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Dexamethasone decreases phospholipase C β1 isozyme expression in human vascular smooth muscle cells

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Abstract

The molecular characterization of the human *PLC β1* gene was just reported by Peruzzi et al. [Biochim. Biophys. Acta 1582 (2002) 46]. This prompted us to investigate the effects of dexamethasone on *PLC β1* expression in two types of human vascular smooth muscle cells—coronary artery smooth muscle cells (hCASMC) and aortic smooth muscle cells (hAoSMC), since glucocorticoids are known to affect the signaling pathways of Gprotein coupled receptors. Semi-quantitative RT-PCR was used to analyze mRNA expression and Western-blot for protein expression. Dexamethasone treatment in the two types of cells studied decreased (mRNA and protein) *PLC β1* isozyme expression. A rapid (2 h) fall in mRNA occurred in hCASMC after treatment, and hCASMC were more sensitive to dexamethasone (1 nM versus 100 nM) than hAoSMC. The major reduction (80%) was observed after 48 h of exposure in both VSMC. Treatment with mifeprisone, an antagonist of glucocorticoid receptors, blunted the dexamethasone effect on *PLC β1* mRNA and showed that this effect was mediated by glucocorticoids receptors.
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Keywords: Human VSMC; *PLC β1*; Dexamethasone

1. Introduction

Glucocorticoids are known to affect the signaling pathway of G protein coupled receptors (GPCR). By example, the pressure responsiveness to angiotensin II is increased in cortisol-induced hypertension in human [2]. Studies using rat cultured vascular smooth muscle cells (VSMC), suggest that adrenal steroids act directly to potentiate angiotensin II action, in that corticosteroids up-regulate angiotensin membrane receptors [3]. If many studies involving glucocorticoids focused on the transduction pathway of angiotensin II, most of them were related to the effects on angiotensin II type 1 (AT1) receptors. AT1 receptors mediate many of the physiological and behavioral actions of angiotensin II. More little studies have been performed on G proteins (αq and α11) and fewer on the *PLC β1*. Moreover, most of the previous reports were done in vivo in animal species, so little information is available for human models and specially for cultured cells.

In human blood cells, which possess the two subtypes of AT1 receptors, mRNA levels of $AT1_A$ receptors were only elevated by an excess of cortisol [4]. The rat AT_1 receptor has been shown in various conditions to be capable of coupling to various G proteins α-subunits (G_q, $G_{q/11}$, and $G_{i/o}$) [5,6], which may provide insights into the potential mechanism by which a single AT_1 receptor stimulates various signaling cascades. It was recently reported that dexamethasone treatment increased Gα11 mRNA and protein in the osteoblastic UMR 106-01 cell line [7], but as yet, no data were done on human VSMC. In human aortic VSMC, PLC β1 was detected both by RT-PCR and immunoblot analysis [8]. Incubation of permeabilized VSMC with anti-PLC β1 or anti-Gαq antibodies inhibited in hAoSMC the angiotensin II-dependent inositol phosphate production [8]. Thus, PLC β1 is the isoform that is critical for angiotensin II-regulated PLC signaling in human VSMC.

The lack of glucocorticoid responsive element in the putative promoter region of the human *PLC β1* gene [1] prompted us to study by semi-quantitative RT-PCR and Western-blot the effects of dexamethasone on *PLC β1* in human VSMC. Mifeprisone was used to demonstrate that dexamethasone effects on *PLC β1* involved an interaction with glucocorticoid receptors. mRNAs of the AT1 receptor and Gαq and Gα11 were also measured in human VSMC after dexamethasone treatment to extend our observations

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to the main components of the transduction pathway of angiotensin II.

2. Material and methods

2.1. Material

Dexamethasone (Dex) and Mifeprisone (RU-486) were obtained from sigma Aldrich (St-Quentin Fallavier, France). Human coronary artery smooth muscle cell (hCASMC) and reagents for cell culture were obtained from Clonetics (Biowhittaker, Verviers, Belgium), they were used on the 7th passage. Human aortic smooth muscle cell (hAoSMC) and reagents for cell culture were obtained from Promocell (Heidelberg, Germany), and used on the 8th passage.

2.2. Cell culture

The human coronary VSMC and human aortic VSMC were maintained in smooth muscle growth medium supplemented with 5% fetal calf serum, 0.5 μg/l hEGF, 2 μg/l hFGF, 5 mg/l insulin and a mixture of antibiotic (50 mg/l gentamicin + 50 μg/l amphotericin-B). Cultures were maintained at 37 °C with atmospheric air and 5% CO_2. The medium was changed every 48 h and cells were subcultured after treatment with trypsin and EDTA. Subconfluent human coronary and aortic VSMC in their seventh passage were used for the experiments. Dexamethasone and Mifeprisone (RU-486) were dissolved in absolute ethanol and then diluted in culture medium to reach a final ethanol concentration of 0.01%. Controls with the same volume of vehicle were used in each case.

2.3. RT-PCR

Total RNAs were isolated by the Trizol method (Invitrogen, Cergy-Pontoise, France). The RNA pellets were dissolved in sterile distilled water, and quantified by optical density at 260 nm.

cDNA were synthesized from 1 μg of total RNA. The reaction mixture had a final volume of 20 μl, and contained 75 mM KCl, 50 mM Tris–HCl (pH 8.3), 3 mM $MgCl_2$, 10 mM DTT, 20 U of RNAsin (Promega), 200 U Superscript II reverse transcriptase (Invitrogen, Cergy-Pontoise, France), 1 mM of each dNTP (Promega, Charbonnière, France), and 0.25 μg of random primers (Amersham Pharmacia Biotech). Annealing and primer extension were performed at 42 °C for 1 h. Then PCR were performed on a aliquot (2 μl) of this mix by adding 18 μl of a mix containing 22 mM Tris–HCl (pH 8.4), 55 mM KCl, 1.4 mM $MgCl_2$, 10 pmol of each specific primer (Invitrogen, Cergy-Pontoise, France), 2 nmol of each dNTP, 1 U of *Taq* DNA polymerase (Invitrogen, Cergy-Pontoise, France), and 1 mCi of [α-^{33}P]dCTP.

Semi-quantitative PCR was established by determination of the exponential phase of the PCR products. Thus, amplifications were routinely performed as shown in the Table 1. The thermal cycling protocol was 94 °C for 45 s, 60 °C for 60 s, and 72 °C for 60 s. During the first cycle, the 94 °C step was extended to 5 min, and on final cycle the 72 °C step was extended to 5 min. Control reactions were carried out as previously described [9] in a T Gradient Thermocycler 96 (Biometra, Vysis, Voisins le Bretonneux, France). In some experiments, amplified products were analyzed by electrophoresis in 2% agarose gels and visualized by ethidium bromide. The gel was fixed on 7% TCA for 30 min and then dried before direct exposition to Kodak Biomax autoradiographic (Kodak, France).

2.4. Western-blot

The human coronary and aortic VSMC were prepared by adding a membrane lysis buffer containing a detergent (16 mM CHAPS, 20 mM Tris–HCl (pH 7.5), 1 mM Na_2EDTA, 1 mM DTT) with proteases inhibitors (1 mM benzamidine, 1 μg/ml leupeptine, 10 μg/ml soybean trypsine inhibitor, 0.5 mM PMSF (SIGMA, St. Quentin

Table 1
Oligonucleotides used for the amplification

Target		Sequence	Size (bp)	Cycles	Accession no.
PLC β1	Sens (2539-2555) Antisens (3009-2991)	5′-GAT CCT GGG GAA ACA CC-3′ 5′-GGC ATC CAG GGC AGC AAG G-3′	470	27	163521
hGαq	Sens (402-427) Antisens (765-742)	5′-GGT CTC TGG TTT TGA GAA TCC ATA TG-3′ 5′-CTC CAC GAG GAC TTG ATC ATA TTC-3′	364	22	1181670
hGα11	Sens (549-574) Antisens (901-886)	5′-GAC CAC CTT CGA GCA GCA TCA GTA CGT CA-3′ 5′-CTC CAC CAG GAC TTG GTG TCG TAT TC-3′	363	23	4504036
AT1	Sens (252-271) Antisens (506-487)	5′-GAT GAT TGT CCC AAA GCT GG-3′ 5′-TAG GTA ATT GCC AAA GGG CC-3′	255	30	XM_051470
GAPDH	Sens (244-264) Antisens (817-798)	5′-ATC ACC ATC TTC CAG GAG CG-3′ 5′-CCT GCT TCA CCA CCT TCT TG-3′	573	20	M17701

Expected sizes of the amplicons and the Genbank accession no. for each gene are also shown.

Fallavier, France)) as already described [10]. The samples were then centrifuged at 15,000 g for 30 min and the supernatants collected. The protein content of the lysates was estimated using the Bradford method (Biorad, France). The amounts of proteins used for Western-blot analysis were 10 μg for human coronary and aortic VSMC to detect PLC β1. The Proteins were loaded on to 8% SDS–Tris–glycine polyacrylamide gels and after electrophoresis the proteins were blotted on to PVDF membranes. The membrane was saturated for 2 h in TBS containing 0.1% Tween and 5% non-fat dry milk. The primary rabbit polyclonal antibodies against PLC β1 (sc-205, Santa Cruz Biotechnology Inc., Heidelberg, Germany) were used at 1:500 dilution. A peroxydase conjugated rat anti-rabbit antibody (Sigma, St. Quentin Fallavier, France) was used at 1:15,000 dilution as secondary antibody. The ECL Western-blotting system (Amersham Pharmacia Biotech, les Ulis, France) was used for detection. Autoradiographies were then scanned using a densitometer (Biocapt, Villmer Lourmat, Torcy, France) and the PLC β1 contents were normalized using actin.

2.5. Statistical analysis

For the RT-PCR and Western-blots experiments, all data are expressed as mean ± S.E.M. Statistically significant data were evaluated with a Fisher's *F*-test (SuperAnova, Abacus Concepts, USA). Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Kinetic effects of dexamethasone s on mRNA levels of PLC β1

To evaluate the mRNA expression, a semi-quantitative RT-PCR involving ^{33}P incorporation in PCR products was used during the exponential phase of PCR product accumulation. We have performed RT-PCR using specific primers designed in Table 1. All these mRNA were detected in basal culture conditions in the two cell types of VSMC (hCASMC and hAoSMC).

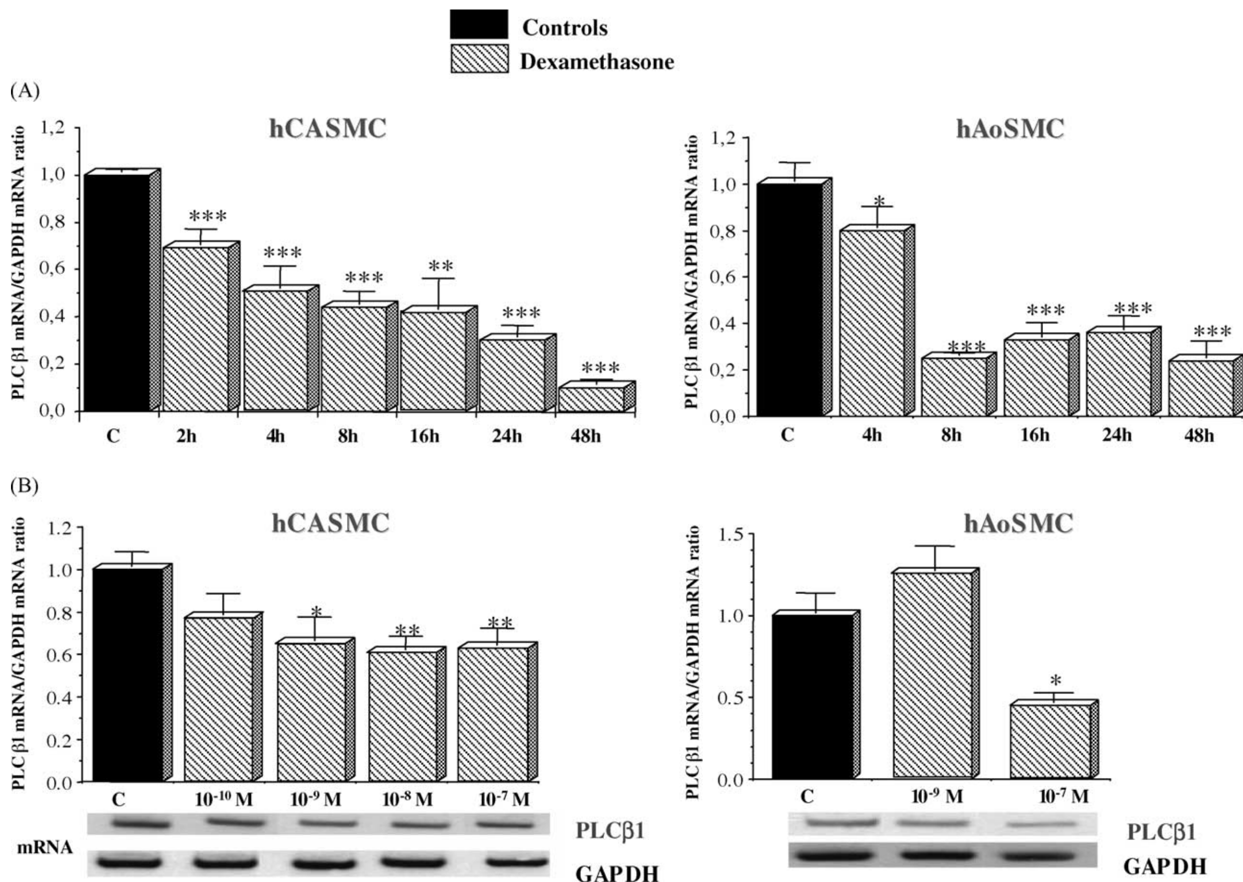

Fig. 1. (A) Time-dependent effects of 10^{-7} M dexamethasone (Dex) on *PLC β1* mRNA levels in human coronary artery smooth muscle cells (hCASMC), and in human aortic smooth muscle cells (hAoSMC). Cells were treated with Dex for the indicated times (C: controls). (B) Log-dose effect of 10^{-7} M Dex on *PLC β1* mRNA levels in hCASMC treated for 4 h with doses ranging from 10^{-10} to 10^{-7} M, and in hAoSMC treated by Dex for 24 h with doses ranging from 10^{-9} to 10^{-7} M. Semi-quantitative RT-PCR was performed using (α^{33}P)dGTP incorporation in the PCR products. Mean ± S.E.M. of scan ratios (four determinations in hCASMC, and six determinations for hAoSMC) between the x mRNA and GAPDH mRNA used as a control probe. $P < 0.05$ (∗); $P < 0.01$ (∗∗); $P < 0.00$ (∗∗∗) from controls using the Fisher's *F*-test.

We have observed that 10^{-7} M dexamethasone reduced significantly the *PLC β1* mRNA amount in hCASMC (Fig. 1A). This decrease was significant after 2 h of treatment, and was maximal at 48 h (90% of reduction). In hAoSMC, dexamethasone decreased significantly *PLC β1* mRNA levels only after 4 h of exposure, and this decrease was maximal from 8 to 48 h (80% of reduction) (Fig. 1A).

3.2. Log-dose effects of dexamethasone on PLC β1 mRNA levels

Log-dose effects of dexamethasone were performed in the two types of human VSMC (Fig. 1B), 4 h after addition of dexamethasone in hCASMC and, after 24 h of treatment in hAoSMC.

In hCASMC, a fall in *PLC β1* mRNA was noted with doses ranging from 10^{-9} to 10^{-7} M dexamethasone. However, hAoSMC appeared less sensitive to dexamethasone since *PLC β1* mRNA were decreased only for large doses (10^{-7} M), no effect being observed with 1 nM (Fig. 1B).

3.3. The glucocorticoid receptor is involved in dexamethasone effects on PLC β1 mRNA expression

The effects of glucocorticoids and mineralocorticoids are mediated by intracellular receptors. The mineralocorticoid receptors (MR) bind both aldosterone and corticosterone with high affinity ($K_d = 0.5$ nM), whereas the glucocorticoid receptor (GR) preferentially binds glucocorticoids, although at a lower affinity ($K_d = 5$ nM). To see the specificity of the effects of glucocorticoids on *PLC β1* mRNA, hAoSMC were treated with several concentrations of aldosterone (10^{-7} to 10^{-11} M) for 24 h. No variation on *PLC β1* mRNA levels were observed for all doses tested (data not shown).

To assess the implication of glucorticoid receptors in the dexamethasone action on *PLC β1* mRNA expression, mifeprisone (RU-486), a specific antagonist of glucocorticoid receptors was used. As shown in Fig. 2, hCASMC were pretreated with 10^{-6} M of mifeprisone during 1 h and, then both 10^{-7} M dexamethasone and RU-486 were added for 48 h. The mifeprisone abolished the decrease of *PLC β1* mRNA induced by 10^{-7} M dexamethasone. No change in the *PLC β1* mRNA expression was observed after the sole administration of mifeprisone (Fig. 2). Therefore, the reduction of *PLC β1* mRNA expression under dexamethasone involved the interaction with glucocorticoids receptors.

3.4. Effect of dexamethasone on PLC β1 protein expression in hCASMC and in hAoSMC

Western-blots were done under reducing and denaturing conditions in both VSMC. In basal conditions of culture, the PLC β1 isoform (150 kDa) was detected both in hCASMC and hAoSMC. To evaluate the dexamethasone effect on PLC

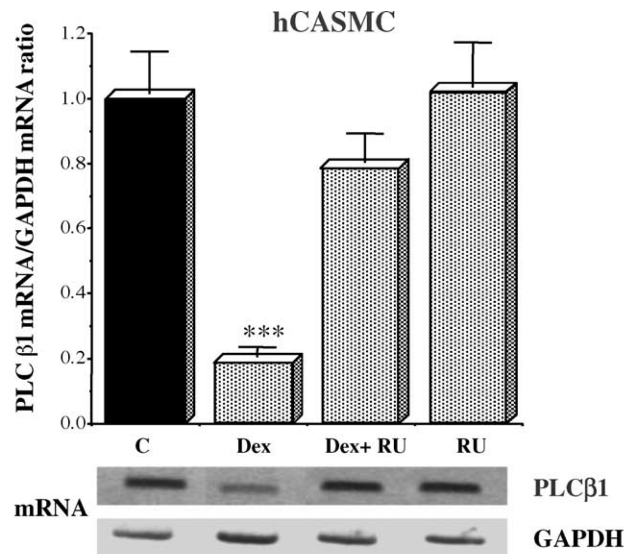

Fig. 2. Mifeprisone (RU-486) blunted the effect of dexamethasone (Dex) on *PLC β1* mRNA levels in human coronary artery smooth muscle cells (hCASMC). Cells were preincubated 1 h with RU-486 and treated for 48 h with 10^{-7} M Dex + RU-486. Semi-quantitative RT-PCR was performed using (α^{33}P)dGTP incorporation in the PCR products. Mean ± S.E.M. of scan ratios of six determinations between the x mRNA and GAPDH mRNA used as a control probe. $P < 0.001$ (***) from controls using the Fisher's *F*-test.

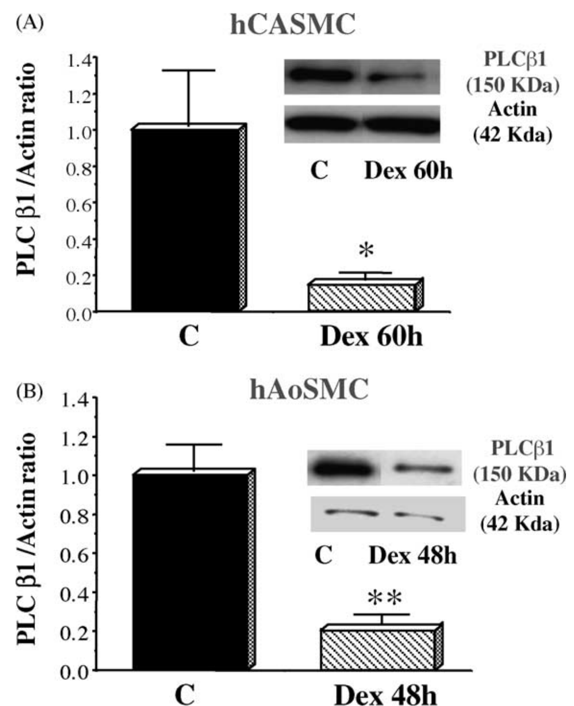

Fig. 3. Effect of Dexamethasone (Dex) on PLC β1 protein. Quantitative determinations by Western-blot in human Coronary Artery Smooth Muscle Cells (hCASMC) (A) and in human Aortic Smooth Muscle Cells (hAoSMC) (B). Cells were treated with 10^{-7} M Dex for 60 h in hCASMC and for 48 h in hAoSMC. Data are shown as means ± S.E.M. of four determinations for hCASMC and three determinations for hAoSMC. Each value was corrected by use of a control actin probe. $P < 0.05$ (*); $P < 0.01$ (**) from controls using the Fisher's *F*-test.

β1 protein expression, we treated the CASMC with 10^{-7} M of dexamethasone for 60 h and the AoSMC with 10^{-7} M of dexamethasone for 48 h. In both cases, the PLC β1 protein was markedly reduced by the dexamethasone treatment (about 80% of reduction) (Fig. 3).

3.5. Effects of dexamethasone on mRNA levels of AT1, Gαq and Gα11

As shown in Fig. 4, we have determined the mRNA levels of the angiotensin II type I receptor (AT1 receptor). The hCASMC and the hAoSMC, were exposed to 10^{-7} M of dexamethasone and time course studies were performed (0, 4, 8, 16, 24, and 48 h). Administration of dexamethasone caused a significant increase of AT1 receptor mRNA at 4, 16 and 48 h in hAoSMC. No change was detected at 8 h (Fig. 4A) and 24 h. An increase in AT1 mRNA levels occurred in CASMC after 48 h of dexamethasone treatment (Fig. 4B).

AT1 receptors are coupled to Gαq and Gα11, so, we have investigated the effects of dexamethasone on Gαq and Gα11 mRNA levels. No change of Gαq and Gα11 mRNA levels occurred after dexamethasone (10^{-7} M) treatment of hCASMC during 24 and 48 h (Fig. 4B).

4. Discussion

Glucocorticoids are known to affect the transduction pathway of angiotensin II. Dexamethasone application in rat VSMC induces a decrease in formation of inositol phosphate in spite of an increase in angiotensin II receptors [11]. Angiotensin II receptors (AT1) are coupled with Gq/G_{11} heterotrimeric proteins and PLCs. As yet, no data were reported on the possible effects of dexamethasone on PLC expression in human VSMC. So, we have investigated the possible action of this synthetic glucocorticoid on mRNA and protein expression of PLC β1 in two types of human VSMC. Different isoforms of PLC β are expressed in a cell line of human VSMC, Western-blotting had characterized the β1, β2, β3, and β4 isoforms [12]. In our model, dexamethasone reduced significantly the *PLC β1* mRNA and protein expression in the two types of human VSMC used (hAoSMC and hCASMC). The fall in mRNA level was rapid occurring 2 h after dexamethasone treatment in hCASMC, and observed with 1 nM of the glucocorticoid. The effect was specific of glucocorticoids, since aldosterone treatment was without effect (data not shown). The use of an antagonist of glucocorticoid receptors (RU-486) showed that dexamethasone effect on *PLC β1* mRNA involved an

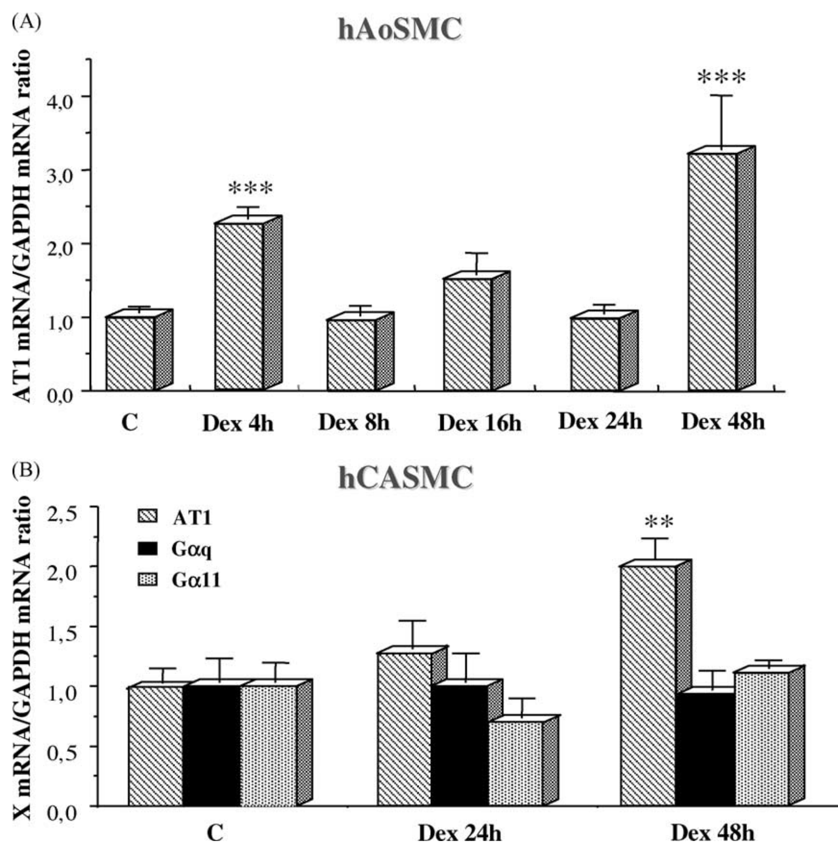

Fig. 4. (A) Time-dependent effects of 10^{-7} M dexamethasone on AT1 mRNA levels in human aortic smooth muscle cells (hAoSMC). (B) Time-dependent effects of 10^{-7} M dexamethasone on AT1, Gαq and Gα11 mRNA levels in human coronary artery smooth muscle cells (hCASMC). Semi-quantitative RT-PCR was performed using (α^{33}P)dGTP incorporation in the PCR products. Mean ± S.E.M. of scan ratios (four determinations in hCASMC, and six determinations for hAoSMC) between the x mRNA and GAPDH mRNA used as a control probe. $P < 0.01$ (**); $P < 0.001$ (***) from controls using the Fisher's *F*-test.

interaction with glucocorticoid receptors. The decrease in *PLC β1* mRNA levels in human VSMC was accompanied by a marked reduction of the protein level as shown after 48 h of dexamethasone treatment. The recent characterization of the 5'upstream region of the human *PLC β1* gene [1] provides new insights about the possible transcriptional effects of glucocorticoids on the *PLC β1* gene. No GRE was found in the predicted promoter region, but 3 AP1 site were localized [1] suggesting an indirect effect of the GR through protein interactions with c-jun and/or c-fos as already known for the collagenase gene [13]. An effect of dexamethasone on *PLC β1* mRNA and protein expression was already reported in rats where repeated administrations for 10 days increased the PLC expression in both membrane and cytosol fractions of the hippocampus [14].

Since glucocorticoids modulate the transduction pathway of angiotensin II in rat VSMC, we look for an effect of dexamethasone on angiotensin II receptors and on $G\alpha_q/_{11}$. We showed here that dexamethasone, both in hCASMC and in hAoSMC increases AT1 receptor mRNA levels by induction of $AT1_A$ mRNA, a subtype of the AT1 receptor (data not shown). Such an effect was already reported by Shibata et al. [4] in human blood cells and by Sato et al. [15] in rat VSMC who demonstrated that dexamethasone induced $AT1_A$ mRNA expression. Glucocorticoids are known to increase Gαq and Gα11 protein expression in a rat osteosarcoma cell line [7]. Here, no change on Gαq and Gαq mRNA expressions in hCASMC treated with dexamethasone was observed. In conclusion, our data emphasized the potential role of glucocorticoids in the control of GPCR transduction pathways in human VSMC involving PLC since dexamethasone decreased the *PLC β1* expression in basal culture conditions.

Acknowledgements

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