Role of D2 dopamine receptor in adrenal cortical cell proliferation and aldosterone-producing adenoma tumorigenesis

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Abstract

Aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia are the two characteristic types of primary aldosteronism. Dysregulation of adrenal cortical cell proliferation contributes to both diseases. We previously demonstrated that APA expressed less dopamine D2 receptor than the respective non-tumor tissue and might contribute to the overproduction of aldosterone. As activation of D2 receptor inhibits the proliferation of various cells, downregulation of D2 receptor in APA may play a role in the tumorigenesis of APA. In this study, we demonstrate that D2 receptor plays a role in angiotensin II (AII)-stimulated adrenal cortical cell proliferation. The D2 receptor agonist, bromocriptine, inhibited AII-stimulated cell proliferation in primary cultures of the normal human adrenal cortex and APA through attenuating AII-induced phosphorylation of PKC-stimulated cyclin D1 protein expression and cell proliferation. D2 receptor also inhibited AII-induced ERK1/2 phosphorylation. Our results demonstrate that, in addition to inhibiting aldosterone synthesis/production, D2 receptor exerts an anti-proliferative effect in adrenal cortical and APA cells by attenuating PKC and ERK phosphorylation. The lower level of expression of D2 receptor in APA may augment cell proliferation and plays a crucial role in the tumorigenesis of APA. Our novel finding suggests a new therapeutic target for primary aldosteronism.

Key Words

- dopamine receptor
- aldosterone-producing adenoma
- protein kinase C
- cell proliferation

Introduction

There is growing evidence that the prevalence of primary aldosteronism is much higher than previously thought (Rossi et al. 2006). The two main subtypes of primary aldosteronism are bilateral adrenal hyperplasia and aldosterone-producing adenoma (APA). The pathogenesis of both diseases involves the over-proliferation of adrenal cortical cells. However, the molecular mechanism remains unclear. Although angiotensin II (AII) stimulates aldosterone production through the angiotensin type 1 receptor (AT1R), its effect on the proliferation of the aldosterone-producing cells is controversial (McEwan et al. 1990, Mazocchi et al. 1997, Otis et al. 2004, 2005). There was no difference in the level of AT1R between APA and the non-tumor part of the adrenal (Chang et al. 2007), and a linkage study failed to demonstrate a role of AT1R in the pathogenesis of primary aldosteronism (Torpy &
Subjects and methods

Patients and tissue samples

The diagnosis of APA was established by clinical manifestations of primary aldosteronism and confirmed histologically. Adrenal samples were obtained from surgical specimens of 12 APA patients and three patients with renal cell carcinoma without adrenal disease, after obtaining informed consent. Adrenal glands from APA patients were separated into tumor and non-tumor portions after the adrenal medulla was dissected. Nodules surrounding the main tumor were not included in the non-tumor portion. The study was approved by the Ethical Committee of the National Taiwan University Hospital (NCT00173446).

Cell culture

Primary cultured cells from human normal adrenal cortex (NAC) were prepared from surgical specimens of patients who were free from adrenal disease. The primary NAC culture was prepared as described by Liu et al. (1995). Briefly, small pieces of tissue were washed in Hank's Balanced Salt Solution and minced in culture medium (DMEM–Ham’s F-12 medium containing 10% FCS, 2 mmol/l glucose (Gibco), 1.25 x 10^5 IU/l penicillin, and 0.125 g/l streptomycin sulfate (Gibco)). The minced tissues were dissociated with 0.3% collagenase-IA and 20 mg/l deoxyribonuclease-I (Sigma–Aldrich Co.) in culture medium at 37 °C. Digestion was carried out over two 2-h periods. The dispersed cells were washed once with medium and plated on six-well plastic cell culture dishes. The cultures were maintained at 37 °C in humidified 95% air at 5% CO2, with replacement of medium every 3 days until the cells achieved subconfluency on days 5–10 of culture. The NAC cells cultured at this stage expressed both DRD2 and AT1R and preserved the physical function to secrete aldosterone after addition of AII. Ten nanomolar AII (Sigma–Aldrich Co.), 1 μmol/l bromocriptine (BMC) (Sigma–Aldrich Co.), and 1 μmol/l raclopride (Racl) (Sigma–Aldrich Co.) were used in all experiments unless otherwise mentioned. All experiments were performed at least in triplicate; for each experiment, the data analyzed was the mean of three measured samples.

Chemicals

AII, BMC, Rac, tetrazole 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and PD98059 were purchased from Sigma–Aldrich Co. and phospho-PKC-specific antibodies, PKC subtype-specific antibodies, and phospho-MAPK-specific antibodies from Cell Signaling Technology, Inc. (Danvers, MA, USA). AT1R (sc1173), P21 (sc817), P27 (sc1641), and P53 (sc71784) were obtained from Santa Cruz Biotechnology, Inc. D2 receptor antibody and its immunizing peptide were from Chemicon (Temecula, CA, USA). Cyclin D1 (NB110-2308) antibody was from Novus Biologicals, Inc. (Littleton, CO, USA).

Immunoblotting

APA and NAC were separated from surgical specimens and solubilized in lysis buffer. For western blots, equal amounts of protein (10 μg for human tissue) were separated on a 10% polyacrylamide gel and were transferred to Immobilon P membranes. Blots were probed with different antibodies, followed by a HRP-conjugated anti-rabbit secondary antibody. Immunoreactive proteins were visualized with enhanced chemiluminescence (Pierce, Rockford, IL, USA).
MTT test
The MTT cell proliferation assay was purchased from the American Tissue Culture Collection (Manassas, VA, USA) and performed according to the manufacturer’s instructions. Briefly, NAC or APA cells were incubated in the presence of 0.5 mg/ml MTT reagent for 4–8 h, after which cells were lysed by the addition of one volume of solubilization solution (40% dimethylformamide and 10% SDS, pH 4.0). Absorbance (A) in the wells was read with a microplate reader with a 570 nm cutoff filter. Viable cells metabolizing MTT were quantified at 570 nm by spectrophotometer and then translated into the cell number by normalizing the measurement against the 570 nm absorption of a known number of directly countable viable cells.

Quantitative real-time PCR
Quantitative real-time PCR was carried out in an Applied Biosystems 7900 Real-Time PCR System using TaqMan gene expression assays for AT1R and DRD2 as described in the user manual (assay ID, Hs00258938_m1 and Hs00241436_m1 respectively). GAPDH (assay ID, Hs99999905_m1) was used as an endogenous control in the TaqMan human endogenous control plate (Applied Biosystems; Volpe et al. 1997). Cycle to threshold (Ct) was recorded for statistical analysis. Sample dilutions contained 100 ng template cDNA. All samples were tested in a total volume of 20 μl in triplicate.

Short hairpin RNA (shRNA) for PKCμ
The sense strand for synthesis of shRNA targeting PKCμ was CCAGAGCACATAACGAAGTTT. The control plasmid expressing an shRNA against EGFP was purchased from Open Biosystems, Inc. (Huntsville, AL, USA). Lentiviruses carrying the target nucleotides were generated by cotransfecting 9 μg lentiviral vector (Plko1), 4.5 μg pHCMV-G (envelope system: VSV-G), and 6.75μg of pCMVΔR8.2 (packaging vector: env, tat, rev, vpr, vpu, vif, and nef) into 293T cells using calcium phosphate reagent (2×PBS 750 μl, 2.5 M CaCl2 75 μl, and H2O 750 μl) (Dull et al. 1998). Supernatants were collected 48 and 72 h after transfection, filtered through a 0.45 μm membrane (Amicon Ultra-15 100K, Millipore, Billerica, MA, USA), centrifuged at 4000 g for 30 min at 4 °C, and used to directly infect NAC. The level of PKCμ was semi-quantified by SDS–PAGE and immunoblotting with a PKCμ-specific antibody. The experiments were carried out 48 h after infection.

Bromodeoxyuridine assay
Cell proliferation was determined by bromodeoxyuridine (BrdU) incorporation analysis. NAC cells were plated in 96-well plates (100 cells/well, counted with a hemocytometer). After incubation for 12 h, BrdU was added to the medium for 2 h. (HTS01, Calbiochem EMD Chemicals, Inc., Billerica, CA, USA). Plates were analyzed using a fluorometer at 320 nm excitation and 460 nm emission.

Cell apoptosis assay
DNA fragmentation was detected by an ELISA specific for nucleosome-associated cytosolic DNA. Apoptosis ELISA was performed using the Roche Cell Death Detection ELISA Kit (Roche Diagnostics). This assay is based on the sandwich-enzyme immunoassay principle using mouse MABs directed against DNA and histones respectively. This technique allows the specific determination of mononucleosomes and oligonucleosomes in the cytoplasmic fraction of the cell. Cell lysates isolated as described above were used for ELISA. Sample absorbance was read with a spectrophotometer at a wavelength of 405 nm.

Statistical analysis
Statistical analysis was performed with the Mann–Whitney U test using the Stat View Software Package (Abacus Concepts, Inc., Berkeley, CA, USA). Statistical significance was accepted for P<0.05. All the statistical analyses were aided by the advice of the statistics specialist of the Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine.

Results

DRD2 activation attenuated All-induced cell proliferation
Three cell populations of APA and three clones of NAC were obtained from APA and renal cell carcinoma patients respectively. The viability of the cells was assayed using the MTT test at 4, 8, 12, 24, and 48 h after subculture with 2000 cells in each well of 96-well plates. The increase in the number of viable cells after 24 h is illustrated in Fig. 1a. Under all conditions, treated or untreated, the cell numbers for APA were greater than those for NAC (P<0.01). After 24-h culture, the increased cell number for APA was 30% more than that for NAC cells. All doubled the cell numbers of both APA and NAC, but the difference remained 30%. Treatment with DRD2 agonist (BMC) attenuated the All-stimulated viable cell number of both NAC and APA (P<0.005). The BMC effect...
could be partially reversed by DRD2 antagonist (Racl) for both NAC and APA, 12 and 23% respectively ($P < 0.05$).

Our previous study showed that DRD2 was expressed much less in APA than in the respective non-tumorous adrenal cortex. Whether DRD2 has a role in the regulation of the proliferative adrenal cortical cells should be clarified. Treatment with BMC (1 μmol/l) attenuated the AII-induced increase in cell number (Fig. 1a and b) but did not alter the basic growth rates for either NAC or APA cells. This attenuating effect of BMC was more obvious in APA than in NAC cells and was partially reversed by treatment with Racl (Fig. 1a and b). The rate of cell proliferation decelerated when cells became confluent (Fig. 1b).

In order to understand whether the increase in the number of viable cells stimulated by AII resulted from increased cell proliferation or decreased cell death, BrdU incorporation was performed to measure the rate of DNA synthesis, which is known to parallel the rate of cell division. Because the significant differences in the numbers of the viable cells were observed by 24 h after treatment, the cell division rates were measured at 12 h. Treatment with All significantly enhanced BrdU incorporation of NAC cells; this effect was attenuated by BMC and was partially reversed by adding Racl (Fig. 1c). On the other hand, in the apoptosis experiments, the accumulations of BrdU-incorporated DNA fragments in cell extracts at 24 h were not affected by the experimental conditions (Fig. 1d); additionally, no differences were observed in the supernatants (data not shown). These results indicate that the effect of BMC on the All-stimulated increase in cell viability was due to the inhibition of cell proliferation but not the induction of cell apoptosis.

**DRD2 level in APA, non-tumorous tissue, and in the NAC**

Consistent with our previous study, the level of DRD2 in APA was much lower than in the respective non-tumorous adrenal cortex or NAC. The levels of AT1R were not different among...
the three different tissue samples (Fig. 2a). The mRNA expression of DRD2 and AT1R in these three different samples paralleled the findings from western blotting, namely that DRD2 mRNA level was downregulated in APA, and no difference in AT1R mRNA expression was observed, in comparison with the non-tumor portion and NAC (Fig. 2b).

**Effect of DRD2 on PKC\(\mu\) phosphorylation and cell proliferation**

As demonstrated in our previous study, APA expressed more abundant phosphorylated PKC\(\mu\) than the non-tumor portion or NAC (\(P<0.05\); Fig. 3a). The levels of total PKC\(\mu\), total PKC\(\alpha\), total PKC\(\beta\)II, and phosphorylated PKC\(\alpha/\beta\)II were not different among three types of tissue samples however. In primary cultured cells of NAC, AII increased the phosphorylation of PKC\(\mu\) (Ser916), PKC\(\mu\) (Ser744/748), PKC\(\alpha/\beta\)II, and PKC\(\alpha\), but not PKC\(\alpha\) or PKC\(\gamma\). BMC treatment attenuated only the AII-induced PKC\(\mu\) (Ser916) phosphorylation, which was reversed by adding Racl (Fig. 3b). Depletion of PKC\(\mu\) protein expression in NAC via transfection of targeted shRNA reduced the level of AII-stimulated cell proliferation by over 50% (Fig. 3c).

**DRD2 and MAPK phosphorylation**

There was a higher level of phospho-ERK in APA than in non-tumor tissue and NAC; however, there was no difference in the levels of phospho-JNK and phospho-p38 among these tissue samples (Fig. 4a).

To investigate whether the anti-proliferative effect of DRD2 is mediated by modification of MAPK activation, the effects of BMC on the phosphorylation of ERK1/2, JNK, and p38 were studied. All stimulated the phosphorylation of these three molecules, but only the phosphorylation of ERK1/2 was attenuated by BMC (Fig. 4b), more than 30%. PD98059, an inhibitor of ERK phosphorylation, dose dependently inhibited AII-stimulated cell proliferation (Fig. 4c). This result indicates that the inhibitory effect of DRD2 on AII-induced proliferation of NAC cells was mostly due to the attenuation of ERK phosphorylation. Depletion of PKC\(\mu\) reduced AII-stimulated ERK phosphorylation by more than 50% (Fig. 4d).

**Cyclin D1 as a target for AII-stimulated cell cycle progression**

All treatment increased cyclin D1 protein expression in NAC cells, and BMC significantly attenuated it; this effect was reversed by adding Racl (Fig. 5a). Depletion of PKC\(\mu\) by infection with lentiviruses PKC\(\mu\) shRNA reduced protein expression of AII-induced cyclin D1 by 30% (Fig. 5b). Inhibition of ERK phosphorylation by PD98059 also decreased AII-stimulated cyclin D1 protein expression (Fig. 5c).

**Discussion**

PKC\(\mu\) (PKD) has been proven to play a role in AII-induced aldosterone production (Romero et al. 2006, Shapiro et al. 2010). In a previous study, we demonstrated that DRD2 inhibits AII-stimulated aldosterone synthesis/secretion through attenuation of PKC\(\mu\) phosphorylation.
In this study, we further found that DRD2 inhibited AII-stimulated proliferation of NAC and APA cells, and that phosphorylation of PKCε was responsible for the signaling mechanism.

In fact, we cannot separate the zona fasciculata (ZF) cells from zona glomerulosa (ZG) cells completely as many previous reports. It is highly possible that some of our cultured cells were ZG cells and some were ZF cells. However, there were over 70% CD56-positive cells in our primary cultured NAC and APA cells. It has been noted that ZG cells rather than ZF cells expressed CD56 (Caroccia et al. 2010). In addition, the mRNA expressions of CYP11B2 over CYP11B1 were about 6.86 and 8.78 in our primary cultured NAC and APA cells respectively, indicating that most of the cultured cells were ZG cells. In a previous report (Greco et al. 2002), AII also stimulates the proliferation of choriocarcinoma cells through the AT1R and its downstream signals, including PKC and MAPK (Ino et al. 2003). The mitogenic response of adrenal glomerulosa cells to AII is mediated via AT1R activation (McEwan et al. 1990, Tian et al. 1995). However, studies analyzing the expression of AT1R in APA have not shown significant differences in AT1R mRNA levels in comparison with non-tumorous tissue (Kitamura et al. 1998, Chang et al. 2007). In our previous study, we found that AII-stimulated PKCζ/βII, PKCε, and PKCμ phosphorylation and translocation to membrane in human adrenal cortical cells and DRD2 activation only attenuated AII-stimulated PKCμ phosphorylation and translocation to membrane (Chang et al. 2007). Therefore, the tumorigenesis of APA can be partially attributed to the loss of DRD2 inhibitory tone under concomitant stimulation by AII. An interactive balance between DRD2 and AII signaling appears to underlie the regulation of adrenal cortical cell proliferation.

In this study, we demonstrated that the inhibitory effect of DRD2 on adrenal cortical cell proliferation was mediated by attenuation of PKCμ phosphorylation. PKCμ is involved in many physiological functions including cell proliferation, migration, protein transport, survival, and apoptosis (Wang 2006). Over-expression of PKD1, the mouse homolog of PKCμ, has been noted in mouse skin
Dopamine receptor in primary aldosteronism

Figure 4

(a, Upper panel) Immunoblotting of phosho-MAPK expression, lanes 1–5, APA; lanes 6–10, corresponding non-tumor tissues; lanes 11–13, NAC; (a, lower panel) quantification of the immunoblotting results by densitometry, results for APA (black bars), the corresponding non-tumorous tissue (gray bars), and human normal adrenal cortex (NAC, white bars). *P < 0.05 vs non-tumor portion and NAC. (b) Immunoblotting of MAPK phosphorylation in primary cultured human adrenal cortical cells 5 min after various treatments; C, control; AII, 10 nM angiotensin II; BMC, 1 µM bromocriptine; Rac1, 1 µM raclopride. †P < 0.05 vs AII. (c) Dose effect of PD98059 on induction of cell proliferation at 24 h after AII treatment (10 nM) in primary cultured NAC cells; §P < 0.05, vs PD98059 treatment (10 nM) (d) Immunoblotting of phospho-ERK1/2 expression in primary cultured human adrenal cortical cells. WT, vector: the vector for expression of PKCθ; shRNA. †P < 0.05, vs All experiments have been performed in at least triplicate.

carcinoma (laccarino et al. 2002) as well as in basal cell carcinoma and psoriatic skin lesions (Ristich et al. 2006). Inhibition of PKCθ by Go6976 decreased the proliferation of primary mouse epidermal keratinocytes (Hausser et al. 2001). VEGF-induced angiogenesis of primary human umbilical vein endothelial cells has been shown to require PKCθ phosphorylation (Rennecke et al. 1999). In Swiss 3T3 cells, over-expression of PKCθ potentiates DNA synthesis and cell growth induced by bombesin and vasopressin (Zhukova et al. 2001, Sinnett-Smith et al. 2004). Our study showed a greater level of phosphorylated PKCθ in tumor areas of APA than in non-tumor areas or in the NAC. The inhibition of All-stimulated cell proliferation by depletion of PKCθ indicates the involvement of PKCθ in the mitogenesis of adrenal cortical cells. Therefore, the decreased DRD2 level in APA may augment All-stimulated PKCθ phosphorylation and in turn contribute to APA tumorigenesis.

ERK1/2 is a signaling molecule that responds to several mitogenic stimuli (Werry et al. 2006, Katz & Yarden 2007). We demonstrated that AII stimulated the phosphorylation of ERK1/2, JNK, and p38 in human adrenal cortical cells, but only ERK1/2 was modulated by DRD2. The effects of DRD2 on MAPK/ERK activation are controversial. In mouse endothelial cells, dopamine inhibits VEGF-induced phosphorylation of VEGFR2, MAPK, and focal adhesion kinase (Sarkar et al. 2004). However, laccarino et al. (2002) observed increased phosphorylation of ERK1/2 in lactotroph cells at 15 min after addition of BMC, followed by a return to the baseline levels at 16 h. Over-expression of the short variant of DRD2 in mice led to the activation of MAPK and levels of phosphorylated ERKs decreased in DRD2-null mice (Asa et al. 1999). Despite the opposite effect of MAPK activation, ligand activation of DRD2 results in decreased proliferation of lactotroph cells and leads to pituitary hypoplasia (laccarino et al. 2002). In addition, ablation of DRD2 resulted in hyperprolactinemia and prolactinomas (Asa et al. 1999). The current study showed that a D2 agonist selectively attenuated

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AII-stimulated phosphorylation of ERK1/2 but not JNK and p38 in human adrenal cortical cells. In comparison with NAC, APA showed downregulation of DRD2 that was accompanied by the elevated phosphorylation of PKC$_\mu$ and ERK1/2. Therefore, loss of the inhibitory effect of DRD2 on AII-stimulated PKC$_\mu$ and ERK1/2 phosphorylation may play a role in the tumorigenesis of APA.

There is evidence for the relationship between PKC$_\mu$ and ERK1/2. Transient expression of constitutively active PKC$_\mu$ led to activation of Raf-1 kinase and phosphorylation of ERK1 but not of JNK and p38 MAPKs (Hausser et al. 2001). PKC$_\mu$ may also mediate endothelial proliferation and ERK activation induced by vascular endothelial growth factor (Wong & Jin 2005). PKC$_\mu$ upregulates Ras activity and ERK1/2 signaling by phosphorylating Ras-binding protein RIN1 to release Ras for subsequent activation in the Raf–MEK–ERK pathway (Lint et al. 1995, Wang et al. 2002). In this context, the anti-proliferative effect of DRD2 in adrenal glomerulosa cells probably resulted from the attenuation of PKC$_\mu$ and ERK1/2 phosphorylation.

Some may argue that the rennin–angiotensin system is blunt in primary aldosteronism and therefore the exploration of the role of DRD2 in the proliferogenic response to AII is odd. Although the RAS activity may be lower in PA, the AII-responsive subtype of APA is well documented (Gordon 1995) and characterized by an increase in aldosterone secretion on assuming an upright posture and after ambulation. Our previous study has shown an inverse correlation of DRD2 level in APA with PAC and CYP11B2 mRNA expression. This finding indicates that the different amount of DRD2 may contribute to the heterogeneous sensitivity to AII stimulation in APA (Chang et al. 2007). It is possible that downregulation of DRD2 may increase the sensitivity of the proliferation response to aldosterone-producing cells under the same plasma concentration of AII. As the tumor grew and plasma aldosterone increased, AII decreased in some of these patients, but not all. The reduced AII stimulation slows the tumor growth rate to a steady-state condition. Besides, the AT1R level is not reduced in APA (Chang et al. 2007) and some of APA patients do not have reduced plasma AII levels. Therefore, AII could retain its stimulating effect on the proliferation of these aldosterone-producing cells.

In summary, similar to its inhibition aldosterone synthesis/production, DRD2 also antagonizes the AII proliferating effect of adrenal glomerulosa cells as well as APA. PKC$_\mu$ and ERK1/2 are the main signaling pathways mediating this anti-proliferative effect. Our findings provide a new therapeutic target for the medical treatment of human APA and perhaps other types of primary aldosteronism.
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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