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Internalization and distribution of three α_1-adrenoceptor subtypes in HEK293A cells before and after agonist stimulation[1]

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Key words

adrenoceptor; agonist stimulation; confocal image; enzyme-linked immunosorbent assay; [^{3}H]-prazosin binding assay; internalization; subcellular distribution; subtype

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Abstract

Aim: To examine the subcellular distribution of the 3 α_1-adrenoceptor (α_1-AR) subtypes and their internalization and trafficking upon agonist stimulation in human embryonic kidney 293A cells. **Methods:** Confocal real-time imaging, enzyme linked immunosorbent assay (ELISA) and whole cell [^{3}H]-prazosin binding assay were applied to detect the distribution and localization of the 3 α_1-AR subtypes. **Results:** α_{1A}-AR was found both on the cell surface and in the cytoplasm; α_{1B}-AR, however, was predominantly detected on the cell surface, while α_{1D}-AR was detected mainly in the intracellular compartments. After stimulation with phenylephrine, localization changes were detected by confocal microscopy for α_{1A}- and α_{1B}-AR, but the localization of α_{1D}-AR were unaffected. Phenylephrine stimulation promoted a more rapid internalization of α_{1B}-AR than α_{1A}-AR. α_{1D}-AR internalization was detected only by ELISA. Whole cell [^{3}H]-prazosin binding assay showed that α_{1A}-AR functional receptors were detected both on the cell surface and in the cytoplasm; α_{1B}-AR, however, were detected predominantly on the cell surface, while α_{1D}-AR were detected mainly in intracellular compartments. Phenylephrine stimulation promoted internalization of α_{1A}- and α_{1B}-AR. **Conclusion:** Phenylephrine stimulation induced changes in the localization of the 3 α_1-AR.

Introduction

α_1-adrenoceptors (α_1-AR) are $G_{q/11}$-coupled receptors that respond to the neurotransmitters and hormones norepinephrine and epinephrine to mediate physiological effects such as prostate smooth muscle contraction and myocardial hypertrophy by activating phospholipase C and generating second messengers that release stored intracellular Ca^{2+} and stimulate protein kinase C[1,2]. α_1-AR are also involved in the regulation of growth-promoting responses via the mitogen-activated protein kinase family[3].

Three human α_1-AR subtypes have been cloned: α_{1A}-, α_{1B}-, and $_{1D}$-AR. Although α_1-AR family members are highly homologous (eg 75% amino acid identity in transmembrane domains), they share little homology at their amino and carboxyl termini[3,4], suggesting that α_1-AR subtypes have different expression, function, and subcellular distribution.

α_1-AR expression was found in many human tissues such as the brain, heart, and vascular smooth muscles[5]. Several reports have shown that α_{1B}-AR was mainly localized on the cell surface[6–9], and agonists induced its phosphorylation and internalization. G-protein-coupled receptors (GPCR), kinases 2 and 3, seem to be involved in the phosphorylation of agonist-bound α_{1B}-AR during homologous desensitization[10,11]. The phosphorylation sites involved have been located at Ser404, Ser408, and Ser440 in the receptor C terminus[11]. Several researchers found that α_{1A}-AR was mainly located on the cell surface and the intracellular compartments[9]. However, α_{1D}-AR was mainly localized in the intracellular compartments[12], but could be induced to translocate to the cell surface by co-expressed α_{1B}-AR[13]. Unfortunately, the molecular determinants of desensitization and internalization for α_{1A}-AR and α_{1D}-AR were largely unknown. Vazquez-Prado *et al*[14] showed that α_{1A}-AR could undergo

agonist-mediated phosphorylation, but not to the same extent as α_{1B}-AR. Yang and colleagues[15] stably transfected fibroblasts with each α_1-AR and observed that increases in inositol phosphates mediated by α_{1A} and α_{1B}-AR could be desensitized, whereas the increase mediated by α_{1D}-AR was refractory to agonist-mediated desensitization. In contrast, Garcia-Sainz *et al*[16] found that α_{1D}-AR could be phosphorylated and desensitized. Recent reports showed that cell trafficking (desensitization and redistribution) was an important step in the regulation of GPCR, particularly in response to receptor stimulation by agonists. The ultimate effect on receptor signaling and the fate of sequestered receptors varies with receptor type, duration of agonist exposure, and cellular environment[17]. In this study, we examined subcellular distribution of the 3 α_1-AR subtypes and their internalization and trafficking upon agonist stimulation in human embryonic kidney (HEK) 293A cells. We also compared the distribution and trafficking property between total receptors and functional receptors.

Materials and methods

Flag-tagged human α_{1A}-, α_{1B}-, and α_{1D}-AR were gifts from Prof KP MINNEMAN (Emory University, Atlanta, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Rockville, MD, USA). Geneticin, an anti-flag antibody, and phenylephrine were obtained from Sigma-Aldrich (St Louis, MO, USA). [^{125}I]-BE2254 and [^{3}H]-prazosin were obtained from Amersham Biosciences (Buckinghamshire, UK). α_1-AR green fluorescent protein (GFP) vectors were constructed by ligating the coding region of human α_{1A}-,α_{1B}-, and α_{1D}-AR into the *EcoR* I–*BamH* I site of the basic pEGFP-C2 protein fusion vector (Clontech, USA) as described previously[7].

Cell culture and transfection HEK293A cells were cultured in DMEM with sodium pyruvate supplemented with 10% FBS, 100 U/mL streptomycin, and 100 U/mL penicillin in a humidified atmosphere with 5% CO_2. Cells for transient transfection were grown in culture dishes with a glass coverslip. When grown to approximately 60% confluence, cells were transfected with 5 μg cDNA encoding α_{1A}-, α_{1B}-, α_{1D}-AR/GFP fusion protein using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively. Cell lines stably transfected with α_{1A}-, α_{1B}-, and α_{1D}-AR/Flag, which were cloned and established before[18], were grown on 6-well plates and maintained with geneticin (200 mg/mL). As previously described, the B_{max} of α_{1A}-AR/Flag, α_{1B}-AR/Flag, and α_{1D}-AR/Flag were 4.8, 20.8, and 0.24 pmol/mg protein, respectively, on cell membranes as determined by [^{125}I]-BE2254 binding assay[18].

Western blot assay HEK293A cells transiently expressing α_{1A}-, α_{1B}-, or α_{1D}-AR were treated with phenylephrine (10 μmol/L) or prazosin and phenylephrine or vehicle. Cells were lysed with ice in cold lysis buffer containing 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2.5 mmol/L edetic acid, 50 mmol/L NaF, 0.1 mmol/L $Na_4P_2O_7$, 1 mmol/L Na_3VO_4, 1% TritonX-100, 10% glycerol, 0.1% SDS, 1% deoxycholic acid, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mg/mL aprotinin for 20 min, sonicated for 20 s, and centrifuged at 12 870×*g* for 15 min. The supernatant is the whole cell protein. The lysate [30 μg, extracellular signal-related kinase (ERK) assay] was separated by electrophoresis using 10% SDS-PAGE and transferred onto nitrocellulose membrane or polyvinylidene difluoride membrane. Nonspecific IgG was blocked with 5% fat-free milk, and the membrane was incubated with the antibody to phospho-ERK (Cell Signaling Technology, Inc, Beverly, MA, USA); horseradish peroxidase-conjugated anti-mouse IgG was used as a second antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The peroxidase reaction products were visualized by LumiGLO Chemiluminescent Substrate (New England Biolabs, Beverly, MA, USA). The same membrane was then stripped and reprobed with anti-ERK to determine the total protein.

Confocal microscopy and image quantification HEK293A cells transiently transfected with GFP-tagged construct were grown on sterile coverslips, fixed for 30 min with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, and rinsed 3 times with PBS. Cells were imaged with a Spectra-Physics laser scanning confocal microscope with a Plan-Apo 40× oil immersion objective lens (Leica, Wetzlar, Germany). The software used to collect the images was the Leica TCS NT, version 1.6.587. The images were transferred to a computer and analyzed with Adobe Photoshop version 5.0 (Adobe Systems, Mountain View, CA, USA). The setting on the laser was constant for all experiments. Fluorescence was excited using an argon laser at a wavelength of 488 nm, and the absorbed wavelength was detected at 510–520 nm. The extent to which the α_1-AR/GFP was internalized after exposure to agonists was quantified by the intracellular immunofluorescence intensity. The extent of α_1-AR/GFP receptor internalization was defined as the rate of the fluorescence intensity before and after exposure to agonists.

Internalization assay The HEK293A cells stably expressing α_1-AR were seeded at a density of 2×10^5/well onto poly-*L*-lysine-coated 24-well plates. The next day, the cells were pre-incubated with 1 μg of mouse anti-flag antibody for 1 h

in DMEM free of serum at 37 °C and washed twice using serum-free DMEM. Cells were then treated with 10 μmol/L phenylephrine for different time periods in the serum-free DMEM. Subsequently, the cells were fixed and incubated with peroxidase-conjugated anti-mouse antibody (1:1000, Santa Cruz, CA, USA) for 2 h at room temperature. After washing, the plates were developed with 250 μL of o-phenylenediamine dihydrochloride (OPD) solution (4 mg OPD in 10 mL PBS, 15 μL 30% H_2O_2). After 10–30 min, 200 μL of the substrate solution from each well was transferred to 96-well plates and analyzed at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Whole cell binding assay To further determine the subcellular distribution of the 3 α_1-AR subtypes prior to, or after incubation with phenylephrine for 1 h, radioligand binding was performed on whole cells stably transfected with the flag-tagged 3 α_1-AR constructs as described by Eason MG *et al*[19]. The cells were re-suspended in ice-cold DMEM medium, then incubated with increasing concentrations (0.01–2 nmol/L) of [^{3}H]-prazosin alone, or in the presence of the lipophilic competitor, phentolamine (10 μmol/L), or in the presence of the hydrophilic competitor, adrenaline (100 mmol/L), for 4 h at 4 °C. With the intact cells, specific binding, as defined with phentolamine, represented the total cellular amount of α_1-AR, because it is able to penetrate the plasma membrane and scatter about the whole cell, whereas specific binding, as defined with adrenaline, represents only cell surface receptors owing to its hydrophilic properties, that is, adrenaline is unable to penetrate the plasma membrane, especially at 4 °C. The intracellular pool of receptors is defined as the total number of receptors minus those on the cell surface. Saturation and displacement curves were analyzed by non-linear regression analysis with Prism (GraphPad Software, San Diego, CA, USA).

Statistical analysis Data from confocal quantity were analyzed using the unpaired t-test with statistical significance defined as $P<0.05$. Data from Western and internalization assays were analyzed by GraphPad Prism using the 2-tailed paired *t*-test. Significant differences were defined as $P<0.05$.

Results

ERK 1/2 were activated by phenylephrine in the HEK293A cells transiently transfected with α_1-AR/GFP fusion protein To demonstrate that the α_1-AR/GFP fusion proteins expressed in HEK293A cells were functional, the HEK293A cells transfected with α_{1A}-, α_{1B}-, and α_{1D}-AR/GFP, respectively, were treated with phenylephrine, and the phosphorylation of ERK 1/2 was measured by Western blot. The levels of the phospho-ERK1/2 in HEK293A cells transfected with α_{1A}-, α_{1B}-, or α_{1D}-AR/GFP significantly increased after treatment with phenylephrine (PE, 10 μmol/L), an agonist of α_1-AR, for 30 min. Pretreatment with prazosin (1 μmol/L), an antagonist of α_1-AR, however, greatly attenuated the effects of phenylephrine on the activation of ERK1/2 in the transfected HEK293A cells. These results suggested that the α_1-AR/GFP fusion proteins expressed in the HEK293A cells were biologically active (Figure 1).

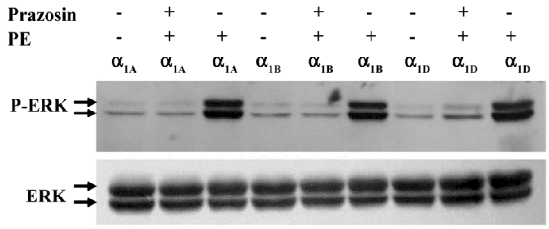

Figure 1. Activation of ERK1/2 phosphorylation. HEK293A cells transiently expressing the 3 α_1-AR subtypes were pretreated with or without 1 μmol/L prazosin for 30 min and stimulated with phenylephrine (PE) for 30 min. Whole cell lysates were immunoblotted with anti-phospho-ERK. Membranes were stripped and reprobed using anti-ERK antibody. Three separate experiments showed similar results. $^bP<0.05$ *vs* control.

Agonist-induced localization changes of α_1-AR subtypes in HEK293A cells By real-time confocal microscopy, we found that the incubation of HEK293A cells transiently expressing α_{1A}- or α_{1B}-AR/GFP with 10 μmol/L phenylephrine caused changes of receptor localization (Figures 2, 3). An accumulation of α_1-AR in vesicle-like structures was found in the cytoplasm, and the membrane receptors became uneven. Because coated vesicles are the prelude of GPCR internalization by agonist stimulation, these results suggested that agonist stimulation could induce α_{1A}- and α_{1B}-AR internalization. The receptor localization in cells expressing α_{1D}-AR, however, was unchanged after phenylephrine stimulation (Figure 4).

We then measured the signal intensity of intracellular fluorescence to estimate the rate of receptor internalization. The fluorescent intensity of α_{1A}- and α_{1B}-AR was significantly increased in the cytoplasm after phenylephrine stimulation, and α_{1B}-AR occurred in a very rapid manner (20 min after phenylephrine stimulation, Figure 5A), while α_{1A}-AR occurred in a slower manner (25 min, Figure 5B). HEK293A cells expressing α_{1D}-AR, however, showed no translocation of the receptors on the cell surface after phenylephrine stimulation (Figure 5C). These results indicated that the location

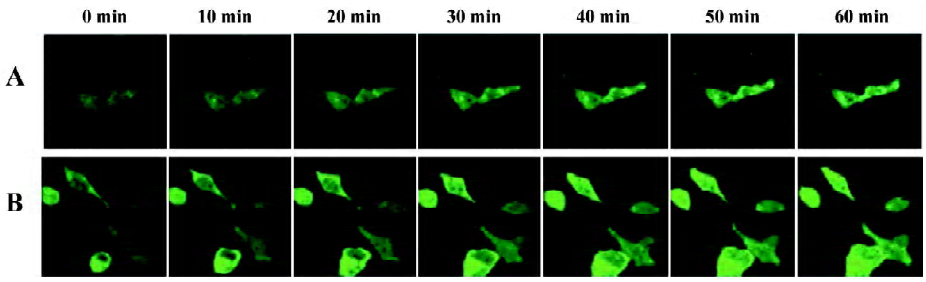

Figure 2. Effects of 10 μmol/L phenylephrine (PE) on the cellular localization of α_{1A}-AR/GFP transiently transfected into HEK293A cells. Real-time images were captured before and after PE addition at different time points with or without prazosin. Images are representative of 4–5 independent experiments. (A) prazosin+PE; (B) PE alone. Magnification×600.

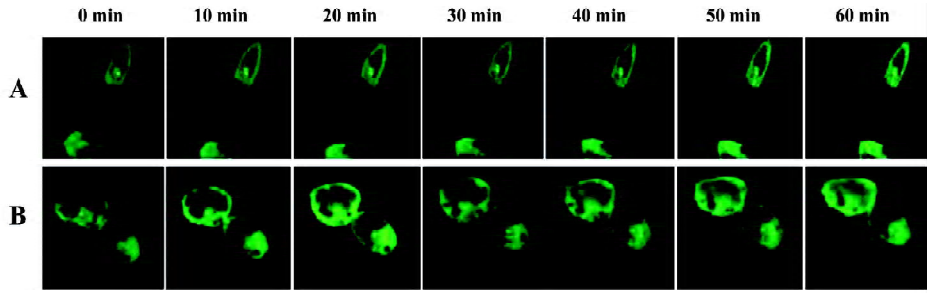

Figure 3. Effects of 10 μmol/L phenylephrine (PE) on the cellular localization of α_{1B}-AR/GFP transiently transfected into HEK293A cells. Real-time images were captured before and after PE addition at the different time points with or without prazosin. Images are representative of 4–5 independent experiments. (A) prazosin+PE; (B) PE alone. Magnification×600.

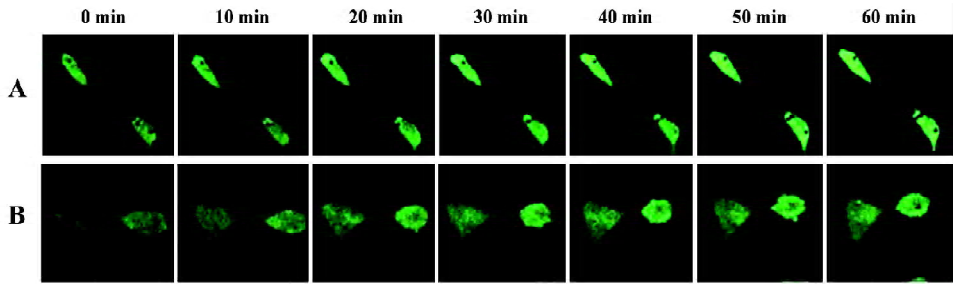

Figure 4. Effects of 10 μmol/L phenylephrine (PE) on the cellular localization of α_{1D}-AR/GFP transiently transfected into HEK293A cells. Real-time images were captured before and after PE addition at different time points with or without prazosin. Images are representative of 4–5 independent experiments. (A) Prazosin+PE; (B) PE alone. Magnification ×600.

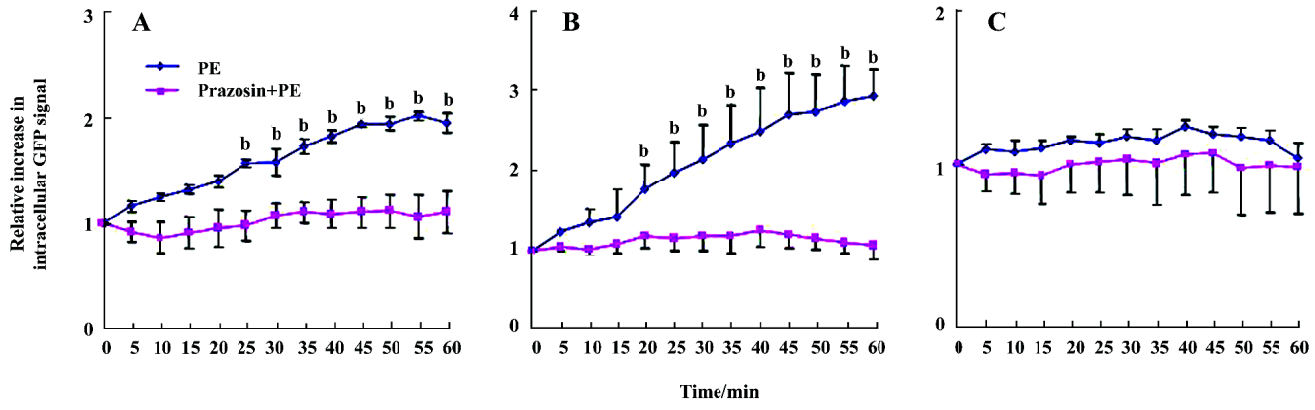

Figure 5. Comparison of the effect of 10 μmol/L phenylephrine (PE) on changes of intracellular fluorescence intensity in cells transiently transfected with the α_{1A}-AR/GFP or α_{1B}-AR/GFP or α_{1D}-AR/GFP with or without prazosin. (A) relative fluorescence intensity of α_{1A}-AR; (B) relative fluorescence intensity of α_{1B}-AR; (C) relative fluorescence intensity of α_{1D}-AR. Data represent the mean and standard error of the mean values of 3–5 independent experiments. $^{b}P<0.05$ *vs* prazosin and PE cotreatment.

changes of the receptors after phenylephrine stimulation were due to receptor internalization.

Pretreatment with prazosin prior to phenylephrine stimulation inhibited α_{1A}- and α_{1B}-AR translocation and decreased the fluorescent intensity of cytoplasmic receptors in the transfected HEK293A cells (Figure 5), suggesting that phenylephrine acted directly on α_{1A}- and α_{1B}-AR to induce receptor translocation, a phenomenon called homologous desensitization or internalization.

α_1-AR internalization induced by phenylephrine in HEK293A cells We next examined the effects of phenylephrine stimulation on cell surface receptor internalization using HEK293A cells stably expressing α_{1A}-, α_{1B}-, or α_{1D}-AR by intact cell enzyme-linked immunosorbent assay (ELISA). Five minutes after phenylephrine stimulation, the amounts of α_{1B}-, α_{1A}-, and α_{1D}-AR on the cell surface decreased by about 29.2%, 9.7%, and 11.3%, respectively. One hour after the stimulation, they further decreased by about 64.8%, 36.4%, and 32.1%. These results indicated that phenylephrine could induce the internalization of α_{1A}, α_{1B}, and α_{1D}-AR, and the internalization of α_{1B}-AR occurred faster than that of α_{1A}- and α_{1D}-AR (Figure 6).

Agonist-induced distribution changes in binding of functional α_1-AR subtypes in HEK293A cells To test whether membrane and cytoplasm functional receptors changed under phenylephrine stimulation, whole cell [^{3}H]-prazosin binding assay was used. Functional α_{1B}-AR was mainly expressed on the surface of transfected HEK293A cells, which was about 92.4% of the total receptor. α_{1D}-AR was mainly expressed in the cytoplasm, about 69.5% of total receptor, and α_{1A}-AR was expressed both on the cell surface and in the cytoplasm, which was consistent with the results of the confocal analysis. One hour after phenylephrine stimulation, functional α_{1D}-AR did not change compared with the control. α_{1A}- and α_{1B}-AR on the cell surface, however, decreased significantly: α_{1A}-AR from 53.7% to 35.5% of the total receptor, and α_{1B}-AR from 92.4% to 30.2% of the total receptor, which suggested that phenylephrine stimulation could induce the internalization of functional α_{1A}-AR and α_{1B}-AR proteins (Table 1).

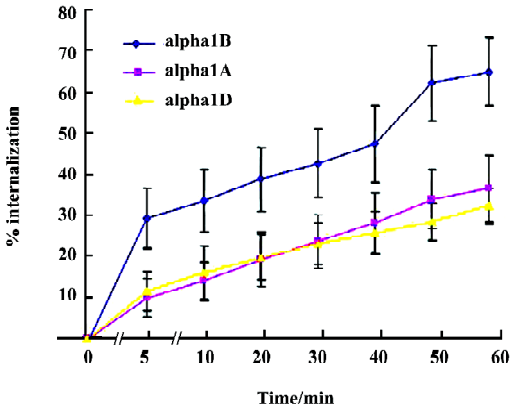

Figure 6. Quantitative analysis of agonist-induced internalization of α_{1A}-AR or α_{1B}-AR or α_{1D}-AR stably expressed in HEK293A cells by ELISA. Percentage of loss of cell surface receptor at different times in phenylephrine (PE)-treated cells. n=4 independent experiments. Mean±SEM.

Discussion

In this report, we investigated the trafficking profile of the 3 α_1-AR. We created a fusion construct consisting of N-terminal GFP-tagged α_1-AR and transfected this construct into HEK293A cells, which allowed us not only to identify the 3 subtype receptors by confocal microscopy, but also to compare the surface and total receptor by intact cell ELISA and [^{3}H]-prazosin binding assay before and after phenylephrine stimulation.

As described in the Methods and in our previous work, the B_{max} of α_{1B}-AR on the membrane was found to be the highest, followed by α_{1A}-AR and α_{1D}-AR, respectively[18]. These results are consistent with the previous report in which the functional binding of wild type α_1-AR was examined[20].

Previous studies with the α_{1B}-AR/GFP construct demonstrated that it was fully functional and internalized in the

Table 1. Effect of phenylephrine (PE) on the localization of functional α_1-AR subtypes in whole cell, cell surface, and cytoplasma of transfected HEK293A using [^{3}H]-prazosin binding assay. n=3. Mean±SD. ^bP<0.05 compared with control.

	B_{max} (fmol per 1×10^6 cells)					
	Total receptors		Receptors on cell surface		Intracellular receptor	
	Control	PE	Control	PE	Control	PE
α_{1A}	763±239	2251±26	1483±300	798±133^b	1280±72	1453±108
α_{1B}	3232±391	3781±434	2986±396	1140±325^b	246±141	2641±511
α_{1D}	3324±513	3165±286	1048±358	1091±108	2276±160	2074±370

same manner as a non-GFP tagged α_{1B}-AR construct[21]. Both the α_{1A}- and α_{1B}-AR were associated with the activation of ERK[22]. In this study, all the α_{1A}-, α_{1B}-, and α_{1D}-AR, when coupled to GFP, promoted the increase in ERK1/2 phosphorylation, suggesting that the α_1-AR were functional and retained their ability to activate cellular signaling when conjugated to the GFP.

Cellular localization and trafficking is very important for α_1-AR to accomplish their physiological functions. Results obtained over the past years have shown that there is a significant difference in the subcellular distribution among the α_1-AR subtypes. Fonseca *et al*[6] observed that α_{1B}-AR was expressed predominantly on the cell membrane and α_{1A}-AR was expressed both on the membrane and cytoplasm in the stably-transfected fibroblasts by immunocytochemistry. This was confirmed by Awaji *et al*[21] who used α_{1B}-AR/GFP fusion proteins to verify cell membrane localization of α_{1B}-AR in COS-7 cells. McGrath *et al*[23] used BODIPY-FL-labeled prazosin to image α_1-AR subtypes in cultured prostate smooth muscle cells and fibroblasts stably transfected with each subtype and found that the 3 α_1-AR subtypes were expressed in the cytoplasm of fibroblasts. Very little α_{1D}-AR expression was detected on the cell surface. In this study, we transfected HEK293A cells with cDNA encoding α_1-AR/GFP. Living cells were then visualized by real-time laser scanning confocal microscopy. We found that α_{1B}-AR/GFP fluorescence was predominant on the cell surface, whereas the α_{1A}-AR expression was detected both on the cell surface and in the cytoplasm. α_{1D}-AR was mainly localized in intracellular compartments, suggesting that the localization of the 3 α_1-AR subtypes was different in the transfected HEK293A cells.

α_1-AR are subject to dynamic regulation by a variety of mechanisms, including phosphorylation, protein-protein interactions, protein trafficking, and transcription. One of the most intensive studies of these mechanisms is internalization and desensitization, a general phenomenon in which the intensity of a biological response wanes over time, despite continued stimulus. Desensitization can be further characterized as either homologous, where receptor response wanes upon continuous exposure to its agonist, or heterologous, where agonist-mediated stimulation of a receptor can attenuate the response by other receptors mediating similar cellular events[24]. With regard to the α_1-AR subtypes, the desensitization, downregulation, and internalization characteristics of the α_{1B}-AR have been extensively examined. For example, agonist-mediated phosphorylation and internalization of the α_{1B}-AR have been demonstrated, and the domains of the receptors involved in internalization have been identified[6,10,11]. We know much less about the molecular determinants of desensitization, downregulation, and internalization for α_{1A}- and α_{1D}-AR. Here, we transfected HEK293A cells with cDNA encoding α-AR/GFP. Living cells were then visualized by real time laser scanning confocal microscopy under phenylephrine stimulation. Stimulation of α_1-AR with phenylephrine induced the changes of localization of α_{1A}-AR and α_{1B}-AR, but not the localization of α_{1D}-AR.

Confocal image quantification analysis found that stimulation of α_{1B}-AR with phenylephrine promoted a rapid internalization of α_{1B}-AR, which began at about 20 min, and a slower internalization of α_{1A}-AR, which began at about 25 min. However, α_{1D}-AR internalization did not occur after phenylephrine stimulation. We only assayed the changes of fluorescence intensity of the plasma, but α_{1D}-AR was mainly localized in intracellular compartments. Therefore, this method can not determine whether α_{1D}-AR was internalized under phenylephrine stimulation.

To confirm the results of confocal microscopy, the internalization of membrane receptors was detected by intact cell ELISA. This does not change the permeability of cell membrane, so the antibodies to α_1-AR subtypes only bind to the membrane surface of stably-transfected HEK293A cells. The internalization percentage of α_{1A}-AR was about 9.7% at 5 min and about 36.4% at 1 h after phenylephrine stimulation. The internalization percentage of α_{1B}-AR was about 29.2% at 5 min and about 64.8% at 1 h after phenylephrine stimulation. Intact cell ELISA assay also demonstrated α_{1D}-AR internalization after phenylephrine stimulation. The internalization percentage of α_{1D}-AR was about 11.3% at 5 min and about 32.1% at 1 h after phenylephrine stimulation. Although Garcia-Sainz *et al*[22] showed that α_{1D}-AR could be phosphorylated and internalized, many other studies reported that α_{1D}-AR could not be internalized under agonist stimulation. Our results by ELISA are the same as Garcia-Sainz *et al*. As mentioned earlier, cell-intact ELISA only detects the receptors located on the outside surface of the membrane, but confocal imaging analysis mainly assesses the changes of fluorescence intensity of the plasma. When internalization occurs, α_{1D}-AR may just move from the outside to the inside of the membrane. Although some α_{1D}-AR may translocate from the membrane to the plasma, the internalized α_{1D}-AR are negligible compared with the total α_{1D}-AR because most α_{1D}-AR are expressed in the plasma. This may explain why α_{1D}-AR internalization can be detected by ELISA rather than confocal imaging analysis. However, further studies are needed to elucidate this discrepancy.

The confocal imaging and ELISA analyses demonstrated

the localization changes and internalization of the 3 α_1-AR subtypes. Receptor proteins include receptors with binding activity and receptors without binding activity. Past studies generally did not differentiate these 2 categories. In this study, we detected the B_{max} of the total cell surface and intracellular functional α_1-AR by whole cell [^{3}H]-prazosin binding assay. We found that the total B_{max} were the same in the 3 subtypes, and not different before and after phenylephrine stimulation. α_{1A} functional receptors were not only on the cell surface, but also in intracellular compartments. α_{1B} functional receptors were predominantly on the cell surface; α_{1D} functional receptors were mainly in intracellular compartments. In general, AR are G-protein-coupled membrane receptors. From the previous point of view, only the receptors on the membrane have the binding function, whereas the receptors in the cytoplasm are immature and do not have binding ability. However, the present study demonstrated that there were functional receptors in the cytoplasm. Stimulation of α_{1A}- and α_{1B}-AR with phenylephrine for 1 h promoted internalization of α_{1A}- and α_{1B}-AR, but not α_{1D}-AR. This apparent discrepancy in α_{1D}-AR internalization, as determined by different methods, may be partly explained by the different observed parameters. Both confocal imaging and internalization assay determine receptor protein, whereas whole cell binding assay determines functional receptors. Since only a few α_{1D}-AR are located on the cell surface, in order to maintain the reactivity as strongly as possible, most of them are probably functional receptors. On the contrary, since both α_{1A}- and α_{1B}-AR on the cell surface are quite abundant, the amount of their functional receptors would be far more than that of α_{1D}-AR, even though their functional receptors may only account for a small fraction of the whole receptors. It is supposed that when internalization occurs, there would be as many mature functional α_{1D}-AR as possible sorted on the cell surface to complement the internalized ones in order to maintain the maximal reactivity. However, even if quite a lot of functional α_{1A}- or α_{1B}-AR were internalized upon phenylephrine stimulation, the residual functional receptors on the cell surface would be enough to maintain the same reactivity as before, so it would not be necessary to sort the mature functional receptors on the cell membrane. Therefore, internalization could only be observed on α_{1A}- and α_{1B}-AR, but not α_{1D}-AR by confocal imaging. However, the exact mechanism remains to be explored.

These results also explain why the total amount of the 3 α_1-AR subtypes was equal by whole cell [^{3}H]-prazosin binding assay, while their densities on the membrane were quite different when detected by traditional [^{125}I]-BE2254 binding assay. Because [^{125}I]-BE2254 binding assay only reflected the receptors on the membrane, a part of the whole cell receptors, [^{3}H]-prazosin binding assay reflected the total amount of receptors, including both receptors on the cell membrane and those in the cytoplasm. The discrepancy between the 2 methods offers evidence that the 3 α_1-AR subtypes have different distribution, localization, and trafficking characteristics, which is consistent with that examined by other methods in this study.

In summary, in transfected HEK293A cells, α_{1A}-AR evenly distribute in the cytoplasm and membranes, but phenylephrine stimulation causes their internalization. α_{1B}-AR are mainly located on the membrane, however, phenylephrine can result in rapid internalization, and the number of internalized α_{1B}-AR is more than α_{1A}-AR. α_{1D}-AR predominantly exist in the cytoplasm, and their internalization upon phenylephrine stimulation could be detected only by intact-cell ELISA, but not by other methods. Therefore, a definitive answer regarding the internalization characteristics of α_{1D}-AR requires additional study. Furthermore, we compared the distribution between functional receptors and total receptor proteins. Because there were functional receptors in the cytoplasm, we suggested that the receptors in the plasma act as a receptor's storage pool.

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