Published in final edited form as:
J Mol Endocrinol. 2014 April ; 52(2): 159–167. doi:10.1530/JME-13-0248.

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 (CPA; n=15) and idiopathic hyperaldosteronism cases (IHA; n=5). APA samples (n=45) were also submitted to quantitative RT-PCR (qPCR) of PCP4, CYP11B1, and CYP11B2, as well as DNA sequencing for KCNJ5 mutations. Transient transfection analysis using PCP4 siRNA was also performed in

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6. AUTHOR CONTRIBUTIONS

S.J.AF. 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 totality.
Y. Ono was partly involved in the study design, and played a role in qPCR experiments.
K. Kitamura was partly involved in the study design, and was responsible for part of the immunohistochemical analysis.
K. Kikuchi was responsible for the ELISA analysis set-up and performance.
Y. Onodera 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 insight 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.

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 (ZG) 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 wild-type ($P=0.005$). 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.

Keywords

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

1. 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 have 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 (CaMK-II) (Putkey et al. 2003, Kleerekoper et al. 2009, Wei et al. 2011). It has been also reported that PCP4 can prevent cellular degeneration and apoptosis, as neuroprotection in the central nervous system (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 to 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 to that of adrenal steroidogenic enzymes. In addition, we also evaluated the correlation between the status of PCP4 and KCNJ5 somatic mutations in aldosterone producing adenomas (Choi et al. 2011, Zennaro et al. 2011).

2. MATERIALS AND METHODS

2.1 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, 15 non-pathological adrenal glands (NA), 5 idiopathic hyperaldosteronism (IHA) and 30 adrenocortical tumor specimens (15 APA, 15 CPA) were retrieved from 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 sample (AVS) 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 3β-HSD immunostaining, in contrast to the adjacent adrenal cortex of APA, which expresses very low levels of 3β-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 prior to surgery.

2.2 Immunohistochemical analysis (IHC)

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

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 3 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. Kitamura) and the mean values were used for analysis.

2.3 RNA isolation and quantitative RT-PCR (qPCR)

RNA isolation with subsequent cDNA production and qPCR technique were performed as previously reported (Felizola et al. 2013b, Felizola et al. 2014). The primer sequences used in our study were: *PCP4* forward 5′-TGA CAT GGA TGC ACC AG-′3, reverse 5′-GTG TGG ATT GTG TGT GG-′3; *CYP11B1* forward 5′-CCC AGC ACA AAT GGA ACT CCC GA-′3, reverse 5′-CCG CTT AAT GAC TCT GAC AGT CTG CG-′3; *CYP11B2* forward 5′-TCC AGG TGT GTT CAG TAG TTC C-′3, reverse 5′-GAA GCC ATC TCT GAG GTC TGT G-′3; *RPL13A* forward 5′-CCT GGA GGA GAA GAG GAA AG-′3, 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 ΔΔCt method as reported (Nogueira et al. 2007). *RPL13A* was used as an endogenous control gene.

2.4 Cell culture

Human adrenocortical carcinoma cells H295R (Bird et al. 1995) were cultured in DMEM/Eagle's F12 medium (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% Cosmic Calf Serum (CCS) (Hyclone laboratories Inc., Nampa, ID, USA), 1% penicillin/streptomycin (Invitrogen), and 0.01% gentamycin (Sigma-Aldrich). Cells were maintained in a 37°C humidified atmosphere (5% CO_2).

2.5 H295R cell line assays and following qPCR analysis

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

2.6 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 40ng/μl concentration using a Nucleofector-4D electroporator machine (Lonza, Koln, Germany). After transfection, the cells were transferred to 12 wells dishes in groups of 600,000 cells per well, and after 48h RNA and protein were harvested from one set of cells. Remaining cells were either treated with angiotensin-II (10nM) or vehicle from this point. After 60h from transfection, RNA was collected from: 1- cells transfected with PCP4 siRNA plus vehicle; 2- cells transfected with PCP4 siRNA plus angiotesin-II; 3- cells transfected with negative control siRNA plus vehicle; and 4- cells transfected with negative control siRNA plus angiotensin-II, respectively. In addition, cell media were collected 96h 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.

2.7 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.

Negative control 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 24h. The media were changed to DMEM supplemented with 1% stripped FBS, and the cells were incubated either with or without angiotensin-II (100nM) for 6 h. Following, cell extracts were prepared using Glo Lysis Buffer (Promega, Madison, WI, USA). Luciferase activity was measured using Bright-Glo reagents (Promega), and protein concentration was measured using protein assay kit (Bio-Rad, Hercules, CA, USA). Data were normalized by protein concentration.

2.8 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, Buckinghamshire, UK) following the incubation with an anti-rabbit or anti-mouse second-antibody for 1h. 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.

2.9 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, reverse 5′-AGG GTC TCC GCT CTC TTC TT-′3) with annealing temperature of 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 analyser (Applied Biosystems, Foster City, CA, USA), and mutations at the G151R and L168R regions of the KCNJ5 gene were analysed as described (Choi et al. 2011).

2.10 Statistical analysis

Data from the immunohistochemical analysis of NAC and IHA samples were evaluated in groups of 3 (ZG, ZF, 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 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 tests. A correlation coefficient of $R>0.3$ and/or $P<0.05$ were considered significant. The statistical analysis of patient gender and its correlation to KCNJ5 mutations was assessed by contingency table, and a chi-square value of $P<0.05$ was considered significant. Cell experiments *in vitro* data 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.

3. RESULTS

3.1 Immunohistochemical analysis

Results of H-scores of nuclei and positive cytoplasm of NA and IHA are summarized in Figure 1A and B respectively, and adrenocortical tumors in Figure 1C and D. PCP4 immunoreactivity was detected in the nuclei of the ZG, the zona fasciculata (ZF) and the zona reticularis (ZR) cells of NA (Figure 1A and Supplementary Figures 1–3) and IHA (Figure 1B and Supplementary Figures 4–6), with a significantly higher nuclear immunoreactivity in the ZG when compared to the other 2 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 to the other zones ($P<0.001$). The immunoreactivity of PCP4 in nuclei and cytoplasm was significantly higher in APA compared to CPA ($P<0.001$) (Figures 1C and D).

3.2 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 ($P<0.0001$; $R>0.6$: Figure 2A), although not between PCP4 and CYP11B1 (Figure 2B). Also, no significant correlation was detected between CYP11B2 and CYP11B1 (Figure 2C). KCNJ5 mutations were detected in 28 out of 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 data, Table 1. 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 (Figures 2D, E and F).

3.3 Stimulation of H295R cells by angiotensin-II and forskolin

Results of the time course of H295R cells following the treatment with angiotensin-II and forskolin are summarized in Figure 3. The mRNA levels of CYP11B1 and CYP11B2 reached a peak at 12h, after both angiotensin-II and forskolin treatments (Figure 3A and 3B, respectively), but PCP4 mRNA levels peaked at 6h of treatment under the same conditions (Figure 3C). With angiotensin-II, PCP4 mRNA levels increased up to 3 fold relative to basal levels (mean ±0.4 s.d.), while with forskolin, PCP4 levels increased up to 2 fold at its peak (mean ±0.3 s.d.). The detection of maximum levels of PCP4 mRNA at the 6h time point coincided with the elevation period of CYP11B1 and CYP11B2 mRNA levels in H295R cells.

3.4 Transient transfection and luciferase assays

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

3.5 Transient siRNA transfection, plasmid DNA, Western blotting and ELISA

Results of the siRNA transfection of H295R cells and subsequent Western blotting and ELISA were summarized in Figure 5. A mean of 90% knockdown of PCP4 mRNA levels (P<0.0001; negative controls 100%±13 s.d.; PCP4 siRNA 10%±4 s.d.) (Figure 5A) and a mean of 70% decrement in PCP4 protein levels (P=0.031; negative controls 100%±36 s.d.; PCP4 siRNA 30%±7 s.d.) (Figure 5B) were detected in three independent experiments. Following the incubation with angiotensin-II for 12h, the PCP4 siRNA transfected H295R cells demonstrated approximately 30% less CYP11B2 mRNA (negative controls 100%±30 s.d.; PCP4 siRNA 70%±13 s.d.) than those transfected with negative control siRNA (P=0.012) (Figure 5C). However, no statistical significance was detected in CYP11B1 mRNA levels between PCP4 and negative control siRNA transfected H295R cells (Figure 5D). When cell culture media were analyzed through ELISA, a significant decrease in aldosterone production levels was detected in PCP4 siRNA transfected cells treated with angiotensin-II (P=0.011) when compared to negative control siRNA transfected cells treated with angiotensin-II (Figure 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 N.C. siRNA, either in Ang-II treated or non-treated samples. (Figure 5F).

4. DISCUSSION

Results of our present study demonstrated high expression levels of PCP4 protein in normal, hyperplastic and neoplastic aldosterone-producing adrenocortical cells and tissues. Previous studies did 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, Romero et al. 2007, Nogueira et al. 2007, Nogueira and Rainey 2010, Choi et al. 2011, Uruno et al. 2011, Zennaro et al. 2011). Results of our present *in vitro* study revealed an increment of CYP11B2 mRNA levels after 6h of angiotensin-II treatment, with a peak at the 12h time point, as previously reported (Romero et al. 2004, Xing et al. 2012). We detected the highest mRNA levels of PCP4 after 6h of angiotensin-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 angiotensin-II receptor and aldosterone production in adrenocortical cells. Therefore, we hypothesized that PCP4 is regulated by angiotensin-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 to 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 60h of transient knockdown of PCP4 and 12h of angiotensin-II treatment of H295R cells, there was a significant decrease in CYP11B2 mRNA levels compared to 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 and 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 Ca^{2+} homeostasis have been well studied in mammalian CNS tissues, and we hypothesized that similar mechanisms might function in human adrenal cortex (Putkey et al. 2003, Johanson et al. 2000, Kleerekoper et al. 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 Ca^{2+} channels, resulting in elevation of the intracellular levels of Ca^{2+} in APA (Choi et al. 2011, Zennaro et al. 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 to plasmatic aldosterone levels (PAC) in APA patients, which suggests an indirect correlation between PCP4 and aldosterone levels *in vivo*.

The elevation of intracellular Ca^{2+} 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 Ca^{2+} channels signal that can be controlled by angiotensin-II and extracellular K^+ (Hu et al. 2012), but further investigations are required for clarification.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was partly supported by the Takeda Science Foundation.

The first author (S.J.A.F.) received scholarship support from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT).

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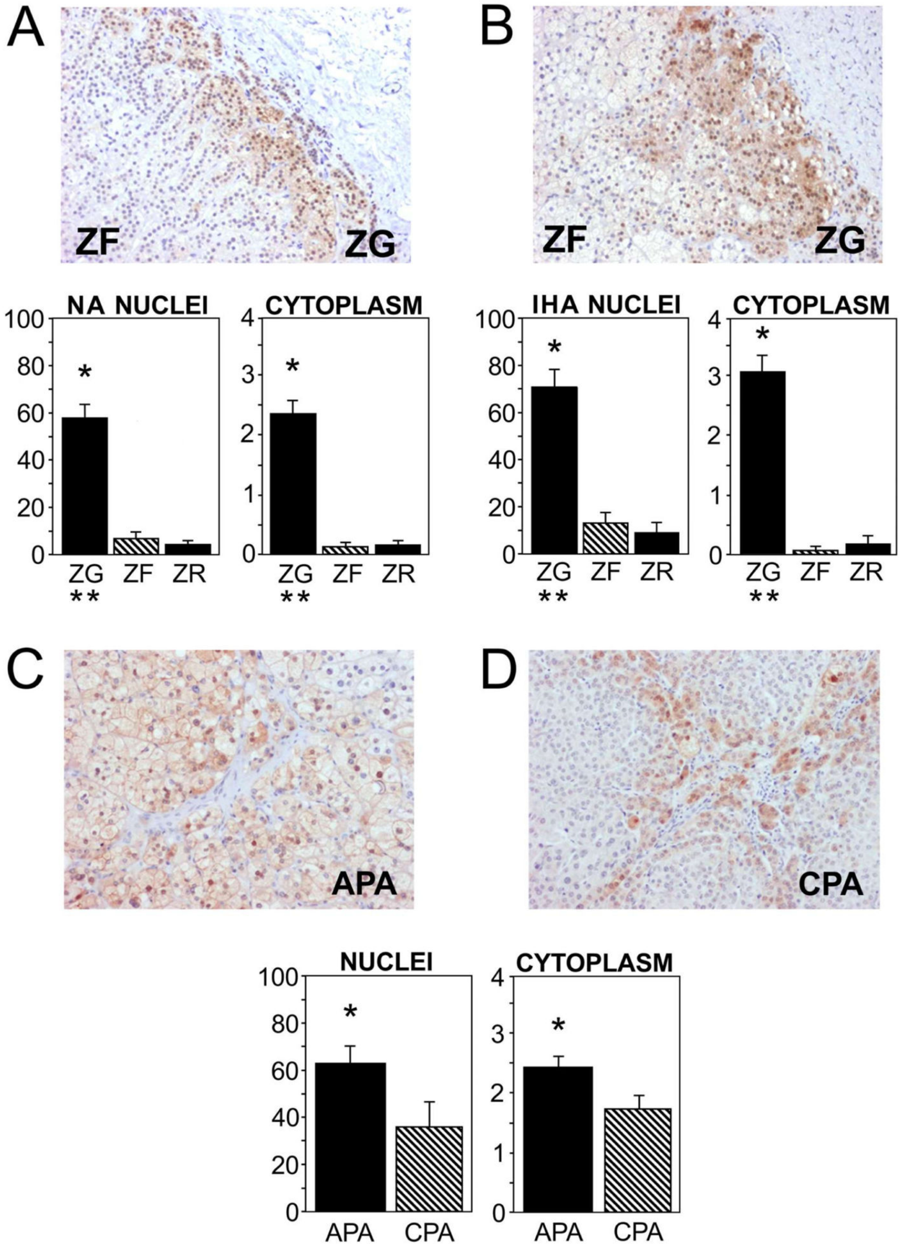

FIG. 1. Immunohistochemistry of PCP4

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, [D] cortisol producing adenoma. Standard deviations are indicated as error bars in the graphics. *$P<0.001$.

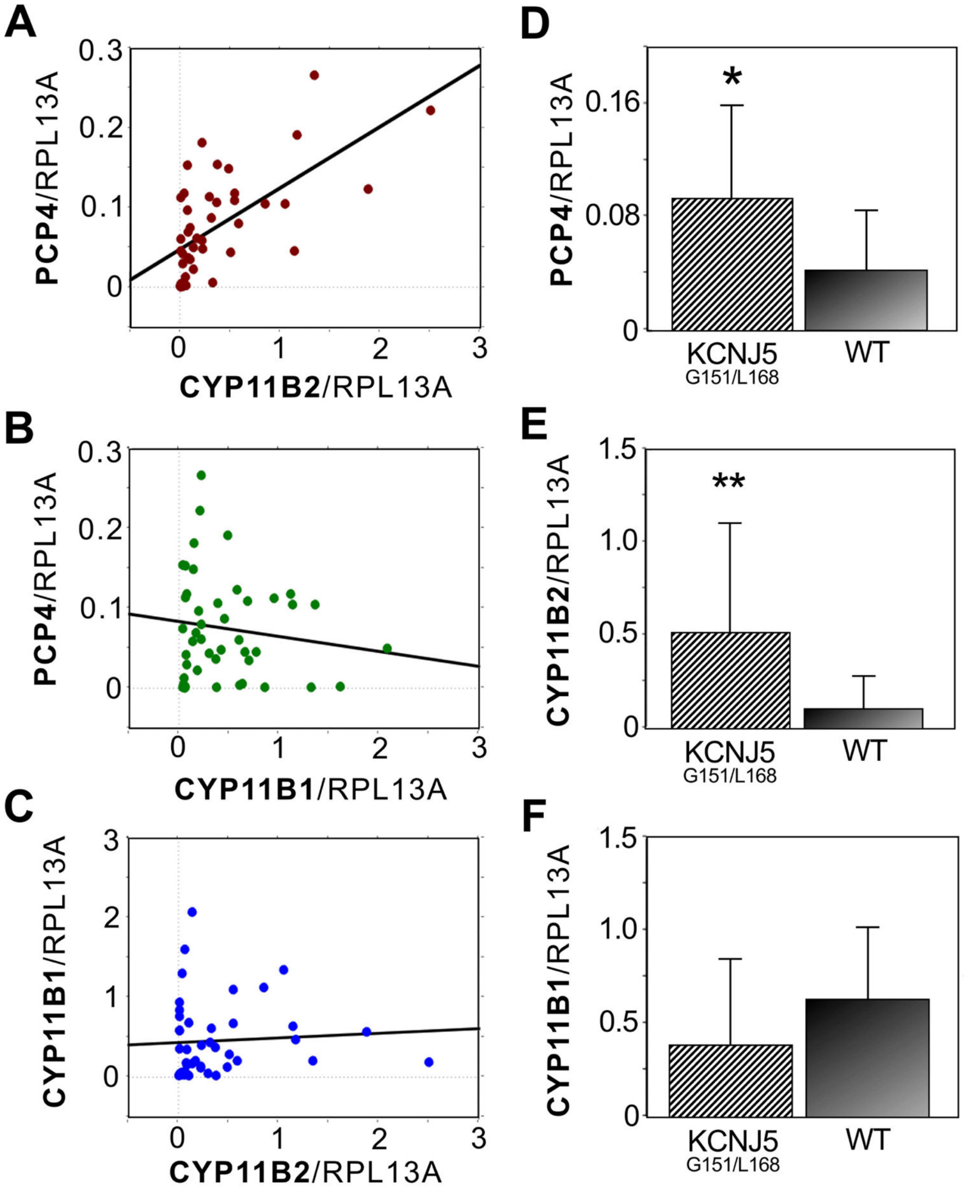

FIG. 2. Real time quantitative PCR (qPCR) analysis of PCP4, CYP11B2, and CYP11B1, and DNA sequencing of KCNJ5 in 45 APA samples

[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 wild-type (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.

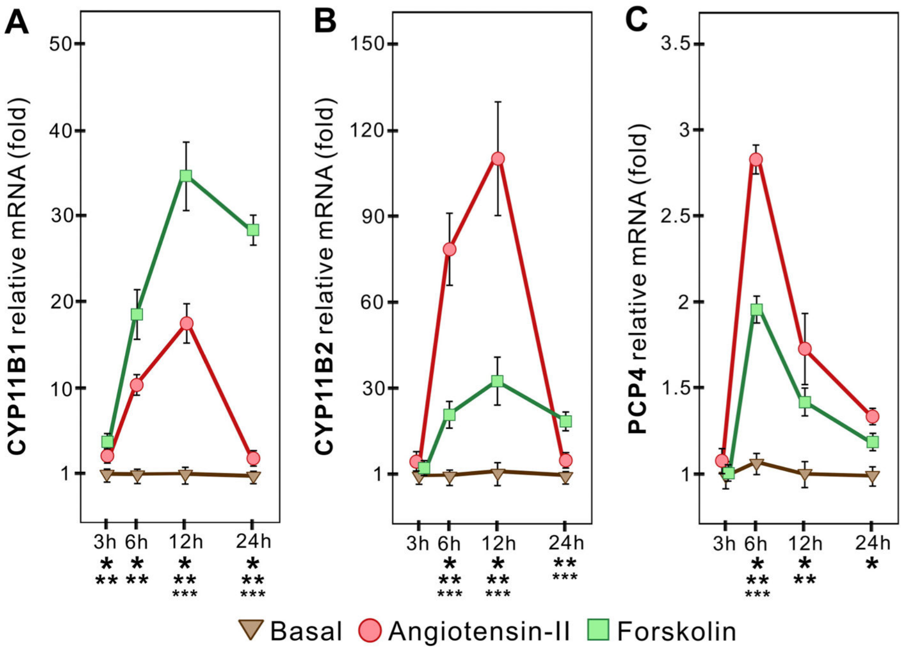

FIG. 3. PCP4, CYP11B2, and CYP11B1 drug treatment time course in H295R cells

[A] CYP11B1 mRNA levels after treatment with angiotensin-II and forskolin in time-dependent manner. [B] CYP11B2 mRNA levels after treatment with angiotensin-II and forskolin. [C] PCP4 mRNA levels after treatment with angiotensin-II and forskolin. *P<0.01 (angiotensin-II vs. basal); **P<0.01 (forskolin vs. basal); ***P<0.01 (angiotensin-II vs. forskolin).

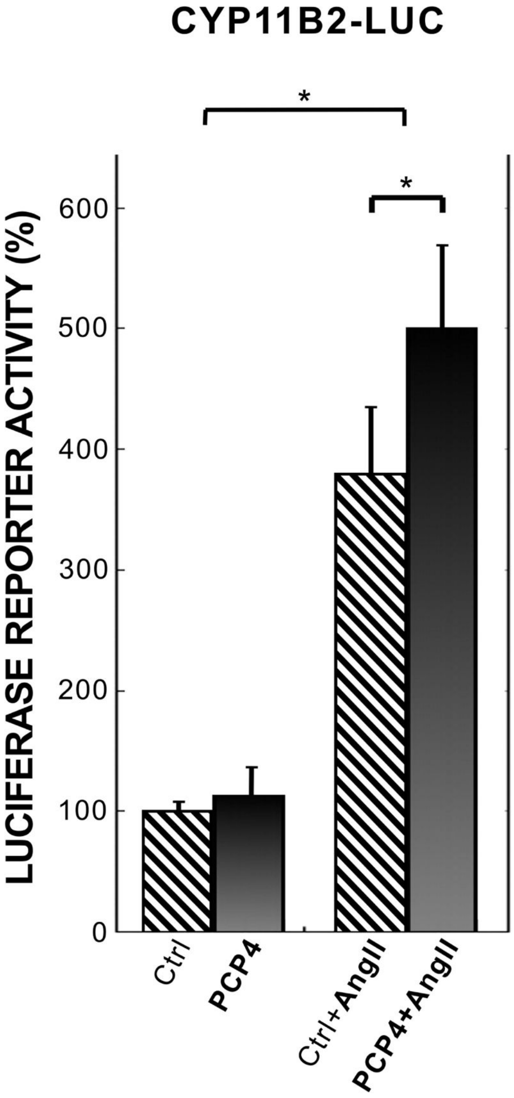

FIG. 4. PCP4 transient DNA transfection and luciferase assays

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

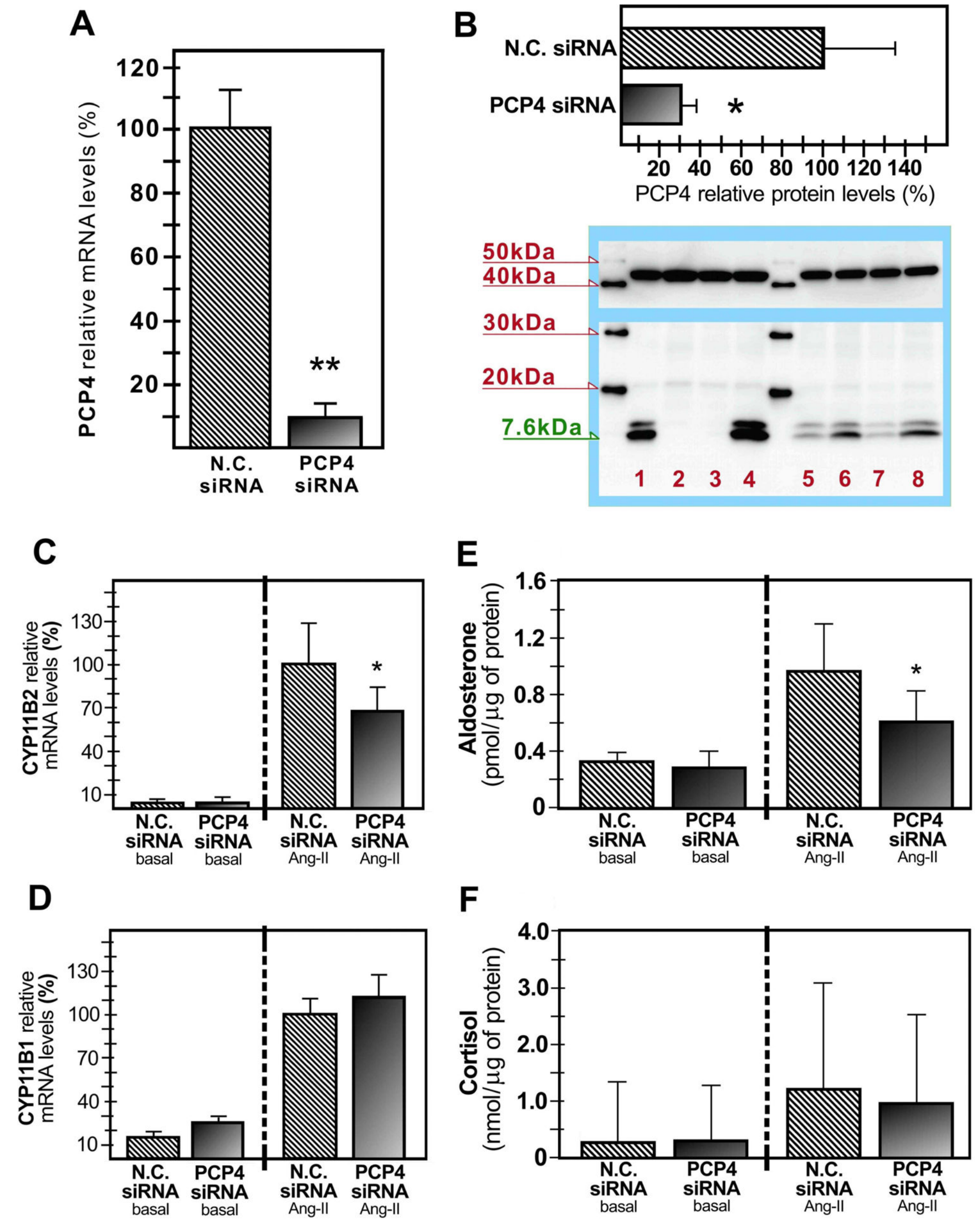

FIG. 5. Transient PCP4 siRNA transfection

[A] After 48h from transfection with PCP4 siRNA, the mRNA levels of PCP4 in H295R cells decreased by 90% compared to negative control (N.C.) siRNA (mean, N.C. 100%±13; PCP4 siRNA 10%±4 s.d.; ** $P<0.0001$). **[B]** Immunoblotting analysis revealed a mean of 70% decrement in PCP4 protein levels (N.C. 100%±36; PCP4 siRNA 30%±7 s.d.; *$P<0.05$); the bands between 40 and 50kDa represent beta-actin, while the bands below 20kDa in the expected band-size of 7.6kDa 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 N.C. siRNA. **[C]** CYP11B2 mRNA levels 60h after transfection: 2 groups of cells were submitted to angiotensin-II (Ang-II) treatment for 12h (N.C. siRNA and PCP4 siRNA) while 2 groups remained as basal controls (no drug treatment); the comparison of Ang-II treated cells reveals a decrement of CYP11B2 mRNA levels in PCP4 siRNA treated cells (mean 30%; N.C. 100%±30; PCP4 siRNA 70%±13 s.d.; * P=0.012). **[D]** CYP11B1 mRNA levels 60h after transfection: no statistical significance could be detected between PCP4 and N.C. siRNA treated cells. **[E]** aldosterone levels were significantly lower in PCP4 siRNA transfected cells after 48h of angiotensin-II (Ang-II), compared to negative control (N.C.) 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 N.C. siRNA, either in Ang-II treated or non-treated samples.