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YPEL4 modulates HAC15 adrenal cell proliferation and is associated with tumor diameter

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ABSTRACT

Yippee-like (YPEL) proteins are thought to be related to cell proliferation because of their structure and location in the cell. The aim of this study was to clarify the effects of YPEL4 on aldosterone production and cell proliferation in the human adrenocortical cell line (HAC15) and aldosterone producing adenoma (APA). Basal aldosterone levels in HAC15 cells over-expressing YPEL4 was higher than those of control HAC15 cells. The positive effects of YPEL4 on cell proliferation were detected by XTT assay and crystal violet staining. YPEL4 levels in 39 human APA were 2.4-fold higher compared to those in 12 non-functional adrenocortical adenomas, and there was a positive relationship between YPEL4 levels and APA diameter ($r = 0.316$, $P < 0.05$). In summary, we have demonstrated that YPEL4 stimulates human adrenal cortical cell proliferation, increasing aldosterone production as a consequence. These results in human adrenocortical cells are consistent with the clinical observations with APA in humans.

1. Introduction

Aldosterone plays a significant pathophysiological role in hypertension and cardiovascular diseases. It is synthesized in the zona glomerulosa of the adrenal and is primarily under the regulation of the renin-angiotensin system (Hattangady et al., 2012). A major site of action of aldosterone is in the distal tubules of the kidney, where it promotes sodium absorption and potassium (K^+) excretion. Angiotensin II (A-II) binds to the angiotensin receptor (AT1R) in the adrenal cortex to stimulate the production of aldosterone; K^+ ions directly stimulate aldosterone production independently of the RAA system (Pralong et al., 1992).

The binding of A-II to the AT1R triggers several intracellular signaling cascades including protein kinase C, calcium/calmodulin-dependent kinases, and mitogen-activated protein kinase (MAPK) (Côté et al., 1998; Hattangady et al., 2012; Hunyady et al., 2004; Otis et al., 2005). The concentration of extracellular K regulates calcium influx into zona glomerulosa cells and activates calcium/calmodulin-dependent kinases (Pralong et al., 1992). Importantly, some of the cascades stimulated by A-II may cause adrenal cell growth or proliferation as well as aldosterone production. Although we and other researchers have investigated mechanisms of adrenal cell growth and aldosterone production (Côté et al., 1998; Hunyady et al., 2004; Otis et al., 2005; Romero et al., 2010; Romero et al., 2007a, 2007b), the pathways have not been fully elucidated.

The human yippee-like (YPEL) gene family consists of 5 genes, YPEL1 to YPEL5 cloned in 2004 (Hosono et al., 2004). These proteins are located at the centrosome, adjacent to nucleolus and mitotic apparatus, and are ubiquitously expressed in all eukaryotes (Hosono et al., 2010; Hosono et al., 2004). Therefore, the YPEL family proteins are likely to have role in cell life cycle. We previously suggested that YPEL4 is one of the most up-regulated mRNA after A-II or K^+ stimulation in rat adrenal cells (Romero et al., 2007a, 2007b), however the roles of YPEL4 in the adrenal gland has not been studied.

We hypothesized that YPEL4 has a role in regulating aldosterone production and/or adrenal cell proliferation. Thus, the aim of our study was to investigate the effects of YPEL4 on aldosterone production and cell proliferation in the human adrenal cortical cell line, HAC15. Herein, we report that YPEL4 potentiates aldosterone production by increasing cell proliferation. We also found that

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YPEL4 expression in aldosterone-producing adenomas (APA) from patients was 2.4-fold higher compared with its expression in non-functioning adrenocortical adenomas (NF) and there was a positive relationship between YPEL4 expression levels and tumor diameter in APA.

2. Methods

2.1. Cell culture and materials

The HAC15 human adrenocortical carcinoma cell line, a subclone of the H295R (Parmar et al., 2008), was provided by W. E. Rainey (University of Michigan). The HAC15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM):F12 (1:1) supplemented with 10% Cosmic Calf serum (HyClone, Logan, UT) at 37C under a humid atmosphere of 95% air and 5% CO2. A-II and potassium chloride were purchased from Sigma Aldrich Co. Ltd. (St. Louis, MO).

2.2. Plasmids

The full length cDNA of YPEL4 was ligated downstream of the cytomegalovirus promoter of a feline immunodeficiency virus based lentivector (System Biosciences, Mountain View, CA), resulting in pCDF-YPEL4. Control plasmid was prepared as pCDF without YPEL4. pCMV-VSV-G [pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid # 8454)], and pCPRDEenv [pCPRDEnv was a gift from Garry Nolan (Addgene plasmid # 1732)] for virus production were obtained from Addgene.org. (Cambridge, MA).

2.3. Lentiviral production and infection

293 TN cell line (System Biosciences, Mountain View, CA) were cultured in DMEM supplemented with FetalClone II serum (HyClone, Logan, UT) until 60–70% confluent, then transfected with lentiviral vector, pCMV-VSV-G, and pCPRDEev (0.28 μg/cm^2, 10:8:5 M ratio) using linear PEI25000 transfection reagent (4.8 μl/μg DNA, Polysciences, Inc Warrington, PA). Medium was replaced after overnight incubation, and then cells were cultured for an additional 48 h when the supernatant from cell culture was collected and centrifuged at ×1500g and 4C for 30 min. This supernatant was mixed with 80 μg/ml of polybrene and 10 μg/ml of chondroitin, and after 30 min centrifuged at 6000 × g and 4C for 45 min (Le Doux et al., 2001). After aspiration of the supernatant, the virus was reconstituted with DMEM/F12 without phenol red (Invitrogen, Carlsbad, CA).

Transduction with YPEL4 or control lentivirus were performed as described previously at 24 h after seeding the HAC15 cells, and the cells were cultured for 72 h after transduction (Romero et al., 2007a, 2007b). followed by serum deprivation with DMEM:F12 supplemented with 0.1% Cosmic Calf serum for 24 h to assess the effect of YPEL4 overexpression on aldosterone production and mRNA expression. The medium was replaced with fresh DMEM:F12 containing 0.1% Cosmic Calf serum with or without various combination of A-II or K^+ at indicated concentrations for another 24 h. Second, to investigate the effect of YPEL4 on cell proliferation and aldosterone production, the cells were selected with 2.0 μg/ml of puromycin. The cells were cultured for at least 4 weeks in the absence of selecting agent before performing the experiments to avoid any confounding effect due to the antibiotic selection.

2.4. Patients and tissue collection

The diagnosis of PA, subtype diagnosis and non-functional adenomas (NF) were performed as previously reported (Matsumoto et al., 2015; Oki et al., 2012a, 2012b), based on the guidelines from the Japan Endocrine Society (Nishikawa et al., 2011). We enrolled 51 consecutive patients (12 NF and 39 APA) for the pathological study. Written informed consent was obtained from all subjects. Our study was approved by the ethics committee of Hiroshima University. Tissue samples were immediately preserved in RNAlater (Life Technology, Delhi, India) and stored at −80 °C until assayed, or fixed in formalin and embedded in paraffin for immunohistochemical analysis. APA was confirmed by detecting the expression of *CYP11B2* by immunohistochemistry and/or quantitative polymerase chain reaction (qPCR) assays as previously reported (Gomez-Sanchez et al., 2014).

2.5. RNA extraction and RT-PCR

Total RNA was extracted with the RNAzol-RT Reagent (Molecular Research Center, Inc., Cincinnati, OH). For reverse transcription, 2.5 μg of total RNA was incubated with SUPERase-In (Applied Biosystems/Ambion, Austin, TX) and SuperScript III (Invitrogen, Carlsbad, CA) following the manufacture's protocol.

Table 1 shows real-time PCR primers designed to generate amplicons of approximately 100-bp as we previously reported (Oki et al., 2012a, 2012b). Aldosterone synthase (CYP11B2) and GAPDH mRNA expression were determined by the Taqman Gene expression assay as previously reported (Romero et al., 2010). mRNA expression of steroidogenic acute regulatory protein (STAR), cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1), 3β-hydroxysteroid dehydrogenase (HSD3B2), cytochrome P450, family 21, subfamily A, polypeptide 2 (CYP21A2), YPEL1-4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified in 1 μl RT product, 1 μl Titanium Taq DNA polymerase (Clontech, Mountain View, CA), 1:20 000 dilution SYBR Green I (Molecular probes, Carlsbad, CA), 10 nM Fluorescein (Bio-Rad, Hercules, CA), 0.2 mM dNTPs, and 0.1 μM of each primer. Real time data were obtained during the extension phase and critical threshold cycle values were calculated on the log phase of each gene amplification curve. Gene expression levels were analyzed as arbitrary units normalized against GAPDH mRNA expression.

2.6. Cell proliferation and viability assay

The XTT cell proliferation assay kit (American Type Culture Collection, Manassas, VA) was used to evaluate the effect of YPEL4

Table 1
Real-time PCR primers.

Gene symbol	Forward primer	Reverse primer
YPEL1	AAGTCCTTTCAGGGGAGCC	TGAGAAGGACCCTCTCCTCTG
YPEL2	ACTAATTTCCAAGTCATTCCAAGG	CAGTCCTGTTAGCAACACTCG
YPEL3	TCAGGCCTACTTGGATGATTG	ACGTTCACCACTGAGTTGAAGAG
YPEL4	TTAACTCCGTGGTCAACGTG	CCTTCACCATGTGTGACATTTC
YPEL5	TTTCCTTGATCATATCGGTGG	ACTGTACTGCAGGTTAACTACCTTG
GAPDH	CCCCTTCATTGACCTCAACTAC	GATGACAAGCTTCCCGTTCTC