Cancer Letters 406 (2017) 54–63

Contents lists available at ScienceDirect

Cancer Letters

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

Original Article

Cofilin is a cAMP effector in mediating actin cytoskeleton reorganization and steroidogenesis in mouse and human adrenocortical tumor cells

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ARTICLE INFO

Article history:
Received 15 May 2017
Received in revised form
20 July 2017
Accepted 27 July 2017

Keywords:
Adrenocortical adenomas
Cofilin
Cytoskeleton
cAMP
Cortisol

ABSTRACT

cAMP pathway plays a major role in the pathogenesis of cortisol-producing adrenocortical adenomas (CPA). cAMP-induced steroidogenesis is preceded by actin cytoskeleton reorganization, a process regulated by cofilin activity.

In this study we investigated cofilin role in mediating cAMP effects on cell morphology and steroidogenesis in adrenocortical tumor cells.

We demonstrated that forskolin induced cell rounding and strongly reduced phosphorylated (P)-cofilin/total cofilin ratio in Y1 ($-52 \pm 16\%$, $p < 0.001$) and human CPA cells ($-53 \pm 18\%$, $p < 0.05$). Cofilin silencing significantly reduced both forskolin-induced morphological changes and progesterone production (1.3-fold vs 1.8-fold in controls, $p < 0.05$), whereas transfection of wild-type or S3A (active), but not S3D (inactive) cofilin, potentiated forskolin effects on cell rounding and increased 3-fold progesterone synthesis with respect to control ($p < 0.05$). Furthermore, cofilin dephosphorylation by a ROCK inhibitor potentiated forskolin-induced cell rounding and steroidogenesis (2-fold increase vs forskolin alone).

Finally, we found a reduced P-cofilin/total cofilin ratio and increased cofilin expression in CPA vs endocrine inactive adenomas by western blot and immunohistochemistry.

Overall, these results identified cofilin as a mediator of cAMP effects on both morphological changes and steroidogenesis in mouse and human adrenocortical tumor cells.

Introduction

Cortisol-producing adrenocortical adenomas (CPA) are the most common cause of ACTH-independent Cushing's syndrome (CS).

Genetic alterations involving cAMP pathway are found in a consistent proportion of CPA demonstrating a crucial role for cAMP pathway in the pathogenesis of these tumors. The first genetic alterations described were inactivating mutations of phosphodiesterase 11A (PDE11A) [1], but the more frequent ones are activating mutations of catalytic subunit of protein kinase A (PKA) [2–4].

Although the central role of cAMP as second messenger mediating the action of ACTH in adrenal cells is well recognized, the molecular mechanisms that lead to cortisol secretion remain largely unknown. cAMP/PKA activation effects include increased activity of cholesterol ester hydrolase and steroidogenic acute regulatory protein (StAR), and enhanced transcription of StAR and

Abbreviations: CPA, Cortisol-producing adrenocortical adenomas; EIA, endocrine inactive adrenocortical adenomas; CS, Cushing's syndrome; LD, lipid droplets.

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http://dx.doi.org/10.1016/j.canlet.2017.07.025
0304-3835/

enzymes involved in steroidogenesis [5,6]. The rate-limiting step and the key control point of adrenal steroidogenesis is the transport of intracellular cholesterol, stored in cytoplasmic cholesterol ester droplets, towards inner mitochondrial membrane, where steroidogenesis begins.

Experimental evidence suggests that cAMP pathway regulates this early step of cortisol biosynthesis through the modulation of cell cytoskeleton. Indeed, it is well established that cAMP/PKA pathway activation induces morphological changes accompanied by cell rounding and disassembly of actin filaments and focal adhesion in different steroidogenic cell types, including adrenal [7–11], testicular [12,13] and ovarian granulosa cells [14–16].

Moreover, different inhibitors of actin polymerization affect steroids production, and blocking cAMP-induced cell rounding also inhibit steroidogenesis in mouse Y1 adrenocortical tumor cells [7,10,17,18]. It has been proposed that cytoskeleton plays a prominent role in the delivery of cholesterol from lipid droplets (LD) to the mitochondrial membrane [19]. However, the biology that underpins these mechanisms is a matter of controversy.

To date, the molecular players that mediate the cAMP-induced cytoskeleton rearrangements, their relationship with cortisol synthesis and their possible role in the pathogenesis of CPA have not been investigated.

Actin cytoskeleton reorganization is regulated by cofilin, an actin binding protein that severs actin filaments and initiates actin-polymerization by increasing the number of actin-free barbed ends, from which actin filaments (F-actin) polymerize, and by providing actin monomers (G-actin) for polymerization. Cofilin activity is negatively regulated by Rho GTPases-induced phoshorylation at Ser3, a residue located within the cofilin actin binding domain, with a consequent loss of its ability to bind actin [20], whereas different phosphatases participate in cofilin reactivation. Our group and others have recently provided evidence suggesting that alterations in the cofilin pathway might be involved in tumor invasiveness [21–24], in agreement with the key role of cofilin in promoting cell migration. In addition, it has been shown that cofilin is required to initiate progesterone secretion by preovulatory granulosa cells [16].

Interestingly, cofilin phosphorylation is reduced by cAMP in ovarian granulosa cells [16], suggesting a link between cAMP pathway and cofilin.

In this work we investigated a possible role of cofilin in mediating the morphological changes and cortisol production induced by cAMP in an adrenocortical tumor cell line, Y1, and in primary cultured cells derived from human adrenocortical tumors. Moreover, we compared cofilin phosphorylation and expression levels in tissue samples from human CPA and endocrine inactive adrenocortical adenomas (EIA).

Materials and Methods

Mouse and human adrenocortical cell cultures

Y1 mouse adrenocortical tumor cell line (CCL-79) was purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in F12-K Nut Mix medium (Gibco, Invitrogen, Life Technologies Inc., Carlsband, CA) supplemented with 15% HS (horse serum), 2,5% FBS (fetal bovine serum), 2 mM glutamine, 100U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Invitrogen, Life Technologies Inc., Carlsband, CA) at 37 °C in humidified atmosphere of 95% air-5% CO_2.

After obtaining written informed consent, sixteen frozen tissue samples (CPA n = 8, EIA n = 8) were subjected to protein extraction, and fresh tissues (CPA n = 6) were used to obtain primary cell cultures.

The diagnosis of CPA was made in the presence of signs and/or symptoms of cortisol excess (i.e. striae rubrae, moon facies, buffalo hump and skin atrophy) and in the presence (in at least 2 out of 3 different determination) of cortisol levels after 1 mg overnight dexamethasone suppression (1 mg-DST) >5.0 μg/dL (138 nmol/L) or in the presence of ≥2 out of the following biochemical features: 1 mg-DST >3.0 μg/dL (83 nmol/L), adrenocorticotroph hormone (ACTH) levels <10 pg/mL (2.2 pmol/L), 24-h urinary cortisol levels (UFC) > 70 μg/24-h (193 nmoL/24-h). The diagnosis of EIA was made in the absence of signs and/or symptoms of cortisol excess and in the presence of no more than 1 out of the following biochemical features: 1 mg-DST >3.0 μg/dL (83 nmol/L), ACTH levels <10 pg/mL (2.2 pmol/L), UFC >70 μg/24-h (193 nmoL/24-h) [25]. In EIA patients, the surgical option was considered mandatory on the basis of the increasing dimensions (>1 cm increase during 12 months of follow up) or a size larger than 4 cm at the diagnosis. In all subjects, before the study inclusion, the determination of 24-h urinary fractionated metanephrines and aldosterone/plasma renin activity ratio excluded the presence of pheochromocytoma and aldosteronoma, respectively.

Fresh tissues were dissociated in Dulbecco's modified Eagle's medium (DMEM) containing 2 mg/mL collagenase (Sigma Aldrich, St. Louis, MO) at 37 °C for 2 h to obtain primary cell cultures. The digested tissue was passed on a 100-μm filter (nylon cell strainer, BD Transduction Laboratories, Lexington, UK) to remove undigested material. The obtained cell suspension was centrifuged (1000 rpm, 10 min). After this centrifugation step, cells were pelleted at the bottom of the tube, while adipocytes were found on the surface as a ring due to their low density. At this step, the adipocyte ring was transferred to a new tube, and discarded. Pelleted cells were cultured in DMEM (Sigma Aldrich, St. Louis, MO) supplemented with 20% FBS, 2 mM glutamine and 100U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Invitrogen, Life Technologies Inc., Carlsband, CA, USA).

Morphological analysis

Mouse Y1 cells and human adrenocortical tumor cells were treated with 10 μM forskolin for 30 min and the degree of cell rounding was estimated by counting cells displaying spherical shape in a given field, and expressing them as a percentage of the total cells in view [10,26,27]. Each field contained a minimum of 30 cells, and the observations were taken on five separate, random fields. The counting was performed by two independent operators. Y1 cells, transfected with wild type, S3A, S3D cofilin, or empty vector or silenced for cofilin were stimulated for 3 h with 10 μM forskolin, since time course experiments showed no effects of cell rounding before, and then analysed as described above.

For actin staining, Y1 cells and adrenocortical tumor cells were fixed with 4% paraformaldehyde for 10 min and stained with Alexa Fluor 555 conjugated-phalloidin (Invitrogen, Life Technologies Inc., Carlsbad, CA) for 20 min at room temperature. After wash with PBS, cells were mounted on glass slides with ProLong Diamond Antifade mounting medium (Life Technologies, Carlsbad, CA) and analysed by both fluorescence microscopy (Axio Vert.A1, Zeiss) and TCS SP2 laser scanning confocal microscope with a HeNe 543 nm laser and a 63 × objective (HCX PL APO 63X/1.4–0.60 OIL) (Leica Microsystem, Deerfield, IL).

Western blot analysis

Western blot analysis was carried out on total proteins extracted from Y1 cells, human primary cultured cells or adrenocortical tumor tissues. Cells were stimulated with 10 μM forskolin and/or 2.5 μM Y27632 dihydrochloride ROCK inhibitor at different time points (10, 30, 60, 120 min). Proteins were quantified by BCA, separated on SDS/polyacylamide gel and transferred to a nitrocellulose filter. To detect phosphorylated cofilin, an anti-phospho-cofilin primary antibody (1:1000, Cell signalling, Danvers, MA) and an anti-rabbit peroxidase-linked secondary antibody were used. The presence of total cofilin was analyzed by stripping and reprobing with an anti-cofilin antibody (1:1000, Cell signalling, Danvers, MA). GAPDH was used as housekeeping (1:4000, Ambion, Life Technologies Inc., Carlsband, CA). Chemiluminescence was detected using the Chemidoc-IT Imaging System (UVP, Upland, CA) and densitometrical analysis was performed with NIH ImageJ software.

Cofilin silencing and transfection

Small interfering RNAs (siRNAs, SMARTpool) for murine cofilin were synthesized by Dharmacon (Chicago, IL). Y1 cells were transfected with Dharmafect reagent (Dharmacon, Chicago, IL), according to the manufacturer's instruction, for 72 h prior to perform experimental treatments. In order to obtain the best efficiency of cofilin silencing, four different Dharmafect reagents were tested. Preliminary experiments to determine the optimal concentration of siRNAs and the kinetics of silencing of cofilin were performed. A negative control siRNA, a non-targeting sequence without significant homology to the sequence of mouse, human, or rat transcripts, was used in each experiment.

Transient transfection of expression vectors containing GFP-tagged wild-type or mutated cofilin (phosphodeficient S3A and phosphomimetic S3D) [21] was performed in Y1 cells using Lipofectamine 3000 reagent (Invitrogen, Carlsband, CA) according to the instruction of the manufacturer. Transfection efficiency was evaluated by fluorescence microscopy in each experiment. Only experiments with a transfection efficiency >50% were accepted. Empty vector was used in each experiment as control. Cells were transfected for 72 h prior to perform experimental treatments.

Steroidogenesis assay

For progesterone determination, transfected and silenced Y1 adrenocortical tumor cells were incubated for 24 h with forskolin 10 μM. To test the effects of Y27632, cells were treated with 10 μM forskolin with or without 2.5 μM Y27632 for