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Basal adrenocorticotropin (ACTH) secretion is negatively modulated by protein phosphatase 5 in mouse pituitary corticotropin AtT20 cells

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Abstract

siRNA oligonucleotides for protein phosphatase 5 (PP5) were designed and transfected into mouse corticotroph AtT20 cells to induce lower PP5 expression levels. PP5-siRNA transfections (at 3 days) produced a ~50% down-regulation in targeted protein levels. PP5-underexpressing cells released significantly more ir-ACTH (10–12-fold) relative to baseline levels and promoted POMC release into the media. Neither CRF-mediated ACTH release nor dexamethasone-induced ACTH repression were affected in PP5-siRNA transfected cells. In summary, our observations suggest that endogenous PP5 can exert a negative modulatory effect on basal ACTH release in neurosecretion-competent AtT20 cells through a mechanism as yet unknown but which does not directly involve regulated CRF or glucocorticoid receptor-dependent pathways. However, PP5 may cause mis-sorting of POMC and POMC-derived peptides at the constitutive-like secretory pathway level in an unregulated manner. Such a missorting could lead to impaired processing of POMC.

Keywords: Antisense oligonucleotide; Adrenocorticotropic hormone; Dexamethasone; POMC; CRF

1. Introduction

Protein phosphatase 5 (PP5) is a member of the PPP family, but unlike its related members it is less abundant in vivo with a low basal activity [1,2]. PP5 consists of a single polypeptide chain containing a ser/thr phosphatase C-terminal catalytic domain and four regulatory N-terminal tetratricopeptide repeat (TPR) domains that mediate a variety of protein-protein interactions. The TPR domain and a region at the C terminus negatively regulate PP5 phosphatase activity [3,4], whereas polyunsaturated fatty acids and CoA esters stimulate its activity [2,3].

PP5 is known to participate in signal transduction pathways controlling cell proliferation, differentiation and programmed cell death [1,5,6]. Several proteins thought to have important CNS functions have been reported to interact with PP5 in vitro. These include cryptochrome proteins which are implicated in the central control of mammalian circadian rhythms [7], apoptosis signal-regulating kinase 1 (ASK1) [8], PP2A [9], Galpha12 and Galpha13 [10], ERα and Erβ [11], atrial natriuretic peptide/guanylate cyclase [12], which modulate neuronal excitation and neuroendocrine secretion via cGMP production, and glucocorticoid-receptor-heat-shock protein 90 (Hsp90) heterocomplexes [13,14]. In the CNS, PP5's heterogenous distribution indicates that it may serve specific functions [15]. In particular, the presence of PP5 in structures such as the supraoptic nucleus of the hypothalamus and pituitary suggest its participation in neuroendocrine secretion.

In our view, the siRNA technique provides a unique opportunity for targeted protein down-regulation at the cellular level. In the case of PP5, the siRNA approach contributes several technical advantages such as 1) the ability to selectively interfere with PP5 without effecting other structurally related PPases (e.g PP1 and PP2A) or the use of non-selective toxins, and 2) an alternative method to PP5 inactivation avoiding transfections of dominant-negative forms (e.g. TPR and C-terminal catalytic domains)

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which could interfere with unrelated protein families containing similar domains.

In this study, we have used antisense 2′-OH form oligonucleotides capable of inhibiting the expression of PP5 to induce lowered production levels of PP5 in adrenocorticotropin (ACTH)-producing AtT20 cells. The AtT20 cell line represents a model for a HPA-axis component since they are of pituitary origin, can modulate the release of ACTH upon stimulation with corticotropin-releasing-hormone and are dexamethasone-sensitive [16,17]. For the first time, our results show that the amounts of basal ACTH released following PP5 down-regulation are increased in a secretion-competent cell line, suggesting that PP5 may play a modulatory role in neuroendocrine secretion.

2. Materials and methods

2.1. Mouse corticotroph AtT20 cell line

The mouse AtT20 cell line was obtained from American Tissue Type Collection (ATCC, Manassasa, VA, USA). The cell line was maintained in Dubelcco's modified Eagle's medium containing 10% fetal calf serum (FCS) and 4 mM L-glutamine, in a 5% CO2-humidified atmosphere at 37 °C. All cell cultures were routinely passed when 90–95% confluent.

2.2. siRNA generation

Two siRNA duplexes for mouse PP5 were designed and synthesized (antisense #166 and #493) according to siRNA design guidelines; a scrambled sequence termed 'sc' was also supplied and used for control transfections (Dharmacon Research). The PP5-siRNA working sequences were 5′>3′ AAG ACA CAG GCC AAC GAC UAC for mPP5-#166 (i.e. cDNA start sequence position 166) and AAG AUU GUG AAG CAG AAG GCC for mPP5-#493 (i.e. cDNA start sequence position 493).

2.3. Production of AtT20 cells transduced with PP5-siRNA

AtT-20 cells were plated 1×10^4 cells/well and cultured in DMEM plus 10% FCS for 2 days. At 70% confluency, cells were washed with DMEM; 400 ng of siRNA was added to each well (as appropriate) in accordance with the Lipofectamine Plus protocol (Invitrogen). After incubating the cells for 3 h at 37 °C, the cells were washed and maintained in fresh DMEM containing 10% FCS.

Preliminary studies were performed to determine the time course for PP5 protein knockdown with each siRNA design between 48 and 72 h. At the appropriate time point cells were lysed in RIPA buffer and lysate analyzed for PP5 protein content by Western blotting using PP5 antibody (Transduction Laboratory, mAb). Anti-Actin antibody (Sigma, mAb) was used to monitor protein loading to the gel. The blots were developed by ECL and relative optical density measurements performed on the film using a GS710 calibrated imaging densitometer (Molecular Dynamics). Percentage PP5 knockdown was determined relative to scrambled control (sc) values. Cell viability assays were performed utilizing the MTS method of Promega, Inc. to determine condition of the cells over time.

2.4. ACTH release experiments

At 3 days post-transfection in the presence or absence of PP5-siRNA, AtT20 cells were washed twice and replaced with serum-free DMEM. Cells were incubated for 3 h under basal conditions for studies requiring the addition of CRF or mifepristone, or 24 h in the case of dexamethasone (added 2 days post-transfection with PP5-siRNA). The cell populations were lysed and ir-ACTH amounts were measured as picograms of secreted ir-ACTH per well. All experiments were conducted with duplicate cultures. Media were collected and stored at −80 °C until titrated by RIA for immunoreactive (ir)-ACTH (MP Biomedicals). The sensitivity of the ACTH RIA was 1 pg/tube. Final reported values for secreted ir-ACTH were corrected for sample dilutions (1/100). Results were expressed as picograms of ir-peptide per well for the specified incubation time.

2.5. Electrophoresis and immunoblotting

The AtT20 cells were grown to confluence in T25 flasks and transfected with either scrambled or PP5-siRNA for 3 days as described earlier. Media was removed, cells washed twice with buffer, 0.01 M phosphate, 0.15 M NaCl, pH 7.4 (PBS), and incubated with serum-free medium for 3 h. The conditioned medium was removed and quantification of total protein of the media was determined. Samples (20 μl) were resolved on SDS–PAGE gels (12% polyacrylamide) and proteins on the gels were transfered onto a nitrocellulose membrane and analyzed by quantitative enhanced chemiluminescence (ECL) Western blot (Amersham) with antibody specific for POMC (Cat. No.: G-029-30; Phoenix Pharmaceuticals, Inc). The advantage of this detection system was that the membrane could be exposed to the film for increasing times to detect both high-concentration (short exposure) and low-concentration (long exposure) protein levels. Bands were quantified by densitometry using a GS710 calibrated imaging densitometer (Molecular Dynamics). Statistical analysis of data derived from Western blots was done using the arbitary units for the band quantitated by the scanner.

2.6. Statistical analysis

Values are reported as mean±SE and statistical differences were determined by a two-way ANOVA and a Tukey's multiple comparisons test (Graphpad Prism) or