Cancer Letters xxx (2017) 1–10

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

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3 h. Progesterone concentration in cell media was measured by Progesterone EIA Kit (Cayman Chemical, Michigan USA) according to the manufacture's instruction.

For cortisol determination, human primary adrenocortical tumor cells were treated with 10 μM forskolin with or without 2.5 μM Y27632 for 18 h and then analysed by a specific immunoassay (Cortisol II, Cobas, Roche, Basel, Switzerland).

A pre-incubation with Y27632 for 30 min was performed prior to Y27632 incubation.

Oil Red O staining and quantification assay

Oil Red O staining was performed as previously described [28]. Briefly, cells were washed with PBS, fixed by 10% formalin in PBS for 10 min at room temperature, washed with PBS, and then stained with 60% filtered Oil Red O stock solution (0.35 g Oil Red O (Sigma-Aldrich, Milan, Italy) in 100 ml isopropanol) for 20 min, washed three times with water and then analyzed under a microscope (Axio Vert.A1, Zeiss). Area of LD was measured by ISCapture software. At least 100 LD were analysed for each condition.

Immunohistochemistry

Representative 4 μm-thick formalin-fixed paraffin embedded (FFPE) adrenocortical tumor sections (CPA n = 10, EIA n = 15) were stained with antibodies against cofilin and phospho-cofilin (Ser3) (1:1000 and 1:50 respectively, both from Cell Signaling, Danvers, MA) along with positive and negative controls. Human adrenocortical tumors primary cells were fixed by 10% formalin in PBS for 10 min at room temperature, washed with PBS, and then stained with S100 antibody (prediluted from Ventana Medical Systems, Inc., Roche Group, AZ, USA).

Reactions were revealed using the ultraView Universal DAB (Ventana Medical Systems Inc), as previously described [29]. Each stained slide was digitalized with 200X original magnification [30] and analyzed independently by two pathologists (NF and SF) using a semi-quantitative system. Discordant results were reviewed over a high-resolution monitor during a consensus meeting.

Statistical analysis

The results were expressed as the mean ± standard deviation (SD). A paired two-tailed Student's *t*-test was used to detect the significance between two series of data. One-way ANOVA by Dunnett's test was used to determine the statistical differences between treatments and the control group in multiple comparison.

CPA vs EIA densitometric data were analysed by the non-parametric Mann-Whitney *U* test. In all analyses, a probability value $p < 0.05$ was accepted as statistically significant. Biostatistics data were analysed using GraphPad Prism 5.0 software.

Results

Forskolin induced morphological changes and cofilin dephosphorylation (activation) in Y1 cells and primary adrenocortical tumor cells

Y1 mouse adrenocortical tumor cells are flat, adherent cells with polyhedral shape in basal culture condition. Forskolin treatment induced cell rounding, with some rounded cells displaying long and thin protrusions extending from the cell body (Fig. 1A). The percentage of rounded cells after 30 min of 10 μM forskolin was 74.2 ± 3.2% vs 11.8 ± 0.4% in control untreated cells ($p < 0.001$). In basal condition, Y1 cell shape was maintained by a network of stress fibers and focal adhesions, as shown by actin cytoskeleton staining, whereas forskolin incubation induced the loss of actin fibers and a redistribution of actin staining at cell periphery (Fig. 1A).

Similar effects on cell morphology and actin cytoskeleton were also observed in primary cortisol-secreting adrenocortical tumor cells (Fig. 1B). Primary cell cultures were characterized by staining with Oil Red O, that revealed the presence of intracellular LD with the size and distribution typical of the steroidogenic cells (recently reviewed in Ref. [31]) (Fig. 5C). The absence of a contamination of adipose precursors was confirmed by the absence of staining for marker of preadipocytes S100 [32] (data not shown) (see Fig. 6).

Time course experiments showed that 18 h incubation with forskolin led to a significant increase of the percentage of rounded cells (73.7 ± 11% *vs* 49.6 ± 17% in control untreated cells, $p < 0.05$). It is worth noting that in basal condition the percentage of rounded cells was higher compared to what observed in Y1 cells.

To investigate the mechanism by which cAMP induces actin cytoskeleton reorganization, we measured the effects of forskolin on the phosphorylation of cofilin by western blot. Forskolin incubation strongly reduced cofilin phosphorylation in Y1 cells, starting from 10 min, with a maximum effect after 30 min (52 ± 16% reduction of P-cofilin/total cofilin ratio, $p < 0.001$ *vs* basal), maintained at 60 min, and reverted at 120 min (Fig. 1C). A significant reduction of P-cofilin/total cofilin ratio was also observed in primary cultured secreting adrenocortical tumor cells (−53 ± 18% *vs* basal, $p < 0.05$) (Fig. 1D).

Cofilin silencing in Y1 cells reduced forskolin effects on both cell morphology and progesterone production

Since cofilin is a key protein in regulating the actin dynamics and we found that it is positively regulated by cAMP, we hypothesized that it might play a role in the cAMP-induced actin reorganization in Y1 cells. To test this hypothesis, we knocked-down cofilin expression in Y1 cells by genetic silencing.

Transient transfection of Y1 cells with siRNAs specific for cofilin resulted in a reduction of 80% of cofilin expression with respect to negative control siRNAs transfected cells (Fig. 2A). Cofilin silencing did not alter basal cell morphology, but forskolin effects on cell rounding were strongly impaired in cofilin silenced cells *vs* control cells (37 ± 8% rounded cells in cofilin silenced cells vs 63 ± 7% in control cells, $p < 0.05$) (Fig. 2B).

We then investigated a possible effect of cofilin silencing on Y1 steroidogenesis by measuring progesterone secretion, since Y1 cells do not produce corticosterone [9,33]. Forskolin increased the progesterone amount 1.8-fold over basal in negative control cells culture media, and 1.3-fold in cells lacking cofilin ($p < 0.05$) (Fig. 2C), suggesting that cofilin silencing significantly impaired the ability of forskolin to stimulate progesterone production. A slight but not statistically significant reduction of the basal progesterone production was also observed in cofilin silenced cells (Fig. 2C).

Overexpression of wild type or phospho-deficient S3A mutant cofilin, but not S3D cofilin, potentiated forskolin effects of on both cell morphology and progesterone production in Y1 cells

We then tested the effects of cofilin overexpression and the role of cofilin phosphorylation by transfecting Y1 cells with a phospho-deficient mutant cofilin in which Ser3 is substituted with Ala (S3A cofilin), a phospho-mimicking, inactive mutant of cofilin, namely S3D, or wild type cofilin.

Forskolin-induced cell rounding was enhanced in wild type or phospho-deficient S3A cofilin expressing cells (80 ± 6% and 85 ± 14% rounded cells, respectively) vs control cells transfected with empty vector (69 ± 6% rounded cells, $p < 0.05$), but not in phospho-mimicking S3D cofilin transfected cells (60 ± 3% rounded cells) (Fig. 3A). No changes in Y1 cell morphology were observed in the transfected cells under basal condition (Fig. 3A).

As expected, transfection of wild type or S3A cofilin, but not inactive S3D cofilin, strongly increased the ability of forskolin to induce steroidogenesis compared to control cells (2.7 and 2.8-fold increase vs control cells, $p < 0.05$) (Fig. 3B).

Y27632 reduced cofilin phosphorylation and potentiated forskolin effects on both cell morphology and steroidogenesis in Y1 cells and primary cultured adrenocortical tumor cells

We tested whether alterations in cofilin phosphorylation status, without modification of cofilin expression levels, were able to affect the cellular responses to cAMP pathway activation. Since to date no pharmacological inhibitors of phosphatases that dephosphorylate

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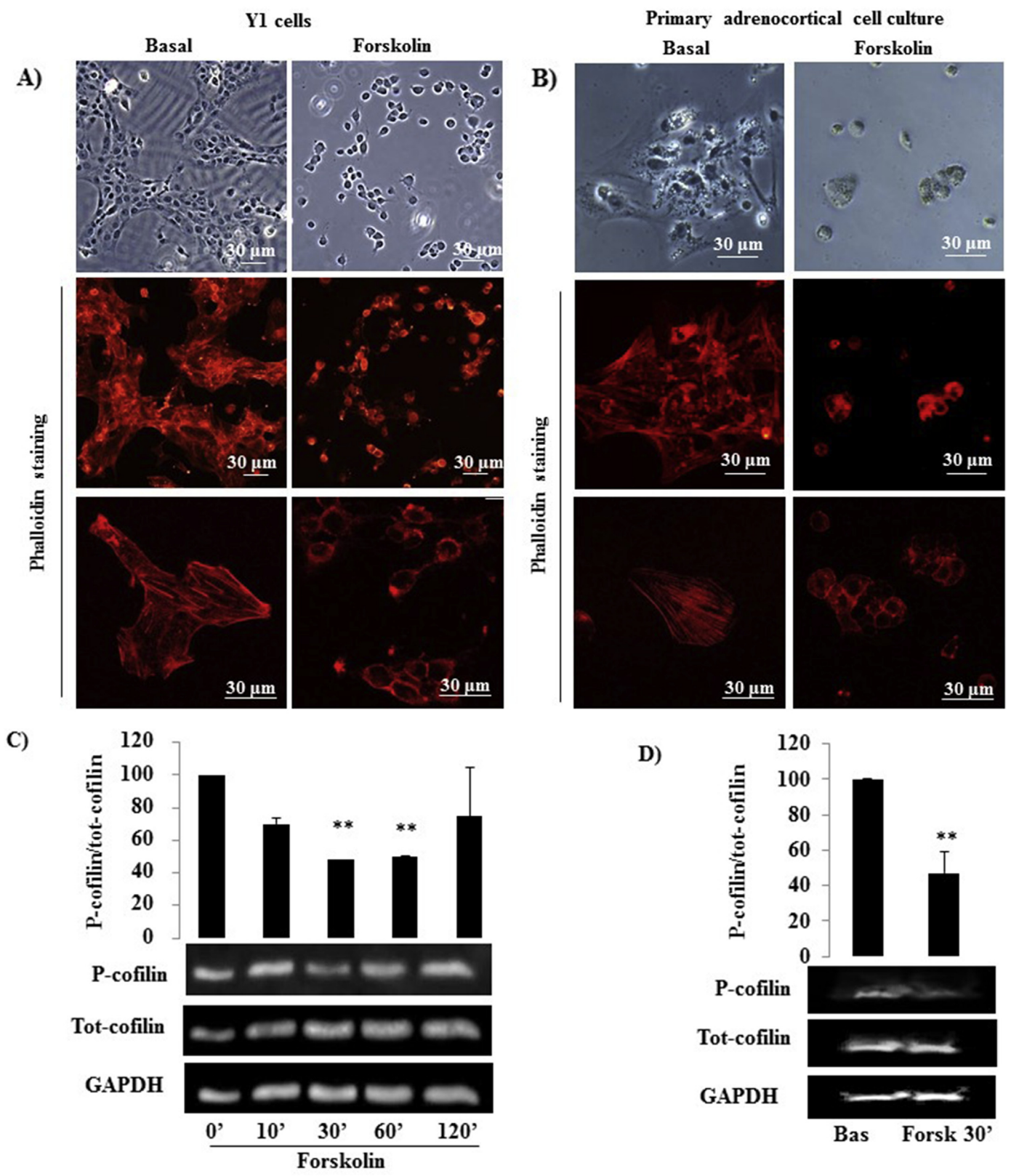

Fig. 1. Forskolin treatment induced morphological changes and cofilin dephosphorylation in Y1 cells and primary adrenocortical tumor cells. A) Y1 cells were treated for 30 min with 10 µM forskolin and actin cytoskeleton was stained with phalloidin (red). Representative pictures show bright field (phase-contrast microscopy, 20X, upper panel), fluorescence (20X, middle panel) and confocal (63X, lower panel) images. B) Primary cortisol-secreting adrenocortical tumor cells were treated for 18 h with 10 µM forskolin. Actin cytoskeleton was stained with phalloidin (red). Representative pictures show bright field images (upper panel) and fluorescence images (lower panel). C&D) Forskolin reduced cofilin phosphorylation in Y1 cells (C) and primary adrenocortical tumor cells (D). Cells were incubated for the indicated time with 10 µM forskolin. The graphs show the quantification of P-cofilin/tot cofilin normalized *vs* respective basal (mean value ± SD from 4 independent experiments in Y1 cells and from 3 primary cultures of 3 different adrenocortical tumors, ** = $p < 0.01$ vs basal). GAPDH was used as housekeeping. The figure shows representative immunoblots performed with phospho-cofilin, total cofilin and GAPDH antibodies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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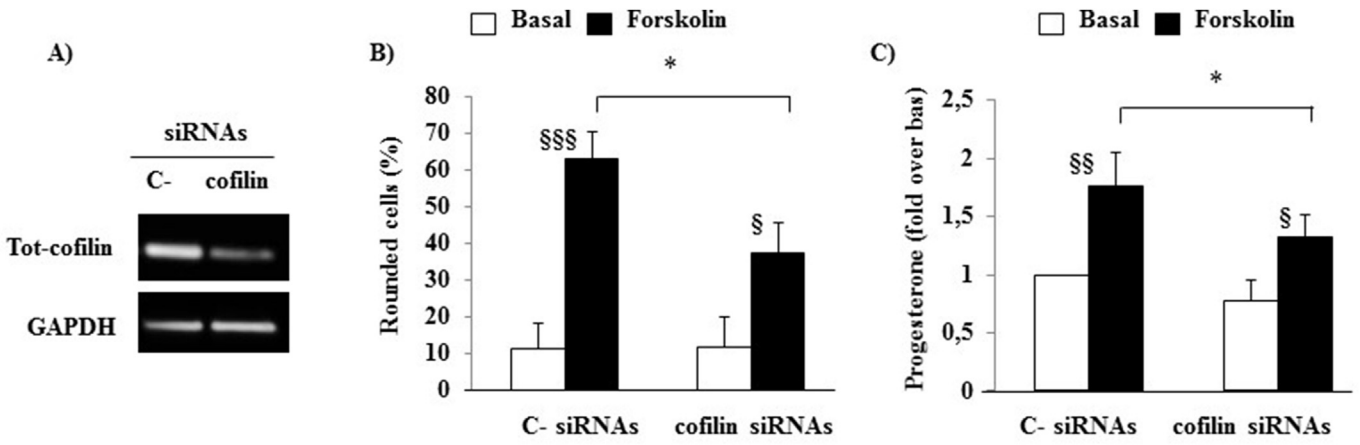

Fig. 2. Cofilin silencing in Y1 cells reduced forskolin effects on both cell morphology and progesterone synthesis. A) Y1 cells were transfected for 72 h with negative control siRNAs (C) or siRNAs specific for mouse cofilin. The figure shows representative immunoblot performed with total cofilin and GAPDH antibodies. B) After 72 h of transfection with negative control (C) or cofilin siRNAs, Y1 cells were treated for 3 h with 10 μM forskolin. Cell rounding was expressed as the percentage of round shaped cells (mean value ± SD from 3 independent experiments, * = $p < 0.05$ vs negative control cells, § = $p < 0.05$ vs bas, §§§ = $p < 0.001$ vs bas). C) Y1 cells were transfected for 72 h with negative control (C) or cofilin siRNAs, treated with 10 μM forskolin for 3 h and progesterone secretion was measured in cell media. The graph shows mean value normalized over the basal of negative control cells ±SD from 4 independent experiments. Each determination was done at least in triplicate. * = $p < 0.05$ vs negative control cells, § = $p < 0.05$ vs bas, §§ = $p < 0.01$ vs bas).

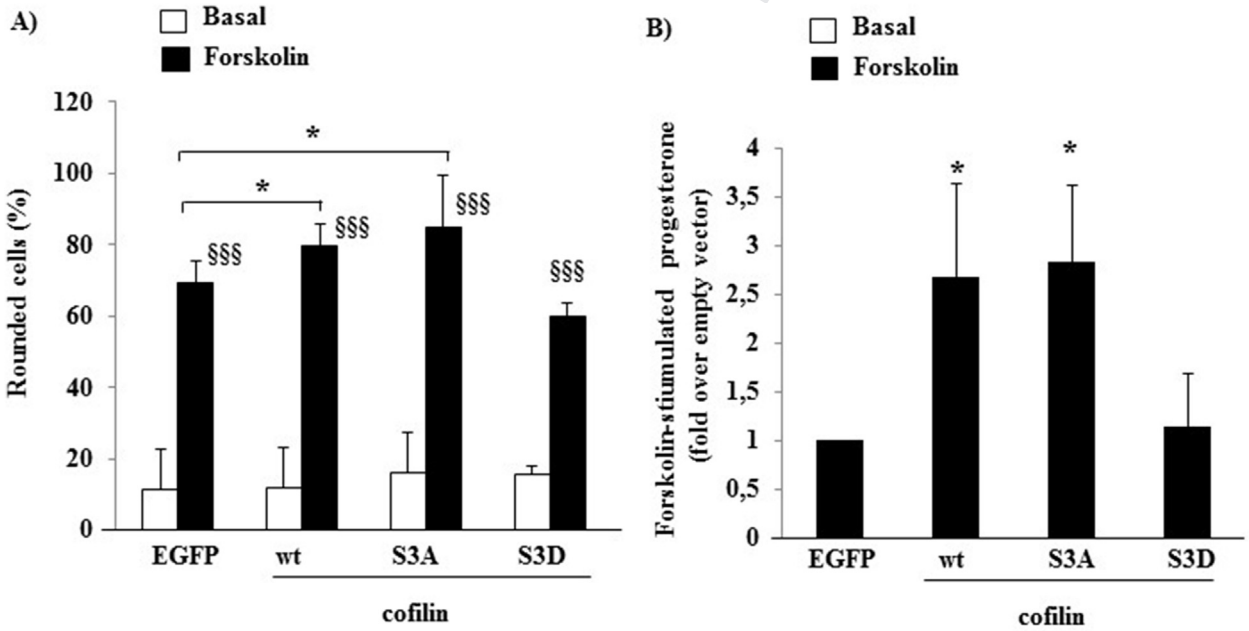

Fig. 3. Effects of transfection with wild type, phosphodeficient S3A or phosphomimicking S3D mutant cofilin on cell morphology and progesterone production in Y1 cells. **A)** After 72 h of transfection with the indicated constructs, Y1 cells were treated for 3 h with 10 μM forskolin. Cell rounding was expressed as the percentage of rounded cells (mean value ± SD from 3 independent experiments, * = $p < 0.05$ vs empty vector (EGFP) transfected cells, §§§ = $p < 0.001$ vs corresponding bas). B) Y1 cells were transfected for 72 h with the indicated constructs, treated with 10 μM forskolin for 3 h and progesterone secretion in cell media was measured. The graph shows the mean value normalized over the forskolin treated empty vector control cells ±SD from 3 independent experiments. Each determination was done at least in triplicate. * = $p < 0.05$ vs forskolin treated control cells.

cofilin are available, we evaluated the effects of blocking cofilin phosphorylation. Y27632 is a cell-permeable, highly potent and selective inhibitor of ROCK [21,34] a kinase that promotes cofilin phosphorylation. Time course experiments in Y1 cells showed that 2.5 μM Y27632 induced a reduction of cofilin phosphorylation comparable to that obtained with forskolin (59.45 ± 4% reduction after 30 min incubation, $p < 0.01$) (Fig. 4A), a result replicated in primary adrenocortical tumoral cells (Fig. 4B). Moreover, Y27632 exerted a synergistic effect together with forskolin in reducing cofilin phosphorylation (−83 ± 16% vs bas, $p < 0.05$, Fig. 4B).

Y27632 alone did not induce any detectable change in cell morphology, but if used in combination with forskolin it acted synergistically in promoting cell rounding both in Y1 cells (90.9 ± 2.2% rounded cells *vs* 74.2 ± 3.2% with forskolin alone, $p < 0.01$) (Fig. 4C) and in human adrenocortical tumor cells (90 ± 6% rounded cells vs 73.7 ± 11% with forskolin alone, $p < 0.05$) (Fig. 4D).

Accordingly, with the synergistic effect observed on cell morphology, incubation with Y27632 strongly potentiated forskolin effects on steroidogenesis. Indeed, in Y1 cells progesterone secretion was increased by 4-fold over basal with forskolin alone and 7.9-fold over basal in cells coincubated with forskolin and Y27632 (Fig. 4E). In primary cultured cortisol-secreting adrenocortical tumor cells we found that forskolin-stimulated cortisol production was strongly increased in the presence of Y27632 (3.3-fold over basal in cells incubated with forskolin+Y27632 *vs* 1.9-fold in cells incubated with forskolin alone; Fig. 4F).

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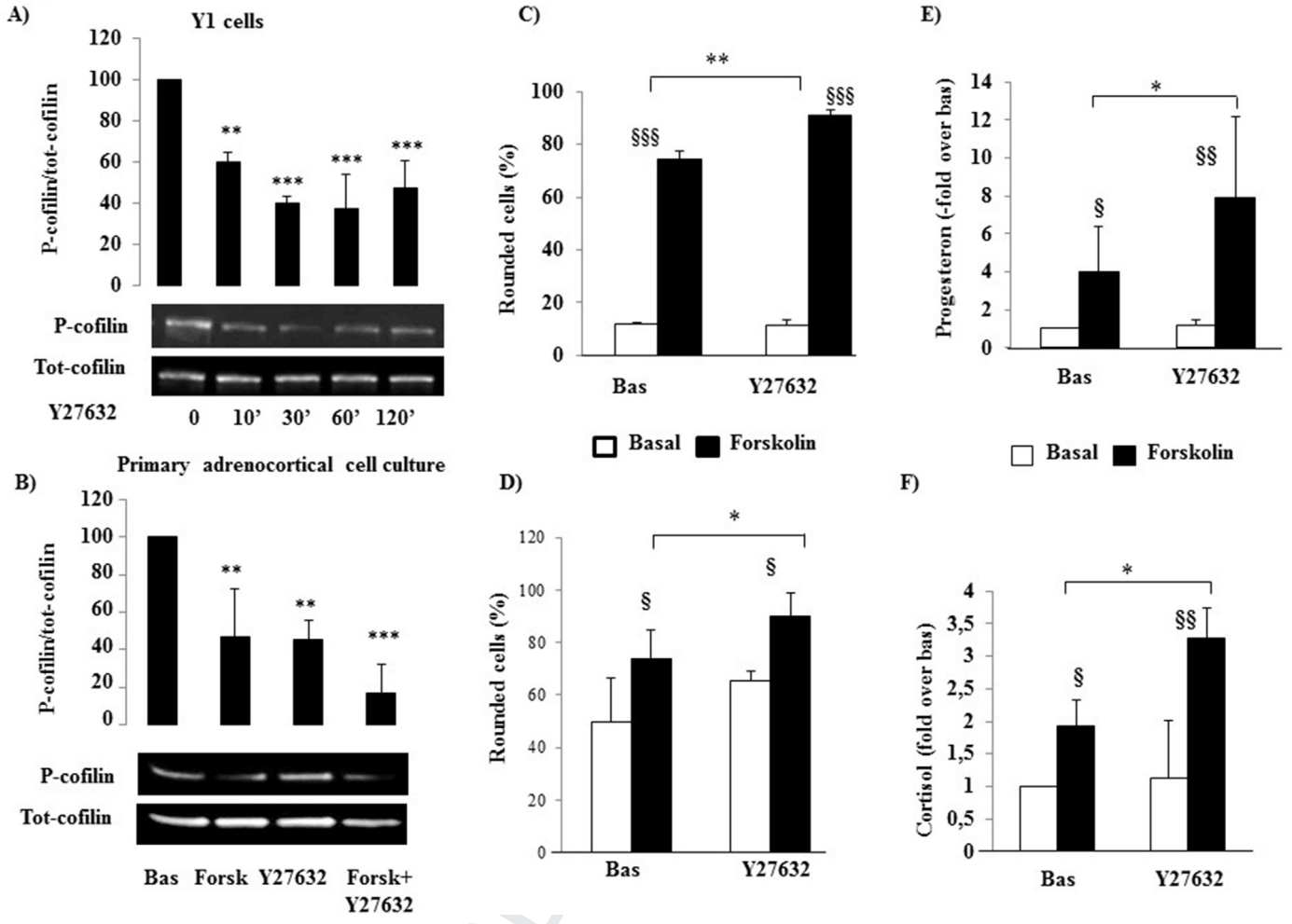

Fig. 4. A&B) ROCK inhibitor Y27632 reduced cofilin phosphorylation in Y1 cells (A) and primary cultured adrenocortical tumor cells (B). Cells were incubated for the indicated time with 2.5 μM Y27632 with or without 10 μM forskolin. Primary adrenocortical tumor cells were incubated for 30min. The graphs show the quantification of P-cofilin/tot cofilin normalized *vs* basal (mean value ± SD from 3 independent experiments in Y1 cells and from 3 adrenocortical tumors, ** = $p < 0.01$ vs basal, *** = $p < 0.001$ vs basal). The figures shows representative immunoblots performed with phospho-cofilin and total cofilin antibodies. C&D) Y27632 potentiated forskolin effects on cell morphology of Y1 cells and primary cultured adrenocortical tumor cells. Y1 cells were preincubated for 30 min with Y27632 as indicated and treated with 2.5 μM Y27632 and 10 μM forskolin for 30 min. Cell rounding was expressed as the percentage of round shaped cells (mean value ± SD from 3 independent experiments, * = $p < 0.05$ ** = $p < 0.01$ vs control cells incubated with forskolin but not Y27632, § = $p < 0.05$ §§§ = $p < 0.001$ vs respective bas). E&F) Y27632 potentiated forskolin effects on steroidogenesis in Y1 cells and primary cultured adrenocortical tumor cells. E) Y1 cells were treated with Y27632 and forskolin as described in Materials and Methods for 3 h and progesterone amount in cell media was measured. The graph shows the mean value normalized over basal ±SD from 5 independent experiments. Each determination was done at least in triplicate. * = $p < 0.05$ vs forskolin alone treated cells, § = $p < 0.05$ vs bas, §§ = $p < 0.01$ vs bas. F) Primary cultured adrenocortical tumor cells were preincubated 30 min with 2.5 μM Y27632 and treated with 2.5 μM Y27632 and 10 μM forskolin for 18 h. Cortisol in cell media was measured by immunoassay. The graph shows the mean value normalized over basal ±SD from 3 independent experiments. Each determination was done at least in triplicate. * = $p < 0.05$ vs forskolin alone, § = $p < 0.05$ vs respective bas, §§ = $p < 0.01$ vs respective bas.

No differences in basal progesterone or cortisol secretion were observed in Y27632 treated cells.

Y27632 potentiated forskolin effects on LD reduction in Y1 cells and primary cultured adrenocortical tumor cells

To investigate a potential effect of cofilin in the delivery of cholesterol from LD to the mitochondrial membrane, we measured Oil Red O staining variations in Y1 cells and human primary cultures of adrenocortical tumors after incubation with forskolin with or without Y27632. We found that in both cell models forskolin induced a significant reduction of LD area (−55% in Y1 cells and −64.6% in primary cells) that is further reduced by cotreatment with Y27632 (−65.1% in Y1 cells and −77.9% in primary cells) (Fig. 5A–C).

Human CPA show increased cofilin expression and reduced P-cofilin/total cofilin ratio in comparison to EIA

Finally, we tested a possible correlation with cofilin phosphorylation status in human tissues of CPA or EIA. Western blot analysis of the expression of P-cofilin and total cofilin in CPA (n = 8) and EIA (n = 8) showed that P-cofilin/total cofilin ratio was higher in EIA vs CPA (mean P-cofilin/total cofilin ratio 2.44 and 0.76 respectively, $p < 0.05$) (Fig. 5). Moreover, total cofilin/GAPDH was lower in EIA vs CPA (mean total cofilin/GAPDH ratio 0.54 and 1.20, respectively, $p < 0.01$).

In agreement, immunohistochemistry revealed that CPA express higher levels of cofilin than EIA (mean percentage of positive cells 67.8 ± 16.7% and 40.5 ± 30.7%, respectively, $p < 0.05$, Fig. 5B),

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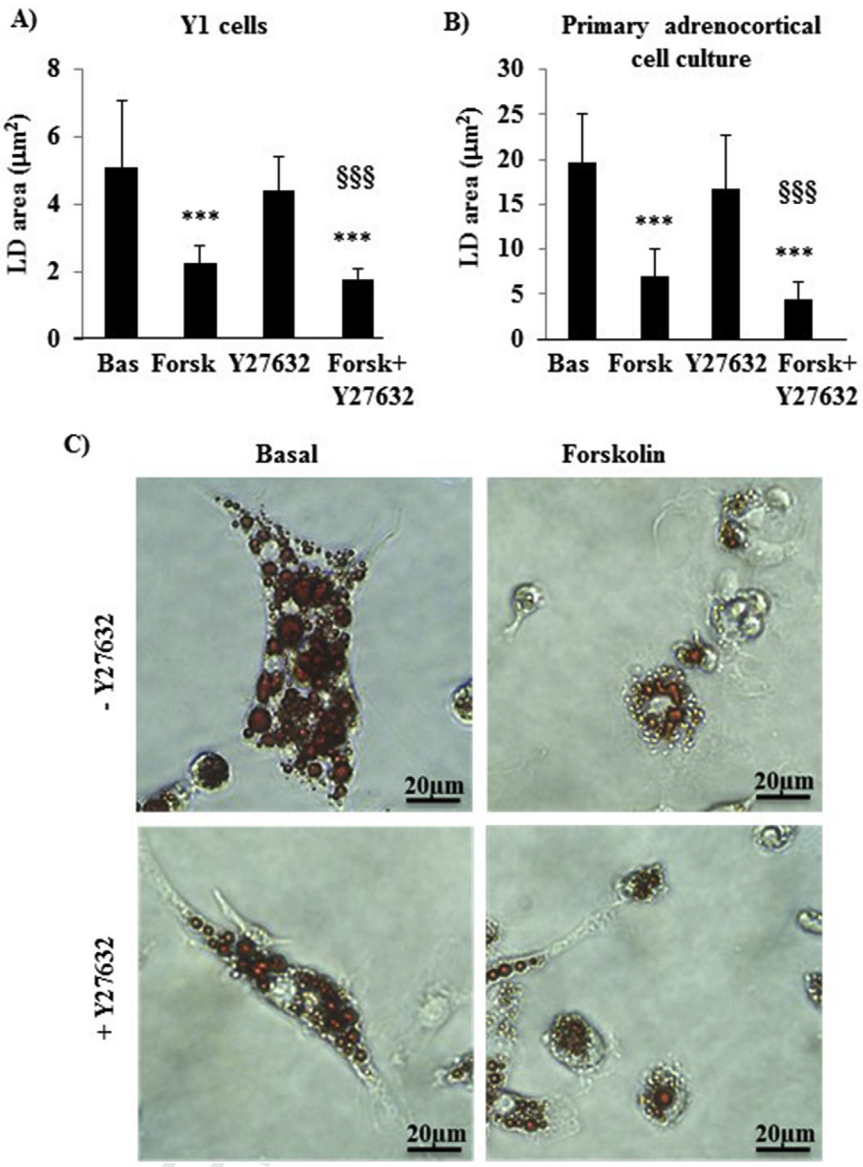

Fig. 5. Effects of forskolin and Y27632 on LD size. The graphs show LD area (mean value ± SD) in Y1 cells (A) and human primary adrenocortical cells (B). Cells were incubated with 10 µM forskolin with or without 2.5 µM Y27632 for 18 h and stained with Oil Red O. Area of LD was measured by ISCapture software. At least 100 LD were analysed for each condition. *** = $p < 0.001$ vs basal, §§§ = $p < 0.001$ vs forskolin alone. C) Representative pictures of human primary adrenocortical tumor cells treated as indicated for 18 h and stained with Oil Red O. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

whereas a low percentage of P-cofilin positive cells was found in both groups.

Overall, the increased cofilin expression and reduced cofilin phosphorylation observed in CPA suggest that these tumors have an increased activity of cofilin in respect to EIA.

Discussion

Despite the recognized leading role of the cAMP pathway in CPA pathogenesis, the molecular mechanisms that lead to cortisol secretion are poorly understood. The hypothesis of an active contribution of cell cytoskeleton in the early steps of steroidogenesis has been strengthened by the widely recognized morphological changes induced by cAMP, the most evident of which is cell rounding that precedes steroidogenesis in different steroidogenic cell types, although a clear causal correlation between these events has yet to be proven.

To the best of our knowledge, this is the first work that provides strong evidences to suggest that cofilin is a protein that mediate cAMP effects on both actin cytoskeleton reorganization and steroidogenesis in mouse and human adrenocortical tumor cells.

In agreement with literature data, forskolin treatment of Y1 cells induced the characteristic cell rounding, accompanied by actin cytoskeleton rearrangements. We first replicated this action on human primary cultured cells derived from adrenocortical lesions. This morphological effect was associated with a transient reduction of cofilin phosphorylation in both cell models. A cofilin phosphorylation inhibition mediated by cAMP/PKA activation was previously described in preovulatory granulosa cells [16], whereas a cAMP-induced increase of cofilin phosphorylation was observed in other cell types (mouse sperm and mouse embryonic fibroblasts) [35,36], suggesting that the inhibitory pathway may be peculiar of steroidogenic cell type.

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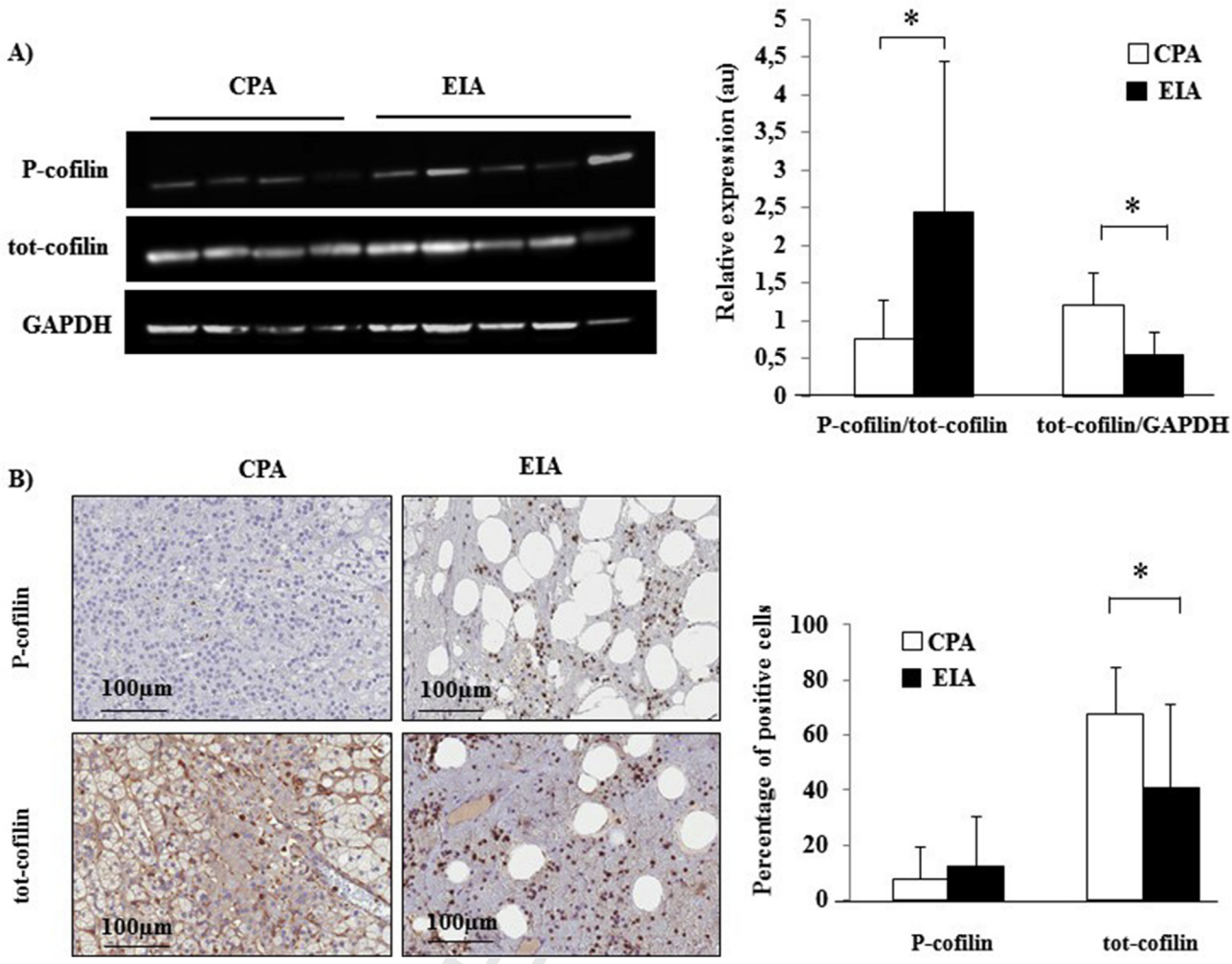

Fig. 6. Human CPA showed increased cofilin expression and reduced P-cofilin/total cofilin ratio with respect to EIA. A) Representative immunoblot performed with phospho-cofilin, total cofilin and GAPDH antibodies in tumor tissues (4 CPA and 5 EIA). The graph show the quantification of P-cofilin/tot cofilin and total cofilin/GAPDH in 8 CPA and 8 EIA (mean value ± SD, * = $p < 0.05$). B) Immunohistochemistry representative pictures showing P-cofilin and total cofilin staining in one CPA and one EIA tissue (20X magnification). The graph shows percentage of P-cofilin and cofilin positive cells (mean ± SD, 10 CPA and 15 EIA, *$p < 0.05$).

To test a possible causal role of cofilin dephosphorylation in mediating forskolin effects we used three different approaches, with consistent results: 1) genetic silencing of cofilin; 2) transient transfection of wild type or mutated S3A and S3D cofilin; 3) pharmacological inhibition of cofilin phosphorylation.

The morphological effects of cAMP, evaluated as percentage of rounded cells, were impaired in cofilin silenced cells and increased in wild type or phospho-deficient cofilin expressing cells or in cells treated with Y27632, that potentiates forskolin effects by reducing cofilin phosphorylation.

The same results observed on cell rounding after cofilin manipulations were reproduced on steroid synthesis, suggesting the involvement of a common intracellular molecular mechanism.

It should be noted that cofilin silencing efficiency reached about 80%, with the remaining 20% of endogenous cofilin that might explain the only partial suppression of forskolin induced cell rounding and progesterone production.

The actions of cofilin on actin cytoskeleton reorganization depend on its ability to bind actin, which in turn is regulated by cofilin phosphorylation at Ser3 induced by Rho/ROCK pathway. In agreement with these notions, we demonstrated that the overexpression of wild-type or S3A cofilin by transient transfection in Y1 cells potentiated forskolin effects, due to an increased cofilin activity. On the contrary, the overexpression of S3D cofilin did not exert any effect on forskolin actions, probably because it does not function as a dominant negative mutant, being not competitive with endogenous dephosphorylated cofilin for the binding of actin, as previously observed [21].

Accordingly, we found that altering only cofilin phosphorylation status without affecting the expression level of total cofilin, by using a specific inhibitor of ROCK [21,34], potentiated the cellular responses to the cAMP pathway activation. It would be of great interest to test a possible impairment of cAMP actions after stimulation with pharmacological inhibitors of phosphatases that dephosphorylate cofilin, but unfortunately these compounds are not available to date. Y27632 acted synergistically with forskolin in promoting cell rounding and steroidogenesis in both Y1 cells and in human adrenocortical tumor cells.

Since none of the cofilin manipulations itself induced any effect on basal cell morphology nor on basal steroidogenesis without forskolin co-incubation, we can hypothesize that cofilin activity is necessary to mediate cAMP effects but is not sufficient to reproduce cAMP actions, that involve a complex cascade of activation of different molecular components.

Although we could test only few primary cultures of human adrenocortical tumors, and silencing or transfection experiments could not be performed due to technical limitation regarding the number of viable cells that can be obtained from each tumor, this

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work clearly demonstrated that the data obtained in Y1 cell model are well replicated in human cultures.

To investigate how the cAMP-induced cofilin activation increases steroidogenesis we tested the effects of cAMP increase and cofilin activation on intracellular cholesterol accumulation in LD. Interestingly, Oil Red O staining for intracellular lipid accumulation was decreased after foskolin treatment, with a synergistic effects of Y27632, suggesting an increased mobilization of cholesterol from its storage in LD. Our data are in agreement with previous observation supporting a role for cytoskeleton in the delivery of cholesterol from LD to the mitochondrial membrane. Indeed, mitochondrial cholesterol content in adrenal tissues was lower in the cytoskeleton protein vimentin null mice, characterized by a defective steroidogenesis [37]. Moreover, phosphotyrosin phosphatase inhibitors, that block steroidogenesis in Y1 cells, have no effect on steroid production from 22(R)-hydroxycholesterol, the hydrosoluble cholesterol derivative that can freely cross the mitochondrial membranes [10]. A possible role for actin in regulating lipid transport is also supported by the beta-actin association with intracellular LD observed in rat adrenocortical cells [38].

The strong impact of cofilin activity on cortisol production was supported by the analysis of cofilin expression and phosphorylation status in CPA *vs* EIA. Western blot analysis demonstrated a significant increase of total cofilin expression levels and a reduction of P-cofilin/total cofilin ratio in CPA *vs* EIA, accordingly with the proposed role of active, dephosphorylated cofilin as mediator of cAMP-induced cortisol overproduction in adrenocortical adenomas. It should be noted that the reduction of cofilin phosphorylation may be due to the observed increase of the expression of total cofilin in CPA *vs* EIA without a proportional increase of expression/activity of the kinases that phosphorylate cofilin. Immunohistochemistry analysis confirmed a significant increase in cofilin expression in CPA *vs* , further supporting the hypothesis of high levels of cofilin activity in CPA with respect to EIA.

The reduced phosphorylation of cofilin in CPA is consistent with the constitutive activation of cAMP pathway in this type of tumor, mainly due to genetic alterations involving the catalytic subunit of PKA. On the contrary, EIA have been associated with genetic alterations involving Wnt/β-catenin signaling pathway [39]. Since cofilin promoter is predicted to contain a cAMP-response element (CRE) [40], also the high levels of cofilin expression might be explained by the constitutive cAMP pathway activation in CPA, but further studies are required to test this hypothesis.

Since the available inhibitors of adrenal steroidogenesis that may control hypercortisolism in CS have variable efficacy and significant side effects, to date the management of CS remains challenging [41]. Dissecting the molecular mechanism involved in cortisol synthesis might hopefully open the way to the discovery of new molecular targets.

Conflicts of interest

None.

Acknowledgements

This work was supported by E-RARE (European Research Projects on Rare Diseases) grant to A.S. [GOSAMPAC], AIRC (Associazione Italiana Ricerca Cancro) grant to G.M. [IG 2014-15507] and Ricerca Corrente Funds from the Italian Ministry of Health.

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