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Activation of β_3 Adrenergic Receptor Decreases DNA synthesis in Human Skin Fibroblasts Via Cyclic GMP/Nitric Oxide Pathway

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Key Words

β adrenoceptors • DNA synthesis • Skin fibroblast • Cyclic nucleotides • Nitric oxide synthase

Abstract

Background: Evidences have shown that β_1 and β_2 adrenoceptors co-exist in human fibroblasts, but it is not yet clear the functional expression of β_3 adrenoceptor in these cells. The aim of this study was to investigate the expression and biological effect of β_3 adrenoceptor activation in human skin fibroblast and the different signaling pathways involved in its effect. Methods: For this purpose *in vitro* cultures of human skin fibroblast were established from human foreskin and grown in Dulbecco's modified Eagle's medium. The effect of ZD 7114 (β_3 agonist) on cell DNA synthesis, radioligand binding assay, cyclic GMP and cyclic AMP accumulation and nitric oxide synthase (NOS) activity were evaluated. Results: ^{3}H-CGP binding to human fibroblast membranes was a saturable process to a single class of binding site. The equilibrium parameters were: Kd 20±3 pM and Bmax 222±19 fmol/mg protein. Ki values showed that these cells express a high number of β_3 adrenoceptor subtypes. ZD 7114 stimulation of β_3 adrenoceptor exerts a concentration-dependent inhibition of DNA synthesis and cAMP accumulation with parallel increase in NOS activity that led to cGMP accumulation. All these effects were blocked by the β_3 adrenoceptor antagonist (SR 59230A). The effect of ZD 7114 on DNA synthesis significantly correlated with its action either on cAMP or NOS-cGMP signaling system. Inhibitors of NOS activity and NO-sensitive guanylate cyclase prevented the inhibitory effect of ZD 7114 on DNA synthesis. In addition, the β_3 adrenoceptor-dependent increase in cGMP and activation of NOS were blocked by the inhibition of phospholipase C (PLC), calcium/calmodulin (CaM), endothelial NOS activity and cGMP accumulation. Conclusions: β_3 adrenoceptor activation exerts inhibitory effect on human fibroblast DNA synthesis as a result of the activation of NO-cGMP pathway and the inhibition of adenylate cyclase activity. The mechanism appears to occurs secondarily to stimulation of PLC and CaM. This in turn triggers cascade reaction leading to increase production of NO-cGMP with decrease in cAMP accumulation.

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Introduction

The human fibroblasts have been useful in the clinical investigation of a variety of conditions due to its ease of access and its potential for reflecting alterations in central and systemic diseases. The fibroblast is the cell central effective to wound healing and tissue remodeling and is tightly regulated by many hormones and neurotransmitters that govern its migration, extracellular matrix remodeling and proliferation [1, 2].

As well as adrenergic receptors are implicated in the modulation of normal cell proliferation, exerting a negative or a positive control of cell proliferation depending on the different cell types and the β adrenoceptor subtypes expressed in the cells. It is also well known, that stimulation of β_1 or β_2 adrenoceptors releases of $G_{s\ alpha}$ which then activates adenylate cyclase to produce cAMP accumulation [3, 4], that in turn is able to modulate cell proliferation [5]. Elevated levels of cAMP increases cell proliferation via activation of cAMP-dependent protein kinase A (PKA) and this PKA are responsible to cause phosphorylation of β_1 or β_2 adrenoceptors leading to their inactivation [6].

Substantial evidence exists that increase in cyclic AMP modulates fibroblasts proliferation mediated by β adrenergic receptors [7]. The positive action has been observed on human cardiac fibroblasts [7-9] and the negative action was previously reported on human lymphocytes [10]. The human β_2 adrenoceptor stimulation has been the prototype for elucidating mechanisms of human fibroblast proliferation [11]. But, even less is known about β_1 adrenoceptor stimulation that is also expressed in active state on fibroblasts stimulating DNA synthesis [12]. Atypical β adrenergic responses has been identified with a novel receptor subtype, β_3 adrenoceptor; first found in brown fat cells [13], colon and gall bladder [14] and in human ventricle [15]. Recently, a promiscuous coupling between β_3 adrenoceptor and G_s/G_i protein was demonstrated [16]. There is an unusual biphasic effect on cAMP production in response to β adrenoceptor agonist; in which the agonist could either stimulate or inhibit adenylate cyclase activity in adipocyte [16]. Furthermore, in some cell types, although β_3 adrenoceptors are prominent, increases in cAMP occur primarily through β_1 or β_2 adrenoceptors. The β_3 adrenoceptor coupling to G_i could serve to restrain G_s mediated activation of adenylate cyclase and to initiate additional signal transduction pathways [16]. However, there is little information on the additional signal transduction pathway involved β_3 in adrenoceptor-mediated modulation of fibroblasts proliferation.

Cyclic GMP (cGMP) and substances that increase cellular cGMP and nitric oxide (NO) – generating molecules, inhibit the stimulation of DNA synthesis in vascular smooth muscle cells, endothelial cells, mesangial cells and cardiac fibroblasts [17-19]. Also, evidence exits that cGMP is an antagonist of mitogen action in murine fibroblasts decreasing cell proliferation [1]. A number of years ago were demonstrated that fibroblast cell lines contain particularly high activity of a guanylate cyclase receptor and some fibroblast cell lines, also, appear to contain a soluble guanylate cyclase responsive to NO [20].

The purpose of the present study was to investigate the effect of β_3 adrenoceptor agonist on the human skin fibroblast DNA synthesis. An additional purpose of this study, was to examine the possibility that growth-inhibiting β_3 adrenoceptor activation is mediated by a cross talk between the decrease in cAMP and the increase in cGMP / NO accumulation.

Materials and Methods

Cell Culture

Pure cell cultures of neonatal human skin fibroblast 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 37ºC described by Varani et al [21]. The medium was replenished every 3-4 days. Confluent cells were sub cultured by detaching the monolayer with 0.25% trypsin in phosphate buffered saline solution (PBS). Cells of passages 12-14 were used throughout. To identify the fibroblast cultures and assay conditions, a series of experiments was carried out to established incubation time and cell numbers. The optimal conditions (sharp peak) for [^{3}H] thymidine incorporation were at 3-6 hours and was $2x10^4$ the number of cell growth. All the studies involving human subjects were conducted according to the tenets of the Declaration of Helsinki.

Measurement of DNA Synthesis

DNA synthesis was estimated by measurement of [^{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 ZD 7114 concentrations for 3 hours and [^{3}H]-thymidine (0.1 μCi/ml) was added during the last hour. Cells were precipitated twice with ice-cold 10% TCA. When antagonistic drugs and enzymatic inhibitors were used, they were added 15 min before ZD 7114. Cells were then removed with trypsin/PBS and radioactivity was determined by liquid scintillation counting.

Binding Assay

For the radioligand binding assays, cells were grown to confluency in tissue culture dishes, quantitatively harvested and collected by means of low-speed centrifugation (250 g). The cells were resuspended in 5 volumes of 10 mM potassium phosphate, 1 mM $MgCl_2$, 0.25 M sucrose pH 7.5 (buffer A), supplemented with 0.1 mM phenylmethylsulphonylfluoride (PMSF), 2 μg/ml leupeptin and 1 μM pepstatin A and homogenized in an Ultraturrax at 4ºC. Then, the homogenate was centrifuged twice for 10 min a 3000 g, then at 10000 g and 40000 g at 4ºC for 15 and 90 min respectivelly. 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 described previously [22]. Aliquot of the membrane suspension (50 μg protein) were incubated with increasing concentrations of [^{3}H]-CGP (Dupont New England Nuclear, Boston, MA, USA; Sp.Ac. 48.6 Ci/mmol) for 60 min at 25ºC in a total volume of 150 μl of buffer B. 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 spectometer. Nonspecific binding was determined in the presence of 1 μM propranolol and never exceeded 10% of total binding. Radioactivity bound was lower than 10% of total counts. For competition binding assays, membranes were incubated with about 0.51 nM [^{3}H]-CGP alone and in the presence of increasing concentration of adrenoceptor antagonists (nonspecific and specific).

Determination of nitric oxide synthase activity (NOS)

Nitric oxide synthase (NOS) activity was measured in cells by production of [U-^{14}C]-citrulline from [U-^{14}C]-arginine according to the procedure described by Bredt, Ferris & Snyder [23] and by Sterin-Borda et al. [24]. 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% in the presence of 0.5 mM N^G-monomethyl-L-arginine (L-NMMA). 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.

Cyclic GMP (cGMP) and Cyclic AMP (cAMP) assay

Cells were incubated in 1 ml KRB for 30 min and the β agonist (ZD 7114) was added in the last 5 min. When blockers were used, they were added 25 min before the addition of ZD 7114. After incubation, cells were homogenized in 2 ml of absolute ethanol and centrifuged at 6.000 g for 15 min at 4°C. Pellets were then rehomogenized in ethanol-water (2:1) and supernatants collected and evaporated to dryness as indicated above. Cyclic AMP or cyclic GMP in the residue was dissolved in 400 μl of 0.05 M sodium acetate buffer pH 6.2. Aliquots of 100 μl were taken for the nucleotide determination using RIA procedure with a cyclic AMP^{3}H or cyclic GMP^{125}I - RIA KITS from Dupont New England Nuclear [25].

Table 1. Inhibition of [^{3}H]-CGP binding to human skin fibroblast membranes. The inhibition constants (Ki) for the competing agents were calculated from the equation of Cheng and Prussoff: $Ki = IC_{50}/a$ ([3H]-CGP) / Kd, where IC_{50} is the concentration of the competing drug to inhibit 50% of the specific radioligand binding present at a concentration of 0.5 nM. IC_{50} values were obtained from competition experiments performed in duplicate at several concentrations of each drug.

Adrenoceptor Antagonist	Ki (x 10^{-8} M)
Propranolol (full)	0.81 ± 0.03
SR 59230A (β_3)	2.27 ± 0.08
ICI 118,551 (β_2)	3.77 ± 0.09
Atenolol (β_1)	78 ± 6.7

Drugs

Aminoguanidine, L-arginine, 8-bromo-cyclic GMP, forskolin, L-NMMA, propranolol, atenolol, BAPTA and trifluoperazine (TFP) (Sigma Chemical Company, Saint Louis, Mo, USA); U-73122, ODQ and staurosporine (ICN Pharmaceuticals Inc., Costa Messa, CA, USA); ZD 7114, SC 59230A, ICI 118,551, L-NIO and N-propyl-L-arginine (Tocris Bioscience, Ellisville, Mo, USA). Stock solutions were freshly prepared in the corresponding buffers.

Statistical Analysis

Student's t-test for unpaired values was used to determine the levels of significance. Analysis of variance (ANOVA) and post hoc test (Dunnett´s Method and Student-Newman-Keuls test) were employed when pair wise multiple comparison procedure was necessary. Differences between means were considered significant if $P < 0.05$.

Results

To analyze the expression of β adrenoceptor in skin human fibroblasts, we studied the ability of [^{3}H]-CGP to bind to fibroblast membranes. Figure 1 shows the results obtained from saturation binding assays (A) and Scatchard analysis (B) indicated the presence of a single class of high affinity specific binding site. The maximal number of binding site (Bmax) was 222 ± 19 fmol/mg protein and the equilibrium dissociation constant (Kd) was 20 ± 3 pM. The Ki values calculated from competitive binding assays showed that fibroblasts express a high number of β_3 adrenoceptor subtypes (Table 1).

To define the physiological action of β_3 adrenoceptors in these cells, the effects of a β_3 adrenoceptor agonist on DNA synthesis and cAMP production were studied. As shown in Figure 2, ZD 7114 induced a concentration-dependent decrease in both DNA

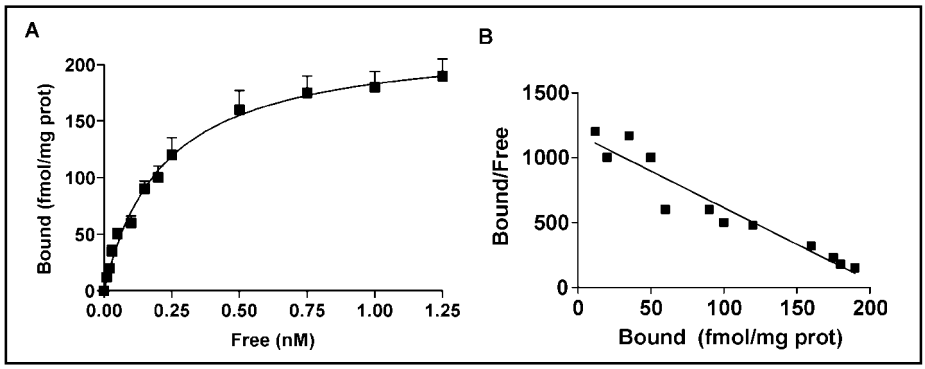

Fig. 1. ^{3}H-CGP binding on human skin fibroblast membranes. (A) membranes (50 µg protein) were incubated in the presence of increasing ^{3}H-CGP concentrations. (B) equilibrium parameters calculated from Scatchard plots were: Bmax 222 ± 19 fmol/mg protein and Kd 20 ± 3 pM. Results are mean ± SEM of five experiments performed in duplicate

synthesis (A) and cAMP production (B). These effects were prevented by the specific β_3 adrenoceptor antagonist SR 59230A (1x10^{-7} M); indicating that the inhibition of DNA synthesis and the decrement in basal cAMP are direct effect triggered by the same receptor subtype. As control, the direct activation of adenylate cyclase by forskolin (1x10^{-4} M) was able to stimulate both DNA synthesis (+48±2.3 %, n=5) and cAMP production (+92.5±7%, n=6).

To determine if an endogenous nitric oxide (NO) signaling system participates in the DNA synthesis inhibition triggered by β_3 adrenoceptor agonist, the effect of different inhibitors of the enzymatic pathways involved in NO and cGMP production were studied. Figure 3A shows that ZD 7114 effect was prevented by the inhibition of nitric oxide synthase (NOS) activity by L-NMMA (1x10^{-5} M). The natural substrate of NOS, L-arginine (5x10^{-5} M), reversed the L-NMMA effect. Figure 3B shows that β_3 adrenoceptor agonist increase in a concentration-dependent manner NOS activity and this effect was selectively blocked by SR 59230A.

Additionally, to examine the possibility that the following steps of NO cascade activation would be involved, the effect of a NO-sensitive guanylate cyclase inhibitor (ODQ 5x10^{-5} M) was explored. Figure 4A shows that ODQ 5x10^{-5} M impaired the inhibitory effect of ZD 7114 on DNA synthesis. The same Figure (4B) shows the ability of the β_3 adrenoceptor agonist to increase production of cyclic GMP (cGMP) and the specific inhibition by SR 59230A (1x10^{-7} M). As control, 1x10^{-4} M 8-bromo-cGMP (analogue to cGMP) was able to inhibit fibroblast DNA synthesis (-45 ± 3.8%, n=5).

Figure 5 demonstrates a significant correlation between decrease in DNA synthesis and decrease in cAMP or increase in cGMP or stimulation of NOS activity. This result indicates that β_3 adrenoceptor agonist-induced decrease in the DNA synthesis resulted in NOS/guanylate cyclase stimulation and adenylate cyclase inhibiton.

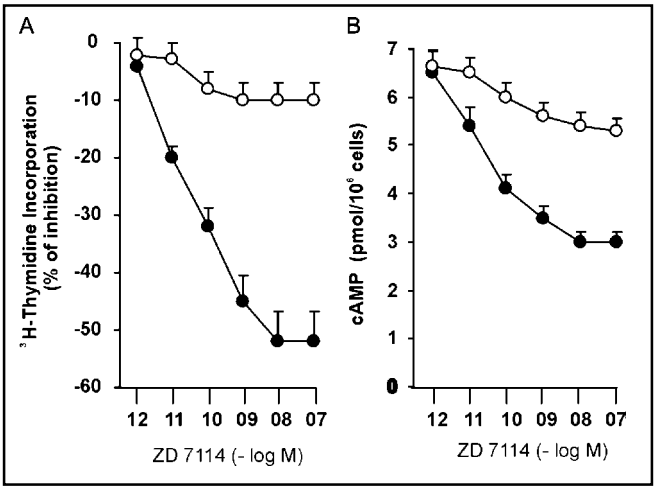

Fig. 2. Decrease on ^{3}H-Thymidine Incorporation (A) and in cAMP (B) by increasing concentration of ZD 7114 (●). The inhibitory action of SR 59230A (1x10^{-7} M) is also shown (○). Values represent the mean ± SEM of six experiments in each group performed by duplicate. Tissues were incubated for 30 min in presence or absence of SR 59230A and then ZD 7114 was added. Values of ^{3}H-Thymidine Incorporation were expressed as percentage of changes calculated by comparison with the absolute values prior to the addition of ZD 7114. No effects were observed with SR 59230A alone at the concentration used either in DNA synthesis or in basal cAMP.

To determine whether enzymes involved in the NO/cGMP signaling system could participate in the ZD 7114 inhibitory effect on DNA synthesis, the action of U-73122 and TFP were studied. Table 2 shows that the inhibition of PLC by U-73122 (5x10^{-6} M) , calcium/calmodulin (CaM) by TFP (5x10^{-6} M) prevented ZD 7114 effect. Also, Table 2 shows that BAPTA (1x10^{-5} M) was able to attenuate the inhibitory effect of ZD 7114 on DNA synthesis. In order to assess whether the enzyme activities are dependent on each other, fibroblasts were incubated with different inhibitors of enzymatic pathways involved in β_3 adrenoceptor activation. Table 3 shows that U-73122, TFP and L-NMMA inhibited the stimulatory action of

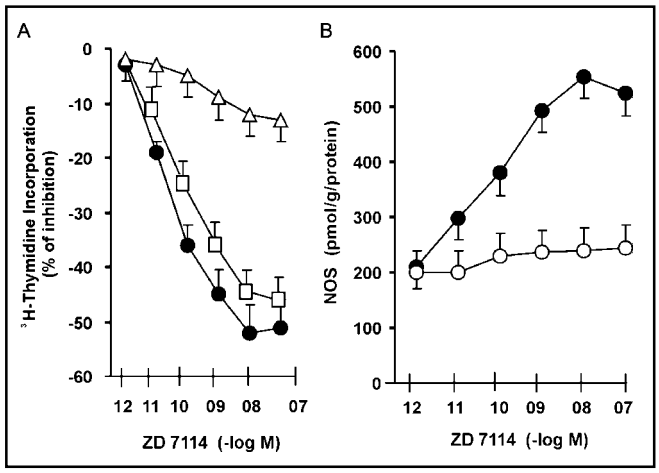

Fig. 3. (A) Effect of $1x10^{-5}$ M L-NMMA (△) on the action of ZD 7114 (●) upon DNA synthesis. Reversion of L-arginine ($5x10^{-5}$ M) (□). No effects of L-arginine or L-NMMA alone were obtained at the concentration used (data not shown). (B) Stimulation in NOS activity by increasing concentration of ZD 7114 (●) and the inhibition by treated fibroblasts with $1x10^{-7}$ M SR 59230A (○). Values are mean ± SEM of six experiments in each group performed by duplicate. For other details see legends of Figure 1.

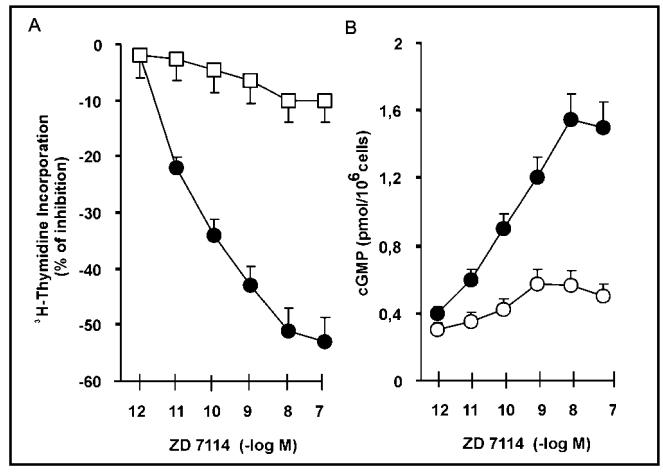

Fig. 4. (A) Effect of $5x10^{-5}$ M ODQ (□) on the dose-response curve of ZD 7114 (●) upon ^{3}H-Thymidine Incorporation. (B) The stimulation of cGMP accumulation by ZD 7114 alone (●) or in the presence of SR 59230A (○) is also shown. Values are mean ± SEM of six experiments in each group performed by duplicate. For other details see Figure 1.

ZD 7114 ($1x10^{-7}$ M) upon NOS activity and cGMP accumulation. The inhibition of PKC by staurosporine ($1x10^{-9}$ M) was without effect. Table 3 also shows that $1x10^{-4}$ M L-NIO (eNOS inhibitor) but not N-propyl-L-arginine (nNOS inhibitor) or aminoguanidine (iNOS inhibitor), prevented the stimulatory action of ZD 7114 on both NOS activity and cGMP accumulation. Additionally, the inhibition of NO-sensitive guanylate cyclase by ODQ ($5x10^{-5}$ M) inhibited the ZD 7114 induced increase in cGMP (-98±3%, n=5) but reverted the cAMP inhibition (+19.7±2.2%, n=5). No effects were observed with enzymatic inhibitors at the concentration used (data not shown).

Discussion

The major new finding of this study is that ZD 7114, a β_3 agonist, exerted an inhibitory action on human skin fibroblasts DNA synthesis. The ZD 7114 on DNA synthesis was accompanied by a decrease on cAMP production and with the increase amount of cGMP levels. These effects were receptor-mediated action demonstrated by the virtue of blockade by the selective β_3 adrenoceptor antagonist SR 59230A.

Our findings demonstrated that β_3 adrenoceptor are expressed in human fibroblast cells, displaying different β adrenoceptor populations, i.e. $\beta_3 > \beta_2 > \beta_1$ as demonstrated in binding assays with a β adrenoceptor radiolabelled antagonist [^{3}H]-CGP. Selective β adrenoceptor antagonist subtypes (β_3: SD 59230A; β_2: ICI 118,551; β_1: atenolol) displaced with different potencies [^{3}H]-CGP binding; indicating by the competition curves (Ki values) that fibroblast express a high number of β_3 and β_2 adrenoceptors.

Functional evidences have shown that β_1 and β_2 adrenoceptors co-exist in fibroblasts and that their stimulation produces increase in DNA synthesis and in cAMP production [26, 27]. To our knowledge there have been no previous reports as to a direct biological effect of β_3 adrenoceptor stimulation on human fibroblasts.

The mechanism of β_3 adrenoceptor-induced inhibition on skin fibroblast DNA synthesis was accompanied by a decreased on cAMP production. In stark contrast with the effect of ZD 7114, the stimulation of intracellular cAMP production by forskolin, enhanced DNA synthesis; indicating that cAMP generation (*per se*) provide a sufficient stimulus for DNA synthesis of human skin fibroblasts. The effect of cAMP on fibroblast DNA synthesis is still unclear. Previous studies have demonstrated a growth-inhibitory effect of cAMP [28,29] although a stimulatory effect has also been reported [30].

β_3 adrenoceptor differ from the β_1 adrenoceptor and β_2 adrenoceptor subtypes by their molecular structure and pharmacological profile. The β_3 adrenoceptor gene contains two introns [31, 32] leading to alternative splice

Drugs	DNA synthesis (% inhibition)
ZD 7114 ($1x10^{-7}$M)	- 52.3 ± 4.1
U-73122 ($5x10^{-6}$M) + ZD 7114	- 9.2 ± 0.7*
TFP ($5x10^{-6}$M) + ZD 7114	- 10.4 ± 1.1*
BAPTA ($1x10^{-5}$M) + ZD 7114	- 11.5 ± 1.3*

Table 2. Effect of different blockers in β_3-agonist-induced inhibition of DNA synthesis Results are mean ± SEM of five experiments in each group. * $p < 0.001$ comparing with ZD 7114 alone.

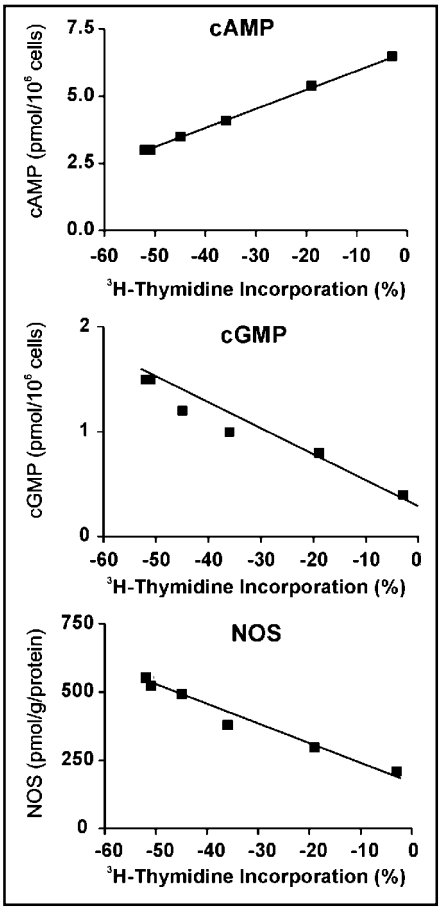

Fig. 5. Correlation in the effect of ZD 7114 ($1x10^{-12}$ to $1x10^{-7}$ M) on DNA synthesis, on c-AMP (R^2 0.9994, P value 0.0001), cGMP (R^2 0.9647, P value 0.0005) and NOS (R^2 0.9724, P value 0.0003) production. Inhibition of DNA synthesis was plotted as a function of cAMP or cGMP production or NOS activity. Values are mean ± SEM of six experiments in each group.

isoforms, whereas β_1 adrenoceptor and β_2 adrenoceptor genes are introless. β_3 adrenoceptors are G-protein-coupled receptors that interact with either G_s or G_i [33]. Depending on the tissue, β_3 adrenoceptor stimulation produces functional effects that are either comparable with or opposite to those produced by β_1 adrenoceptor and β_2 adrenoceptor stimulation. In adipose tissue, β_3 adrenoceptor stimulation increases intracellular cAMP production [34] but in the human heart, β_3 adrenoceptor mediate negative inotropic effects that is unlikely to be related to stimulation of the cAMP production but rather to stimulation of the cGMP pathway [33, 35].

In the present study, in which the expression levels of β_3 adrenoceptors is high, the inhibition of DNA synthesis triggered by ZD 7114 on fibroblasts significantly correlated with the decrement of cAMP and with the increment of NO/cGMP production. These results suggest that β_3 adrenoceptor stimulation-induced inhibition of DNA synthesis as a resulted of adenylate cyclase inhibition and NO/guanylate cyclase stimulation. The ability of cGMP to induce inhibition of fibroblast DNA synthesis was evidence by the results obtained with 8-bromo-cGMP that mimicked ZD 7114 action and with ODQ that impaired its. Our results also described that cGMP-elevating agents like NO, suppress fibroblast DNA synthesis. The effect of L-NMMA inhibiting the ZD 7114 agonist action on fibroblast DNA synthesis, point to the role of NO-mediated pathways on β_3 adrenoceptor activation.

To investigate the nature of NOS involved, we examined the influence of selective NOS inhibitors. Detailed experimentation with selective inhibitors of different NOS isozymes revealed that only L-NIO causes a substantial inhibition of the ZD 7114 on NOS activity. Inhibitors of iNOS and nNOS have no significant effect. Recent studies [36] in the gastrointestinal tract are in general agreement with the concept that eNOS is not restricted to endothelial cells and that it also occurs in other cells such as interstitial cells of Cajal [37], smooth muscle cells [38] and cardiac myocytes [39].

In addition, our results propose that among intracellular events triggers by ZD 7114 to inhibit DNA synthesis, the activation of phosphoinositides turnover via PLC occurs. In accord with this, experiments with U-73122 prevented the inhibitory action of ZD 7114 on fibroblast DNA synthesis. Moreover, the fact that CaM inhibitor (TFP) and BAPTA abrogated the β_3 adrenoceptor agonist action on DNA synthesis, point to the release of calcium from cytoplasmatic stores being responsible for the inhibitory action of ZD 7114. In contrast, staurosporine, a PKC inhibitor, was unable to alter ZD 7114 action on fibroblast. It was observed that the increase of cytoplasmatic calcium resulted in inhibition of cAMP production in brown adipocytes, through the activation of calmodulin-controlled phosphodiesterase activity. Under such conditions, fibroblast can produce NO and NO alters the activities of adenylate cyclase and phosphodiesterases, thus functional linking-the cAMP/NO-cGMP pathways has been demonstrated [40, 41]. A diagram to tie together the various systems studied and proposing a model for the mechanisms where ZD 7114 down-regulates fibroblast DNA synthesis through NO/cGMP pathway is shown in Figure 6.

In conclusion, this work demonstrates that β_3 adrenoceptor stimulation decreases human skin fibroblast

Table 3. Effects of ZD 7114 upon NOS activity and cGMP accumulation. Influence of enzymatic inhibitors. Values are mean ± SEM of five experiments in each group performed in duplicate. NOS activity and cGMP accumulation were measured after incubation for 30 min fibroblasts with or without enzymatic inhibitors and with an additional 1 min with $1x10^{-7}$ M ZD 71214. The following concentrations were used: $5x10^{-6}$ M U-73122 and TFP, $1x10^{-9}$ M staurosporine, $1x10^{-5}$ M L-NMMA or $1x10^{-4}$ M L-NIO or N-propyl-L-arginine or aminoguanidine and $5x10^{-5}$ M ODQ. *p<0.001 comparing with basal values (none); **p<0.001 comparing with ZD 7114 alone.

Drugs	NOS (pmol/g/protein)	cGMP (pmol/10^6 cells)
None	218 ± 18	0.42 ± 0.03
ZD 7114	587 ± 22*	1.56 ± 0.08*
ZD 7114 + U-73122	250 ± 20 **	0.51 ± 0.05**
ZD 7114 + TFP	210 ± 15**	0.54 ± 0.05**
ZD 7114 + L-NMMA	46 ± 4**	0.50 ± 0.04**
ZD 7114 + staurosporine	609 ± 30*	1.65 ± 0.1*
ZD 7114 + L-NIO	59 ± 5**	0.53 ± 0.05**
ZD 7114 + N-propyl-L-arginine	550 ± 20	1.42 ± 0.07
ZD 7114 + aminoguanidine	590 ± 28	1.60 ± 0.10
ZD 7114 + ODQ	578 ± 23*	0.29 ± 0.02**

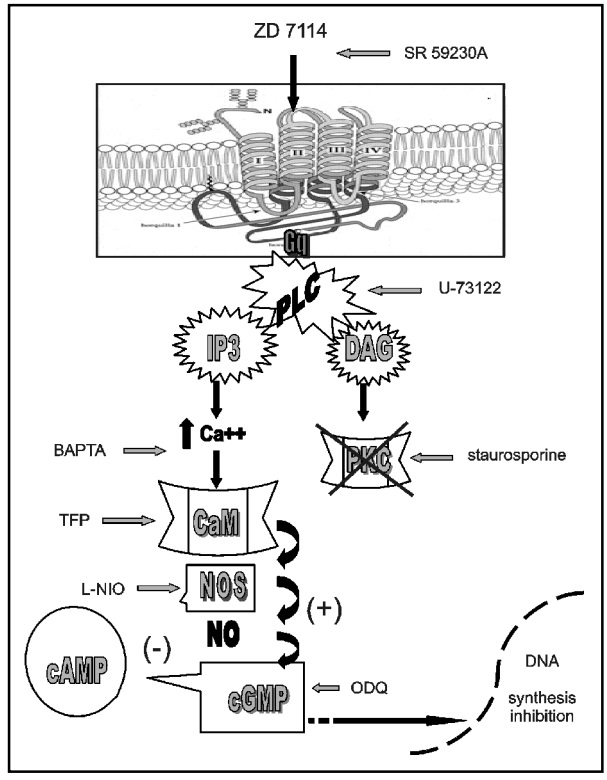

Fig. 6. Proposed model for the mechanism where ZD 7114 down-regulates fibroblast DNA synthesis through NO/cGMP pathway. ZD 7114 acting on G-protein-β_3 adrenoceptors activates PLC, mediating production of inositol-tri-phosphate (IP_3) and 1-2 diacylglycerol (DAG). IP_3 triggers intracellular release of calcium (Ca^{++}). Free Ca^{++} binds to calmodulin and the calcium/calmodulin (CaM) activates eNOS with NO production. NO in turn stimulates soluble guanylate cyclase with increase production of cyclic GMP (cGMP). The increment of cGMP (+) and the decrement in cAMP (-) triggers fibroblast DNA synthesis inhibition. The DAG-protein kinase C (PKC) pathway appears not to participate in this mechanism. Grey arrows indicate the site where the inhibitory agents act.

DNA synthesis through activation of cGMP/NOS pathway with an inhibition of adenylate cyclase coupled to β_3 adrenoceptor activation. These data indicate that constitutive β_3 adrenoceptor coupling to G_i protein in human skin fibroblast could serve both to restain G_s - mediated activation by β_2 and β_1 adrenoceptor and to initiate additional signal transduction pathways including NO-cGMP. Changes in the expression of this pathway may alter the balance between positive and negative effect of catecholamines upon fibroblast DNA synthesis; so, potentially altering the coordination required for wound healing and tissue remodeling.

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