Molecular and Cellular Endocrinology 419 (2016) 252–258

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Protein Kinase C Delta restrains growth in ACTH-secreting pituitary adenoma cells

Erica Gentilin [a, b], Carmelina Di Pasquale [a], Teresa Gagliano [a], Federico Tagliati [a], Katiuscia Benfini [a], Maria Rosaria Ambrosio [a], Marta Bondanelli [a], Ettore C. degli Uberti [a, b], Maria Chiara Zatelli [a, b, *]

[a] Section of Endocrinology & Internal Medicine, Dept. of Medical Sciences, University of Ferrara, Italy
[b] Laboratorio in rete del Tecnopolo "Tecnologie delle terapie avanzate" (LTTA) of the University of Ferrara, Italy

ARTICLE INFO

Article history:
Received 4 September 2015
Received in revised form
21 October 2015
Accepted 26 October 2015
Available online 30 October 2015

keywords:
Protein Kinase C Delta
Epithelial growth factor receptor
ACTH
Pituitary

ABSTRACT

Protein Kinase C Delta (PRKCD) has been highlighted among disrupted pathways in corticotroph adenomas. PRKCD is expressed at low level in human corticotroph adenomas and controls cell cycle in vitro. Therefore, PRKCD may play an important role in the development/progression of corticotroph adenomas, warranting further studies to understand the role of PRKCD and related pathways in restraining pituitary cell growth. We evaluated PRKCD role in influencing cell behavior in terms of cell viability, hormone expression and protein expression profile, by silencing PRKCD in AtT-20/D16v-F2 cells. PRKCD silencing increases cell viability, enhances hormone expression and induces morphological changes associated with deregulation of adhesion molecules. PRKCD silencing is associated with an increase in Epithelial Growth Factor Receptor (EGFR) expression, a marker of tumor aggressive behavior, and sensitivity to anti-EGFR molecules. PRKCD might restrain corticotroph adenoma cells from acquiring an aggressive behavior, candidating PRKCD as a possible molecular target for the treatment of corticotroph adenomas.

1. Introduction

The mechanisms underlying the pathogenesis of adrenocorticotroph hormone (ACTH) secreting pituitary adenomas is a matter of great interest, since clinical effects of uncontrolled hypercortisolism are very grim and associated with high morbidity and mortality (van Haalen et al., 2015). Pituitary surgery is always referred to as the first-line therapy, but it is burdened with a high recurrence/persistence rate (Fleseriu, 2012). Therefore, effective pharmacological treatments are still needed, and are even more necessary for patients with high surgical risk and/or acute hypercortisolism. Currently available medical treatment for Cushing's disease (CD) allows to achieve disease control in a subset of patients (Eckstein et al., 2014), representing a great therapeutic advantage. However, some are not adequately controlled, therefore current research aims at discovering new effective drugs and identifying novel mechanisms that may represent promising pharmacological targets. Along this line, the research for new putative molecular targets has become very intensive, highlighting the importance of abnormal cell signaling (Dworakowska and Grossman, 2012). Recently, PRKCD has been highlighted among the pathways that are disrupted in ACTH-secreting pituitary adenomas (Gentilin et al., 2013a). PRKCD is a serine–threonine kinase that regulates proliferation, cell cycle, differentiation and apoptosis in several cellular models (Kikkawa et al., 2002). Alterations in its activity and/or expression levels are crucial events in the development of a malignant phenotype in many tumors (Koike et al., 2006; Haughian et al., 2006; D'Costa et al., 2006; Castilla et al., 2013). Recently, we demonstrated that PRKCD is expressed at low level in human ACTH-secreting pituitary adenomas and in a murine ACTH-secreting pituitary adenoma cell line, where it mediates the powerful effects of microRNA 26a on cell cycle, down-regulating cyclin E and cyclin A expression (Gentilin et al., 2013a). These results support the hypothesis that PRKCD may play an important role in the development and progression of ACTH pituitary adenomas, and therefore prompt us to investigate the role of PRKCD and related pathways in restraining corticotroph cell growth.

The aim of this study was to evaluate the role of PRKCD in influencing corticotroph cells behavior in terms of cell viability,

* Corresponding author. Section of Endocrinology and Internal Medicine, Dept. of Medical Sciences, University of Ferrara, Via Savonarola 9, 44100 Ferrara, Italy.
E-mail address: ztlmch@unife.it (M.C. Zatelli).

http://dx.doi.org/10.1016/j.mce.2015.10.025
0303-7207/

proopiomelanocortin (POMC) expression and protein expression profile, by silencing PRKCD in a murine ACTH-secreting pituitary adenoma cell line.

2. Materials and methods

2.1. Cell culture

The mouse ACTH secreting pituitary adenoma cell line, AtT-20/D16v-F2, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell line was maintained as previously reported (Tagliati et al., 2010; Gentilin et al., 2013b).

2.2. Transfection with PRKCD shRNA

Cells were transfected with PRKCD shRNA plasmid (OriGene, Rockville, MD, USA), by using Nucleofector II (Amaxa, Gaithersburg, MD, USA) according to the manufacturer's protocols, as previously reported (Gentilin et al., 2013a). Then, stably transfected clones were selected by incubation in medium containing puromycin 2 μg/ml. The parental AtT-20/D16v-F2 cells are referred to as "wt"; scrambled oligonucleotide transfected AtT-20/D16v-F2 cells are referred to as "control cells"; PRKCD shRNA transfected AtT-20/D16v-F2 cells are referred to as "sh cells".

2.3. Cell studies

Cell viability was assessed by the ATPlite assay (PerkinElmer, Waltham, Massachusetts, USA) as previously reported (Zatelli et al., 2010). Changes in cell morphology were observed as described previously (Molè et al., 2015).

2.4. Tissue collection

Tissue samples of human ACTH secreting pituitary adenomas were collected in accordance with the guidelines of the local committee on human research. Patients provided informed consent. Primary cultures were then prepared as described previously (Zatelli et al., 2006).

2.5. Western-blot analysis

Protein isolation from frozen tissues and cell lines was performed as described previously (Gagliano et al., 2013). Total protein extracts were measured by the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Thirty μg of proteins were fractionated on SDS–PAGE and transferred by electrophoresis to nitrocellulose membranes (Schleicher & Schuell Italia SRL, Milano, Italy), as described previously (Tagliati et al., 2006). The following primary antibodies were employed: 1/200 rabbit anti-PRKCD antibody (Santa Cruz Biotechnology, Santa Cruz, California), 1/200 anti-POMC/ACTH antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:1000 anti-E cadherin antibody (Abcam, Cambridge, MA, USA), 1/1000 phospho-β-catenin (Ser33/37) antibody (Cell Signaling Technology, Danver, MA, USA), 1/1000 β-catenin (D10A9) XP rabbit mAb (Cell Signaling Technology), 1/1000 phospho-GSK-3α/β (Ser21/9) antibody (Cell Signaling Technology), 1/1000 GSK-3β (27C10) rabbit mAb (Cell Signaling Technology), 1/200 EGFR antibody (Santa Cruz Biotechnology), 1/1000 rabbit β-actin antibody (Cell Signaling Technology) and 1/1000 cyclophilin A antibody (Cell Signaling Technology). Immunoreactive bands were detected using species-specific horseradish peroxidase-conjugated secondary antibodies (Dako Italia, Milano, Italy) and visualized using the ECL Western Blotting Detection Reagents (GE Healthcare Europe GmbH, Milano, Italy). Protein bands were quantified by using ImageJ software (http://rsbweb.nih.gov/ij/).

2.6. Statistical analysis

Results are expressed as ± standard error of the mean (SEM). Shapiro–Wilk and Levene tests were employed to verify normality and homoscedasticity, respectively. One-way ANOVA with post-hoc Bonferroni t test was used to test statistical significance.

3. Results

3.1. PRKCD silencing increases AtT-20/D16v-F2 cell viability

AtT-20/D16v-F2 cells were transfected with a shPRKCD plasmid (sh cells). As shown in Fig. 1, sh cells showed lower PRKCD protein levels (~60%) as compared to control cells.

For each cell line, cell viability was monitored after 24 and 48 h (Fig. 2A). Cell lines displayed unique growth profiles over time, since sh cells doubling time is significantly shorter (26.7 h) as compared to control cells (51.8 h; http://www.doubling-time.com/compute.php). Cell viability was significantly greater in sh cells as compared to control cells both after 24 and 48 h ($p < 0.01$). These data indicate that PRKCD influences the mechanisms regulating pituitary adenoma cell viability, supporting our previous findings (Gentilin et al., 2013a). Therefore PRKCD may play a crucial role in restraining cell growth in corticotroph adenoma cells.

3.2. PRKCD silencing increases POMC and ACTH expression in AtT-20/D16v-F2 cells

POMC and ACTH expression was assayed by Western blot. As shown in Fig. 2B, sh cells showed higher protein levels of both POMC and ACTH (~2-fold and ~3-fold, respectively) as compared to control cells. These data indicate that PRKCD is involved in POMC and ACTH protein expression regulation and confirm the identity of these cells as corticotroph cells.

3.3. PRKCD silencing modifies AtT-20/D16v-F2 cell morphology

Examining the morphology of the cells in culture (Fig. 3), we observed that control cells have a regular epithelial-like shape, while sh cells have an elongated and fibroblast-like shape with several cytoplasmic extroflessions. These data indicate that PRKCD affects morphological appearance of AtT-20/D16v-F2 cells, therefore PRKCD may influence adhesion mechanisms in ACTH-secreting pituitary adenoma cells.

3.4. PRKCD silencing reduces E -cadherin protein levels of AtT-20/D16v-F2 cells

E-cadherin protein expression levels were assessed in control and sh cells (Fig. 4A). Sh cells displayed lower E-cadherin levels (~3-

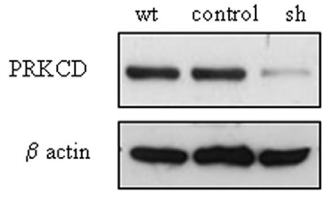

Fig. 1. PRKCD silencing. Total proteins were isolated from AtT-20/D16v-F2 (wt), scrambled oligonucleotide transfected AtT-20/D16v-F2 (control) and PRKCD silenced AtT-20/D16v-F2 (sh) cells and PRKCD protein expression was assessed by Western blot analysis. β-actin is shown as a loading control.