

PCP4: a regulator of aldosterone synthesis in human adrenocortical tissues

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Abstract

Purkinje cell protein 4 (PCP4) is a calmodulin (CaM)-binding protein that accelerates calcium association and dissociation with CaM. It has been previously detected in aldosterone-producing adenomas (APA), but details on its expression and function in adrenocortical tissues have remained unknown. Therefore, we performed the immunohistochemical analysis of PCP4 in the following tissues: normal adrenal (NA; $n=15$), APA ($n=15$), cortisol-producing adenomas ($n=15$), and idiopathic hyperaldosteronism cases (IHA; $n=5$). APA samples ($n=45$) were also submitted to quantitative RT-PCR of PCP4, CYP11B1, and CYP11B2, as well as DNA sequencing for *KCNJ5* mutations. Transient transfection analysis using PCP4 siRNA was also performed in H295R adrenocortical carcinoma cells, following ELISA analysis, and CYP11B2 luciferase assays were also performed after PCP4 vector transfection in order to study the regulation of PCP4 protein expression. In our findings, PCP4 immunoreactivity was predominantly detected in APA and in the zona glomerulosa of NA and IHA. In APA, the mRNA levels of *PCP4* were significantly correlated with those of CYP11B2 ($P<0.0001$) and were significantly higher in cases with *KCNJ5* mutation than WT ($P=0.005$). Following PCP4 vector transfection, CYP11B2 luciferase reporter activity was significantly higher than controls in the presence of angiotensin-II. Knockdown of PCP4 resulted in a significant decrease in *CYP11B2* mRNA levels ($P=0.012$) and aldosterone production ($P=0.011$). Our results indicate that PCP4 is a regulator of aldosterone production in normal, hyperplastic, and neoplastic human adrenocortical cells.

Key Words

- ▶ Purkinje cell protein 4 (PCP4)
- ▶ adrenal cortex
- ▶ aldosterone
- ▶ calmodulin (CaM)
- ▶ CYP11B2

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Introduction

Purkinje cell protein 4 (PCP4), also known as PEP-19, is a 7.6 kDa protein with an IQ-motif that binds to calmodulin (CaM) (Wei *et al.* 2011). PCP4 is abundant in Purkinje cells of the cerebellum, and plays an important role in

synaptic plasticity (Sangameswaran *et al.* 1989, Wei *et al.* 2011). PCP4-null mice were reported to exhibit impaired locomotor learning and markedly altered synaptic plasticity in cerebellar Purkinje neurons (Wei *et al.* 2011).

PCP4 accelerates both the association and dissociation of calcium (Ca^{2+}) with CaM, which is postulated to influence the activity of CaM-dependent enzymes, especially CaM kinase II (Putkey *et al.* 2003, Kleerekoper & Putkey 2009, Wei *et al.* 2011). It has been also reported that PCP4 can prevent cellular degeneration and apoptosis, as neuroprotection in the CNS (Erhardt *et al.* 2000, Johanson *et al.* 2000, Slemmon *et al.* 2000). Recently, PCP4 expression has been reported to be up-regulated in adrenocortical aldosterone-producing adenoma (APA), compared with APA-adjacent adrenal gland (Wang *et al.* 2011). However, details of its expression within the adrenocortical layers, and its possible functions in normal or pathological adrenocortical tissues, have remained unclear.

Therefore, in this study, we evaluated PCP4 immunoreactivity in normal and neoplastic adrenocortical tissues, explored a possible function using the H295R adrenocortical carcinoma cell line, and correlated its expression with that of adrenal steroidogenic enzymes. In addition, we also evaluated the correlation between the status of PCP4 and *KCNJ5* somatic mutations in APA (Choi *et al.* 2011, Zennaro & Jeunemaitre 2011).

Materials and methods

Human adrenal tissues

The research protocols were approved by the Ethics Committee at Tohoku University Graduate School of Medicine (Sendai, Japan). All patients read and signed informed consent documents regarding the diagnostic and scientific use of tissue samples and clinical data.

For immunohistochemical analysis (IHC), 15 non-pathological adrenal glands (normal adrenal (NA)), five idiopathic hyperaldosteronism (IHA), and 30 adrenocortical tumor specimens (15 APA and 15 cortisol-producing adenomas (CPA)) were retrieved from the surgical pathology files of Tohoku University Hospital (Sendai, Japan). NA tissues were obtained from nephrectomy cases due to renal carcinoma, and were subsequently evaluated to confirm the absence of neoplastic invasion, necrosis, or other histopathological abnormalities.

APA and IHA patients in our study underwent adrenal vein sampling at Tohoku University Hospital, following the previously reported protocol (Satoh *et al.* 2007). The IHA patients were relatively young, and unilateral adrenalectomy was performed to lower circulating aldosterone levels, in order to further prevent future organ damage (Sukor *et al.* 2009, Nakamura *et al.* 2011).

Therefore, all IHA patients in this study agreed to surgical treatment. The histopathological diagnosis of IHA was based on the presence of hyperplastic zona glomerulosa (ZG) with a marked β -HSD immunostaining, in contrast to the adjacent adrenal cortex of APA, which expresses very low levels of β -HSD in the ZG (Nakamura *et al.* 2011).

For quantitative RT-PCR analysis (qPCR) and DNA sequencing, 45 APA samples were obtained from Tohoku University Hospital, with clinical data retrieved from the respective clinical records. All APA patients were treated with spironolactone before surgery.

Immunohistochemical analysis

Rabbit polyclonal antibody of human PCP4 was purchased from Sigma–Aldrich and used at 1:2000 dilution. IHC technique was carried as previously reported (Felizola *et al.* 2013a,b).

After completely reviewing the slides, relative immunoreactivity of PCP4 in each zone of the adrenocortex as well as in tumor specimens was evaluated by a modified *H*-score of nuclei and the counting of positive cytoplasm, carried out by examining three high-power fields and counting 1000 cells (Lai *et al.* 2007). Regarding cytoplasmic immunoreactivity, a semiquantitative evaluation was used, and a scoring of 0–4 corresponded to 0–1, 1–25, 25–50, 50–75, and 75–100%, as described (Hui *et al.* 2009). The nuclear and cytoplasmic evaluations were independently and blindly carried out by two of the authors (S J A F and K K) and the mean values were used for analysis.

RNA isolation and qPCR

RNA isolation with subsequent cDNA production and qPCR technique were performed as previously reported (Felizola *et al.* 2013a,b, 2014). The primer sequences used in our study were *PCP4* forward, 5'-TGA CAT GGA TGC ACC AG-3' and reverse, 5'-GTG TGG ATT GTG TGT GG-3'; *CYP11B1* forward, 5'-CCC AGC ACA AAT GGA ACT CCC GA-3' and reverse, 5'-CCG CTT AAT GAC TCT GAC AGT CTG CG-3'; *CYP11B2* forward, 5'-TCC AGG TGT GTT CAG TAG TTC C-3' and reverse, 5'-GAA GCC ATC TCT GAG GTC TGT G-3'; and *RPL13A* forward, 5'-CCT GGA GGA GAA GAG GAA AG-3' and reverse, 5'-TTG AGG ACC TCT GTG TAT TT-3'. The cDNA produced from a human brain specimen was used as a positive control in the PCP4 and RPL13A qPCR experiments, while the cDNA from H295R adrenocortical carcinoma cells was used as a positive control for *CYP11B1* and *CYP11B2*.

The relative gene expression was calculated as previously reported (Felizola *et al.* 2014). For the analysis of cell experiments data, the relative gene expression was calculated by the $\Delta\Delta\text{Ct}$ method as reported (Nogueira *et al.* 2007). *RPL13A* was used as an endogenous control gene.

Cell culture

Human adrenocortical carcinoma cells H295R (Bird *et al.* 1995) were cultured in DMEM/Eagle's F12 medium (Invitrogen) and supplemented with 10% Cosmic Calf Serum (CCS; Hyclone Laboratories, Inc., Nampa, ID, USA), 1% penicillin/streptomycin (Invitrogen), and 0.01% gentamycin (Sigma–Aldrich). The cells were maintained in a 37 °C humidified atmosphere (5% CO₂).

H295R cell line assays and following qPCR analysis

H295R cells were transferred to 12-well dishes in groups of 600 000 cells/well, and maintained at the conditions described. After 24 h passage, DMEM/Eagle's F12 medium supplemented with 0.1% CCS and, after 48 h, DMEM/Eagle's F12 media containing angiotensin-II (Ang-II; Tocris, Bristol, UK; 10 nM), and forskolin (Tocris; 10 μM) were added to different groups of cells, each group comprising three wells. A basal group, to which no drug was added, was used as the control. RNA was extracted at 3, 6, 12, and 24 h time points (RNeasy Mini Kit, Qiagen). All the cell experiments were independently conducted in triplicate, with cells raised at different times.

PCP4 transient siRNA knockdown and ELISA

Human PCP4 MISSION siRNA (Sigma–Aldrich) and MISSION siRNA Universal Negative Control 1 (Sigma–Aldrich) were transfected into H295R cells at 40 ng/ μl concentration using a Nucleofector-4D electroporator machine (Lonza, Koln, Germany). After transfection, the cells were transferred to 12-well dishes in groups of 600 000 cells/well, and after 48 h RNA and protein were harvested from one set of cells. The remaining cells were either treated with Ang-II (10 nM) or vehicle from this point. After 60 h from transfection, RNA was collected from i) cells transfected with PCP4 siRNA plus vehicle; ii) cells transfected with PCP4 siRNA plus Ang-II; iii) cells transfected with negative control (NC) siRNA plus vehicle; and iv) cells transfected with NC siRNA plus Ang-II respectively. In addition, cell media were collected 96 h after transfection and were submitted to ELISA analysis of aldosterone and cortisol with ALPCO ELISA Kits

(ALPCO Diagnosis, Salem, NH, USA). These ELISA data were adjusted by protein concentration at these time points. All the experiments were independently performed in triplicate.

PCP4 transient DNA transfection and luciferase assays

MCF7 breast cancer cells were transfected with the plasmid produced in *Escherichia coli* using the following DNA primers: forward, 5'-GGG GCT AGC ATG AGT GAG CGA CAA GGT GCT G-3' and reverse, 5'-CGC AAG CTT CAC TAG GAC TGA GAC CCA GCC-3'. The pcDNA3.1(-) vector (Invitrogen) was used as a backbone for the PCP4 plasmid. NC MCF7 cells were transfected with empty vector. Protein was collected and western blotting performed in order to confirm and evaluate the transient transfection.

After confirmation of plasmid activity, H295R cells were grown to 80% confluence in 24-multiwell plates, and transiently transfected with 200 ng -1521/+2-luc harboring the 5'-flanking region of *CYP11B2* (CYP11B2-LUC), and 300 ng pcDNA of PCP4 or control pcDNA using Lipofectamine LTX and Plus Reagent (Invitrogen) for 24 h. The media were changed to DMEM supplemented with 1% stripped FBS, and the cells were incubated either with or without Ang-II (100 nM) for 6 h. Following incubation, cell extracts were prepared using Glo Lysis Buffer (Promega). Luciferase activity was measured using Bright-Glo Reagents (Promega), and protein concentration was measured using protein assay kit (Bio-Rad). Data were normalized by protein concentration.

Western blotting analysis

Both H295R and MCF7 cell lines were submitted to total protein extraction with the M-PER mammalian protein extraction reagents (Thermo Scientific, Rockford, IL, USA) after the addition of Halt protease inhibitor (Thermo Scientific). Semi-dry immunoblotting was carried out using a nitrocellulose membrane after SDS–page electrophoresis. The immunoblot membrane was incubated overnight with rabbit polyclonal anti-human PCP4 antibody (Sigma–Aldrich) in a 1:400 dilution or mouse monoclonal anti- β -actin antibody (Sigma–Aldrich). Chemiluminescence was performed using the Amersham ECL Prime western blotting detection reagent (GE Healthcare Life Sciences, Little Chalfont, Bucks, UK) following the incubation with an anti-rabbit or anti-mouse second-antibody for 1 h. The immunoreactive bands were analyzed through dark-chamber photography

with a Fujifilm luminescent image analyzer model LAS-1000CH (Fujifilm, Tokyo, Japan), and quantification performed with integrated analysis software.

DNA sequencing and mutations in *KCNJ5*

APA specimens ($n=45$) were submitted to PCR using a *KCNJ5* primer (forward, 5'-CGA CCA AGA GTG GAT TCC TT-3' and reverse, 5'-AGG GTC TCC GCT CTC TTC TT-3') with annealing temperature at 65 °C. After cooling, all samples were submitted to electrophoresis in agarose gel 0.1% and DNA purification with a QIAquick gel extraction kit (Qiagen). Analysis of the purified DNA was carried out with a ABI PRISM 310 Genetic Analyzer (Applied Biosystems), and mutations at the G151R and L168R regions of the *KCNJ5* gene were analyzed as described (Choi *et al.* 2011).

Statistical analysis

Data from the IHC of NAC and IHA samples were evaluated in the three groups (ZG, zona fasciculata (ZF), and zona reticularis (ZR)) using Mann–Whitney multiple comparison tests with the significance level set to $\alpha=0.05$. The Bonferroni inequality was used to correct multiple comparisons, with $0.05/3=0.0167$ resulting in $P<0.0167$ as the statistically significant value. The statistical difference between the cortical layers of NAC and IHA,

as well as between APA and CPA samples, was evaluated using Mann–Whitney *U* tests, and $P<0.05$ was considered statistically significant.

Data from the qPCR of human tissues were evaluated using regression analysis and/or Mann–Whitney *U* tests. A correlation coefficient of $R>0.3$ and/or $P<0.05$ was considered significant. The statistical analysis of patient gender and its correlation to *KCNJ5* mutations was assessed by contingency table, and a χ^2 value of $P<0.05$ was considered significant. The data from cell experiments *in vitro* were evaluated using either ANOVA *post-hoc* tests or *t*-tests, with the significance level set to $\alpha=0.05$, and the Bonferroni inequality was used to correct multiple comparisons when applicable.

Results

Immunohistochemical analysis

Results of *H*-scores of nuclei and positive cytoplasm of NA and IHA are shown in Fig. 1A and B respectively, and adrenocortical tumors in Fig. 1C and D. PCP4 immunoreactivity was detected in the nuclei of the ZG, the ZF, and the ZR cells of NA (Fig. 1A and Supplementary Figs 1, 2 and 3, see section on supplementary data given at the end of this article) and IHA (Fig. 1B and Supplementary Figs 4, 5 and 6), with a significantly higher nuclear immunoreactivity in the ZG compared with the other two

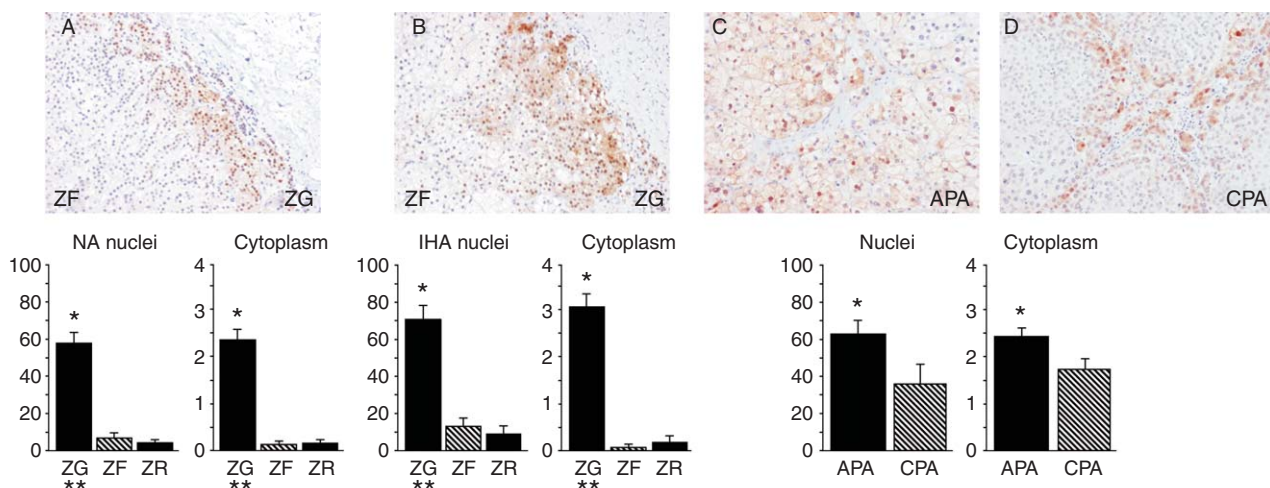


Figure 1

Immunohistochemistry of PCP4. Nuclei were evaluated by modified *H*-score and cytoplasm by semi-quantitative scoring. (A) Normal adrenal (NA), (B) idiopathic aldosteronism (IHA). Results on *H*-score (nuclei) and semi-quantitative evaluation (cytoplasm) are below the respective photographs and indicate the zona glomerulosa (ZG), zona fasciculata (ZF), and the zona reticularis (ZR). Significantly higher nuclear and cytoplasmic

immunostaining was detected in the ZG of IHA than the ZG of NA (** $P<0.05$), but no statistical difference was observed when comparing the other layers. (C) Aldosterone-producing adenoma and (D) cortisol-producing adenoma. s.d.s are indicated as error bars in the graphics, * $P<0.001$. A full colour version of this figure is available via <http://dx.doi.org/10.1530/JME-13-0248>.

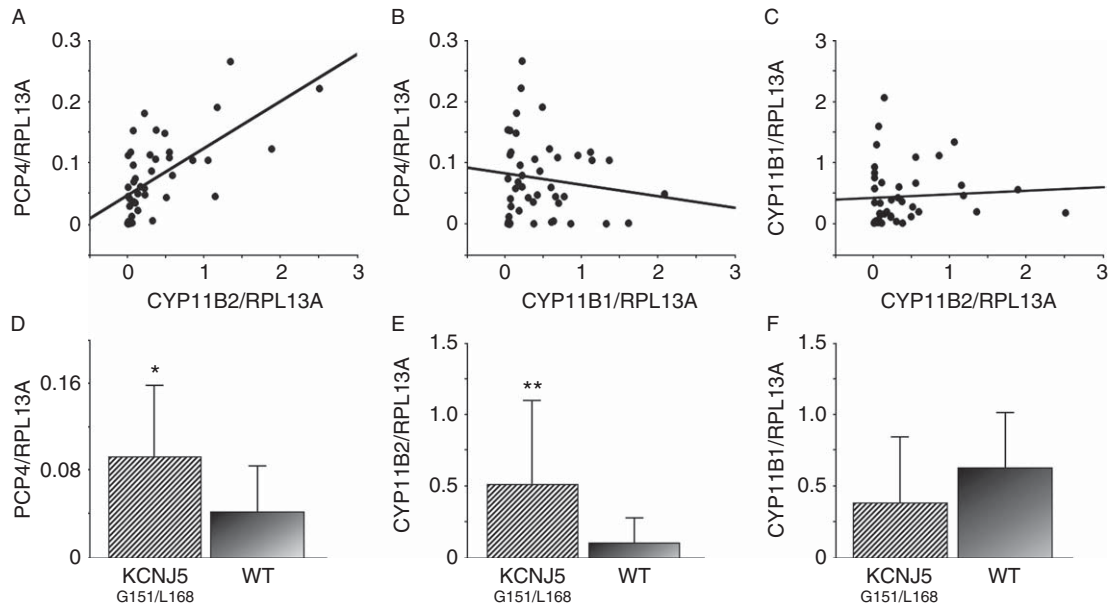


Figure 2

Real-time quantitative PCR (qPCR) analysis of PCP4, CYP11B2, and CYP11B1, and DNA sequencing of KCNJ5 in 45 APA samples. (A) A significant statistical correlation was detected between PCP4 and CYP11B2 relative mRNA levels ($P < 0.0001$; $R > 0.6$). (B) No statistical significance was observed between PCP4 and CYP11B1. (C) No statistical significance was detected

between CYP11B1 and CYP11B2 mRNA levels. (D) PCP4 levels were significantly higher in KCNJ5 mutated samples than WT ($*P = 0.005$). (E) CYP11B2 were also higher in KCNJ5 mutated specimens ($**P < 0.005$). (F) No statistical relationship was detected between CYP11B1 and KCNJ5 mutations.

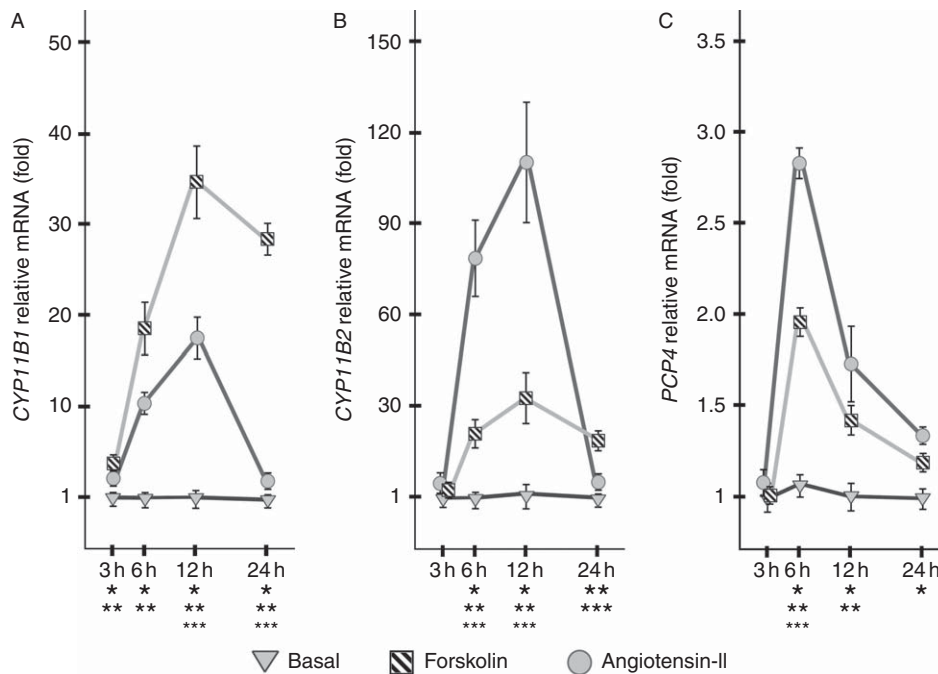


Figure 3

PCP4, CYP11B2, and CYP11B1 drug treatment time course in H295R cells. (A) CYP11B1 mRNA levels after treatment with angiotensin-II (Ang-II) and forskolin in a time-dependent manner. (B) CYP11B2 mRNA levels after

treatment with Ang-II and forskolin. (C) PCP4 mRNA levels after treatment with Ang-II and forskolin. $*P < 0.01$ (Ang-II vs basal); $**P < 0.01$ (forskolin vs basal); and $***P < 0.01$ (Ang-II vs forskolin).

adrenocortical layers ($P < 0.001$). The semi-quantitative evaluation of cytoplasm immunoreactivity also revealed a higher number of PCP4 positive cells in the ZG of NA and IHA compared with the other zones ($P < 0.001$). The immunoreactivity of PCP4 in nuclei and cytoplasm was significantly higher in APA compared with CPA ($P < 0.001$; Fig. 1C and D).

QPCR analysis of APA samples

When the qPCR results of *PCP4*, *CYP11B1*, and *CYP11B2* mRNA levels in APA cases were analyzed with polynomial regression, a significantly positive correlation was detected between PCP4 and CYP11B2 ($P < 0.0001$; $R > 0.6$; Fig. 2A), although not between PCP4 and CYP11B1 (Fig. 2B). Also, no significant correlation was detected between CYP11B2 and CYP11B1 (Fig. 2C). *KCNJ5* mutations were detected in 28 of the 45 APA cases (62.2%), and the data obtained from the clinical records of the 45 APA patients and the respective statistical correlations to *KCNJ5* mutations are summarized in the Supplementary Table 1, see section on supplementary data given at the end of this article. Both PCP4 ($P = 0.005$) and CYP11B2 ($P < 0.005$) were significantly higher in *KCNJ5* mutated cases, while no statistical significance of *CYP11B1* mRNA levels between mutated and WT APA cases (Fig. 2D, E and F).

Stimulation of H295R cells by Ang-II and forskolin

Results of the time course of H295R cells following the treatment with Ang-II and forskolin are summarized in Fig. 3. The mRNA levels of *CYP11B1* and *CYP11B2* reached a peak at 12 h, after both Ang-II and forskolin treatments (Fig. 3A and B respectively), but *PCP4* mRNA levels peaked at 6 h of treatment under the same conditions (Fig. 3C). With Ang-II, *PCP4* mRNA levels increased up to threefold relative to basal levels (mean ± 0.4 s.d.), while with forskolin, *PCP4* levels increased up to twofold at its peak (mean ± 0.3 s.d.). The detection of maximum levels of *PCP4* mRNA at the 6 h time point coincided with the elevation period of *CYP11B1* and *CYP11B2* mRNA levels in H295R cells.

Transient transfection and luciferase assays

Results of luciferase assays were summarized in Fig. 4. The transient transfection with PCP4 DNA vector produced significant rise of CYP11B2-LUC in PCP4-transfected H295R when Ang-II was added to cell media, compared with cells transfected with control vector ($P < 0.0001$).

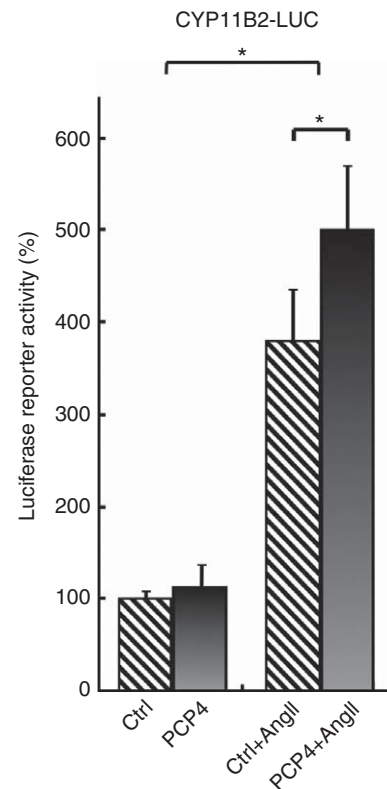


Figure 4

PCP4 transient DNA transfection and luciferase assays. Following the transient transfection with PCP4 DNA vector, CYP11B2 luciferase (CYP11B2-LUC) assays were performed. When angiotensin-II (Ang-II) was added to cell media, a significant increment in CYP11B2-LUC was detected in PCP4-transfected H295R cells than empty vector cells (Ctrl). However, without Ang-II treatment, no statistically significant differences were detected, $*P < 0.0001$.

Transient siRNA transfection, plasmid DNA, western blotting, and ELISA

Results of the siRNA transfection of H295R cells and subsequent western blotting and ELISA are summarized in Fig. 5. A mean of 90% knockdown of *PCP4* mRNA levels ($P < 0.0001$; NC 100% ± 13 s.d.; PCP4 siRNA 10% ± 4 s.d.; Fig. 5A) and a mean of 70% decrement in PCP4 protein levels ($P = 0.031$; NC 100% ± 36 s.d.; PCP4 siRNA 30% ± 7 s.d.; Fig. 5B) were detected in three independent experiments. Following the incubation with Ang-II for 12 h, the PCP4 siRNA-transfected H295R cells demonstrated $\sim 30\%$ less *CYP11B2* mRNA (NC 100% ± 30 s.d.; PCP4 siRNA 70% ± 13 s.d.) than those transfected with NC siRNA ($P = 0.012$) (Fig. 5C). However, no statistical significance was detected in *CYP11B1* mRNA levels between PCP4 and NC siRNA-transfected H295R cells (Fig. 5D). When cell culture media were analyzed through ELISA, a significant decrease in aldosterone production levels was detected in PCP4 siRNA

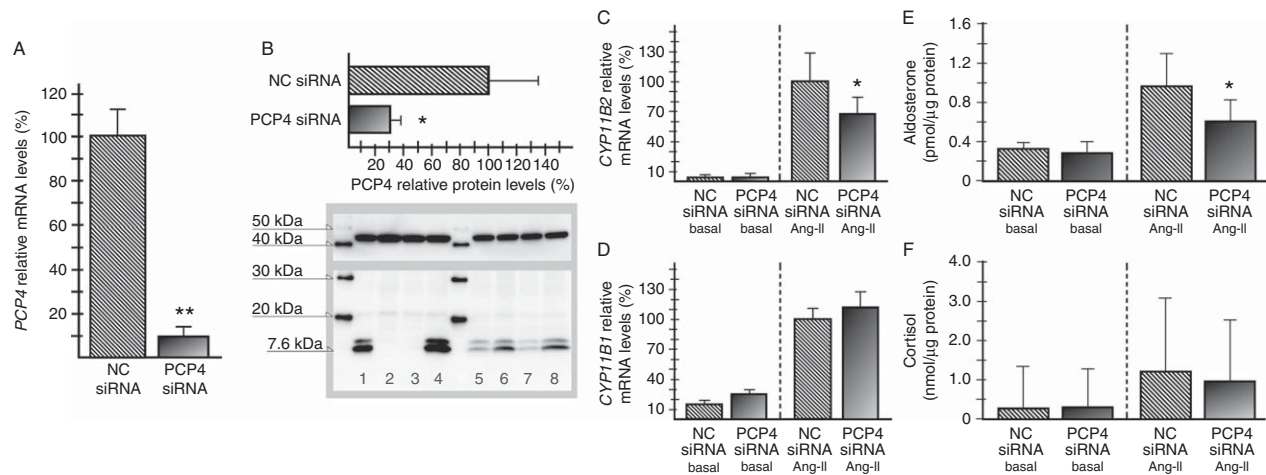


Figure 5

Transient PCP4 siRNA transfection. (A) Forty-eight hours after transfection with PCP4 siRNA, the mRNA levels of PCP4 in H295R cells decreased by 90% compared with negative control (NC) siRNA (mean, NC 100% \pm 13; PCP4 siRNA 10% \pm 4 s.d.; ** P < 0.0001). (B) Immunoblotting analysis revealed a mean of 70% decrement in PCP4 protein levels (NC 100% \pm 36; PCP4 siRNA 30% \pm 7 s.d.; * P < 0.05); the bands between 40 and 50 kDa represent β -actin, while the bands below 20 kDa in the expected band-size of 7.6 kDa represent PCP4, as follows: (1) and (4) MCF7 transfected with PCP4 plasmid DNA (positive controls), (2) and (3) MCF7 transfected with empty vector, (5) and (7) H295R cells transfected with PCP4 siRNA, (6) and (8) H295R cells transfected with NC siRNA. (C) *CYP11B2* mRNA levels 60 h after transfection: two groups of cells were submitted to angiotensin-II (Ang-II) treatment for

transfected cells treated with Ang-II (P = 0.011) compared with NC siRNA transfected cells treated with Ang-II (Fig. 5E); however, no statistical significance was detected between the basal, non-treated samples. In addition, no significant changes could be detected in cortisol levels between cells transfected with PCP4 siRNA and NC siRNA, either in Ang-II treated or non-treated samples (Fig. 5F).

Discussion

Results of this study demonstrated high expression levels of PCP4 protein in normal, hyperplastic, and neoplastic aldosterone-producing adrenocortical cells and tissues. Previous studies suggest a role for Ca^{2+} and CaM in aldosterone synthesis; however, the detailed intracellular mechanisms of aldosterone production still remain to be clarified (Kotchen *et al.* 1977, Fakunding *et al.* 1979, Kojima *et al.* 1985, Mantero *et al.* 1986, Kramer 1988, Laird *et al.* 1991, Ganguly *et al.* 1995, Pezzi *et al.* 1997, Condon *et al.* 2002, Nogueira *et al.* 2007, Romero *et al.* 2007, Nogueira & Rainey 2010, Choi *et al.* 2011, Uruno *et al.* 2011, Zennaro & Jeunemaitre 2011). Results of our present *in vitro* study revealed an increment in *CYP11B2* mRNA levels after 6 h of Ang-II treatment, with a peak at the 12 h time point, as

12 h (NC siRNA and PCP4 siRNA) while two groups remained as basal controls (no drug treatment); the comparison of Ang-II treated cells reveals a decrement in *CYP11B2* mRNA levels in PCP4 siRNA-treated cells (mean 30%; NC 100% \pm 30; PCP4 siRNA 70% \pm 13 s.d.; * P = 0.012). (D) *CYP11B1* mRNA levels 60 h after transfection: no statistical significance could be detected between PCP4 and NC siRNA treated cells. (E) Aldosterone levels were significantly lower in PCP4 siRNA transfected cells after 48 h of Ang-II, compared with NC siRNA cells treated at the same conditions (* P = 0.011); however, there were no statistical significant differences between the basal, non-treated samples. (F) There were no statistically significant differences in cortisol levels between cells transfected with PCP4 siRNA and NC siRNA, either in Ang-II treated or non-treated samples.

previously reported (Romero *et al.* 2004, Xing *et al.* 2012). We detected the highest mRNA levels of *PCP4* after 6 h of Ang-II and forskolin treatments in H295R cells, which coincides with the rising period of *CYP11B2* in the time course experiments. As reported, the Ca^{2+} /CaM pathway is suggested to be related to the Ang-II receptor and aldosterone production in adrenocortical cells. Therefore, we hypothesized that PCP4 is regulated by Ang-II as part of the Ca^{2+} /CaM pathway.

In order to prove this hypothesis, we first performed luciferase assays following the transient transfection of PCP4. As our results demonstrate, the *CYP11B2* luciferase was up-regulated in PCP4 transfected cells compared with controls. Then, in order to further confirm the evidence and explore whether PCP4 does serve as a key protein in aldosterone-producing pathways, we performed siRNA experiments. After 60 h of transient knockdown of PCP4 and 12 h of Ang-II treatment of H295R cells, there was a significant decrease in *CYP11B2* mRNA levels compared with controls, which, together with the time course and luciferase assays data, indicated the regulation of *CYP11B2* by PCP4. The lack of significant changes in the mRNA levels of *CYP11B1* also indicated a specific correlation of PCP4 with the aldosterone-producing pathways in adrenocortical cells.

We then evaluated the aldosterone production of H295R cells, and confirmed that while aldosterone production significantly decreased in PCP4 siRNA-transfected cells, cortisol production remained the same. Therefore, the results of our *in vitro* studies indicated that PCP4 is actually involved in the regulation of CYP11B2 and aldosterone production in human adrenocortical cells.

As a complement to *in vitro* data, and in order to further clarify the role of PCP4 in human adrenocortical tissues, we evaluated NA, IHA, APA, and CPA cases. The quantification of nuclear and cytoplasmic PCP4 revealed that this protein correlates with the phenotypes of aldosterone-producing cells, and our analysis suggests that PCP4 can work in conjunction with CaM in order to activate the CREB transcription factor (Deisseroth *et al.* 1998, Thorogate & Török 2004). In addition, when analyzed in several APA tissue samples, the mRNA levels of *PCP4* were significantly correlated with those of *CYP11B2* but not with *CYP11B1*, which was also consistent with our *in vitro* results.

In this study, we attempted to clarify the mechanisms by which PCP4 could act in the aldosterone production pathway *in vivo*. The actions of PCP4 on CaM and intracellular Ca²⁺ homeostasis have been well studied in mammalian CNS tissues, and we hypothesized that similar mechanisms might function in human adrenal cortex (Johanson *et al.* 2000, Putkey *et al.* 2003, Kleerekoper & Putkey 2009). The somatic mutations in the amino acids G151 and L168 of the *KCNJ5* potassium (K⁺) channels have been reported to produce the loss of ion selectivity by the cellular membrane, and hyper-activation of voltage-gated Ca²⁺ channels, resulting in elevation of the intracellular levels of Ca²⁺ in APA (Choi *et al.* 2011, Zennaro & Jeunemaitre 2011), and we demonstrate that these *KCNJ5* mutations are significantly correlated with the expression status of both *CYP11B2* and *PCP4* in APA, but not with the status of *CYP11B1*. In addition, our results demonstrate that *KCNJ5* mutations are significantly correlated with plasmatic aldosterone concentration levels in APA patients, which suggests an indirect correlation between PCP4 and aldosterone levels *in vivo*.

The elevation of intracellular Ca²⁺ in conjunction with mutations in *KCNJ5* could result in a hyperactivity of PCP4, reflected as the up-regulation of its intracellular levels in APA. However, despite the lack of *KCNJ5* mutations, some APA and the ZG of NA and IHA do show higher levels of PCP4 than the adrenocortical ZF or ZR. A reason for that may be explained by the generation of spontaneous membrane potential oscillations of low periodicity by the adrenocortical ZG cells, providing a

platform for the production of a recurrent Ca²⁺ channels signal that can be controlled by Ang-II and extracellular K⁺ (Hu *et al.* 2012), but further investigations are required for clarification.

In summary, PCP4 is considered as a regulator of aldosterone synthesis, and its inhibition could suppress hyperplastic and neoplastic aldosterone production in human adrenocortical tissues.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-13-0248>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S J A F and Y N were responsible for the design of the study, of which S J A F provided the details and performed the experiments, in part or totally. Y O was partly involved in the study design and played a role in qPCR experiments. K K was partly involved in the study design and was responsible for part of the immunohistochemical analysis. K K was responsible for the ELISA analysis set up and performance. Y O was responsible for vector DNA and qPCR primer design. K I was responsible for the histotechnical procedures and human tissue management. K T performed the radiological diagnosis of the adrenocortical tumors used in the study. A S performed the cell transfections and the luciferase assays. N H provided insights on adrenocortical physiology and intracellular pathways. W E R, F S, and H S provided expertise and valuable opinion on the study design and interpretation of the results.

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