Characterization of cAMP accumulation mediated by three α_1-adrenoceptor subtypes in HEK293 cells[1]

SONG Yao, HUANG Yan, DONG Er-Dan, HAN Qi-De, ZHANG You-Yi[2]

Institute of Vascular Medicine, Third Hospital, Peking University,
The Reference Laboratory of Ministry of Education on Molecular Cardiovasology, Beijing 100083, China

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ABSTRACT

AIM: To investigate the characterization of cAMP response mediated by α_1-adrenoceptor (α_1-AR) subtypes in HEK293 cells. **METHODS**: (1) Full-length cDNA encoding three α_1-AR subtypes were transfected into HEK293 cells by the calcium phosphate precipitation method, respectively. (2) The densities of α_1-AR subtypes expressed in HEK293 cells were measured by radioligand binding assay. (3) cAMP accumulation was measured by [^{3}H] adenine prelabeling method. **RESULTS**: (1) Activation of each of three subtypes resulted in an increase of cAMP accumulation in HEK293 cells in a dose-dependent manner, which was inhibited by selective α_1-AR antagonist prazosin. (2) Comparing the pharmacological property, the maximal responses of α_{1A}-AR to agonists were the most potent, while the sensitivity of α_1-AR subtypes to norepinephrine (NE) was the highest. **CONCLUSION:** Each of three α_1-AR subtypes can mediate cAMP accumulation in HEK293 cell line, and there are differences in pharmacological property.

INTRODUCTION

α_1-Adrenoceptor (α_1-AR) is a member of G-protein-coupled receptor superfamily. According to different pharmacological properties, signaling pathways, biological effects, and genetic structures, α_1-AR is divided into three subtypes: α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR[1,2]. After being stimulated, α_1-AR preferentially couples with $G_{q/11}$ protein and then stimulates phosphatidylinositol turnover, which is known as its classical signaling pathway[3,4]. However, some evidence indicates that α_1-AR is also associated with many other signal transduction pathways as well as molecules. It was reported that cAMP accumulation increased upon stimulation of α_1-AR in rat liver[5-8] and cerebral cortex of some mammals[9-15]. However, there has been no report on the differences among the three subtypes in mediating cAMP response up to now. Therefore, in this study HEK293 cells were transfected with full-length cDNA encoding three α_1-AR subtypes and they stably expressed each α_1-AR subtype respectively, and then we compared the difference of cAMP responses mediated by each α_1-AR subtype in HEK293 cells.

MATERIALS AND METHODS

Norepinephrine (NE), phenylephrine (PE), methoxamine (ME), prazosin (Praz), propranolol (Prop),

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[2] Correspondence to Prof ZHANG You-Yi.
Phn 86-10-6201-7691, ext 2739. Fax 86-10-6201-7700.
E-mail zhangyy@bjmu.edu.cn
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Triton X-100, adenosine 3':5'-cyclic monophosphate (cAMP), 3-isobutyl-1-methyl-xanthine (IBMX), pyruvic acid, histidinol, hygromycin B, and geneticin were bought from Sigma Chemical Co; 2, 5-diphenyloxazole (PPO) was from FARCO Co; fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) were products of Hyclone Co; HEK293 cells and full-length cDNA of bovine α_{1A}-AR, hamster α_{1B}-AR, and rat α_{1D}-AR (pREP8, pREP4, and pREP9) were gifts from Prof Kenneth P MINNEMAN (Emory University, USA).

Transfection of HEK293 cells by calcium phosphate precipitation method HEK293 cells were cultured in DMEM containing 10 % FBS at 5 % CO_2, 37 °C. When being 70 % confluent, the cells were transfected with pREP8/α_{1A}-AR, pREP4/α_{1B}-AR, or pREP/α_{1D}-AR by calcium phosphate precipitation and selected under pressure of histidinol 2 g/L, hygromycin B 0.05 g/L, and geneticin 0.15 g/L, respectively. Three days later the cells were diluted and planted in 96-well dishes, with 0-2 cells in each well. After 2 to 3 times of cloning, the cell lines stably expressing each of three α_1-AR subtypes were obtained. The cells were continuously cultured and passaged in DMEM containing selective antibiotics mentioned above.

Saturation radioligand binding assay The cells growing in 75-cm^2 flasks were harvested in PBS and centrifuged twice, first at 3000×*g*, 4 °C for 10 min and then at 21 000×*g*, 4 °C for 20 min. The deposit was resuspended with 4 mL PBS, and kept on ice. α_1-AR antagonist BE2254 was radioiodinated to theoretical specific activity as described by Engel and Hoyer[16]. Measurement of specific ^{125}I-BE2254 binding was performed in PBS in a final volume 250 mL with increasing concentrations of ^{125}I-BE2254 (250-8333 dpm) at 37 °C for 20 min. Nonspecific binding was determined in the presence of phentolamine 50 μmol/L. The reactions were terminated by adding 7 mL of 10 mmol/L Tris-HCl buffer (pH 7.4) and were filtered onto glass fiber filters. Filters were washed twice with 7 mL of 10 mmol/L Tris-HCl buffer and then dried. Bound radioactivity was measured using a gamma counter. Binding data were analyzed by the nonlinear regression and Scatchard analysis (graphPad Prizm Software) on the computer and thus dissociation constant (K_D) between receptor and antagonist and maximal bound capacity (B_{max}) could be obtained. Protein content was determined by Coomassie protein quantification method.

cAMP determination in intact cells HEK293 cells transfected and non-transfected with α_1-AR subtypes were cultured in 24-well dishes respectively at 37 °C, 5 % CO_2, with 2.5×10^8 cells/L medium. When the cells were 80 % confluent, medium was changed, and 1.85×10^4 Bq [^{3}H]adenine was added in each well, then the cells were cultured at 37 °C, 5 % CO_2. Four hours after incorporation, the medium was discarded and the cells were washed twice with warm Krebs' solution. After addition of antagonists in 1 mL Krebs' solution containing IBMX 200 μmol/L and incubation for 30 min, the cells were incubated with different concentrations of agonists for further 20 min. The reaction was stopped by addition of 100 mL 77 % trichloroacetic acid, followed by a centrifugation at 3000×*g*, 4 °C for 20 min. Then 50 μL supernatant was removed in 3 mL scintillation liquid to measure radioactivity as total activity (Bq). The supernatant was applied to Dowex columns and then aluminal columns. The aluminal columns were eluted with 2 mL Tris-HCl (pH 8.0), and the radioactivity of the eluates was measured as newly-produced cAMP. cAMP accumulation was represented by percentage that newly-produced cAMP accounts for total radioactivity. The formula is as follows:

$$\text{cAMP accumulation} = \frac{\text{Radioactivity of newly-produced cAMP (Bq)}}{\text{Total radioactivity (Bq)}\times 22}\times 100\ \%$$

The number of 22 is the volume constant.

Statistical analysis Results were expressed as mean±SD. To compare mean values between two groups, *t*-test was used; ANOVA was used for comparison among three and above groups. Values of $P<0.05$ were considered statistically significant.

RESULTS

Density of three a_1-AR subtypes expressed in HEK293 cells The cell line stably expressing each α_1-AR subtype was obtained through cloning and screening, including a high-expressed- and a low-expressed-α_{1B}-AR cell line. Density of α_{1A}-AR, low-expressed α_{1B}-AR, and α_{1D}-AR were very close ($P>0.05$), whereas density of high-expressed α_{1B}-AR was about three times of the others ($P<0.05$, Fig 1). K_D values of saturation radioligand binding curves for α_{1A}-, low-expressed α_{1B}-AR, and α_{1D}-AR were (168±31), (205±58), and (192±56) pmol/L, respectively, showing no significant difference among them.

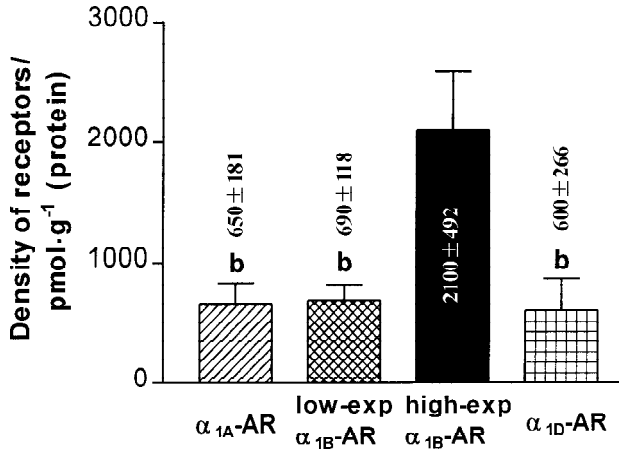

Fig 1. Density of a_{1A}-AR, a_{1B}-AR, and a_{1D}-AR expressed in HEK293 cells. $^{b}P<0.05$ *vs* high-exp a_{1B}-AR.

Comparison of the cAMP response mediated by three subtypes In HEK293 cells non-transfected with any α_1-AR subtype, NE 100 nmol/L-30 μmol/L, PE 100 nmol/L-300 μmol/L, or ME 1 μmol/L-1 mmol/L did not result in increase of cAMP accumulation in the presence of Prop 10 μmol/L (when NE used as agonist) or 1 μmol/L (when PE or ME used as agonist) (data not shown). However, under the same condition, NE 100 nmol/L-30 μmol/L increased cAMP accumulation in a concentration-dependent manner in HEK293 cells with similar expression of three α_1-AR subtypes, respectively (Fig 2), which was inhibited by Praz 100 nmol/L (data not shown), indicating that the cAMP accumulation was mediated by α_1-AR.

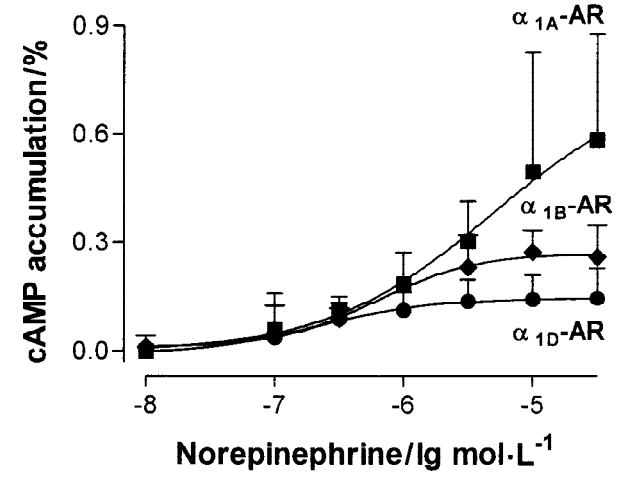

Fig 2. Three a_1-AR subtypes-mediated cAMP accumulation agonized by NE. NE induced an increase of cAMP accumulation in a concentration-dependent manner in HEK293 cells stably transfected with a_{1A}-AR, a_{1B}-AR, and a_{1D}-AR. ■, a_{1A}-AR-mediated cAMP accumulation; ◆, a_{1B}-AR-mediated cAMP accumulation; ●, a_{1D}-AR-mediated cAMP accumulation.

Tab 1. Comparison of three a_1-AR subtypes-mediated cAMP response induced by NE. $^{b}P<0.05$ *vs* a_{1D}-AR. $^{c}P<0.05$ *vs* a_{1B}-AR.

	n	R_{max} / %	pD_2
α_{1A}-AR	8	0.75±0.28^{be}	5.5±0.6
α_{1B}-AR (low exp)	5	0.27±0.07^{b}	6.2±0.6
α_{1D}-AR	8	0.15±0.06	6.7±0.8

As shown in Tab 1, NE produced the cAMP accumulation with the highest maximal response in cells transfacted with α_{1A}-AR ($P<0.05$) and the lowest maximal response in α_{1D}-AR cells ($P<0.05$). However, pD_2 of cAMP response for NE in the three cell lines showed no significant difference ($P>0.05$).

Difference in cAMP responses to NE in cell lines with high- and low-expressed a_{1B}-AR To investigate whether different density of receptor expression affected cAMP accumulation, the cAMP responses in two cell lines expressing different density of α_{1B}-AR were compared. NE (100 nmol/L-30 μmol/L) concentration-dependently generated cAMP accumulation in both cell lines with high- and low-expressed α_{1B}-AR, and the responses were abolished by prazosin. The maximal response generated by NE in high-expressed α_{1B}-AR cells (3.29 %±0.77 %, n=7) was higher than that in low-expressed α_{1B}-AR (0.28 %±0.09 %, n=5, $P<0.05$), whereas their pD_2 values, which were 6.15±0.34 and 6.18±0.78 respectively, were not significant from each other (Fig 3).

Reactivity of a_1-AR subtypes to different agonists The reactivities of α_{1A}-AR and α_{1D}-AR to different agonists were compared. Similar to NE, PE and ME (in the presence of Prop 1 μmol/L to block β_2-AR) both increased cAMP accumulation in a concentration-dependent manner in HEK293 cells transfected with α_{1A}- and α_{1D}-AR respectively (Fig 4A and 4B), which was inhibited by Praz. R_{max} and pD_2 values of the dose-response curve induced by agonists for the two subtypes were shown in Tab 2. The potency order of pD_2 values of the three agonists for α_{1D}-AR was PE>NE>ME ($P<0.05$). As for α_{1A}-AR, pD_2 for ME is much lower than that for NE or PE, and there was no difference between NE and PE.

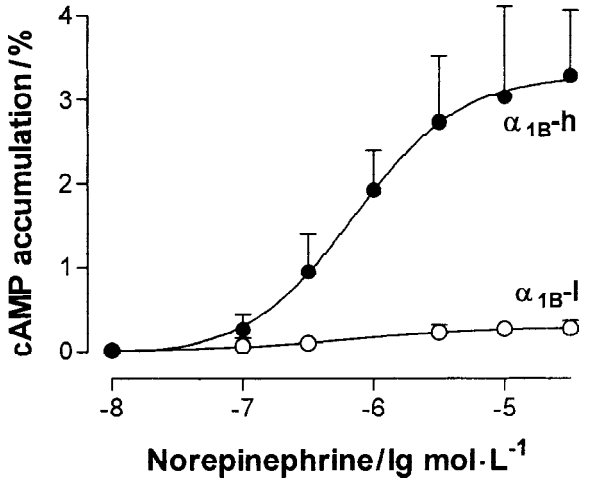

Fig 3. Comparison of cAMP response mediated by low-expressed-a_{1B}-AR and high-expressed-a_{1B}-AR. ○, cAMP accumulation mediated by low-expressed-a_{1B}-AR (a_{1B}-l); ●, cAMP accumulation mediated by high-expressed-a_{1B}-AR (a_{1B}-h).

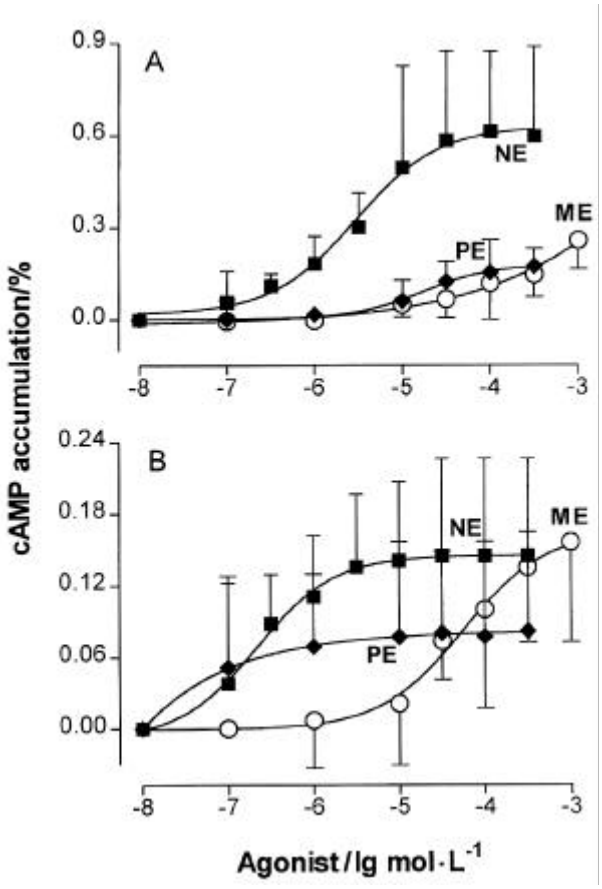

Fig 4. Comparison of cAMP accumulation mediated by different agonists. A. a_{1A}-AR-mediated cAMP accumulation induced by NE, PE, and ME. B. a_{1D}-AR-mediated cAMP accumulation induced by NE, PE, and ME. ■, NE-induced cAMP accumulation; ◆, PE-induced cAMP accumulation; ○, ME-induced cAMP accumulation.

DISCUSSION

In the present study, we applied HEK293 cells transfected with three α_1-AR subtypes as a model to investigate cAMP response upon stimulation of α_{1A}-, α_{1B}-, and α_{1D}-AR, respectively. The results showed that non-selective AR agonist, NE, and α_1-AR selective agonists, PE and ME, all increased cAMP accumulation in a concentration-dependent manner in cells expressing α_1-AR subtypes when β_2-AR was blocked, which was inhibited by α_1-AR antagonist. However, in cells non-transfected with any α_1-AR subtype, none of these agonists increased cAMP synthesis. Thus, we concluded that each α_1-AR subtype could mediate cAMP accumulation in HEK293 cells.

Many previous studies showed that stimulation of α_1-AR mediated not only PI turnover through its classical signaling pathway, but also cAMP synthesis in some tissues and cells. cAMP accumulation was detected in rat liver[5] and in cerebral cortex[9] when α_1-AR was stimulated. Increase in cAMP accumulation was observed upon stimulation of α_1-AR subtypes transfected into COS-1[17], COS-7[18], or HeLa cell lines[1]. We also found that activation of α_{1B}-AR natively expressed in DDT1MF-2 cells resulted in a dose-dependent increase of cAMP accumulation. However, those studies were performed on basis of one subtype instead of all three subtypes, besides, the characteristics of three subtypes in mediating cAMP responses had not been all-around

Tab 2. Comparison of characteristics of a_1-AR subtypes to different agonists. $^bP<0.05$ *vs* ME. $^eP<0.05$ *vs* PE.

	α_{1A}-AR			α_{1D}-AR		
	n	R_{max}/%	pD_2	*n*	R_{max}/%	pD_2
NE	8	0.75±0.28^{be}	5.53±0.65^b	8	0.15±0.06	6.67±0.85^{be}
PE	10	0.18±0.11	4.76±0.76^b	12	0.08±0.01	7.40±0.42^b
ME	6	0.26±0.01	3.72±0.76	7	0.16±0.04	4.26±0.40

compared. Johnson[9] compared the characteristics between α_1-AR-mediated PI turnover and cAMP response in cerebral cortex, and found that they were different in binding property, Ca^{2+} dependence, distribution, and sensitivity to alkylator, chloroethyldonidine (CEC), so he supposed there were probably two different α_1-AR subtypes mediating the two responses. However, our study shows that stimulation of any α_1-AR subtype can cause increase of cAMP synthesis, and the response is inhibited by Praz, suggesting that α_{1A}-, α_{1B}-, and α_{1D}-AR all mediate cAMP synthesis in HEK293 cells.

We also compared the characteristics of the three subtypes in mediating cAMP response in HEK293 cell lines. The results showed that there were differences in maximal response and sensitivity to agonists among the three α_1-AR subtypes. In NE-stimulated cAMP responses mediated by three α_1-AR subtypes with similar expression, maximal response (R_{max}) of α_{1A}-AR was the highest, and α_{1D}-AR was the lowest. However, there was no significant difference in pD_2 values among the three subtypes. Theroux *et al*[20] compared the efficiency in mediating PI turnover among the three α_1-AR subtypes in HEK293 cells and found that with equal expression, the efficiency of α_{1A}-AR was the highest, α_{1D}-AR was the lowest, and pD_2 values of the three subtypes were not different, which was similar to our results.

In addition, α_1-AR-mediated cAMP accumulation was directly correlated to its expression density. The maximal response mediated by high-expressed α_{1B}-AR was significantly higher than that induced by low-expressed α_{1B}-AR, whereas there was no difference between the agonist pD_2 values, suggesting that in HEK293 cells without receptor[21], cAMP response was enhanced along with increase of α_1-AR expression, but its sensitivity was not affected.

The present results also indicate that α_{1D}-AR is the most sensitive to NE and least sensitive to ME; however, although sensitivity of α_{1A}-AR to ME is also the lowest, its sensitivity to NE and PE is similar.

In conclusion, each of the three α_1-AR subtypes can mediate cAMP synthesis in HEK293 cells. The order of maximal response is α_{1A}-AR>α_{1B}-AR>α_{1D}-AR. Furthermore, α_1-AR subtypes have different sensitivity to different agonists, and for the same α_1-AR subtype, cAMP synthesis goes up as density of the receptor increases.

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