ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Jan; 21 (1): 55 - 59
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Cell proliferation and Ca^{2+}-calmodulin dependent protein kinase activation mediated by α_{1A}- and α_{1B}-adrenergic receptor in HEK293 cells[1]

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KEY WORDS alpha-1 adrenergic receptors; norepinephrine; cell division; Ca^{2+}-calmodulin dependent protein kinase; HEK293 cells; radioligand assay; plasmids; recombinant DNA; propranolol; thymidine

ABSTRACT

AIM: To examine the ability of α_1-AR subtypes on proliferation and Ca^{2+}-calmodulin dependent protein kinase (CCDPK, formerly called MAPK) activation in transfected human embryo kidney 293 (HEK293) cells. **METHODS**: pREP8/α_{1A}-AR, pREP4/α_{1B}-AR, and pREP9/α_{1D}-AR were transfected, respectively, into HEK293 cells by calcium phosphate precipitation. The expression of α_1-AR was detected by radioligand binding assays. DNA synthesis was measured by [^{3}H]thymidine incorporation. CCDPK activity was determined by immunoprecipitation method and myelin basic protein was used as substrate. **RESULTS**: Three clonal HEK293 cell lines stably expressing α_{1A}- or α_{1B}- or α_{1D}-AR were chosen and characterized by radioligand binding assay with receptor densities of about 0.6 nmol·g^{-1}. Treatment with norepinephrine (NE) in the presence of propranolol for 24 h increased DNA synthesis in HEK293/α_{1A}- or HEK293/α_{1B}-AR cells concentration-dependently, with EC_{50} values of 48.8 nmol·L^{-1} (95 % confidence limits 9.7 - 246 nmol·L^{-1}) and 8.4 nmol·L^{-1} (95 % confidence limits 2.1 - 32.9 nmol·L^{-1}), respectively. The increase of DNA synthesis induced by NE 10 μmol·L^{-1} was 201 % ± 28 % and 269 % ± 44 % of basal, and the activation of CCDPK was 171 % ± 84 % and 292 % ± 92 % of basal in HEK293/α_{1A}-AR and HEK293/α_{1B}-AR cells, respectively. Preincubation with prazosin completely abolished NE-induced CCDPK activation in HEK293/α_{1A}- and α_{1B}-AR cells. Those changes were not found in HEK293/α_{1D}-AR cells. **CONCLUSION**: The activation of α_{1A}- or α_{1B}-AR but not α_{1D}-AR induces cell proliferation.

INTRODUCTION

Catecholamines influence vascular smooth muscle cell (VSMC) growth and vascular hypertrophic diseases. The stimulation of α_1-adrenergic receptors (AR) causes proliferation and hypertrophy of VSMC *in vitro* and *in vivo*. Rat and rabbit aorta VSMC which express all three α_1-AR (α_{1A}-, α_{1B}- and α_{1D}-AR) were usually used to determine the effect of stimulation of α_1-AR subtypes on cell growth[1,2]. The effect of catecholamines appears to be mediated via the activation of α_{1B}-AR that tiggers the phosphoinositide hydrolysis and activates the mitogen-activated protein kinase pathway, leading to DNA synthesis and cell proliferation[3]. However, different conclusions were conducted by different authors because of the lack of specific subtype selective agonists and antagonists[4-7]. Chen *et al*[6] found that stimulation of chloroethylclonidine (CEC)-sensitive, possibly α_{1B}-AR induces hypertrophy of rat aorta VSMC and that stimulation of 5-MU-sensitive, non-α_{1B}-AR antagonizes this response. But Xin *et al*[7] in the same laboratory found that VSMC growth induced by norepinephrine (NE) was mediated by α_{1D}-AR that couple to activation of the Ca^{2+}-calmodulin

[1] Project supported by the National Natural Science Foundation of China (№ 93470268) and the grant from China Medical Board of New York Inc (# 93-951).
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Received 1999-03-08 Accepted 1999-05-20

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Jan; 21 (1)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

dependent protein kinase (CCDPK, formerly called MAPK) cascade using a "protection from alkylation" strategy.

The cloning of different AR subtypes has provided new approaches for studying functional properties of AR. To determine whether all three α_1-AR subtypes mediate cell proliferation, we utilized stable expression of recombinant DNA encoding the α_1-AR subtypes and examined the ability of each receptor subtype on proliferation in transfected human embryo kidney 293 (HEK293) cells. CCDPK are activated during proliferation triggered by a variety of stimuli. The activation of CCDPK plays an important role in gene regulation during cell proliferation. To elucidate the signaling pathways through which NE induced cell proliferation, we examined whether NE induces CCDPK activation also.

MATERIALS AND METHODS

Chemicals Norepinephrine (NE), prazosin (Pra), phentolamine, *l*-propranolol, myelin basic protein, sodium orthovanadate, phenylmethylsulfonyl fluoride, aprotinin, and leupeptin were purchased from Sigma. Dulbecco's modified Eagles' medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL Life Technologies Inc and HyClone Laboratories Inc. 2-β-(4-Hydroxyphenyl)-ethylaminomethyl)-tetralone (BE 2254) was purchased from Beiersdorf, Hamburg, Germany; [^{125}I]NaI, [γ-^{32}P]ATP, and [^{3}H]thymidine were purchased form China Institute of Atomic Energy, Beijing. Anti-ERK antibody (SC-94) was from Santa Cruz Biotechnology. PANSORBIN cells were purchased from Calbiochem. All other chemicals were reagents of molecular biology grade and were from standard commercial sources.

Cell culture HEK293 cells were grown as monolayers in DMEM supplemented with 5 % FBS, benzylpenicillin 100 kU·L^{-1}, and streptomycin 0.1 g·L^{-1}, in 5 % CO_2 at 37 ℃.

Expression of recombinant DNA encoding α_1-AR subtypes HEK293 cells and the full length DNA encoding the bovine α_{1A}-, hamster α_{1B}- and rat α_{1D}-AR were subcloned into the expression vectors respectively and kindly provided by Dr Kenneth P Minneman, Emory University Medical School, USA. For stable expression of the α_1-AR, pREP8/α_{1A}-AR, pREP4/α_{1B}-AR, and pREP9/α_{1D}-AR were transfected into HEK293 cells respectively, using calcium phosphate precipitation. Clones resistant to the selective antibiotics were selected and tested for their ability to bind ^{125}I-BE 2254.

Ligand binding assay BE 2254 was radioiodinated to theoretical specific activity[8] and stored in methanol at −20 ℃. Membrane preparation of HEK293 cells stably expressing the α_1-AR and radioligand binding assays using ^{125}I-BE 2254 were performed as described[9]. Phentolamine 10 μmol·L^{-1} was used to determine nonspecific binding. For saturation curve analysis, ^{125}I-BE 2254 concentrations ranged from 12 to 360 pmol·L^{-1}[9]. Data were analyzed by computer, using a nonlinear regression program in GraphPad Prism 2.01 (GraphPad Software Inc, San Diego, CA, USA).

[^{3}H]Thymidine incorporation HEK293 cells were seeded in a 24-well tissue culture cluster at the density of 5×10^4 cells per well and were cultured for 12 h in the presence of 5 % FBS. The cells were then placed in the DMEM without serum for 3 d to render them quiescent. Quiescent cells were incubated with NE (1 nmol·L^{-1} − 10 μmol·L^{-1}) in the present of propranolol 10 μmol·L^{-1} in 1mL FBS-free DMEM for 20 h, then [^{3}H]thymidine 37 MBq·L^{-1} was added for pulse labeling. The medium was aspirated 4 h later, and methanol-acetic acid (3:1) 0.75 mL was added for 1 h to fix the cells. The acetic acid-insoluble material was prepared and the radioactivity was determined by scintillation counting, according to the method of Cornwell TL *et al*[10].

Assay of CCDPK *in vitro*[11] Confluent cells on 90-mm plates were incubated without serum for 2 h and pretreated with prazosin for 30 min. Then the cells were treated with NE for 15 min. After treatment, cultures were rinsed and incubated with 1 mL cell lysis buffer. The cell lysate (400 μL) was incubated with antibody against ERK1 (2 μL) with constant agitation for 1 h and then further incubated with 20 μL PANSORBIN cells with agitation at 4 ℃ for 1 h. The washed immunocomplexes were resuspended in 50 μL kinase buffer and myelin basic protein was as a substrate. The reaction mixture was incubated at 30 ℃ for 10 min and was stopped by spotting it onto P-81 phosphocellulose paper (Whatman), which was then washed and dried. ^{32}P incorporation, which represented the

phosphorylation of myelin basic protein by MAP kinase, was measured by scintillation spectrophotometer. Data are expressed as CCDPK activity (% of Basal), in which unstimulated cells were defined as 100 %.

Data analysis Experimental determinations were performed in 2 to 4 replicate cultures. Values are reported as $\bar{x} \pm s$. The statistical significance between groups was assessed by two-side paired t-test.

RESULTS

Densities of α_1-AR subtypes in transfected HEK293 cells Three clonal HEK293 cell lines stably expressing α_{1A}-, α_{1B}-, or α_{1D}-AR were chosen and characterized by radioligand binding assay. Saturation analysis of the binding of the α_1-AR antagonist ^{125}I-BE2254 gave receptor densities of (650 ± 81) pmol·g^{-1} (n = 5), (690 ± 53) pmol·g^{-1} (n = 5), and (600 ± 119) pmol·g^{-1} (n = 5), respectively.

Effects of α_1-AR subtypes on DNA synthesis in transfected HEK293 cells The basal [^{3}H]thymidine incorporation in HEK293/α_{1A}-AR, HEK293/α_{1B}-AR, and HEK293/α_{1D}-AR cells is (5.2 ±2.8) Bq/well (n = 8), (6 ± 5) Bq/well (n = 5) and (6 ± 2) Bq/well (n = 7), respectively. Treatment with NE (1 nmol·L^{-1} – 10 μmol·L^{-1}) in the presence of propranolol (10 μmol·L^{-1}) for 24 h, increased DNA synthesis in HEK293/α_{1A}- or α_{1B}-AR cells concentration-dependently, as measured by [^{3}H]thymidine incorporation, with EC_{50} values of 48.8 nmol·L^{-1} (95 % confidence limits 9.7 – 246 nmol·L^{-1}) and 8.4 nmol·L^{-1} (95 % confidence limits 2.1 – 32.9 nmol·L^{-1}), respectively, and the maximal responses induced by NE 10 μmol·L^{-1} were 201 % ± 28 % (n = 8) and 269 % ± 44 % (n = 4) of basal in HEK293/α_{1A}-AR and HEK293/α_{1B}-AR cells respectively (P < 0.05, HEK293/α_{1B}-AR *vs* HEK293/α_{1A}-AR). However, there was no statistically significant change in HEK293/α_{1D}-AR cells (Fig 1).

Effects of α_1-AR subtypes on CCDPK activity in transfected HEK293 cells To examine whether α_1-AR stimulate CCDPK, we preincubated HEK293 cell with a blocker of β-AR, propranolol (10 μmol·L^{-1}) for 30 min and then exposed cells to NE 10 μmol·L^{-1} for 15 min. The activation of CCDPK induced by NE is 171 % ± 84 % (n = 6) and 292 % ± 92 % (n = 4) of basal in HEK293/α_{1A}-AR and HEK293/α_{1B}-AR cells respectively. (P < 0.01, HEK293/α_{1B}-AR *vs* HEK293/α_{1A}-AR), but no alteration was found in HEK293/α_{1D}-AR cells compared with basal. Preincubation with prazosin (0.5 μmol·L^{-1}) completely abolished NE-induced CCDPK activation in HEK293/α_{1A}- and α_{1B}-AR cells (Tab 1).

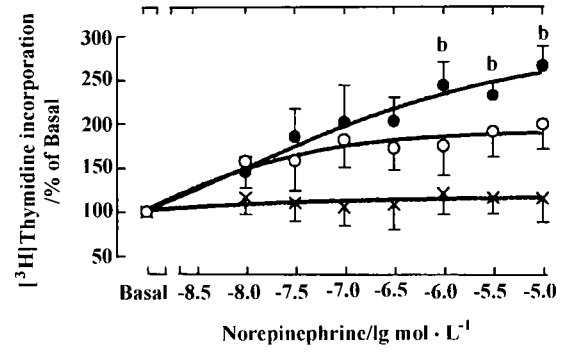

Fig 1. Effects of α_1-AR subtypes on DNA synthesis in transfected HEK293 cells. $\bar{x} \pm s$. n = 5 – 8 experiments. ^bP < 0.05 *vs* HEK293/α_{1A}). ○ HEK293/α_{1A}-AR. ● HEK293/α_{1B}-AR. × HEK293/α_{1D}-AR.

Tab 1. Effect of α_1-AR subtypes on CCDPK activity in transfected HEK293 cells. (NE: norepinephrine, Pra: prazosin, n = 4 – 8 experiments, each experiment with different cells. $\bar{x} \pm s$. ^aP > 0.05, ^bP < 0.05, ^cP < 0.01 *vs* basal. ^eP < 0.05 *vs* NE in HEK293/α_{1A}-AR. ^hP < 0.05 *vs* NE group)

	CCDPK Activity/% of Basal		
	HEK293/α_{1A}-AR	HEK293/α_{1B}-AR	HEK293/α_{1D}-AR
Basal	100	100	100
NE	171 ± 54^b	292 ± 92^{ce}	103 ± 26^a
NE + Pra	116 ± 32^h	133 ± 56^h	116 ± 34

DISCUSSION

The α_1-AR subtypes mediating smooth muscle cells proliferation and the signaling pathway have not been identified, because vascular smooth muscle cells express all three α_1-AR subtypes and because special subtype selective agonists and antagonists are lacking. The respective transfection of α_1-AR subtypes into cell lines is a solution of the above puzzle. HEK293 cells were chosen for expression of α_1-AR subtypes because of the

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Jan; 21 (1)
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ease of transfection and their lack of endogenous α_1-AR. The receptor densities of three clonal HEK293 cell lines stably expressing α_{1A}-, α_{1B}- or α_{1D}-AR are about 0.6 nmol·g^{-1}, which is about 30-fold higher than that of native vascular smooth muscle cells, so the results from this cell line are possibly different from those of native α_1-AR subtypes, but these are suitable to compare with each other.

Since HEK 293 cells express β_2-AR naturally, β-AR selective antagonist propranolol was used to block the β_2-AR in the experiments. Treatment with NE for 24 h, significantly increased DNA synthesis concentration-dependently in HEK293/α_{1A}- or α_{1B}-AR cells. There is no marked alteration in HEK293/α_{1D}-AR cells. So three α_1-AR subtypes are very different in the effect of evoking cell proliferation.

The addition of NE evoked CCDPK activation markedly in HEK293/α_{1A}- and α_{1B}-AR cells, but no alteration in HEK293/α_{1D}-AR cells. Preincubation with prazosin completely abolished NE-induced CCDPK activation. So, the activation of CCDPK by NE was mediated through α_1-AR, and the effects of α-$_1$-AR subtypes on CCDPK activity were paralleled with the effects on DNA synthesis. The partial result is consistent with Della Rocca *et al*'s finding that stimulation of α_{1B}-AR leads to rapid 5 – 10-fold increases in ERK1/2 phosphorylation in HEK293 cells[12].

These data suggest that stimulation of α_{1A}-, α_{1B}-, or α_{1D}-AR transfected in HEK293 cells induce different effects from each other. In the case of similar expression level (about 0.6 nmol·g^{-1}) of three α_1-AR subtypes, the activation of α_{1A}- or α_{1B}-AR induces cell proliferation, with the potency of α_{1B}-AR > α_{1A}-AR, whereas the activation of α_{1D}-AR has no effect on it. The stimulative effect of α_1-AR might be related to the activation of CCDPK.

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α_{1A}-和 α_{1B}-肾上腺素受体介导 HEK293 细胞增殖和 Ca^{2+}-钙调蛋白依赖性蛋白激酶激活[1]

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关键词 α_1 肾上腺素受体；去甲肾上腺素；细胞分裂；Ca^{2+}-钙调蛋白依赖性蛋白激酶；HEK293 细胞；放射配位体测定；质粒；重组 DNA；普萘洛尔；胸苷

目的：研究 α_1-AR 三种亚型对细胞增殖和 Ca^{2+}-钙调蛋白依赖性蛋白激酶(CCDPK)的作用. **方法**：采用磷酸钙沉淀法进行转染，用放射配基结合实验测定 α_1-AR 表达量. 用[^{3}H]胸腺嘧啶参入量测定细胞增殖，用免疫沉淀和髓鞘蛋白底物法测定 CCDPK 的活性. **结果**：三株表达 α_{1A}-，α_{1B}-和 α_{1D}-AR 的细胞株表达受体密度约为 0.6 nmol·g^{-1}. 在普萘洛尔存在下，去甲肾上腺素(NE)作用 24 h 可浓度依赖地刺激 HEK293/α_{1A}-AR 和 HEK293/α_{1B}-AR 细胞 DNA 合成. NE 10 μmol·L^{-1} 可促进 HEK293/α_{1A}-AR 和 HEK293/α_{1B}-AR 细胞 DNA 合成增加，刺激 CCDPK 活性的升高. NE 不引起 HEK293/α_{1D}-AR 细胞 DNA 合成和 CCDPK 活性的显著改变. **结论**：在转染 α_1-AR 亚型的 HEK293 细胞中激动 α_{1A}-或 α_{1B}-AR 可引起细胞增殖，激动 α_{1D}-AR 则无显著改变.

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