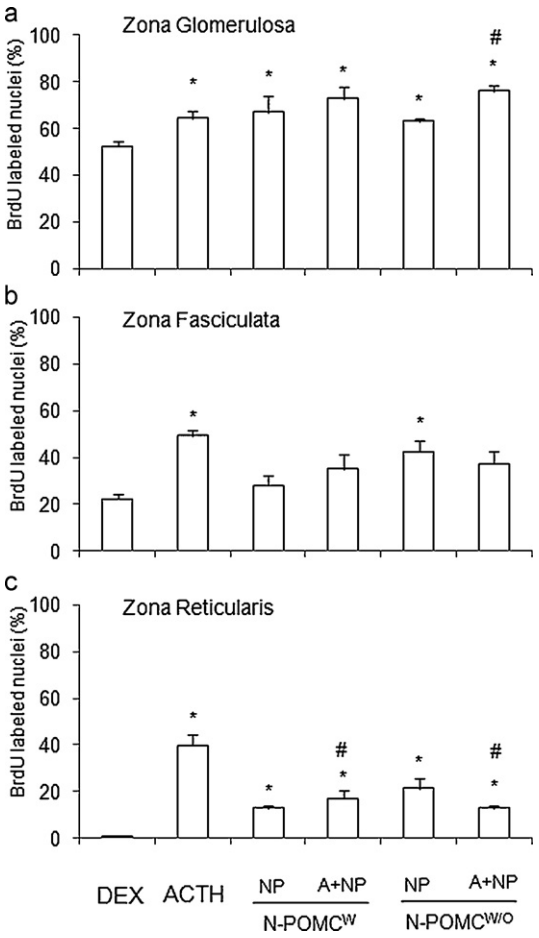

Fig. 1. Effect of N-POMC^{w} on the percentage of BrdU-positive nuclei in the adrenal cortex of dexamethasone (Dex)-treated rats. Values given are the number of BrdU-positive nuclei expressed as a percentage of the total number of nuclei. The differences between values for BrdU incorporation in Dex versus treated rats (*) and ACTH versus ACTH + N-POMC (#) are indicated. Data were analyzed by ANOVA and the Tukey–Kramer multiple comparisons test. *# $p < 0.05$.