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Differential regulation on human skin fibroblast by α_1 adrenergic receptor subtypes

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ABSTRACT

Alpha 1 adrenoceptor (α_1-AR) regulation of DNA synthesis was studied in human neonatal foreskin fibroblast. Saturation assay with a specific radioligand for α_1 adrenergic [^{3}H]-prazosin revealed two saturated and specific binding sites with high or low affinity. Competitive binding assay with different antagonist subtypes, defined pharmacologically three major types of α_1-AR. The α_1-AR agonists (from 1×10^{-10} to 1×10^{-4} M) triggered a biphasic action on DNA synthesis reaching maximal stimulation at 1×10^{-9} M and maximal inhibition at 1×10^{-6} M. Prazosin, abolished the stimulatory (pA_2: 9.24) and inhibitory (pA_2: 8.80) actions of α_1-AR agonists. The α_1-AR stimulation resulted in the activation of phosphoinositide turnover (InsP) via phospholipase C (PLC) involving calcium/calmodulin (CaM) and nitric oxide synthase (NOS) that correlates with the DNA synthesis increment; whereas the inhibition resulted in a decrease of cyclic AMP (cAMP) accumulation via adenylate cyclase inhibition. The potency displayed by the specific antagonists tested in binding, DNA synthesis, InsP and NOS at low agonist concentration suggests that they can be elicited by the activation of the same receptor (α_{1B}-AR subtype); while the decrement in DNA synthesis and cAMP at high concentration account by the activation of α_{1D}-AR coupled to G_i protein. Non-functional α_{1A}-AR in neonatal human foreskin fibroblast was observed. Results suggest that the expression of α_1-AR subtypes on human skin fibroblast may differentially activate signaling pathways that modulate physiological response of the cells.

1. Introduction

α_1 Adrenoceptors (α_1-AR) belong to the seven transmembrane-domain receptor (7-TM) superfamily of G protein-coupled receptors that mediate the functions of catecholamines [1]. Three different native α_1-AR subtypes have been cloned and are referred to as α_{1A}, α_{1B} and α_{1D} [2–5] These subtypes are expressed in many tissues (liver, brain, myocardium, vascular smooth muscle) including fibroblast cell lines [6,7].

The effort to design agents selective for each of the three α_1-AR subtypes has been an active area of research. All three α_1-AR subtypes exhibit similar affinity for endogenous catecholamines [8]; however, the cellular function of these receptors have not been defined. Although the growth/proliferation effects have been studied in vascular smooth muscle cells [9], there is new evidence that indicating α_1-AR are expressed in surrounding fibroblast [10] and may participate in remodeling events. Even though little is known about the signal transduction mechanism by which distinct α_1-AR subtypes regulate cellular fibroblast proliferation [11]. It is now clear that different G-protein family coupled α_1-AR subtypes can stimulate mitogen-activated protein kinase pathways,

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and this activation plays an important role in the regulation of both cell growth and proliferation [11–14].

The α_1-AR subtypes show prevalence for coupling to the G_q family thereby activating phospholipase C (PLC)-dependent hydrolysis of PI-IP_3 [2,15]. On the other hand, G protein coupled α_1-AR acting through G_q and G_s, have all been shown to activate mitogen protein kinase pathways [16,17] in a variety of cell types and play important roles in regulating cell growth and proliferation, although the mechanisms involved appear to be dependent on cell phenotype.

We studied the role of PLC and adenylate cyclase on α_1-AR agonists activated α_1-AR subtypes in human skin fibroblasts. In the course of these studies we obtained pharmacological evidence of an oppositive action on DNA synthesis depending on different α_1-AR subtypes activation. Results suggest that expression of α_1-AR subtypes may differentially activate signaling pathways that modulate physiological responses.

2. Materials and methods

2.1. Cell culture

Pure cell cultures of neonatal human (7 days after birth) skin fibroblast (2×10^5 cells/ml) were established from the foreskin obtained after circumcision and grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and penicillin (100 U/ml) and streptomycin (100 μg/ml) in a 5% CO_2 environment at 37 °C described by Varani et al. [18]. The medium was replenished every 3–4 days. Confluent cells were subcultured by detaching the monolayer with 0.25% trypsin in phosphate buffered saline solution (0.1 M pH 7.2) containing (PBS): Na_2HPO_4 anhydrous 1.09 g; NaH_2PO_4 anhydrous 0.32 g; NaCl 9.00 g and distilled water 100 ml. Only cell of passages 4–6 were used in the experiments. To identify the fibroblast experiments was carry out to establish the incubation time and the number of cell used throughout. Human studies have been performed in accordance with the ethical standards laid down in an appropriate version of the 1964 Declaration of Helsinki.

2.2. Measurement of DNA synthesis

DNA synthesis was estimated by measuring [^{3}H] thymidine (Dupont/New England Nuclear) incorporation into trichloroacetic acid (TCA)-precipitable material. Cells growing in the absence of 10% FBS were treated with different concentrations of adrenergic agonists and antagonists or Pertussis toxin (PTX) for 20 h, and [^{3}H]-thymidine (0.1 μCi/ml) was added during the last 18 h. Cells were precipitated twice with ice-cold 10% TCA. When antagonistic drugs and enzymatic inhibitors were used, they were added 15 min before α_1 adrenergic agonists. Fibroblasts that had been serum-starved for 20 h without any drugs were used as control. Cells were then removed with trypsin/PBS and radioactivity was determined by liquid scintillation counting. The cell cycle distribution of fibroblast used were in resting state and the percentage of confluence at the time of study for all assays was about 95–98%.

2.3. Radioligand binding assay

Membranes were prepared as previously described [19]. In brief, the cells (2×10^6 cell/ml) were homogenized in an Ultraturrax at 4 °C in 6 volumes of potassium phosphate buffer, 1 mM $MgCl_2$, 0.25 M sucrose (buffer A) pH 7.5 supplemented with 0.1 mM phenylmethylsulphonylfluoride (PMSF), 1 mM EDTA, 5 μg ml^{-1} leupeptin, 1 μM bacitracin and 1 μM pepstatin A. The homogenate was centrifuged twice for 10 min at 3000 × *g*, then at 10,000 × *g* and 40,000 × *g* at 4 °C for 15 and 90 min, respectively. The resulting pellets were resuspended in 50 mM phosphate buffer with the same protease inhibitors pH 7.5 (buffer B). Receptor ligand binding was performed as previously described [19]. Aliquots of the membrane suspension (30–50 μg protein) were incubated with different concentrations of [^{3}H]-prazosin (Specific Activity 75 Ci/mmol; Dupont/New England Nuclear, USA) for 60 min at 25 °C in a total volume of 150 μl of buffer B. The binding was stopped by adding 2 ml ice-cold buffer followed by rapid filtration (Whatman GF/c). Filters were rinsed with 12 ml of ice-cold buffer, transferred into vials containing 10 ml of scintillation cocktail and counted in a liquid scintillation spectrometer. No specific binding was determined in the presence of 1×10^{-5} M prazosin and never exceeded 10% of total binding. Radioactivity bound was lower than 10% of total counts. For competition binding experiments, fibroblast membranes were incubated with increasing concentrations of α adrenergic antagonists 5-MU, KMD 3213 and BMY 7378 in the presence of 0.30 nM of [^{3}H]-prazosin. Binding data were analyzed with the computer-assisted curve-fitting program LIGAND. For CEC treatment, fibroblast (2×10^6 cell/ml) were incubated at 30 °C for 30 min with or without 1×10^{-7} M CEC. After the incubation, the cells were washed three times with Krebs Ringer bicarbonate (KRB) solution containing (mM): 113 NaCl; 4.8 KCl; 2.5 $CaCl_2$; 1.2 KH_2PO_4; 1.2 $MgSO_4$; 25 $NaHCO_3$ and 11.7 D-glucose (KRB) and centrifuged at 1000 × *g* for 3 min. The pellet was then homogenized and membranes were prepares as described above.

2.4. Measurement of inositol phosphates (InsP)

Fibroblasts (2×10^6 cell/ml) were incubated for 120 min in 0.5 ml of KRB gassed with 5% CO_2 in O_2 with 1 mCi [myo-^{3}H]-inositol ([^{3}H]-MI) (Sp.Act. 15 Ci/mmol) from Dupont/New England Nuclear and LiCl (10 mM) was added for determination of inositol monophosphate accumulation according to the technique previously described [20]. M 6434 was added 30 min before the end of the incubation period and the blockers were added 30 min before the addition of M 6434. Water-soluble InsPs were extracted after a 120 min incubation period. Tissues were washed with KRB and homogenized in 0.3 ml of KRB with 10 mM LiCl and 2 ml chloroform/methanol (1:2, v/v) to stop the reaction. Then, chloroform (0.62 ml) and water (1 ml) were added. Samples were centrifuged at 3000 × *g* for 10 min and the aqueous phase of the supernatant (1–2 ml) was applied to a 0.7 ml column of Bio-Rad AG (Formate Form) 1 × 8 anion-exchange resin (100–200 mesh) suspended in 0.1 M formic acid that had been previously washed with 10 mM Tris–formic pH 7.4. The resin was then washed with 20 volumes of 5 mM myoinositol followed by 6 volumes of water

and InsP were eluted with 1 M ammonium formate in 0.1 M formic acid. One millilitre fractions were recovered and radioactivity was determined by scintillation counting. Peak areas were determined by triangulation [20]. Results were expressed as a percentage of the total radioactivity incorporated (1st plus 2nd peaks). In order to determine the absence of [^{3}H]-MI in the eluted peaks of InsP, chromatography in silica gel 60 F254 sheets (Merck) was performed using propan-2-ol-6,NH_4 (14:5) as the developing solvent [21]. Spots were located by spraying with freshly prepared 0.1% ferric chloride in ethanol followed, after air drying with 1% sulphosalicylic acid in ethanol. To assay the radioactivity a histogram was constructed by cutting up the sheet gel, placing each sample in Triton-toluene based scintillation fluid and the counting.

2.5. Determination of nitric oxide synthase (NOS) activity

Nitric oxide synthase (NOS) activity was measured in cells (2×10^6/ml) by production of [U^{-14}C]-citrulline from [U^{-14}C]-arginine according to the procedure described by Bredt and Snyder [22]. Briefly, after 20 min preincubation in KRB solution, cells were transferred to 500 μl of prewarmed KRB equilibrated with 5% CO_2 in O_2 in the presence of [U-^{14}C]-arginine (0.5 μCi). Appropriate concentrations of drugs were added and the cells were incubated for 20 min under 5% CO_2 in O_2 at 37 °C. Measurement of basal NOS activity in cells by the above mentioned procedure was inhibited 95 and 78% in the presence of 0.5 mM and 0.05 mM N^G-monomethyl-L-arginine (L-NMMA), respectively. The results (pmol/g/tissue wet wt) obtained for cells were expressed as the difference between values in the absence (218 ± 18; $n = 9$) and in the presence (48 ± 5, $n = 9$) of L-NMMA.

2.6. Measurement of cyclic AMP (cAMP) levels

Cells (2×10^6 ml^{-1}) were incubated in a final volume of 1 ml of KRB with 5% CO_2 in O_2 for 30 min at 37 °C in the presence of 0.1 mmol/l of isobutyl-1-methyl-xanthine (IBMX). Dose–response curve of M 6434 alone and in the presence of prazosin or BMY 7378 or 2×10^{-6} M PTX on cyclic AMP (cAMP) levels was tested. Antagonists were added at the beginning of the incubation period while M 6434 was allowed to react during the last 15 min. Culture cells that were pre treated with PTX were incubated with the toxin for 20 h. After incubation, cells were homogenized in 2 ml absolute ethanol and centrifuged at $6000 \times g$ for 15 min at 4 °C. Supernatants were collected and evaporated to dryness, and residues were resuspended in 5 mmol/Tris–HCl (pH 7.4) containing 8 mmol/l theophylline, 5 mmol/l EDTA and 6 mmol/l 2-mercaptoethanol. cAMP levels were determined used the Biotrak cAMP [^{3}H] assay system (Amersham Life Science-Protocol-cAMP-[^{3}H] assay), based on the competition between unlabeled cAMP and a fixed quantity of the tritium labelled compound for binding to a protein with high specificity and affinity for cAMP.

2.7. Drugs

Phenylephrine, atropine, prazosin, yohimbine, BMY 7378 (8-(2-[4-(2-methoxyphenyl)-l-piperazinyl]-8-azapirol[4.5] decane-7,9-dione dihydrochloride) and pertussis toxin were provided by Sigma Chemical Company and M 6434 (2-[5-chloro-2-methoxyphenyl)azo]-1-H-imidazole), KMD 3213 (−)-R-1-(3-hydroxyproryl)5-[2-[2-(2,2,2-trifluoroethoxy) phenoxyl] ethylamino] propyl] indoline-7-carboxamide dehydrobromide) and oxymetazoline were provided by Tocris Company. Cholorethylclonidine HCl (CEC) and 5-methylurapidil (5-MU) by Research Biochemical Inc. Stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted in the bath to achieve the final concentrations stated in the text. Table 1 shows the α adrenergic receptor (α-AR) agonists and antagonists used throughout the paper.

2.8. Statistical analysis

Student's t-test for unpaired values was used to determine the levels of significance. When multiple comparisons were necessary, after analysis of variance, the Student–Newman–Keuls test was applied. Differences between means were considered significant if $P < 0.05$.

3. Results

Fig. 1A shows the saturation binding with increasing concentration (0.1–5 nM) of [^{3}H]-prazosin to membranes of human skin fibroblast cells revealed the two saturable α binding sites. Scatchard analysis distinguished a α_1-AR population with high affinity (B_{max}: 42.04 ± 8.82 fmol/mg protein; K_d: 0.18 ± 0.02 nM) and a population with low affinity (B_{max}: 430.8 ± 89.24 fmol/mg protein; K_d: 19.5 ± 2.25 nM) within the concentration range examined (Fig. 1B). To determine subtypes of α_1-AR, competition binding studies were performed using 5-MU (α_{1A}-AR/α_{1D}-AR antagonists), BMY 7378 (α_{1D}-AR antagonist) and KMD 3213 (α_{1A}-AR antagonist) to displace the binding of 0.30 nM [^{3}H]-prazosin. As is shown in Fig. 1C and D all three α_1-AR antagonists displaced [^{3}H]-prazosin in a dose-dependent manner. The pK_i values from these data are shown in Table 2. It can be seen that 5-MU and KMD 3213 competed with [^{3}H]-prazosin with two sites model, while BMY 7378 displayed only one site. The high affinity sites for the antagonists 5-MU and KMD 3213 reflect the α_{1A}-AR and the low affinity sites for the antagonists 5-MU and BMY 7378, reflect the α_{1D}-AR subtypes. KMD 3213 competition against 0.30 nM [^{3}H]-prazosin gave 44 ± 7% high affinity (taken as α_{1A}-AR) and 56 ± 9% low affinity (taken a s α_{1B}-AR and/or α_{1D}-AR). The

Table 1 – Alpha adrenergic agents

Name	α Adrenergic agonist	α Adrenergic antagonist
Phenylephrine (phenyl)	α	–
M 6434	α_1	–
Oxymetazoline (oxymet)	α_{1A}	–
Prazosin (praz)	–	α_1
5 Methyl-urapidil (5-MU)	–	α_{1A}/α_{1D}
Clorethyl-clonidine (CEC)	–	α_{1B}
BMY 7378	–	α_{1D}
KMD 3213	–	α_{1A}
Yohimbine (yohim)	–	α_2

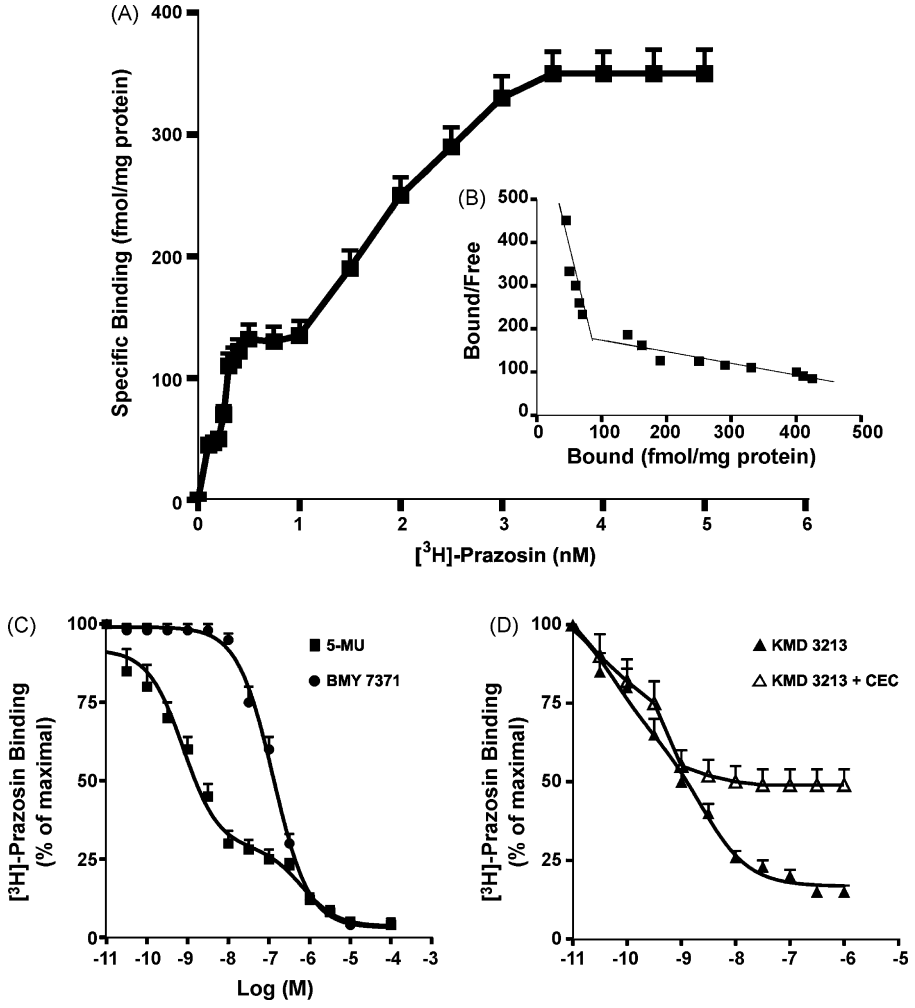

Fig. 1 – Saturation of [^{3}H]-prazosin binding assays of skin fibroblast membranes (A). Scatchard plot (B). Competitive inhibition of [^{3}H]-prazosin by 5-MU (■) and BMY 7371 (●) (C); KMD 3213 (▲) and KMD 3213 + CEC (△) (D) specific binding on fibroblast membranes. Data are means ± S.E. mean of n = #5 separate experiments performed by duplicate.

percentage of high and low affinity [^{3}H]-prazosin binding sites were based on pK_i values of different α_1-AR antagonists; and data also agree closely with functional studies. Since BMY 7378 gave 0% high affinity sites (taken as α_{1D}-AR) and the low affinity KMD 3213 sites are presumed to be the α_{1B}-AR. To further characterize α_{1B}-AR, binding studies were performed in fibroblast cells treated with 1×10^{-7} M CEC before preparing membranes. It can be seen in Fig. 1D that CEC treatment completely eliminated low affinity site for KMD 3213, which was observed in the inhibition of [^{3}H]-prazosin binding by KMD 3213 alone, best fitted to a two site model ($P < 0.05$ versus a one site model). Selective alkylation of α_{1B}-AR by CEC is critically dependent on the concentration of CEC incubation time an temperature; increasing any this factor results in progressive alkylation of α_1-AR and α_{1D}-AR [23]. Therefore, we adopted a protection from alkylation approach using CEC and BMY 7378 to identify the α_{1B}-AR subtype [24].

Table 2 – pK_i of different α_1 adrenergic antagonists

pK_i	5-MU	KMD 3213	BMY 7378
High	9.50 ± 0.42 (α_{1A})**	9.80 ± 0.21 (α_{1A})*	0
Low	6.88 ± 0.35 (α_{1D})	8.50 ± 0.15 (α_{1B})	6.95 ± 0.25 (α_{1D})

Affinity pK_i was determined by displacement of 0.30 nM [^{3}H]-prazosin by different concentration (from 1×10^{-10} to 1×10^{-4} M) of specific α_1 adrenoceptor antagonist subtypes. Values are mean ± S.E. mean of n = #5 in each group taken from Fig. 1. *$P < 0.0010$ between two binding sites with KMD 3213. **$P < 0.0014$ between two binding sites with 5-MU.

To assess the influence of α_1 specific adrenoceptor agonist, concentration–response curves with phenylephrine on [^{3}H]-thymidine incorporation, in human skin fibroblasts were performed. Fig. 2A shows that phenylephrine increased thymidine incorporation reaching the maximal capacity of stimulation at 1×10^{-9} M while at 1×10^{-6} M the α_1 adrenergic agonist exerted it maximal inhibitory action on DNA synthesis. The biphasic effect of phenylephrine was abolished by prazosin (1×10^{-6} M) (an α_1-AR antagonist) and yohimbine (1×10^{-6} M) (an α_2-AR antagonist) was without effect, pointing to the participation of α_1-AR on DNA synthesis. Fig. 2B shows comparatively the biphasic effect

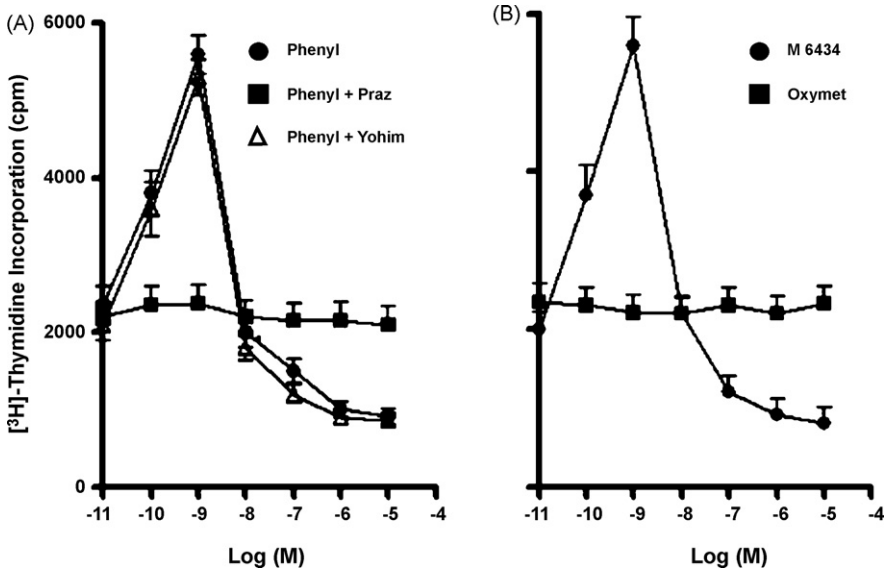

Fig. 2 – (A) Concentration–response curve of phenylephrine alone (phenyl) on [^{3}H]-thymidine incorporation of fibroblast DNA (●) or in the presence of prazosin (praz) 1×10^{-6} M (■) or yohimbine (yohim) 1×10^{-6} M (△). B: Concentration–response curve of α_1 agonist M 6434 (●) and α_{1A} agonist oxymetazoline (oxymet) (■).Values are means ± S.E. mean of n = #6 in each group.

of M 6434 (α_1 agonist) and the lack of the action of oxymetazoline (α_{1A} agonist) upon DNA synthesis.

To determine the α_1 subtypes responsible for the biphasic action on DNA synthesis, the action of prazosin (α_1-AR), CEC (α_{1B}-AR) and 5-MU (α_{1A}/α_{1D}-AR), KMD 3213 (α_{1A}/α_{1B}-AR) and BMY 7378 (α_{1D}-AR) were selectively studied. Fig. 3 shows that prazosin (from 1×10^{-9} to 1×10^{-7} M) (A) shifted to the right the stimulatory dose–response curve of M 6434. Schild plot shows a pA_2 of 9.24 ± 0.3 (D) on DNA synthesis. Moreover, the α_{1B}-AR maximal inhibition with CEC 1×10^{-7} M decreased the α_1-AR agonist stimulatory effect (Fig. 3B). CEC at 10^{-9} and 10^{-8} M inhibited the maximal stimulatory effect of the agonist (M 6434) observed at 5×10^{-9} M in 22 ± 3.1% and 40 ± 3.6% (n = 5), respectively. Neither 10^{-9} M 5-MU that antagonized α_{1A}-AR nor 10^{-5} M 5-MU that antagonized α_{1D}-AR modified the stimulatory action of M 6434 (Fig. 3C). Moreover, the α_{1D}-AR antagonist BMY 7378 from 10^{-8} to 10^{-5} M did not have effect (data no shown); excluding the α_{1D}-AR in the stimulatory effect of the α_1-AR agonist.

On the contrary, Fig. 4 shows experiments with BMY 7378 (A), prazosin (B) and 5-MU (C) from 1×10^{-8} to 1×10^{-6} M, shifted to the right the inhibitory dose–response curve of M 6434. The pA_2 were 8.0 ± 0.2, 8.8 ± 0.7 and 8.1 ± 0.3, respectively (D), pointing to the participation of α_{1D}-AR in the inhibitory effect. Furthermore, α_{1A}/α_{1B}-AR antagonist KMD 3213 at concentration range of 10^{-10} to 10^{-6} M had no effect (data no shown) excluding the implication of α_{1A}/α_{1B}-AR in the inhibitory effect of M 6434.

To elucidate if there were changes in the post-receptor mechanisms involving PLC and NOS activities we studied the participation of InsP and nitric oxide (NO) in the system. With this purpose, we explored the actions of U-73122 (5×10^{-6} M, PLC inhibitor), TFP (5×10^{-6} M, CaM inhibitor) and L-NMMA (5×10^{-5} M, NOS inhibitor) on both, the stimulatory and the inhibitory effects of M 6434. All the inhibitory agents, at the concentrations used, did not have any effect of thymidine incorporation upon basal values (Table 3).

It can be seen after stimulatiom (Fig. 5A) the enzymatic inhibition of PLC, calcium/calmodulin (CaM) and nitric oxide synthase (NOS) activities, prevented as correlated to the maximal stimulatory effect of M 6434 at 1×10^{-9} M to DNA synthesis in human fibroblasts. On the other hand, after inhibition (Fig. 5B) shows that the inhibition of PLC, CaM and NOS activities did not modified the inhibitory action of M 6434 (1×10^{-6} M) on DNA synthesis. However, pertussis toxin (2×10^{-6} M) was able to impaired the inhibitory action of M 6434, but did not modify its stimulatory action (Fig. 5A and B).

To assess if the stimulatory action of M 6434 was related to PLC signaling, InsP accumulation and NOS activity were measured. As shown in Fig. 6, M 6434 (in the concentrations range that increased DNA synthesis) increased InsP accumulation (A) and NOS activity (B). Prazosin (from 1×10^{-9} to 1×10^{-7} M) inhibited the stimulatory effect of M 6434 upon both InsP accumulation (pA_2 9.5 ± 0.6) and NOS activity (pA_2 9.4 ± 0.8). On the contrary, BMY 7378 (from 1×10^{-9} to 1×10^{-7} M) did not prevent the effects of the adrenergic agonist on InsP accumulation and NOS activity. It is important to note that CEC (1×10^{-7} M) decreased the effects of M 6434 on both InsP accumulation (C) and NOS activity (D) (Fig. 6).

For further studies the mechanism by which M 6434 induced inhibition of DNA synthesis, cAMP production was measured. It can be seen in Fig. 7, that M 6434 inhibited basal cAMP production in a concentration-dependent manner. From 1×10^{-8} to 1×10^{-6} M both prazosin (pA_2 8.6 ± 0.4) and BMY 7378 (pA_2 8.1 ± 0.3) but not KMD 3213 were able to

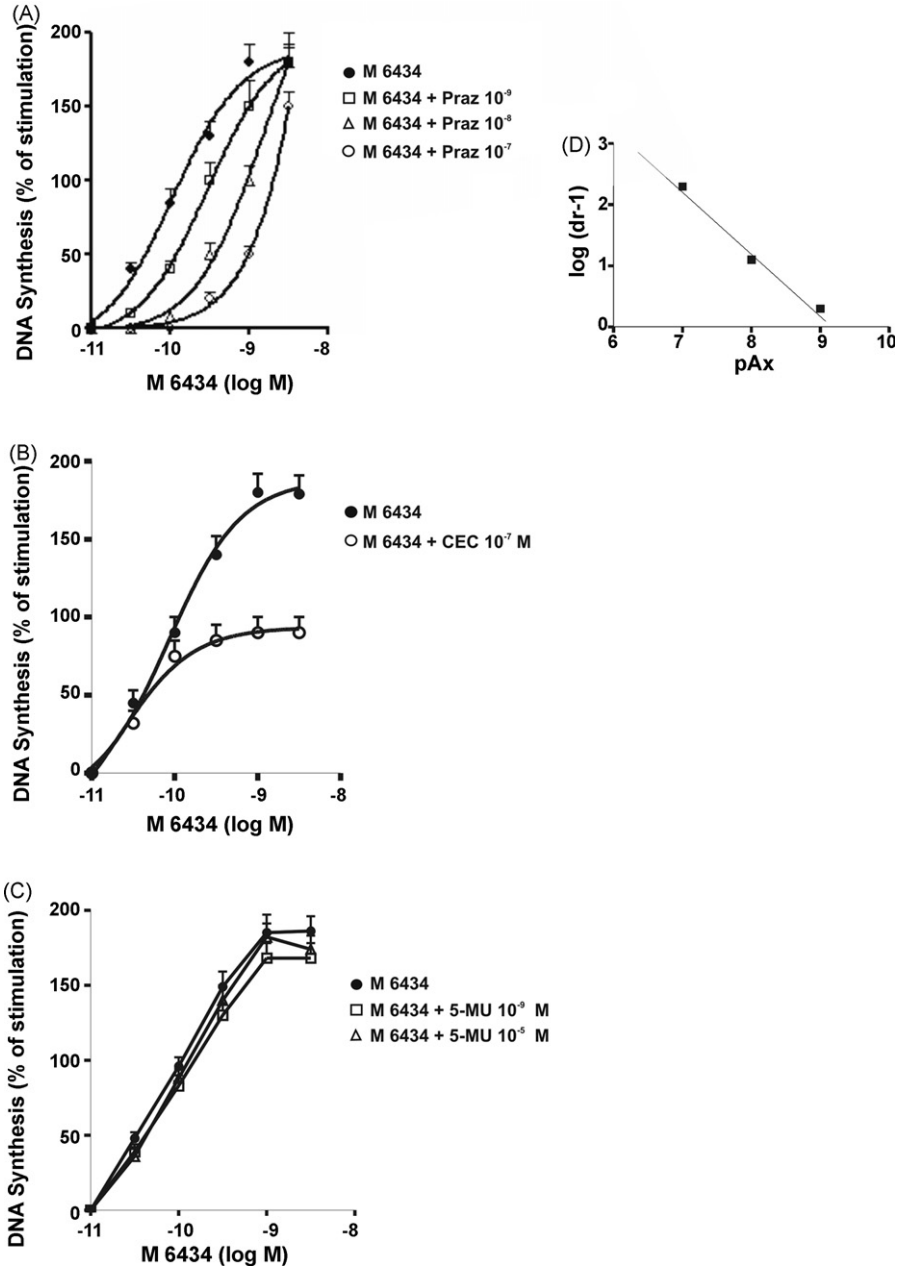

Fig. 3 – Effect of 1×10^{-9} M (□), 1×10^{-8} M (△) and 1×10^{-7} M (○) of prazosin (praz) (A); 1×10^{-7} M CEC (B) and 1×10^{-9} M and 1×10^{-7} M 5-MU (C) on dose–response curve of 6434 (●) induced stimulation on fibroblast of DNA synthesis. Each point represents the mean ± S.E. mean of n = #4 in each group. Schild plots (D) of prazosin (■) antagonism of M 6434-mediated DNA synthesis stimulation.

prevent the inhibition of cAMP production triggered by the specific α_1 agonist in this system. Also, pertussis toxin (2×10^{-6} M) abolished the action of M 6434 on cAMP decrement. Basal values of cAMP fibroblast was 1.4 ± 0.01 ($n = 5$).

Fig. 8A demonstrated a significant correlation between increase in DNA synthesis and increase in InsP accumulation and NOS activity. Also, a significant correlation has observed between decrease in DNA synthesis and decrease in cAMP production (Fig. 8B) and on the decrease of [^{3}H]-prazosin binding triggered by BMY 7378 (Fig. 8C).

4. Discussion

This study is one of the primary novel finding demonstrates the presence of α_1-AR on human neonatal foreskin fibroblasts. Three major types of α_1-AR could be defined pharmacologically on the basis of selective antagonists. We found that the distribution of α_{1A}-AR and α_{1B}-AR are equally expressed in fibroblast cells; while α_{1D}-AR is less prominent. Prazosin has more affinity for the α_{1A}-AR than α_{1B}-AR subtypes. Thus, competition studies with KMD 3213 indicate high affinity binding sites for α_{1A}-AR and low affinity binding sites for

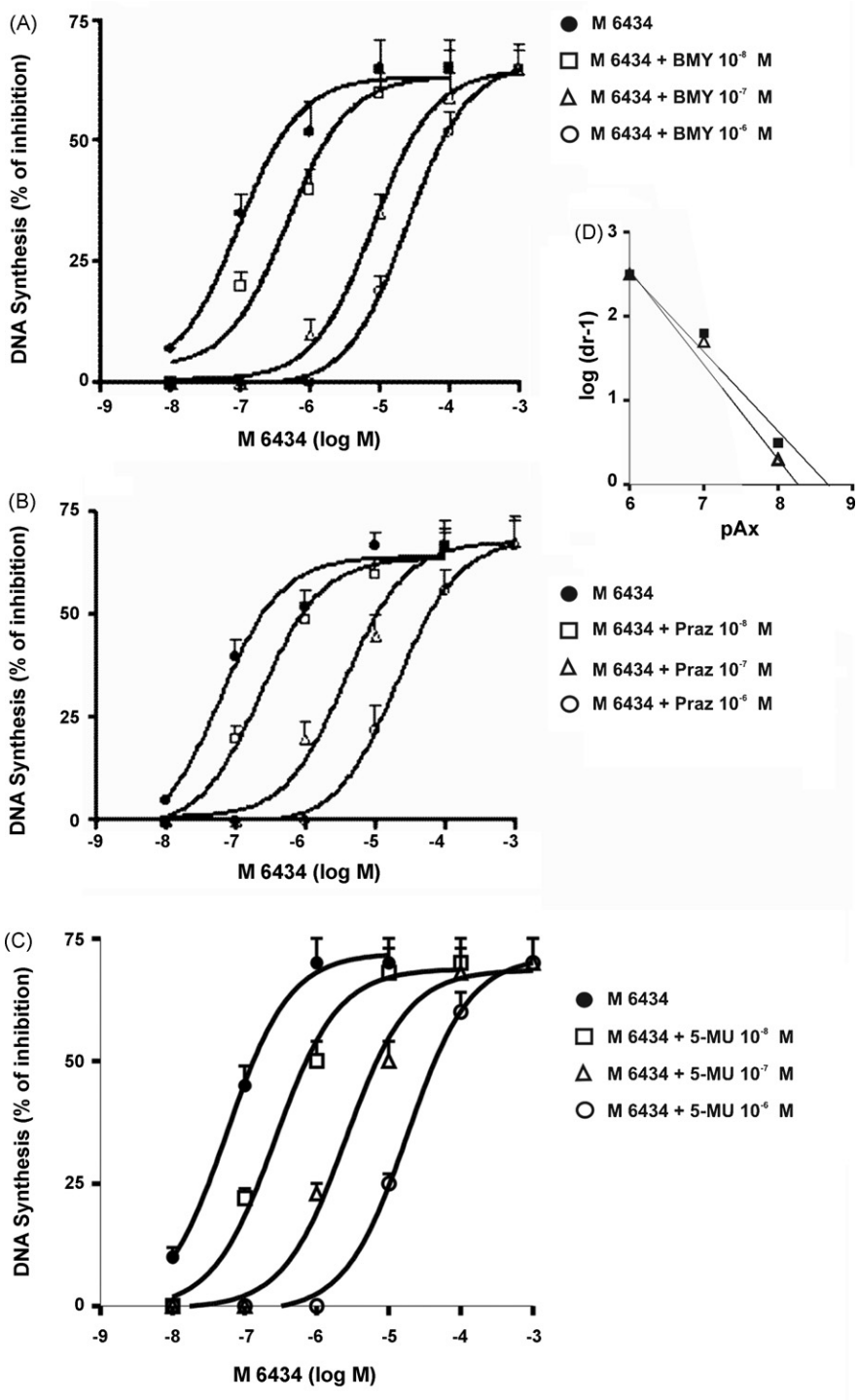

Fig. 4 – Effect of 1×10^{-8} M (□), 1×10^{-7} M (△) and 1×10^{-6} M (○) of BMY 7378 (A); prazosin (praz) (B) and 5-MU (C) on dose–response curve of M 6434 (●) induced inhibition on fibroblast of DNA synthesis. Each point represents the mean ± S.E. mean of n = #4 in each group. Schild plots (D) of BMY 7378 (■) and prazosin (△) antagonism of M 6434-mediated DNA synthesis inhibition.

α_{1B}-AR. The fact that CEC treatment of fibroblast before preparing membrane completely eliminated low affinity sites for KMD 3213 allowed us to identify to α_{1B}-AR subtype in neonatal foreskin fibroblasts. On the other hand, experiments with BMY 7378 indicate a single low affinity binding sites for α_{1D}-AR subtype. Consistent with the identification of the pK_i of α_{1B}-AR and pK_i of α_{1D}-AR, are the observed differences in subtypes functionality of these cells. We not observe α_{1A}-AR functionality in human neonatal foreskin fibroblast. Non-functional truncated α_{1A}-AR isoform have been also observed

Table 3 – Influence of inhibitory agents upon basal [^{3}H]-thymidine incorporation

Inhibitor agents	[^{3}H] thymidine incorporation (cpm)	*n*
Control	2236 ± 230	10
Prazosin (1×10^{-6} M)	2150 ± 198	5
CEC (1×10^{-7} M)	2090 ± 115	5
BMY 7378 (1×10^{-6} M)	2230 ± 216	6
KMD 3213 (1×10^{-6} M)	2370 ± 194	6
U-73122 (5×10^{-6} M)	2150 ± 229	5
TFP (5×10^{-6} M)	2165 ± 234	5
L-NMMA (5×10^{-5} M)	2207 ± 189	5
Yohimbine (1×10^{-6} M)	2195 ± 233	5

Results are means ± S.E. mean for control without drugs. Values showing the [^{3}H]-thymidine incorporation of fibroblast in serum free medium exposed during 20 h to α_1 adrenergic antagonist (prazosin) [27]; α_{1B} antagonist (CEC) [30]; α_{1D} antagonist (BMY 7378) [27]; α_{1A} antagonist (KMD 3213) [10]; PLC inhibitor (U-73122) [38]; calcium/calmodulin inhibitor (TFP) [39], NOS inhibitor (L-NMMA) [40] and α_2 adrenoceptor antagonist (yohimbine) [11]. Control: 20 h without drug.

in human prostate [25], hippocampus [26], heart and liver [27] but the physiological significance of α_{1A}-AR splice variants is currently unknown.

Reports from various laboratory reveal differences with respect to relative α_1-AR subtype expression in different tissue [28,29]. The pattern of expression of α_1-AR and subtypes distribution observed in human fibroblast, resulted similar in magnitude and in potency described in rat preglomerular vessels and in adventitial fibroblasts [10,14].

In this study we determined the specific contribution of α-AR on human fibroblast DNA synthesis. We observed that neonatal human fibroblast responded to α_1-AR agonist in a biphasic fashion that depended on the concentrations in which different α_1-AR subtypes, are functionally activated. α_2-AR appears not to be participated. The α_{1D} antagonist (BMY 7378) blocks the inhibitory effect on DNA synthesis, therefore, indicated that the α_{1D} subtype is linked to the inhibitory response at higher concentration of the α_1-AR agonists. On the contrary, α_{1B}-AR antagonists, inhibited the stimulatory effect on DNA synthesis, suggesting that α_{1B}-AR subtype could be linked to the stimulatory response observed at low concentrations of α_1-AR agonist as described previously [2,30].

The difference in functional regulation of human fibroblast α_1-AR could result from different G protein coupling α_1-AR subtype. The α_{1B}-AR that mediated stimulation of DNA synthesis was dependent on PLC/CaM/NOS signaling whereas α_{1D}-AR inhibition was dependent on decrease cAMP. This promiscuous coupling to G_o/G_i protein observed in this study was reported previously [31]. The actual potency displayed by the antagonists tested on binding, DNA synthesis, InsP accumulation and NOS activity at low agonist concentrations, allow us to propose that they can be elicited by the activation of the same receptors (α_{1B}-AR subtype coupled to G_q). On the contrary, the potency displayed by the antagonists tested indicated that the decrement in DNA synthesis and cAMP at higher concentrations, account by the activation of α_{1D}-AR coupled to G_i protein. This is confirmed by the fact that the

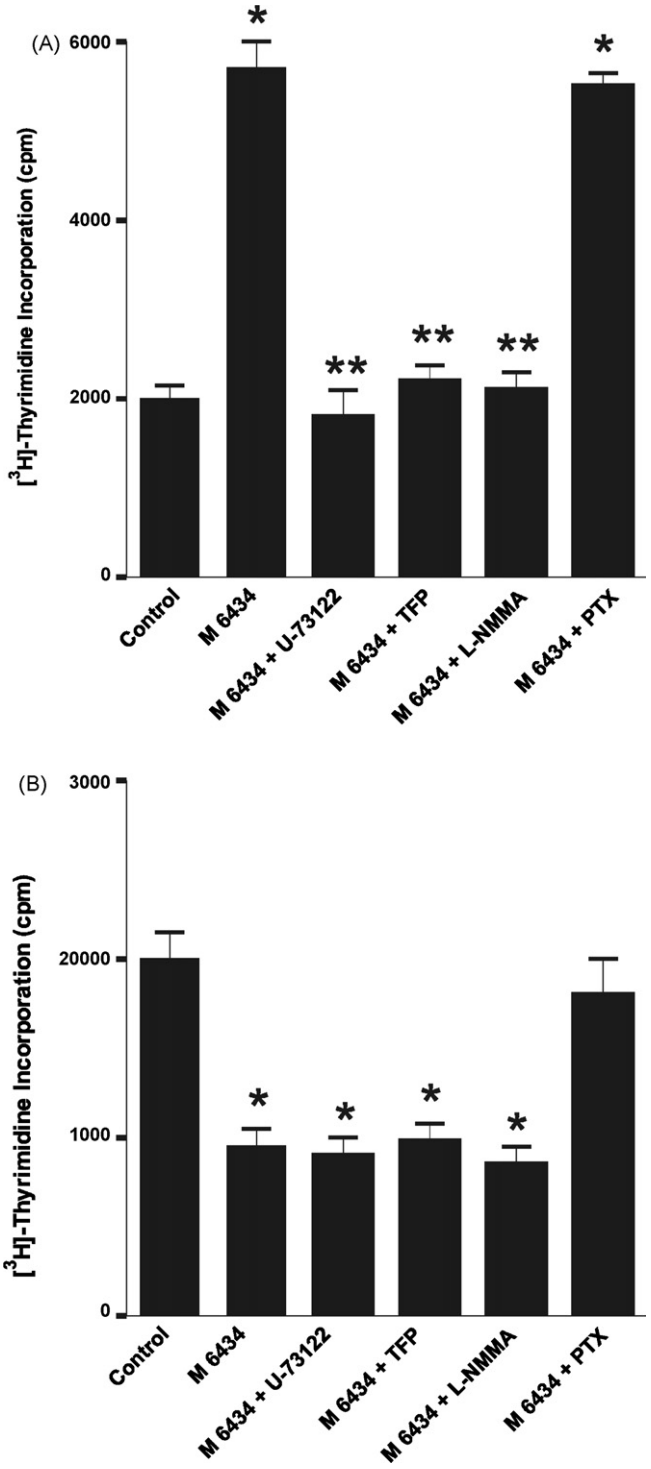

Fig. 5 – Effect of M 6434 alone: 1×10^{-9} M (A) and 1×10^{-6} M (B) or in the presence of U-73122 (5×10^{-6} M, PLC inhibitor), TFP (5×10^{-6} M, CaM inhibitor), L-NMMA (1×10^{-5} M, NOS inhibitor) or pertussis toxin (2×10^{-6} M, PTX) on M 6434-induced ^{3}H-thymidine incorporation on fibroblast cells. Values are means ± S.E. mean of *n* = #6. *They differ significantly from control values with $P < 0.001$. **They differ significantly from M 6434 alone with $P < 0.001$.

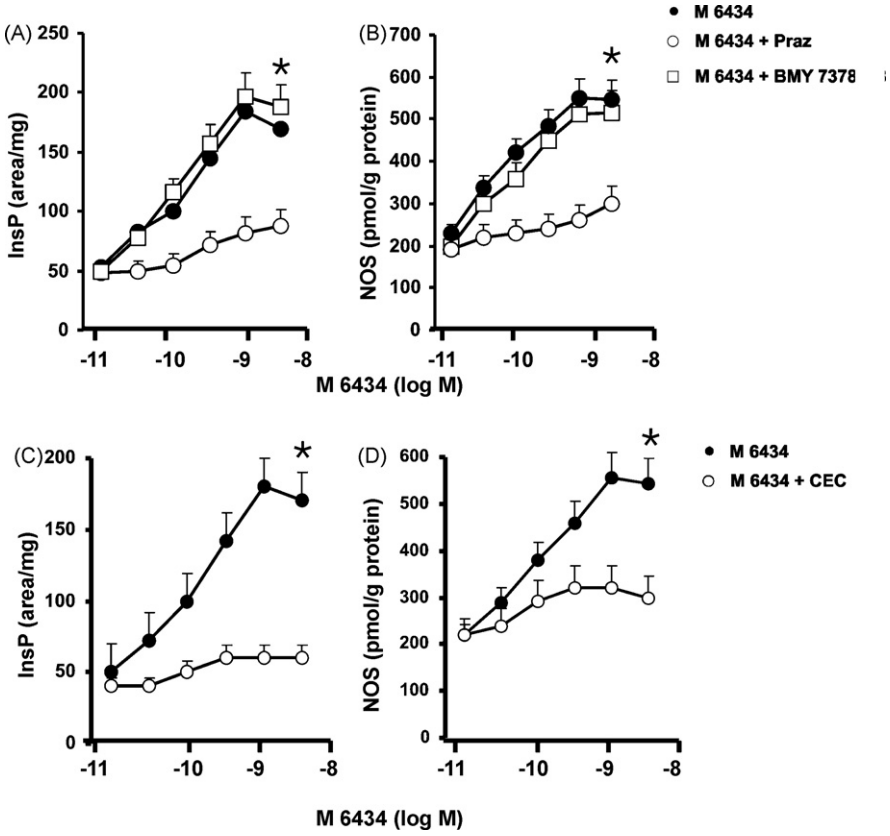

Fig. 6 – Changes in intracellular InsP (A) and NOS (B) in response to incubating fibroblast in serum-free medium in the presence of increase concentration of M 6434 alone (●) or in the presence of 1×10^{-6} M BMY 7378 (□) or 1×10^{-6} M prazosin (○). The action of 1×10^{-7} M CEC (○) on M 6434 dose–response curve of InsP (C) and NOS (D) is also shown. Values are means ± S.E. mean of n = #7. *$P < 0.001$ between M 6434 alone vs. M 6434 plus prazosin or M 6434 alone vs. M 6434 plus CEC.

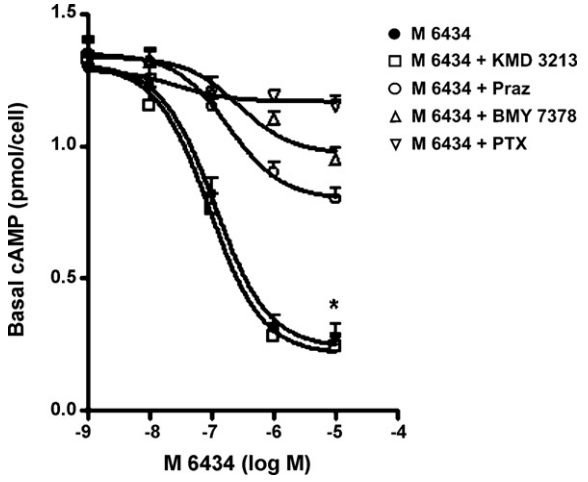

Fig. 7 – Changes in intracellular cAMP in response to incubating fibroblast in serum-free medium in the presence of increasing concentration of M 6434 alone (●) or in the presence of KMD 3213 1×10^{-6} M (□), prazosin 1×10^{-6} M (○), BMY 7378 1×10^{-6} M (△) or pertussis toxin 2×10^{-6} M (▽) on cAMP production. Values are means ± S.E. mean of n = #5. *$P < 0.001$ between M 6434 alone vs. basal or M 6434 alone or in the presence of prazosin or BMY 7378 or pertussis toxin (PTX).

increase in DNA synthesis positively correlated ($r = 0.05$) with an increase in InsP accumulation and NOS activity pertussis toxin insensitive. On the contrary, the inhibition of DNA synthesis correlated ($r = 0.05$) with a decrease in cAMP pertussis toxin sensitive. The rat 1 fibroblast stably expressing either the α_1-AR and α_{1D}-AR subtypes, inhibited DNA synthesis but differ with respect to the MAPK family member involved and the requirement for agonist [11]. In fact, activation of both α_{1B}-AR and α_{1D}-AR inhibited DNA synthesis. In contrast, both AR subtypes stimulated protein biosynthesis and activated extracellular signal-regulated kinases. These responses were agonist-dependent for α_{1B}-AR, but agonist independent for α_{1D}-AR.

The correlation between the receptor subtypes and the signal transduction pathways observed in neonatal skin fibroblast are in accordance with those observed, at least, in rat blood vessels in which α_{1B}-AR mediated a rapid increase in the formation of IP_3 and promotes the release of calcium from intracellular stores [28]. Moreover, activation of α_{1B}-AR in fibroblast up-regulated a set of genes associated with the cell cycle progression and stimulated cell proliferation, a common regulatory mechanism for G protein-coupled receptor [9]. By contrast, the α_{1D}-AR subtype appeared to control the fibroblast cell cycle by not only decreasing the transcription of cell cycle, but also by down-regulating kinase activity [9]. The effect of PKA and cAMP on fibroblast DNA synthesis is still

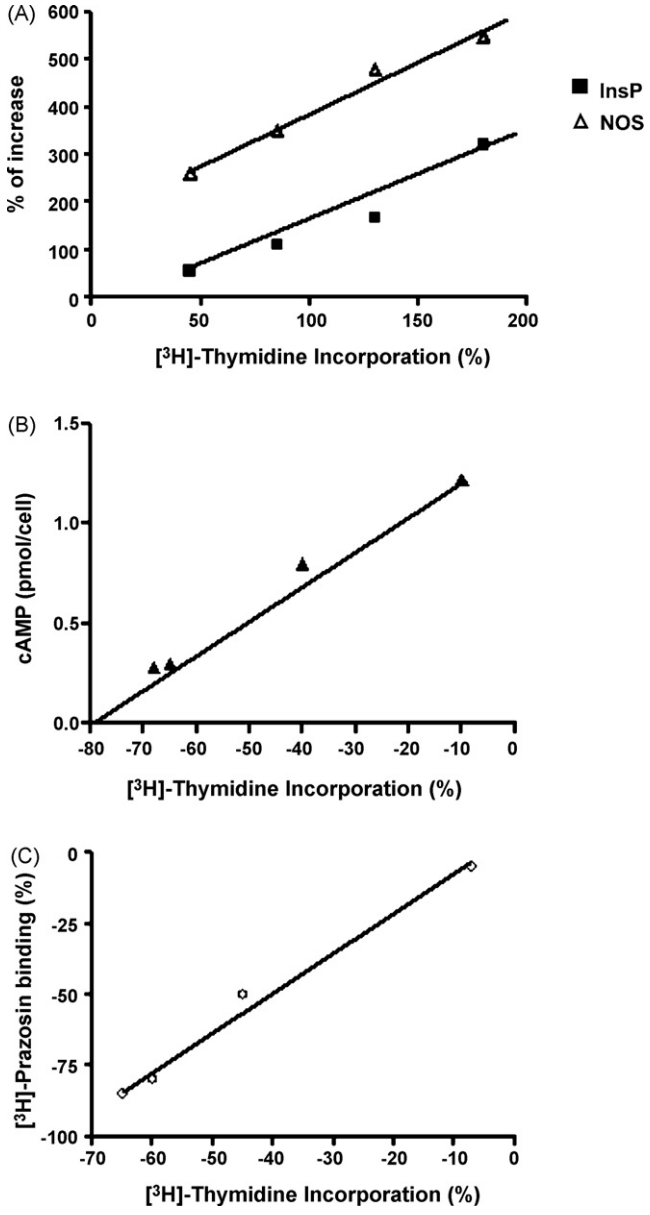

Fig. 8 – Correlation in the effect of M 6434 on fibroblast DNA synthesis on: (A) InsP (■) (R^2: 0.9584; *P*-values 0.0036) and NOS activity (△) (R^2: 0.9832; *P*-values 0.0009); (B) cAMP (R^2: 0.9927; *P*-values 0.0037) production and (C) BMY 7378 competition on [^{3}H]-prazosin binding (R^2: 0.9892; *P*-values 0.0005). Stimulation of DNA synthesis was plotted as a function of InsP and NOS. Inhibition of DNA synthesis was plotted as a function of cAMP and [^{3}H]-prazosin binding. Values are means ± S.E. mean of *n* = #6 in each group.

unclear. Substatial evidence exists that the increase in cAMP production triggers fibroblast proliferation mediated by β adrenergic receptors [32]. Previous studies have demonstrated a growth-inhibitory effect of cAMP [33,34], although a stimulatory effect has also been reported [35].

In addition our results indicated that the accumulation of InsP generated by PLC action is among the intracellular events triggered by the activation of α_{1B}-AR that lead to stimulation of DNA synthesis. The accumulation of InsP via PLC occurs, in accord with this experiments, PLC inhibitor (U-73122) prevented the stimulatory action of the α_1-AR agonist. Moreover, the fact that CaM inhibitor (TFP) abrogates this effect, point to the release of calcium from cytoplasmatic store being responsible for the stimulatory action of the α_1-AR agonist. The increase in intracellular calcium also leads to activation of NOS, that in turn triggers cascade reaction that stimulated DNA synthesis.

Interaction of neuronal NOS and α_{1B}-AR was demonstrated in HEK-293 cells; but direct comparison of α_{1A}-AR, α_{1B}-AR and α_{1D}-AR showed similar degrees of interaction of all three subtypes with neuronal NOS [36]. Related with the functional significance of these interactions, our results demonstrated that α_{1B}-AR-mediated stimulation of DNA synthesis by activation of NOS pathway. Studies on α_{1A}-AR in transfected PC12 cells showed no role for nitric oxide in mitogenic signaling [36].

Our studies gave evidence that within the same cellular context, α_1-AR agonist can either stimulate or inhibit the DNA synthesis depending on the α_1-AR agonist concentrations and the α_1-AR subtypes are expressed, heterogeneously in skin fibroblast as well as the profiles that are pathway-specific. Also, a possible mechanism that could explain the difference in functional regulation of human fibroblast α_1-AR include the subtype specific difference in cellular localization; in which α_{1A}-AR predominantly localize intracellulary whereas most of α_{1B}-AR/α_{1D}-AR localize on cell surface [37]. Our results suggest that α_{1D}-AR mediating decrease in basal cAMP accumulation can be differentiated from α_{1B}-AR mediating increases in inositol phosphate accumulation by their binding property, sensitivity to the alkylating agent CEC and the coupling to G_i/G_o protein, respectively.

To our knowledge there have been no previous reports as to a direct effect of α_1-AR stimulation on human skin fibroblasts. The presence of active α_1-AR subtypes on human skin fibroblast may play a role in the regulation of cell growth and biological activity, since α_1 adrenergic stimulation can change the balance of the intracellular signals involved in triggering cell division and function.

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